

**1**

## THE BASICS OF ENGINEERED NANOMATERIALS FOR TOXICOLOGISTS OR TUTORIAL: THE FUNDAMENTAL CHEMISTRY AND PHYSICS OF ENGINEERED NANOSTRUCTURES.

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Nanotechnology is an emerging multidisciplinary science that deals with the creation and use of molecules a few billions of a meter in size. Assessing the potential hazards of the nanomaterials in this technology, and the products constructed from nanoparticulates is an emerging area in toxicology and health risk assessment. The development of toxicity data sets and exposure assessments for various nanoparticles and nanomaterials is ongoing and evolving as new particles, materials and exposure methodologies are developed. A related issue in toxicology and risk assessment is the extent to which nanoparticle toxicity can be extrapolated from existing toxicology databases for macro and microscale particle-types. Additional information needs that are being addressed include the environmental and biological fate, transport, persistence, and transformation, as well as the recyclability and overall sustainability of manufactured nanoparticles. This sunrise lecture is designed to be a basic primer on the fundamental chemistry and physics of engineered nanomaterials. The understanding of the composition of and source generation of particles becomes fundamentally more important when one considers that the surfaces or surface coatings may comprise > 50% composition of small nanoparticles (< 30 nm), and herein will lie the interactions of nanoparticles with cells. Future studies likely will demonstrate that method of particle synthesis, surface coatings, aggregation potential, surface charge, and shape may be as or more important than particle size in modifying biological/toxicological effects. Methods to form materials such as single-walled carbon nanotubes, fullerenes, quantum dots, nanocrystalline ceramics (e.g., titania), and nanometals will be reviewed. For each material class, the essential properties and applications will be outlined. The tutorial will conclude with a discussion of the actual applications of these high performance materials and projections for the industry growth over the next decade.

**2**

## DOSE CONSIDERATIONS FOR *IN VITRO* STUDIES OF AIR POLLUTANT TOXICITY.

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Technological advances have made possible the investigation of the cellular effects of air pollutants through variety of *in vitro* approaches. However, in order to be relevant to human health effects following ambient exposures, these methods must accurately model biologically relevant exposure pathways, doses and responses. This continuing education course will provide both theoretical and practical information on appropriate dose selection and dosage techniques for *in vitro* studies of air pollutant toxicity. The first presentation will cover the basic concepts of vapor dosimetry including the roles of partitioning, chemical reactivity and local metabolism. Specific information on regional respiratory deposition in laboratory animals and humans and its relevance to cellular microdosimetry will be provided. The second presentation will include specific examples of *in vitro* approaches with a focus on microdosimetric considerations for reactive gases; in particular the potential biological impacts of surface lining layers and their constituents will be emphasized. Basic concepts of size specific regional particle deposition and clearance will be covered in the third presentation. Specific information on regional respiratory deposition efficiencies in laboratory animals and humans and its relevance to cellular microdosimetry will be provided. The final presentation will describe specific examples of *in vitro* approaches on particle toxicity with a cellular dosimetric comparison of effects observed *in vitro* compared to those *in vivo*. Overall the course is aimed at providing information not only on the importance of employing *in vivo* dosimetric considerations in designing *in vitro* studies, but also with providing a firm conceptual foundation for the selection of relevant doses for such work.

**3**

## DEVELOPMENTAL TOXICOLOGY STUDIES: DESIGN, INTERPRETATION AND RISK ASSESSMENT.

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Developmental toxicity studies are among the most complex and challenging in the field of toxicology. They entail multiple and interrelated endpoints and systems that are rapidly changing in characteristics and in their responses to toxic insults over time. These studies inherently generate large data sets. However, because of the de-

cline in relevant training programs, data from developmental toxicity studies are often managed or interpreted by individuals with limited backgrounds in these fields. Although published regulatory agency guidance is available, this course will extend such guidance by presenting in detail current study designs, procedures for study evaluation, and case studies. Lectures will cover fetal endpoints including mortality, growth, visceral exams and skeletal exams, as well as endpoints of maternal toxicity and their relationship to developmental toxicity. Developmental toxicity will be viewed in a broad context, including aspects of postnatal development and multigenerational effects. Proper analysis of developmental toxicity data requires specific statistical considerations, and these will be presented. Finally, putting together all the data from such studies for human risk assessment will be discussed. Considerations of design flexibility, endpoint sensitivities, and use of mode of action analysis and confirmatory studies will be included. Thus, the course will present key information required for understanding the biological and toxicological bases of findings from developmental toxicity studies and will provide guidance for analysis and interpretation.

**4**

## CLINICAL PATHOLOGY AS THE GRANDDADDY OF BIOMARKERS: CURRENT AND FUTURE DIRECTIONS.

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Research for novel biomarkers of toxicity continues to grow at a rapid pace. While biomarkers of tissue injury have a long history in the discipline of clinical pathology, the full value of these preclinical datasets are oftentimes under and/or over interpreted by scientists not extensively trained in this discipline. This basic course will emphasize current practices in clinical pathology utilized in drug discovery and preclinical safety studies and will also highlight advances in more novel biomarkers of toxicity. Basic interpretation of clinical pathology parameters will be presented in addition to factors to consider with respect to animal model, study design and the assays themselves. Examples of hepatic biomarkers and more recently identified biomarkers of toxicity will be highlighted. The applicability and pitfalls of utilizing reference ranges will be discussed, as will the approach in determining biological relevance of results versus statistical significance. Finally, regulatory perspectives on complete datasets and data interpretation will be addressed. This course is intended for the general toxicology community to improve their understanding of clinical pathology data and the role of clinical pathology in biomarker development.

**5**

## IMMUNOLOGY FOR TOXICOLOGISTS.

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The adaptive immune system that is found in mammals comprises a dedicated interacting system of tissues, cells and molecules that work in concert to provide specific immune responses and host resistance to pathogenic microorganisms and transformed cells. Specific immunity is supplemented by, and works in harmony with, the phylogenetically more ancient innate immune system. Immunotoxicology describes the study of adverse health effects that may result from the interaction of xenobiotics with one or more components of the immune system. Such health effects may take a variety of forms. These include frank immunotoxicity where there is functional impairment of the immune system. The concern here is that compromised immune function may translate into an increased susceptibility to infectious and/or malignant disease. A second potential consequence of the interaction of chemicals or proteins with the immune system is allergy; defined as the adverse health effects that may arise from the stimulation of a specific immune response. Allergic disease may take a variety of forms, those of greatest significance for toxicologists being skin sensitization and allergic contact dermatitis, allergic sensitization of the respiratory tract, food allergy and idiosyncratic allergic drug reactions. Finally, xenobiotics have been implicated in the induction or exacerbation of autoimmune reactions and autoimmune disease. This course will provide a grounding in fundamental and clinical aspects of immunology, and will describe the basic elements immunotoxicity, allergy and autoimmunity. The objective is deliver an accessible guide to the immune system and immunotoxicology for general toxicologists.

**6**

## EVALUATION OF CARDIAC DRUG TOXICITY IN PHARMACEUTICAL DISCOVERY AND DEVELOPMENT.

R. L. Phelps<sup>1</sup>, C. S. Louden<sup>2</sup>, Y. J. Kang<sup>3</sup>, and M. J. York<sup>4</sup>. <sup>1</sup>*Toxicology, Allergan, Irvine, CA, 2AstraZeneca Pharmaceuticals, Wilmington, DE, 3School of Medicine, University of Louisville, Louisville, KY and 4Preclinical Safety Sciences, GlaxoSmithKline, Hertfordshire, United Kingdom.*

The heart is an important potential target organ to evaluate in nonclinical and clinical studies during drug development as well as a therapeutic site of action for many cardiovascular diseases. There are examples in almost every therapeutic class of

drugs that produced unanticipated cardiotoxicity leading to market withdrawal or cessation of development. Toxicologists are an integral part of an interdisciplinary group, including physiologists, pharmacologists, pathologists, clinicians, and regulators, which assess cardiac safety. As such, toxicologists play a critical role in the screening of pharmaceutical agents for cardiotoxicity as well as in establishing an adequate margin of safety and working basis for monitoring therapeutic endpoints and clinical safety of trial participants. The goal of this continuing education course is to illustrate the integration of physiology, pharmacology, toxicology, and pathology of the heart addressing both recent scientific advances and practical knowledge in pharmaceutical company setting. This course will cover *in vitro* and *in vivo* models of drug-induced cardiac injury and recent advances in biomarkers of cardiac injury to improve the strategy for detection and nonclinical and clinical monitoring of drug-related cardiotoxicity. This CE course will provide current understanding of the physiology of the heart with a focus on normal and drug-induced disturbances in cardiac electrophysiology and cardiac function, as well as testing strategies for assessing potential cardioactive drug candidates prior to entry into clinical trials. It will also cover basic and advanced knowledge of pathology of the heart, including a review of cardiac pathological evaluation, and provide case examples of the integration of physiological and pathological parameters and risk assessment to humans. Biochemical, cellular and molecular mechanisms of cardiac toxicity, including recent work in experimental animal studies and novel approaches directed toward understanding mechanisms of drug-induced cardiac injury, cardiomyopathy and cardiac hypertrophy will be presented. Finally, biomarkers of drug-induced cardiac injury will be discussed. This will include a review of the findings of the Expert Working Group on Biomarkers of Drug-Induced Cardiac Toxicity, an in-depth look at serum troponins, and related work currently in progress under the ILSI-sponsored Subcommittee on the Development and Application of Biomarkers of Toxicity.

## 7A

### INTERNATIONAL HARMONIZATION OF TECHNICAL REQUIREMENTS FOR CONDUCTING NON-CLINICAL SAFETY STUDIES OF HUMAN PHARMACEUTICALS: GUIDELINES, CASE STUDIES, AND CHALLENGES.

R. Dixit<sup>1</sup>, S. Galloway<sup>2</sup>, M. Feuston<sup>3</sup> and D. Jacobson-Kram<sup>4</sup>. <sup>1</sup>Safety Assessment, Merck and Co., Inc., West Point, AL, <sup>2</sup>Safety Assessment, Merck and Co., Inc., West Point, PA, <sup>3</sup>Research Division, Sanofi-Synthelabo, Malvern, PA and <sup>4</sup>CDER, USFDA, Rockville, MD.

The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) was established in 1990 to standardize and harmonize technical requirements for the world-wide marketing approval of human pharmaceuticals. The six party ICH comprises the regulatory agencies and research-based pharmaceutical industrial organizations from three major geographical areas, the United States, the European Union and the Japan. The major goals of the ICH process are to minimize unique regional requirements, reduce the duplication of non-clinical toxicology and clinical testing requirements, and to accelerate the global development, registration and marketing of human pharmaceuticals in a cost-effective manner. Under Safety topics (non-clinical safety), 15 major guidelines have been harmonized and implemented through three major global regulatory agencies. These include guidelines on technical requirements for genotoxicity, toxicity, carcinogenicity, reproductive and development toxicity, biotechnology safety, toxicokinetics, safety pharmacology and joint safety and efficacy. This course is designed to provide a thorough understanding of the rationale behind ICH guidelines and the utility of the ICH guidelines in accelerating and global harmonization of safety evaluation of pharmaceuticals. The presentations will also highlight case studies with detailed examples, and experience in conducting non-clinical ICH safety studies. The presentations will also discuss the challenges, and problems encountered due to the differences in the interpretation, and the acceptance of the ICH Guidelines by the practicing regulatory organizations, reviewers and the traditional practices of specific geographical areas.

## 8

### MALE REPRODUCTIVE HAZARD IDENTIFICATION AND RISK ASSESSMENT IN PHARMACEUTICAL DEVELOPMENT (WHAT DO YOU DO NOW THAT YOU HAVE A SIGNAL?).

D. Creasy<sup>2</sup>, P. J. Wier<sup>1</sup> and K. Boekelheide<sup>3</sup>. <sup>1</sup>GlaxoSmithKline, King of Prussia, PA, <sup>2</sup>Huntingdon Life Sciences, East Millstone, NJ, and <sup>3</sup>Department of Pathology and Laboratory Medicine, Brown University, Providence, RI.

In the preclinical development of pharmaceuticals, there are numerous opportunities to observe effects on the male reproductive system. There are relevant endpoints in both routine repeated-dose toxicity studies and in the developmental and reproductive toxicity studies. In recent years methods for identification of male reproductive injury has expanded well beyond the traditional endpoints of fecundity in rodent fertility studies and routine microscopic examination of testes in repeated-dose and reproductive toxicity studies. There is an expectation, for instance, that

pathologists will examine testes with knowledge of and reference to the stages of the seminiferous epithelium. Assessment of number, motility and frequency of morphological abnormalities in epididymal sperm has become routine in rodent fertility studies. Recently, some emphasis has also been placed on identification of biomarkers for testicular injury. The next steps following identification of signal are ill defined. The purpose of this continuing education course is to address what types of signals are commonly observed and to present a rationale for interpreting these data with regard to risk assessment for volunteers and patients. The speakers in this course will place an emphasis on case studies and will provide their proposals for subsequent preclinical and/or clinical investigations.

## 9

### DEVELOPMENT AND INTERPRETATION OF TOXICOKINETIC DATA FOR RISK AND SAFETY ASSESSMENT.

J. C. Lipscomb<sup>1</sup>, J. G. Bessems<sup>2</sup>, R. M. Dixit<sup>3</sup> and H. A. Barton<sup>4</sup>. <sup>1</sup>NCEA, USEPA / ORD, Cincinnati, OH, <sup>2</sup>Food & Chemical Risk Analysis, TNO Chemistry, Zeist, Netherlands, <sup>3</sup>Department of Safety Assessment, Merck Research Laboratories, West Point, PA and <sup>4</sup>NHEERL, USEPA / ORD, Research Triangle Park, NC.

Drug development and environmental health risk assessment activities are based on knowledge of chemical disposition and tissue interactions, that may be separately considered as toxicokinetics (TK) and toxicodynamics (TD). Each activity is initiated with valuation of basic toxicity information, including characterizing effective doses and the dose-response relationship identifying critical organs, tissues and processes; examining metabolic characteristics and the toxic nature of metabolites. Each activity culminates with the assessment of TK and TD relative to the concentration/dose-response relationship. This basic continuing education course is intended for the general toxicologist desiring to increase the use of TK data to build compound dossiers, and to address the needs of scientists designing toxicity and pharmacokinetic studies, preclinical and clinical studies, and conducting safety and/or risk assessments. Four lectures will be presented; content will address the design, conduct and evaluation of studies to inform an understanding of chemical disposition and effectiveness in the biological system. Instruction will be given on the considerations of doses and concentrations used in whole-animal toxicity and ADME studies and in *in vitro* investigations; the biochemical basis of chemical metabolism and the considerations and assumptions necessary to interpret metabolism findings; best use of TK data to inform doses intended for use in TD studies; extrapolations of effective doses between and among species through the use of default and chemical-specific uncertainty/adjustment factors; available guidance on uncertainty factor derivation for human health risk assessment; overview of PBPK modeling and its application to extrapolations of dose, route and duration, between and among species; and the basis and results of choice of classical or PBPK modeling for drug development and risk assessment.

## 10

### PHOTOTOXICITY: CURRENT CONCEPTS, EXPERIMENTAL DESIGNS, AND REGULATORY EXPECTATIONS.

J. F. Nash<sup>1</sup>, J. Harbell<sup>4</sup>, P. D. Forbes<sup>3</sup>, J. Ferguson<sup>4</sup> and A. C. Jacobs<sup>5</sup>. <sup>1</sup>Procter & Gamble Co., Cincinnati, OH, <sup>2</sup>Institute for In Vitro Science, Inc., Gaithersburg, MD, <sup>3</sup>Argus Research-Charles River, Horsham, PA, <sup>4</sup>Nine Welles Hospital and Medical School, Dundee, United Kingdom and <sup>5</sup>USFDA/CDER, Rockville, MD.

Exposure to solar ultraviolet and visible radiation produces acute and chronic skin damage. Chemicals, including pharmaceutical agents, may exacerbate such effects following topical or systemic exposure. The aim of phototoxicological testing is to predict the likelihood of such events using various *in vivo* and *in vitro* models. The purpose of this course is to familiarize toxicologists with the basic concepts of phototoxicological testing. This is especially important in light of recent US and European regulatory guidances relating to photosafety testing. The program will describe the comparative anatomy, physiology, and basic photobiology of the skin, the basic principles and experimental designs used in phototoxicity testing in non-clinical species and people as well as *in vitro* models. The course will also address the most recent regulatory guideline from the US Food and Drug Administration regarding phototoxicity testing.

## 11

### ASSESSMENT OF RENAL INJURY *IN VIVO*: TRADITIONAL AND NOVEL BIOMARKERS.

S. G. Emeigh Hart<sup>1</sup>, A. Lavin<sup>2</sup>, D. I. Bounous<sup>3</sup>, J. T. MacGregor<sup>4</sup> and E. Harpur<sup>5</sup>. <sup>1</sup>SAUS, AstraZeneca Pharmaceuticals, Wilmington, DE, <sup>2</sup>ILSI Health and Environmental Sciences Institute (HESI), Washington, DC, <sup>3</sup>Bristol-Meyers Squibb Company, Laurenceville, NJ, <sup>4</sup>Toxicology Consulting Services, Arnold, MD and <sup>5</sup>Sanofi-Synthelabo, Media, PA.

Because the kidney is an important target organ of toxicity, there is a need for sensitive, specific and non-invasive assays that can be used to detect low-level, potentially reversible renal injury in both animal toxicology studies and the clinical set-

ting. Most of the available techniques do not allow identification of the segment(s) of the kidney affected by a toxicant. Better understanding of the pathophysiology of nephrotoxic processes, combined with information arising from genomic, proteomic and molecular biologic assessment of the effects of nephrotoxic chemicals has led to the identification of several promising new candidates that might serve as sensitive, segment-selective biomarkers of nephrotoxicity that can be applied readily in both the preclinical and clinical settings with adequate testing and validation. This basic CE course will provide an overview of renal structure and function, discuss the appropriate use and limitations of traditional clinical pathology assessment of renal injury, and describe the processes necessary for appropriate validation and subsequent regulatory acceptance of a novel biomarker. The final two speakers will describe the novel biomarker candidates under consideration by the Nephrotoxicity Working Group of the ILSI-HESI Technical Committee on the Development and Application of Biomarkers of Toxicity. The first of these will provide an overview of novel biomarkers for proximal tubular injury while the second will discuss the pathophysiology of renal papillary necrosis and how it relates to the identification of novel biomarkers of papillary injury.

## 12 EVALUATION OF CARDIAC DRUG TOXICITY IN PHARMACEUTICAL DISCOVERY AND DEVELOPMENT.

B. G. Short. *Toxicology, Allergan, Irvine, CA.*

The heart is an important potential target organ to evaluate in nonclinical and clinical studies during drug development as well as a therapeutic site of action for many cardiovascular diseases. Toxicologists are an integral part of an interdisciplinary group that evaluates cardiac safety, including screening of drugs for cardiotoxicity as well as in establishing an adequate margin of safety and working basis for monitoring therapeutic endpoints and clinical safety of trial participants. The goal of this continuing education course is to illustrate the integration of physiology, pharmacology, toxicology, and pathology of the heart addressing both recent scientific advances and practical knowledge in pharmaceutical company setting. This course will cover *in vitro* and *in vivo* models of drug-induced cardiac injury and recent advances in biomarkers of cardiac injury to improve the strategy for detection and nonclinical and clinical monitoring of drug-related cardiotoxicity. This CE course will provide current understanding of the physiology of the heart with a focus on normal and drug-induced disturbances in cardiac electrophysiology and cardiac function, as well as testing strategies for assessing potential cardioactive drug candidates prior to entry into clinical trials. It will also cover basic and advanced knowledge of pathology of the heart, including a review of cardiac pathological evaluation, and provide case examples of the integration of physiological and pathological parameters and risk assessment to humans. Biochemical, cellular and molecular mechanisms of cardiac toxicity, including recent experimental work and novel approaches directed toward understanding mechanisms of drug-induced cardiac injury, cardiomyopathy and cardiac hypertrophy will be presented. Finally, biomarkers of drug-induced cardiac injury will be discussed. This will include a review of the findings of the Expert Working Group on Biomarkers of Drug-Induced Cardiac Toxicity, an in-depth look at serum troponins, and related work currently in progress under the ILSI-sponsored Subcommittee on the Development and Application of Biomarkers of Toxicity.

## 7B INTERNATIONAL HARMONIZATION OF TECHNICAL REQUIREMENTS FOR CONDUCTING NON-CLINICAL SAFETY STUDIES OF HUMAN PHARMACEUTICALS: GUIDELINES, CASE STUDIES, AND CHALLENGES.

R. Dixit<sup>1</sup>, S. Galloway<sup>2</sup>, M. Feuston<sup>3</sup> and D. Jacobson-Kram<sup>4</sup>. <sup>1</sup>*Safety Assessment, Merck and Co., Inc., West Point, AL*, <sup>2</sup>*Safety Assessment, Merck and Co., Inc., West Point, PA*, <sup>3</sup>*Research Division, Sanofi-Synthelabo, Malvern, PA* and <sup>4</sup>*CDE, USFDA, Rockville, MD.*

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## 13 THE FUTURE OF MOLECULAR GENETIC THERAPEUTICS.

D. Monteith and V. Reddy. *Eli Lilly and Company, Greenfield, IN.*

During the last decade the decoding of the human genome has provided exciting possibilities for the treatment of human disease. The promise of gene therapy and antisense have stirred the imagination to the potential of redirecting abnormal cellular processes. These therapies provide potential advantages in specificity to gene targets and selectivity for protein intervention in some disease targets that are often considered 'undruggable' with traditional chemotherapies. To date, these technologies have provided interesting technologies as research tools to gain perspective with respect to effects of various targets; in particular antisense strategies using single-strand and siRNA provide tools to validate molecular targets in disease pathways. These antisense approaches utilize different mechanisms (i.e., RISK complex and RNase H) to reduce a target protein in a manner not achievable with a small molecule approach. Gene therapy has posed the potential to treat and possibly cure disease by replacing lost function with the insertion of a normal gene. Currently, there are numerous antisense and gene therapy clinical candidates in development by corporate sponsors as well as academic centers and investigators. The selection of disease candidates and targets is complex. The administration, delivery, cell permeability, and pharmacokinetic and pharmacodynamic attributes have played a critical role in the development of these therapies. This symposium will cover the biologic and safety hurdles these therapies have faced and the solutions that have been developed to evaluate these molecules in patients. The symposium will consist of an expert panel of presenters involved in the science and development issues surrounding molecular genetic therapies.

## 14 GENE THERAPY: A SIMPLE CONCEPT WITH COMPLEX CHALLENGES.

R. M. Lyons. *Self-Employed, Gaithersburg, MD.*

Gene therapy is a conceptually simple and straightforward approach to the treatment and possible cure of diseases with a defined genetic basis. The reality is more complex and presents significant challenges, including appropriate disease selection. Genetic diseases are logical candidates and many of the first gene therapy clinical trials focused on monogenetic disorders. The majority of current gene therapy clinical trials are for cancer, due in part to an increased appreciation of the genetic basis of cancer, a higher tolerance for risk when no alternative treatment exists, and a more attractive commercial opportunity. The gene transfer systems being investigated include viral vectors, plasmids, liposomes, and synthetic complexes. The most common gene transfer systems use human or animal viruses engineered to take advantage of specific biological properties or viral life-cycle features while minimizing pathogenicity. One of the more significant challenges continues to be efficient and specific gene transfer to target cells and tissues as most viral vectors have broad cell and tissue tropism. Viral vectors targeted to tissue-specific cellular receptors are frequently less robust or require multiple components, further complicating product development. The level and duration of gene expression is not controlled in current gene transfer systems and is likely to require innovative models and study designs to assess the potential for both acute and chronic effects. Integrating viral vectors may offer a curative therapy, but are also associated with the potential for insertional mutagenesis. Preclinical investigations have been unsuccessful in assessing the magnitude of this risk. In addition, recent clinical cases of vector-related insertional mutagenesis suggest that the risk may be disease specific, further complicating the development of relevant preclinical models for informative risk assessment. In light of these complex biological and technical issues as well as the large number of distinct clinical approaches being taken, the development of gene therapy candidates will continue to be science based and product specific.

## 15 DEVELOPMENT OF SHORT INTERFERING RNAs (SIRNAS) AS POTENTIAL THERAPEUTICS.

P. A. Pavco. *Development, Sirna Therapeutics, Inc., Boulder, CO. Sponsor: J. Lockridge.*

The recent discovery of the RNA Interference (RNAi) pathway has resulted in the use of short interfering RNAs (siRNAs) to direct potent and specific post-transcriptional gene silencing. Because siRNA can be designed to target virtually any disease-related mRNA, the potential use of siRNA as a therapeutic agent is quite broad.

However, the application of siRNA technology *in vivo* is not straightforward. Although extremely useful as a laboratory tool, particularly in cell culture, the development of siRNA as a therapeutic must also address the challenges of siRNA stability *in vivo*, delivery to the appropriate tissue and chemical synthesis of therapeutic amounts. The addition of stabilizing modifications to the siRNA greatly improves its pharmacokinetics and will be required for most therapeutic applications. Additional modifications and/or conjugates are possible that target the siRNA to various tissues. Methods for efficient and cost-effective production of siRNA to support clinical use have been developed. Recent progress with respect to these challenges will be reviewed.

## 16 ANTISENSE THERAPEUTICS: PROGRESS IN THE DEVELOPMENT OF A NOVEL THERAPEUTIC PLATFORM.

**S. P. Henry and A. A. Levin.** *Toxicology, Isis Pharmaceuticals, Carlsbad, CA.*

Dedicated research and development of antisense inhibitors for therapeutic use is now more than a decade old. The application of antisense inhibitors of gene expression while simple in concept and potentially broadly enabling, still faces the same challenges as any pharmacologic agent. In context of therapeutic discovery these challenges include selection of potent inhibitors, pharmacologic specificity in early *in vitro* *in vivo* pharmacology models, relevance of gene target, tissue distribution and cell uptake, target organ toxicity and therapeutic index for specific indications. Knowing the properties of compounds in a given platform such as antisense inhibitors should allow for these challenges to be addressed with relative high efficiency. This presentation will highlight the potential contributions of this technology, particularly in terms of regulating the expression of genes that are not easily inhibited with traditional approaches. Examples of disease studied include treatment of viral disease, cancer, inflammation and metabolic disease. Antisense oligonucleotides have also been studied using multiple routes of administration, including oral. The toxicity studies performed cover the full spectrum of regulatory safety studies including chronic administration, reproductive toxicity and genetic toxicity studies. The study design aspects unique to antisense oligonucleotides will be discussed in the context of general preclinical development strategy. The toxicities common to this class of compound will be described in the context of toxicokinetic and toxicodynamic relationships. The impact of these changes on safety assessment depends greatly on the intended indication, route of administration, and duration of treatment. A prospective look at potential for near-term and long-term applications, along with future directions, will be provided.

## 17 CLINICAL CONSIDERATIONS IN THE DEVELOPMENT OF MOLECULAR THERAPIES FOR CANCER.

**M. F. Burgess.** *Lilly Research Centre, Windlesham, Surrey, United Kingdom.* Sponsor: **D. Monteith.**

The exploration and exploitation of differences between the somatic and malignant cell genome has provided much promise for the delivery of new anticancer drugs to the clinic. A wide variety of therapeutic manipulations from small molecules to gene therapy have been encouraging preclinically, only to fail in the clinic. One of the most promising technologies which can be applied to almost any target in antisense therapy. This therapeutic platform highlights many of the difficulties in the translation of a preclinical tool into a clinically useful drug. Consideration needs to be given not only to concerns of the target, but also to concerns associated with drugability. Issues such as the distribution of antisense to the target tissue to achieve therapeutic concentrations have a fundamental effect on study design. The long half-life of these molecules combined with a hierarchy of organ penetration may compromise the ability to effect the target in the absence of toxicity. Additionally, toxicities may only become apparent after prolonged repeated administration leading to difficulties in assigning an MTD. Since the dose response curve is steep reduction in dose as a consequence of toxicity may result in subtherapeutic concentrations in the target and loss of clinical benefit. Implied within this is an adequate assessment of the pharmacodynamic effect as it pertains to malignant and normal cells. This may vary from target to target, but in general some estimate of the magnitude and duration of the effect of treatment is required. Ideally, this should be accompanied by local evaluation of ASO concentration. The penetration of ASO into tumor relative to surrounding normal tissue is largely unknown and makes prediction of therapeutic dose levels complicated. Scheduling of combination chemotherapy needs to be carefully evaluated in the face of the target knockdown data to optimally design combinations to achieve the greatest therapeutic benefit. All of the above needs to be considered within the context of acceptability to the patient which will be judged on the perception of risk vs benefit.

## 18 OVERVIEW INHALATION EXPOSURE AND SYSTEMIC IMMUNOTOXICITY: MECHANISMS LINKING THE LUNG AND IMMUNE SYSTEM.

**M. Selgrade**<sup>1</sup> and **J. Zelikoff**<sup>2</sup>. <sup>1</sup>*NHEERL, USEPA, Research Triangle Park, NC* and <sup>2</sup>*Department of Environmental Medicine, New York University School of Medicine, Tuxedo, NY.*

Although toxicity to the lung and cardiovascular system are the most frequently studied targets following inhalation exposure, suppression of systemic immune responses has been observed following exposure to a number of diverse compounds. Defects in a variety of immune effector mechanisms have been observed. Frequently, these effects are not a direct result of exposure of immune targets to the chemical or its metabolites, but involve instead the production of mediators in the lung that circulate widely and/or interactions with the nervous system. Often these exposures are to complex mixtures and the active components as well as pharmacokinetics are uncertain. Many of these issues have been considered in recent studies of JP-8 Jet fuel, Sarin, Tobacco smoke, and different gasoline formulations, all of which suppress systemic immune response and have the potential to impact susceptibility to infectious disease and tumor challenge in rodents. These studies have implications for public health, homeland security, industrial hygiene, and indoor environments. This symposium is a sequel to a 2004 symposium Modulation of Host Defenses by Ambient and Source Particulate Air pollutants which focused on pulmonary immune responses and infections

## 19 INTRODUCTION: INHALATION EXPOSURE AND SYSTEMIC IMMUNOTOXICITY: MECHANISMS LINKING THE LUNG AND IMMUNE SYSTEM.

**M. Selgrade.** *NHEERL, USEPA, Research Triangle Park, NC.*

Concerns regarding inhaled compounds, immune suppression and increased risk of disease have focused primarily on suppression of local immune responses in the lung and susceptibility to respiratory infections. However, studies also have shown that both gaseous ( $O_3$ ,  $NO_2$ ) and particulate (diesel, Cd-coated fly ash, cigarette smoke) air pollutants, as well as soluble metals ( $NiCl_2$ ,  $CdCl_2$ ) and a pesticide (carbaryl) administered by inhalation suppress systemic immune responses. These studies have most often assessed the antibody response to sheep red blood cells, lymphoproliferative responses to non-specific mitogens, and/or peripheral blood lymphocyte phenotypes. Although limited data are available, enhanced susceptibility to systemic infections has also been observed. Systemic immune suppression following inhalation exposure has been reported in both rodent and human studies. Results often varied depending on the exposure protocol. Because these same chemicals administered via other routes of exposure did not always suppress immune responses, mechanisms linking the lung and systemic immune system have been proposed. Inflammatory responses are thought to be involved. Potential mechanisms include production of long-lived free radicals, production of immunosuppressive mediators such as prostaglandin  $E_2$  and interleukin-10, and a shift in the balance of T helper (Th1) and Th2 lymphocytes in favor of Th2 responses. This latter possibility is attractive, because many of these same agents have adjuvant effects on the induction of allergic sensitization or are themselves allergenic. Also, as described in later talks, mechanisms have been suggested that involve effects on the nervous and/or endocrine system. Whereas reports of systemic immune suppression following inhalation exposure date back 30 years, the papers presented at this symposium represent some of the first studies to systematically assess the mechanisms (immunologic and metabolic) underlying these effects. (This abstract does not reflect EPA policy).

## 20 IMMUNOTOXICITY OF AEROSOLIZED JP-8 JET FUEL EXPOSURE AND ITS PREVENTION BY SUBSTANCE P.

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Exposure of C57Bl/6 mice to JP-8 jet fuel results in immunotoxicity. Short-term, low concentration inhalation of JP-8 suppresses natural killer (NK), cytotoxic T lymphocyte (CTL) and T-helper cell function, and increases susceptibility to tumor and viral challenges. Changes in immune function occurred within 1-hour post-JP-8 exposure and were cumulative, becoming maximal by 4 days post-exposure. Immune recovery to baseline levels did not occur for more than 30 days after exposure ended. JP-8 exposure at the time of tumor induction increased lung tumors 548%; JP-8 exposure prior to tumor induction increased lung tumors 863%. Morbidity and mortality were increased in mice exposed to JP8 for 7 days, infected with a sublethal dose of influenza virus, and sacrificed 1 wk later. There were fewer immune cells in the spleen and lymph nodes, indicating that the immune response to the virus had been suppressed. Immunophenotyping also indicated that T cell numbers and immune cell viability were decreased. *In utero* exposure to JP-8 in-

duced immune dysfunction in offspring that persisted for at least 8 wks after birth, was most pronounced in males, and directly correlated with the degree of immune dysfunction found in the exposed mothers. JP-8 exposure induced rapid increases in serum PGE2 and IL-10 levels. Treatment with a Cox-2 enzyme inhibitor significantly ablated PGE2 levels, but only partially restored immune function. Treatment of mice with substance P (SP) was observed to restore immune function whereas inhibitors of SP function exacerbated immune dysfunction. Immediately after human occupational exposures to JP-8, decreased total leukocyte numbers, increased neutrophils and eosinophils, and increased plasma PGE2 levels were observed. Also, there appeared to be a susceptible subpopulation within the group as a whole. In order to understand the mechanisms of JP-8's effects on the immune system, experiments are underway to identify the cells *in vivo* that secrete immuno-suppressive cytokines and are targets of jet fuel-induced apoptosis.

## 21 IMMUNOTOXICITY OF SARIN AND OTHER CHOLINERGIC AGENTS.

M. Sopori, R. Kalra, R. Langley, S. Razani-Boroujerdi, N. Mishra and R. Henderson. *Immunology, Lovelace Respiratory Research Institute, Albuquerque, NM*.

Chronic inhalation of subclinical doses of sarin (0.2 and 0.4 mg/m<sup>3</sup>, 1 h/day, for 5 days) suppresses the antibody-forming cell response to a T cell-dependent antigen (sheep red blood cells) and impairs T cell function (proliferative responses to Concanavalin A and anti-T cell receptor antibody). Using various cholinergic agents, we observed further that while the immunosuppressive effects of those agents that crossed the blood-brain-barrier (e.g., sarin, physostigmine) were apparent through inhalation or subcutaneous administration, cholinergic agents that did not cross the blood-brain-barrier (e.g., pyridostigmine bromide, edrophonium) required intracerebroventricular administration to induce the immunosuppression. Thus, the immunotoxicity of cholinergic compounds appears to be mediated through the central nervous system. Interestingly, all cholinergic agents significantly reduced serum corticosterone levels, suggesting that the immunosuppression induced by cholinergic compounds did not result from increased glucocorticoid production through activation of the hypothalamus-pituitary-adrenal axis. However, pretreatment of rats with the ganglionic agent chlorisondamine attenuated the sarin and pyridostigmine bromide-induced immunosuppression and partially blocked the drop in serum corticosterone levels. Chronic cholinergic stimulation increased the interleukin (IL)-1 $\beta$  level in the brain but not in the spleen, and chronic intracerebroventricular but not subcutaneous administration of IL-1 $\beta$  suppressed the antibody-forming cell response. Moreover, IL-1 $\beta$  activated the Src-like protein tyrosine kinase Fyn, and this activation was blocked by pretreatment with chlorisondamine. Thus, IL-1 $\beta$  may be critical in cholinergic immunotoxicity and neuroimmune communication. These results suggest that the immunotoxicity of inhaled cholinergic agents is mediated centrally through the autonomic nervous system. Moreover, serum corticosterone levels might be a biomarker for cholinergic toxicity.

## 22 PRENATAL EXPOSURE OF MICE TO CIGARETTE SMOKE ALTERS TUMOR SURVEILLANCE MECHANISM(S) IN THE JUVENILE AND ADULT OFFSPRING.

J. T. Zelikoff and S. P. Ng. *Nelson Institute of Environmental Medicine, School of Medicine, New York University, Tuxedo, NY*.

Over one million infants in America are born each year after exposure *in utero* from active or passive maternal smoke exposure. Since accumulating epidemiologic evidence suggests that prenatal exposure to intact cigarette smoke increases the incidence of cancer in the offspring, it was hypothesized that inhalation exposure of pregnant B6C3F1 mice to mainstream cigarette smoke (MCS) decreases offspring resistance to tumor challenge due to alterations in surveillance mechanisms critical for recognition/destruction of cancers. This study sought to: ascertain whether exposure of pregnant dams to a low-dose of MCS alters tumor development in the offspring; and, assess the degree to which exposure of dams to MCS during gestation modulates tumor surveillance mechanisms such as natural killer cell and cytotoxic T-lymphocyte (CTL) activity. Even at this relatively low level (equivalent to that measured in a home in which two packs of cigarettes were smoked), exposure to MCS from gestational d 4 to parturition reduced litter size and caused a significant shift in gender ratios (compared to those for controls). Prenatally exposed male and female pups challenged at 5 wk of age with neoplastic cells demonstrated a 2- and 4-fold increase in tumor incidence, respectively (relative to age- and gender-matched air exposed offspring); increased tumor incidence was also observed in prenatally exposed 10-wk-old offspring, but only for the males. Effects of smoke on CTL activity may in part be responsible for the increase in tumor incidence observed in the male offspring; CTL activity in the female pups were unaffected by *in utero* MCS exposure. Results demonstrate that inhalation exposure of pregnant

mice to a low dose of MCS increases susceptibility of the adult offspring to nascent tumors. Taken together, these studies provide biological plausibility for the notion that children of mothers who smoke have a greater risk of developing cancer later in life. This research was supported by Philip Morris USA Inc. and Philip Morris International.

## 23 COMPARATIVE INHALATION IMMUNOTOXICITY OF GASOLINE AND GASOLINE PLUS OXYGENATE ADDITIVES IN RATS.

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Comparative immunotoxicity testing was conducted on evaporative emissions of gasoline alone, and gasoline plus ether or alcohol oxygenates in the Sprague-Dawley rat. The inhalation exposures simulate, at much higher exposures and durations, human exposures during self-serve refueling of automobiles. Seven vapor condensates of gasoline alone, or gasoline plus an ether or alcohol oxygenate were evaluated for effects on the humoral component of the immune system using antibody-forming cell (AFC) response to the T-dependent antigen, sheep erythrocyte. Female Sprague-Dawley rats were exposed to the test agents (2000, 10000 and 20000 mg/m<sup>3</sup>) by inhalation for 4 weeks (6 hrs/day, 5 days/week). Vapor condensates of gasoline alone, gasoline plus MTBE, tert-butyl alcohol (TBA), or tertiary amyl methyl ether (TAME) did not affect humoral response in the plaque assay. Vapor condensates of gasoline plus ethanol or diisopropyl ether (DIPE) did result in statistically significant decreases in the AFC response when evaluated as either AFC/106 spleen cells or AFC/spleen at the highest dose level. Gasoline plus ethyl tertiary butyl ether (ETBE) produced a statistically significant dose-dependent decrease in AFC response at the mid- and high-doses. Recently, there has been speculation in the literature that some immune effects are related, or secondary to concurrent neurotoxicity. Comparative neurotoxicity studies (functional observational battery and motor activity) at the same dose levels with these three test materials were negative, suggesting no relationship between immune and nervous system effects. The neat oxygenates (MTBE, TBA, TAME, ethanol, ETBE, DIPE) tested in these gasoline blends share a number of common metabolites (isopropyl alcohol; acetaldehyde, acetate). Although these studies were not definitive, the negative effects for gasoline alone, gasoline/MTBE, gasoline/TBA and gasoline/TAME suggest that neither gasoline components nor the common metabolites of the neat oxygenates are responsible for the positive effects observed for these mixtures.

## 24 THE DEVELOPMENT AND APPLICATION OF BIOMARKERS OF TOXICITY.

J. Dean<sup>2</sup> and A. L. Lavin<sup>1</sup>. <sup>1</sup>*ILSI Health and Environmental Sciences Institute, Washington, DC* and <sup>2</sup>*Sanofi-Synthelabo, Inc., Malvern, PA*.

A biomarker, or biological marker, is defined as a characteristic that is measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention (FDA/NIH Definitions Working Group, 1999). Such biomarkers have long been used in preclinical safety studies during drug development. Nevertheless, many traditional biomarkers lack the sensitivity and specificity required to extrapolate results from preclinical studies to human clinical outcomes. As a result, interest in the discovery and validation of new bridging biomarkers of toxicity has expanded rapidly in recent years. This increase is due both to the advent of new “-omic” technologies, as well as to a shift in focus within the pharmaceutical companies and regulatory agencies towards finding safety biomarkers that can be of greater use in the drug discovery and safety assessment processes. These drivers have contributed to formation of the Health and Environmental Sciences Institute (HESI) Biomarkers Technical Committee, which is conducting a collaborative research program to evaluate possible new biomarkers for use in preclinical drug development. Biomarkers being explored by the committee for use in preclinical safety studies include serum cardiac troponins, a panel of nephrotoxicity biomarkers (the GSTs, PAP-1, KIM-1, and clusterin), and inhibin B as a biomarker of testicular toxicity. The use of such biomarkers will allow for integrated mechanistic and hypothesis-building studies conducted in the laboratory to be confirmed in man and vice versa.

## 25 NEW BRIDGING BIOMARKERS FOR SAFETY ASSESSMENT.

J. T. MacGregor. *Toxicology Consulting Services, Arnold, MD*.

Perhaps the greatest single limitation of current toxicological practice is the uncertainty associated with extrapolation from laboratory models to human outcomes. The dramatic advances in science and technology during the past two decades have

created an unprecedented opportunity to reduce or eliminate this uncertainty through the introduction of new "bridging biomarkers"—biomarkers of identical or analogous processes in laboratory models and in human subjects that can be measured directly in both cases. Such new markers include an extended set of biomarkers of cellular function and integrity (the traditional biomarkers used in safety evaluation) that are applicable to a broader range of cell and tissue targets, but are more specific to the individual targets. An increased understanding of the functional molecular systems of cells and their responses to cell and tissue damage offers the potential for new types of markers, such as those inducible by specific types of molecular damage and those involved in the signaling and cell activation pathways of cellular host-defense systems. Additionally, the ability to characterize molecular variations (polymorphisms) that convey susceptibility to toxicity or limit the efficacy of targeted therapeutics promises to allow greatly improved assessment of individual risks and benefits as well as improved laboratory models that contain molecular targets similar to those in humans. "-Omics" technologies promise to allow simultaneous measurement of very large numbers of such biomarkers in microformats requiring only microliter quantities of sample. This should lead to a shift toward quantitative risk-based regulatory judgments, integrated toxicity/efficacy toxicity assessments, and an improved mechanistic understanding of genetic, cell, and tissue damage. The use of biomarkers in current regulatory practice, the potential impact of such new approaches, and practical considerations for the introduction of new bridging biomarkers into regulatory practice will be discussed.

## 26 SERUM CARDIAC TROPONINS AS BIOMARKERS OF DRUG-INDUCED CARDIOTOXICITY.

M. J. York. *Pathology, GlaxoSmithKline, Ware, Hertfordshire, United Kingdom.* Sponsor: A. Lavin.

The cardiac troponins (cTnI and CtnT) are well established as the clinical gold standard for the detection of acute myocardial injury and their utility is well documented. In comparison, the usefulness of cardiac troponins in laboratory animal species as markers of cardiomyocyte damage is less comprehensive. A recent review of serum troponins (cTn) as biomarkers of drug-induced cardiac toxicity was conducted by the FDA Expert Working Group (EWG). The output from this group formed a proposal made to the ILSI HESI Biomarkers Technical Committee for a multi-laboratory approach to further progress the analytical and biological validation of cTn in laboratory animal species. The Troponins Working Group, comprising individuals from pharma, USFDA, academia and ILSI, was established and this group confirmed the critical data needs identified by the EWG to further assess the preclinical utility of the cardiac troponins: 1. Conduct further analytical and biological validation of selected cTnI assays, establishing degree of immunoreactivity and imprecision 2. Examine the impact of species-specific calibration on cTnI assays in rat and dog 3. Characterise the threshold and release of cTn response in rat, dog and monkey, thereby establishing the diagnostic window following drug-induced acute and chronic cardiomyocyte injury. 4. Examine the correlation of cardiac histopathology with timing and proportionality of troponin release 5. Examine cTn release in laboratory animal species An update of the progress of the Troponins Working Group in addressing these critical data needs will be presented, including the results of a recent analytical validation study and the future plans to further investigate the application of cTn assays in the detection of drug-induced cardiotoxicity.

## 27 INHIBIN B AS A POTENTIAL BIOMARKER OF TESTICULAR TOXICITY.

J. Stewart. *Safety Assessment, AstraZeneca Pharmaceuticals, Macclesfield, Cheshire, United Kingdom.* Sponsor: A. Lavin.

Inhibin B is a glycoprotein produced by the Sertoli cell which regulates FSH release in a negative feedback loop. In all species studied, circulating inhibin B is a marker of spermatogenesis where severe disturbances in spermatogenesis are associated with detectable decreases in circulating inhibin B. It is being increasingly used in the assessment of infertile males and in population studies evaluating reproductive function where it is proving a useful adjunct to more traditional biomarkers such as serum FSH and semen analysis. Inhibin B has a unique place among testicular biomarkers in that it is of Sertoli cell origin and therefore permits a more direct assessment of the intratubular environment. It could permit linkage between animal studies and monitoring of testicular function in clinical trials. An ILSI HESI Biomarkers sponsored project will evaluate the suitability and limitations of plasma inhibin B as a testicular toxicity biomarker. Although inhibin B can detect significant effects on spermatogenesis, the utility of inhibin B to detect more modest testicular dysfunction using experimental designs typical of a toxicity study is still unproven. Fortunately, the commercially available human inhibin B ELISA kits can detect rat inhibin B. Phase 1 of the HESI project will assess the ELISA kit performance against rat plasma inhibin B and will determine whether the interanimal variability in control animals is compatible with future detection of compound induced

effects. Each laboratory will then undertake experiments with one of seven selected testicular toxicants with aim of generating testicular lesions of variable severity and type. The histopathological lesions in the testes will be graded and compared to the circulating inhibin B concentrations. The panel of reference testicular toxicants will be chosen to have different primary target cells. The overall strategy to facilitate the adoption of inhibin B as a biomarker in regulatory toxicity studies will be described alongside the results of the first phase of the validation study and the choice of reference toxicants.

## 28 BIOMARKERS OF NEPHROTOXICITY.

S. Beushausen<sup>1</sup> and A. Lavin<sup>2</sup>. <sup>1</sup>WWSS, Pfizer, Inc., St. Louis, MO and <sup>2</sup>Health and Environmental Sciences Institute, Washington, DC.

Several classic biomarkers of nephrotoxicity have been developed for clinical use to report or monitor renal damage. Classic serum markers include BUN and creatinine. Urine biomarkers are classified into a number of categories including: urinary proteins (e.g. albumin, transferrin, and IgG), urinary enzymes (e.g. N-acetyl glucosaminidase,  $\gamma$ -glutamyl transpeptidase,  $\beta$ -galactosidase), cytotoxicity markers (tubular antigens, laminin fragments), and biochemical markers (fibronectin, kallikrein activity, scialic acid and glycosaminoglycans). Classical markers are limited by lack of sensitivity, indication of general rather than site-specific damage, and intra/inter variability between individuals. Consequently, these markers have limited utility as reporters of frank tissue damage. Pharmaceutical, medical, and academic professionals have agreed that there is an urgent need to develop safety biomarkers of nephrotoxicity that can predict toxicity before encountering irreversible tissue damage or that can help monitor low-grade compound-induced nephrotoxicity in the clinic. Development of pre-clinical safety biomarkers of nephrotoxicity would also be valuable towards helping curb the high cost of drug development and aid in the development of safer compounds. The ILSI-HESI Nephrotoxicity Working Group has examined a number of recently developed nephrotoxicity biomarkers for increased sensitivity and the ability to predict site-specific renal tissue damage. For example, acute and chronic compound-induced toxicity studies have been designed to examine levels of the GST isoforms  $\alpha$  and  $\mu$  to discriminate between proximal and distal tubule damage, respectively. A novel PAP-1 antibody was evaluated for sensitivity and predictive capability for renal papillary toxicity. The development and application of two novel protein biomarker assays include a clusterin assay to determine whether clusterin has utility as a sensitive biomarker of general nephrotoxicity and a KIM-1 assay as a sensitive and reliable biomarker of proximal tubule damage. A discussion around the utility of these biomarkers will be addressed in the presentation.

## 29 REGULATORY ASPECTS OF NEW BIOMARKERS OF TOXICITY.

F. Frueh. *FDA/CDER, Rockville, MD.* Sponsor: A. Lavin.

Pharmacogenomics introduced a new era in drug development with implications reaching from the discovery of novel chemical entities to the far end of individualized drug therapy. One characteristic that coalesces to the entire process is the availability of predictive biomarkers: from the discovery of genetic markers as novel targets for drug therapy to the use of e.g. genetic variations for the prediction of safety and/or efficacy, the adept use of proper biomarkers is of critical importance. In November of 2003, the FDA has released a draft "Guidance for Industry: Pharmacogenomic Data Submissions". The guidance includes three trees which delineate the decision process of whether a particular genomic data set is required to be submitted under current regulations or can be submitted on a voluntary basis - a new paradigm for FDA-Industry cooperation to break the knot that has hampered the use of pharmacogenomic data in the regulatory decision process. Each data set is evaluated according to the level of validation of its biomarkers and for what, if any, regulatory decision the biomarkers are used. For example, a valid known biomarker constitutes a biomarker that has been widely accepted in the scientific community to be a relevant indicator for establishing a particular safety or efficacy profile. Information based on the use of such a biomarker needs to be submitted to the FDA. Another example is the use of biomarkers in an exploratory nature, for example the use of a DNA array to identify genes associated with the cellular response upon disturbing the system by drug treatment. Without hard scientific evidence that such a data set has implications beyond its use as a discovery tool and, if not used for e.g. patient stratification for a clinical trial, no regulatory action will be taken. However, because the field is moving rapidly and new evidence for the validity of certain biomarkers is generated almost daily, the FDA encourages the submission of such data on a voluntary basis to ensure future scientifically sound reviews of investigational or new drug applications by the Agency. We have received several voluntary genomic data submissions. Examples will be presented.

**30**

## DOSE-ADDITION OF MIXTURES: WHERE ARE WE GOING WITH THE SCIENCE?

**R. Conolly.** CIIT Centers for Health Research, Research Triangle Park, NC.

A growing challenge in human risk assessment is development of methods to evaluate risks posed by low-level exposures to environmentally relevant mixtures. Development of Relative Potency factors (RPFs), which assume fundamental dose additivity, are currently used as the primary method for evaluating the risks of mixtures of compounds acting through similar modes of action. Examples of these are the RPF schemes for dioxins (Toxic Equivalency Factors) and organophosphate and carbamate pesticides. The aim of the workshop is provide a broad overview and discussion of the state of the science for the use and justification of relative potency factors in environmental and human health risk assessment. The workshop will include presentations describing specific examples of how RPFs are currently applied to cancer assessments of dioxin-like compounds and to non-cancer neurotoxicity endpoints such as exemplified by organophosphate and carbamate pesticides. The recently completed National Toxicology Program chronic rodent studies testing critical RPF hypotheses will be discussed as a specific example of the potential impact of experimental findings on RPF assumptions. In addition, the workshop will discuss the central importance of dosimetry and exposure assessments to evaluation of the RPF approach, particularly addressing implications for understanding potential health risks associated with low-level exposures to chemical mixtures. The Workshop presentations will be designed to engender both Panel and Audience discussion focused on defining the scientific justification and limitations of the use of RPF approaches, and identifying future research needs that will improve risk assessment tools used to assess health impacts associated with low-level exposures to a broad spectrum of environmental chemicals.

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## CAN MODE OF ACTION PREDICT MIXTURE TOXICITY FOR RISK ASSESSMENT?

**C. J. Borger<sup>1,2</sup>.** <sup>1</sup>Applied Pharmacology and Toxicology, Inc., Gainesville, FL and <sup>2</sup>Physiological Sciences, University of FL College of Veterinary Medicine, Gainesville, FL.

Recent regulatory guidance for mixture risk assessments and for regulating pesticide chemicals recommend using information about the mode or mechanism of action of individual chemicals to predict dose response characteristics of mixtures. Dose addition is assumed for mixtures of chemicals that have similar mechanisms, and response addition for those with dissimilar mechanisms. Three different sets of criteria have been formulated to guide the selection of an appropriate data set for characterizing a chemical mode of action, but the sufficiency of those criteria to predict dose addition for a mixture has not been validated experimentally. Several examples from the pharmacological and toxicological literature challenge the premise that dose response characteristics of a mixture can be predicted from the modes of action of its components. Detoxification pathways may need to be understood before dose addition in the observable effect range can be extrapolated to mixture concentrations below the no observable effect levels of the mixture components. Because elucidating discreet mechanisms of action may be possible only for chemicals that exhibit a high degree of biological specificity and dose sensitivity, practical limitations on the approach must be defined. In order to reduce the large uncertainties inherent in the recommended approach, future research should be focused on defining the mechanistic features that predict dose additive toxicity in mixtures. A detailed characterization of pharmacodynamics, pharmacokinetics, and slope of dose response curves may be necessary to evaluate whether the toxicity of a mixture can be predicted by the mode of action of its component chemicals.

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## THE USE OF TEFS IN ASSESSING MIXTURES OF DIOXINS, FURANS AND DIOXIN-LIKE PCBs.

**W. H. Farland, M. DeVito and L. Birnbaum.** Office of Research and Development, USEPA, Washington, DC.

Dioxin and related compounds always exist in nature as complex mixtures. These complex mixtures can be characterized through analytic methods to determine concentrations of individual congeners. Exposure to dioxin and related compounds can be quantified and the biological activity of the mixture can be estimated using relative potency values and an assumption of dose additivity. Such an approach has evolved over time to form the basis for the use of toxicity equivalence (TEQ) in risk assessment for this group of compounds. While such an approach is dependent on critical assumptions and scientific judgement, it has been characterized as a useful, interim method to deal with the complex mixture problem and has been accepted by numerous countries and several international organizations. Alternative approaches assessing complex mixtures of dioxin and related compounds, including the assumption that all congeners carry the toxicity equivalence of 2, 3, 7, 8-TCDD, or that all congeners other than 2, 3, 7, 8-TCDD have no activity, have been rejected as scientifically indefensible and inadequate for risk assessment pur-

poses. However, the degree to which the current point estimate TEFs introduce variability and uncertainty into the health risk assessment process cannot be characterized in a quantitative fashion. Such characterizations may be important in settings where numerous PCDD(F) and PCB congeners contribute to potential health risk. Recent work has focused on refining and updating the database underlying the TEFs and evaluating quantitative methods for assessing the variability and uncertainty in TEF estimates.

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## TESTING THE TOXIC EQUIVALENCY FACTOR (TEF) HYPOTHESIS: THE NTP DIOXIN/PCB CANCER BIOASSAYS.

**N. J. Walker<sup>1</sup>, M. E. Wyde<sup>1</sup>, P. W. Crockett<sup>2</sup>, A. Nyska<sup>1</sup>, J. R. Bucher<sup>1</sup> and C. J. Portier<sup>1,2</sup>.** <sup>1</sup>NIEHS, Research Triangle Park, NC and <sup>2</sup>Constella Group, Research Triangle Park, NC.

The dioxin Toxic Equivalency Factor (TEF) approach is currently used worldwide for assessing and managing the risks posed by exposure to mixtures of certain dioxin-like compounds. Use of the TEF approach assumes that the combined effects of dioxin-like compounds in a mixture can be predicted based on a potency adjusted dose additive combination of constituents of the mixture. To test the TEF approach for carcinogenic risk the National Toxicology Program conducted multiple two-year rodent cancer bioassays in female Harlan Sprague-Dawley rats examining the carcinogenicity of several dioxin-like compounds, PCBs, a defined tertiary mixture, and two mixtures of PCBs. Statistically based, dose-response modeling was used to evaluate the dose-response for induction of both neoplastic and non-neoplastic effects seen in these studies, and to test for interactions between compounds within mixtures and interactions between dioxin-like and non-dioxin like PCBs. For the defined mixture of dioxin-like compounds the dose response for induction of carcinogenicity for the mixture was consistent with an additive combination of the potency adjusted doses of the individual compounds, when using administered dose as the dose metric. Overall these data support the use of the TEF approach for potency adjusted dose addition for use in cancer risk assessments for dioxin-like compounds.

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## PHYSIOLOGICALLY BASED PHARMACOKINETIC (PBPK) MODELING AS AN ALTERNATIVE TO RELATIVE POTENCY FACTORS (RPFs) IN CUMULATIVE RISK ASSESSMENT: AN EXAMPLE WITH ACETYLCHOLINESTERASE- (AChE) INHIBITING PESTICIDES.

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RPFs are used to assess health risks associated with exposure to mixtures of chemicals. The RPF approach incorporates the key assumption that dose-response curves for mixture components are parallel. The prediction of cumulative risk based on RPFs is uncertain to the extent that this assumption does not hold. Cumulative risk for AChE inhibiting pesticides, including organophosphates and N methyl carbamates, is calculated from the degree of AChE inhibition due to the mixture, with AChE inhibition being used as a surrogate for frankly neurotoxic effects. PBPK modeling for AChE inhibiting pesticides can be used to predict cumulative risk while avoiding the assumption of parallel dose-response curves. PBPK models are first be developed for the individual components of the mixture and then linked together by describing interactions at sites of metabolism and at AChE. The resulting "mixture model" describes the overall degree of AChE inhibition for the mixture as a function of (1), the individual pharmacokinetic behavior of each chemical and, (2) the chemical-specific kinetics of AChE inhibition. We have developed a prototypical mixture PBPK model based on individual models for disopropylfluorophosphate, chlorpyrifos, and carbaryl. The model predicts dose responses for AChE inhibition for the individual compounds and for arbitrary dosing scenarios for the mixture. Departures from parallel dose-response, reflecting compound-specific differences in pharmacokinetics, are predicted. In addition, the trade off between the greater realism but resource intensiveness of the PBPK approach and the less ambitious RPF approach will be discussed.

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## ROLE OF NUTRIGENOMICS IN SAFETY ASSESSMENT OF FUNCTIONAL FOODS.

**M. G. Soni<sup>1</sup> and T. R. Zacharewski<sup>2</sup>.** <sup>1</sup>Burdock Group, Vero Beach, FL and <sup>2</sup>Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI.

The recent completion of human genome (blueprint) has triggered an explosion in research into how drugs might be individualized to capitalize on each patient's unique genetic code. The line between food and drug is blurring in the era of the

genome. It appears that the food industry is on the verge of a new era where companies will design foods and market them to consumers according to the consumer's genetic makeup. Increased use of bioactive ingredients (so called functional foods) is challenging toxicologists when making safety determinations. This evaluation is different from that of drugs or toxins as efficacy or benefit analysis of the ingredients has not been factored in the safety determination. The evaluation of absorption, body distribution and metabolism will result in a realistic assessment of ranges in target tissue concentrations. Biological effects (both desirable and undesirable) can then be determined based upon genomic and proteomic changes, the result of which will be a bottom up approach rather than a top down methodology that imposes unrealistic safety factors. Thus, data from gene nutrient interaction will open the ways for new concepts of risk-benefit evaluation. The proposed workshop will cover recent discoveries in nutrition, genomics and proteomics and how these developments will change currently used methodology for risk assessment of bioactive/functional foods. This workshop will focus on applied genomic technologies and their impact on nutrition, health sciences and particularly on safety determination of functional foods. The objectives achieved will be: (1) Provide examples of the impact of genotype (cardiovascular diseases, cancer susceptibility, allergy, etc) on the response to foods and food components; (2) Identify relevant biomarkers that are applicable for assessing the benefits and risks of selected foods; (3) FDA's thinking on use of microarray data in regulatory approvals; and (4) Regulatory and other challenges in determining safety of "nutrionic" foods.



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### RISK ASSESSMENT OF FOOD AND FOOD COMPONENTS AT (NEAR) PHYSIOLOGICAL CONCENTRATIONS.

B. van Ommen. *Physiological Sciences, TNO Nutrition and Food Research, Zeist, Netherlands.* Sponsor: M. Soni.

Development of new bioactives for functional foods multiple gene targets exist, which may be modified by food constituents. Functional foods are usually chronically administered and with a possible multitude of biological effects. Since the majority of these responses are believed to be mediated through effector genes, and genomic technologies allow for the determination of an abundance of relevant genes/proteins/metabolites and their effects on metabolism, functional genomics offer great opportunities in this research. Perturbations of homeostasis (as measured by transcriptomics, proteomic and metabolomic technologies) will open the way for a completely new concept of risk-benefit evaluation, based on multiple subtle changes. Analysis of these changes will present a profile or fingerprint of biomarkers and lead to new mechanistic insights. Thus, this type of analysis will allow for the assessment of risk at (or near) physiological concentrations, instead of the classical toxicological approaches with extrapolations from high doses. In combining many subtle changes into new biomarkers, the biomarker becomes much more sensitive and consequently allows for very early detection of changes. This has enormous potential for toxicological and nutritional research. Biomarkers will change from describing a diseased or pathological state (e.g. plaque formation in atherosclerosis) or negative effect (e.g. oxidative DNA damage) into describing subtle changes in health, both in a positive and negative way. In other words, exploiting holistic datasets, healthy homeostasis and related early changes can be carefully described. On top of this, the new science of systems biology is emerging, taking up the challenge of utilizing all available data generated by genomics technology in a complete description of a biological system. As a result, this new paradigm is ideally fit for the evaluation of many subtle changes in biological activity, as triggered by nutrition and toxicology. Here, a multitude of bioactive compounds act simultaneously and chronically in constantly changing combinations.



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### GENE AND PROTEIN EXPRESSION CHANGES DURING IMMUNE RESPONSES TO FOOD ALLERGENS.

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Food allergy is an important health issue. The estimated prevalence among adults in Western Europe is thought to be between 1 and 2%, with the frequency in infants being greater (approximately 5%). Most confirmed food allergies are associated with a relatively limited range of produce, including cow's milk, eggs, tree nuts, peanuts, wheat, fish and shellfish, although the prevalence of allergy to individual foods is known to vary geographically, due largely to differences in dietary practices. There is no doubt that genetic predisposition is also an important determinant. With the increasing interest in novel foods derived from transgenic crop plants, there is a growing need for the development of approaches for the characterization of the allergenic potential of proteins. Although most foreign proteins are immunogenic (able to induce IgG antibody responses), relatively few are important food allergens with the capacity to provoke IgE antibody production and immediate type hypersensitivity responses. Immune responses, including allergic responses, are orchestrated by activated T cell subsets and their cellular products (cytokines).

Characterization in allergen-activated lymph node cells of cytokine gene expression patterns by ribonuclease protection assay and of cytokine secretion profiles, using customized cytokine microarray (Luminex) flow cytometry, demonstrates that exposure of mice to allergenic proteins results in the selective induction of a Th2 cytokine phenotype. Current findings suggest that it may prove possible to use cytokine expression profiling (at either the protein or message level) to identify those proteins with the potential to cause food allergy.



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### NUTRIGENOMICS AND INFLAMMATION.

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The use of genetic and genomic information has various implications for the development of functional foods. Genetic information may be informative relative to both an individual's adverse reactions to dietary substances and the health benefits. Some dietary factors that are known to lead to disease may have a range of adverse health effects in different patients. Certain individuals, due to genetic variations in metabolism or disease causing mechanisms, may benefit from specific dietary substances, whereas others may have a less favorable response. This presentation will consider two examples of how genetic information may influence the response to diet and how this information may be applied to the development of functional foods. Coronary artery disease (CAD) is a complex clinical condition that may result from combinations of multiple biological components. One biological component is lipid metabolism, and specific lipid profiles are influenced by multiple factors, including diet and gene variations. For example, high dietary fat intake adversely influences the lipid profile in some individuals but not others, and the individuals with different biological responses can be distinguished by specific apolipoprotein gene variations. Another major component of CAD is inflammation. Specific cytokine gene variations increase inflammation and the risk for first CAD events, even in individuals with normal cholesterol. One approach to designing functional foods to benefit individuals with a life-long genetic tendency to over-express inflammation will be discussed, including the use of gene arrays in genotype-specific human clinical models to evaluate gene-nutrient interactions.



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### USE OF MICROARRAY DATA IN REGULATORY APPROVAL.

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At present the FDA Center for Food Safety and Applied Nutrition (CFSAN) does not have a policy for the submission and/or acceptance of microarray-based data in support of the approval process for the food-related compounds over which it has jurisdiction. While CFSAN is following the developments in this field, for now this discussion is hypothetical and has no official standing in assessing the safety of functional foods. What are some of the present shortcomings of genomics? When a food additive material is tested for safety, a number of different toxicological tests are applied (intensity of testing differs based on the level of human exposure). Along with characterizing the compound's organ-related toxicologic profile, "effect" and "no-effect" doses are also determined. At present there is no one "omic" technique or series of techniques that can provide this complete characterization of toxicity. So for some indefinite period "omic" techniques will perform a supportive role in mechanism-determination and elucidation of differential responses. Thus, differences in toxicity between the rat and humans might be delineated by toxicogenomic techniques, but there would have to be some other biomarker or mechanistic data (e.g., relative to biosynthetic pathways, occurrence of enzymes, differences in anatomical structures, etc.) to integrate into the overall reasoning and explanation. Case Study: Test results for sucrose acetate isobutyrate left only the issue of species-dependent hepatotoxicity to be resolved. While the dog showed reduced clearance of bromsulfophthalein at low doses, the mouse and rat did not. Eventually the sponsor ran additional studies with primates and human subjects and convinced OFAS that the low-dose hepatotoxicity was only shown by the dog. If the actual mechanism of toxicity had been demonstrated to be strongly correlated with predictive genomic changes, then it might have been possible to substitute genomics testing for one-year feeding studies and special liver function tests to establish the dog as the sole species to manifest this toxicity.



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### IMPACT OF MOLECULAR NUTRITION ON THE SAFETY ASSESSMENT OF FUNCTIONAL FOODS: A SWOT ANALYSIS.

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The powerful tools of molecular nutrition generate massive amounts of information, but not necessarily knowledge and understanding. Using dietary fatty acids as a prototypic functional food, the impact of nutritional genomics and nutritional

proteomics on the safety assessment of functional foods will be presented in terms of Strengths, Weaknesses, Opportunities and Threats, i.e., a SWOT analysis. Fatty acids are particularly useful as a case study as their nutritional pharmacology is based, at least in part, on nuclear receptor activation with related changes in gene expression, modulation of the serum proteome, and perhaps most importantly, they have attendant biomarkers of exposure and clinical efficacy. With respect to the SWOT analysis, the utility of molecular nutrition in the safety assessment of nutritional bioactives encompasses the following: Strengths - the application of systems biology enables a holistic assessment consistent with the multifaceted nature of nutrition; Weaknesses - limited paradigms exist to accommodate the pleiotropic pharmacology of nutritional bioactives, let alone that of whole foods in the broader context of diet; Opportunities - identification of potential adverse effects and vulnerable populations elicited by nutritional products characterized by low potency, limited efficacy, but with significant consumer exposure; and Threats - premature application of promising technologies, some of which have bioethical ramifications.

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#### A NEW GENERATION OF MULTIFUNCTIONAL NANOPARTICLES POSSESSING MAGNETIC MOTOR EFFECT FOR DRUG OR GENE DELIVERY.

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Nanotechnology is expected not only to provide but to may cause risks. Cobalt-ferrite-silica magnetic nanoparticles(-50nm) labelled with organic dye were prepared by a modified PVP method and sol-gel process. The thickness of the silica shell was controlled by varying the ratio between magnetic nanoparticle(MNP) and tetraethoxysilane(TEOS) plus RITC or FITC dyes. The MNP surface was also modified with PEG. Incorporation of the dyes into the MNP enabled us to monitor the movement of nanoparticles under an external magnetic field by optical microscope. Biological distribution study revealed that the nanoparticles could penetrate the blood-brain-barrier(BBB) while not producing any detectable toxicity. Such penetration may provide useful methods for the study of delivery mechanism into BBB, thus, could be applicable to the biological labelling as well as detection, comprehensive drug or gene delivery systems. Supported by NSI-NCRC through KOSEF.

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#### THE AGGREGATION OF SINGLE-WALLED CARBON NANOTUBES IN FRESH WATER AND SEA WATER.

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Single-walled carbon nanotubes (SWNTs) are comprised of nanometer-diameter cylinders. They are formed by deposition as a single graphene sheet and wrapped up to form a tube. They are considered as leading candidates for nanoscale electronic applications. Data on the life cycle assessment of SWNTs is not widely available. The fate, transport, transformation and toxicity of SWNTs are not well investigated. As a first step to study the life cycle assessment of SWNTs, we investigate the aggregation of free particles of SWNT in fresh water and sea water. The SWNTs are hydrophobic and they naturally clump together to form aggregates with diameters in the micro meter range. The size distributions of the aggregates do not change significantly with increasing salinity and temperature. In contrast, the size distribution differs with changing pH in the aquatic environment. Our data suggests that the aggregation of free particles of SWNTs in the aquatic environment will affect their impact on the biota in water.

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#### PROTOCOLS FOR ASSESSING *IN VITRO* TOXICITY OF NANOPARTICULATES.

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The growing development and use of nanoparticulate materials for commercial applications demands a better understanding of the possible effects and mechanisms of interaction of these materials with biological systems. In this study we have paid special attention to the full characterization of the nanomaterials tested. Well-characterized nanomaterials were then used in exposure studies to evaluate their *in vitro* effects at the cellular level. Several biological endpoint assays (LDH release, metabolic activity and apoptosis) were conducted as indicators of cell death, cell viability and cell proliferation. The cell line selected for these experiments is the human lung carcinoma A549 (type II alveolar epithelial cells). Four particulate materials

were evaluated: a nano aluminum powder (Nanotechnologies), two nanosized titanium oxides powders (Degussa P25 and NanoTek®), and crystalline quartz (Min-U-sil 5). The materials were well dispersed and characterized for size, surface properties, and morphology. Concentration and time courses were designed in order to evaluate the extent of cytotoxicity, and to define critical conditions for further research. Treatment consisted of exposing the cells to particles in culture for time periods ranging from 4 to 72 hours and in concentrations from 30 µg/ml to 500 µg/ml. Bioassays were run following standard procedures as outlined in the instructions provided with kits (Roche Diagnostics and BioSource). All measurements were obtained using appropriate controls in order to minimize artifacts such as light scattering by the particles themselves.

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#### ASSESSING THE POTENTIAL HAZARD OF ENGINEERED NANOPARTICLES IN THE WORK ENVIRONMENT.

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Within the arena of nanoparticle development, innovation is running ahead of regulation. There are currently no regulatory guidelines for the protection of workers specifically from nanoparticle exposure. As a result, those organizations concerned must assess the potential hazard of the nanoparticles with which they are working and take the appropriate measures to ensure the safety of their employees. Here we present a simplified matrix that can be used to qualitatively assess the potential hazard associated with specific nanoparticles based on a number of physical and chemical characteristics. When considering the potential human health effects of nanoparticles, the popular press has focused almost exclusively on the influence of size. In reality, any potential human health effects associated with nanoparticle exposure would be the result of a number of factors including route of exposure, shape, surface properties, solubility as well as size. The combined influence of these factors can result in nanoparticles that range from extremely hazardous to relatively benign within the work environment. With such information, organizations can provide their employees with the appropriate protective equipment to ensure that risk of adverse health effects is minimal.

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#### GENE EXPRESSION PROFILING OF NANOSCALE MATERIALS USING A SYSTEMS BIOLOGY APPROACH.

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Nanomaterials are being developed and manufactured on a commercial scale. However, preliminary reports are mixed as to their toxicity. One of the major organ systems affected through environmental exposure is skin. In an effort to assess the risk to humans, human cell cultures and gene expression microarrays were used in a systems biology approach. This approach perturbs a biological system with a possible toxic insult and reiteratively samples it over time. Primary human neonatal epidermal keratinocytes (HEK) were treated *in vitro* with several nanoscale materials, including: silica (Min-U-Sil®5, SiO<sub>2</sub>), titanium dioxide (TiO<sub>2</sub>) and carbon black (Printex 90®). These particulate materials were used to treat randomly-proliferating HEK cultures at 8 time points ranging from 0 to 24 hr. Cell pellets were snap-frozen and stored at -80C. Biotinylated cRNA probes were synthesized from total RNA isolated from the cell pellets and hybridized onto CodeLink™ microarrays containing oligomers from 9, 970 unique human genes. After image analysis, the results were analyzed by statistical methods as well as both supervised and unsupervised methods. Analyzing the data from treating the HEK cultures with each compound at 1mg/mL for 24 hr. by hierarchical agglomerative clustering (Euclidean distance metric, complete linkage), the gene expression patterns for SiO<sub>2</sub> and TiO<sub>2</sub> were more similar to each other as compared to the gene expression pattern observed for Printex90. Approximately 75% of the 9, 970 probes passed a set of stringent quality control criteria. While all three materials regulated similar genes (e.g. several structural proteins, such as laminin), there were differences observed in the gene families of ribosomal proteins and cytokines. These families of genes have previously been reported to be effected by these particulates. Min-U-Sil and Printex90 are registered trademarks of US Silica Company and Degussa Corporation, respectively, and CodeLink is the trademark of GE Healthcare.

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#### *IN VITRO* AND *IN VIVO* EXPOSURE TO ORTHO-SUBSTITUTED PCB 95 AND PCB 170 ALTER NEUROPLASTICITY IN THE RAT HIPPOCAMPAL SLICES.

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There is mounting evidence that exposure to non-coplanar PCB's is associated with significant changes in locomotor activity, spatial learning and memory in rodents. In this study, PCBs 95- and 170-induced changes in synaptic transmission of rat

hippocampal slices were monitored by electrophysiological measurements. Field excitatory postsynaptic potential (fEPSP) was evoked by single pulse stimulation of Schaffer Collateral/commissural fibers at stratum radiatum of the CA1 region in the hippocampus. Following exposure to 100 nM PCB 170, time-dependent changes in the slope and amplitude of fEPSP were seen, with phases of enhancement and depression. To investigate the contribution of inhibitory neurons in the actions of PCB 170, hippocampal slices were pre-treated with the GABAa receptor antagonist, picrotoxin (PTX, 100  $\mu$ M). Pre-treatment with PTX resulted in negligible change in fEPSP slope elicited by single pulse stimuli. Importantly PCB170 introduced in the presence of GABAa blockade produced a threshold response at 1nM and enhanced fEPSP slope of 250% at 100nM, revealing a significant facilitation of synaptic transmission. Non-coplanar PCBs therefore influence both excitatory and inhibitory pathways in CA1 that can mask their potent effects. These results demonstrate that blockade of inhibitory inputs with PTX can unmask the potent actions of ortho-substituted PCB 170 toward facilitating excitatory transmission. Studies of perinatal exposure to PCB 95 (6mg/kg/day) from GD5 to PND 21 were also performed to examine influences on hippocampal excitability in the offspring. PTX (100  $\mu$ M) application to hippocampal slices from PCB-treatment groups displayed a much higher sensitivity to synaptic facilitation in comparison slices isolated from corn oil control groups. These results show that ortho-substituted PCBs are especially potent excitatory neurotoxicants in the presence of GABAergic insufficiency. Highly synergistic xenobiotic mechanisms can dramatically enhance susceptibility of the hippocampus to non-coplanar PCBs.

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#### OVEREXPRESSION OF NQO1 PROTECTS HUMAN DOPAMINERGIC SK-N-MC NEUROBLASTOMA CELLS AGAINST DOPAMINE INDUCED CELL DEATH.

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Dopaminergic neurons are selectively vulnerable to dopamine-quinones and reactive oxygen species generated by dopamine oxidation and destruction of dopaminergic neurons has been implicated in the pathogenesis of Parkinson's disease. NAD(P)H: quinone oxidoreductase (NQO1) can metabolize dopamine derived quinones (DAQ) and a lack of NQO1 due to NQO1\*2 polymorphism has been suggested to be a risk factor for PD. We have recently shown high NQO1 immunoreactivity in the substantia nigra of human Parkinson's brains. In order to confirm the role of NQO1 in the metabolism of dopamine, we have examined the potential role of NQO1 in human neuroblastoma cell line (SK-N-MC), which was transfected with NQO1. The NQO1 activity of stably transfected cells was 350nmole /mg/ min while vector control and parental cells had NQO1 activities of less than 40nmole/ mg/ min using standard activity assays. Incubation of 500 $\mu$ M dopamine for 24 hrs in both parental and vector control SK-N-MC cells resulted in 88% and 72% cell death as assessed by annexin-V/Propidium iodide analysis using flow cytometry. In agreement, 88% and 84% of parental and vector control cells respectively underwent loss of mitochondrial membrane potential (MMP) assessed by tetramethylrhodamine ethyl ester. In contrast, NQO1 transfected cells were resistant to dopamine toxicity and both cell death and loss of MMP were abrogated in NQO1-transfected SK-N-MC cells. Dopamine resistance in NQO1 over-expressed cells may be attributed to the role of NQO1 in the detoxification of DAQ and/or direct scavenging of reactive oxygen species (Siegel et al (2004), Mol Pharmacology 65; 1238-47) and may play an important role in the pathogenesis of Parkinson's disease. (This work is supported by NIH RO1 NS44613)

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#### FORCED EXERCISE ATTENUATES KAINIC ACID-INDUCED NEUROTOXICITY IN THE HIPPOCAMPUS OF C57BL/6J MICE.

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Exercise is considered beneficial to overall health and may enhance the resiliency of the body to insult. Here, we investigated the ability of exercise to modulate neurotoxicity caused by kainic acid in male C57Bl/6J mice. Forced walking was achieved in a motorized exercise wheel (10 s/rev., 30 min., 4:00 P.M.). Mice were trained for three days, then exercised for 14 days prior to kainate treatment. On day 14 of exercise, mice received an injection of saline or kainic acid (20 mg/kg, intraperitoneal, at 12:00 P.M.). Additional non-exercised mice received similar injections for a total of four groups: saline, kainate, wheel saline, wheel kainate. Seizure severity was scored according to the Racine scale. At 24 hours post-injection, mice were decapitated, the brain was removed and bisected; the left hippocampus was dissected for analysis of GFAP by ELISA while the right hemisphere was immersion fixed for histological analysis of neurodegeneration by Fluoro-Jade B staining. Body, thymus, and spleen weights were recorded, and plasma was prepared for analysis of

corticosterone levels. The 24 hour time point for sacrifice was selected based on previous experimentation evaluating kainate-induced neurodegeneration, but preceded the peak of induction of GFAP. Kainic acid treatment caused minimal seizures (mouth and facial movements). Fluoro-Jade B staining revealed fluorescent pyramidal cells in kainate-treated animals indicating neuronal damage; no fluorescent neurons were observed in any exercised mice. GFAP levels were not significantly different between groups, but protein levels were slightly elevated in kainate treated mice, and attenuated in exercised animals. Additional time points will be examined which correlate with the peak of reactive gliosis. No differences in body or organ weights were observed; however, there was a trend of decreasing thymus weight in exercised animals. These data suggest exercise may be protective against kainate-induced excitotoxicity. The generality of these findings for other types of brain insults will be the topic of future investigations.

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#### EVALUATING THE NMDA-GLUTAMATE RECEPTOR AS A SITE OF ACTION FOR TOLUENE, *IN VIVO*.

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*In vitro* studies have demonstrated that toluene disrupts the function of several ion channels localized in the brain, including the NMDA-glutamate receptor. This has led to the hypothesis that effects on ion channel function may contribute to toluene neurotoxicity, CNS depressive behavior, and altered visual evoked potentials observed in animals and humans. However, this hypothesis has not been tested *in vivo*. The present experiment examines potential toluene targets in whole animals by measuring visual evoked potentials (VEPs) during toluene exposure and challenging with a drug that antagonizes toluene's action at the NMDA-glutamate receptor. Therefore the goal of this study was to verify changes in VEPs during toluene exposure and demonstrate that toluene inhibits NMDA-glutamate receptor mediated functions in visual evoked potentials elicited from rats. One week prior to testing, recording electrodes were implanted in the rat skull above left visual cortex. Awake, restrained rats were presented with an onset/offset pattern containing a spatial frequency of 0.16 cpd, temporal frequency of 4.55 Hz, with a 60 percent contrast between bars. Baseline VEPs were recorded and rats were injected with either saline or NMDA (10 mg/kg, i.p.). Ten minutes after injection animals were exposed to air or toluene (2000 ppm). VEP amplitudes were calculated for 2X stimulus frequency (F2) for each rat. Thirty minutes after injection of NMDA or saline, NMDA/air (n=5) treatments decreased F2 by 15 percent, saline/toluene (n=6) decreased F2 by 42 percent and toluene/NMDA (n=6) decreased F2 amplitude by 63 percent, contrary to expectations. These results indicate an unanticipated, additive effect of toluene and NMDA on decreasing F2 amplitude. Thus, there appears to be an interaction between NMDA and toluene in the visual system. (This abstract does not reflect EPA policy)

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#### INVOLVEMENT OF OXIDATIVE STRESS IN POTENTIATION OF NOISE INDUCED HEARING LOSS (NIHL) BY CHEMICAL CONTAMINANTS.

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Permissible workplace exposure limits for noise and for chemical contaminants generally rely upon laboratory and epidemiological investigations that entail exposure to the single agent of interest. However, a growing body of research shows that a variety of chemical contaminants, including those that have no effect upon auditory function, can potentiate noise induced hearing loss. Such potentiation is particularly noteworthy when it occurs for low intensity noise exposures that approach the permissible human noise exposure standards. We predict that chemicals able to impair intrinsic antioxidant mechanisms potentiate NIHL since reactive oxygen species may be generated even at low noise levels. Combined exposure of rats to hydrogen cyanide and mild noise yields significant auditory impairment while at neither agent given alone resulted in hearing loss. Using antioxidant drug treatments to protect auditory function and pro-oxidant drugs to promote oxidative stress we find support for our hypothesis that oxidative stress does play a role in potentiation of noise induced hearing loss by hydrogen cyanide. supported in part by NIOSH OH03481.

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#### MODULATION OF CHOLINERGIC TOXICITY BY CANNABINOIDS.

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Endogenous (e.g., anandamide) and exogenous (e.g., tetrahydrocannabinol) cannabinoids have been reported to decrease or increase acetylcholine release in different brain regions. Both the cholinergic agonist carbachol and the cholinesterase

inhibitor physostigmine increase cannabinoid release in hippocampal slices. Furthermore, some organophosphorus (OP) anticholinesterases block specific binding to cannabinoid CB1 receptors. We studied the effects of repeated exposure to the synthetic cannabinoid WIN 55, 212-2 (WIN) on acute toxicity of the potent OP anticholinesterase, paraoxon. First, motor activity and CB1 binding were studied in rats (n=8/group) treated (daily for 7 days) with 0.5, 1.5 or 5.0 mg/kg WIN. The low dosage (0.5 mg/kg/day) increased locomotor activity, but apparent habituation was noted in both vehicle and 0.5 mg/kg/day WIN groups over time. In contrast, 1.5 mg/kg/day WIN elevated motor activity with no apparent habituation, while rats treated with 5 mg/kg/day WIN initially showed reduced motor activity but hyperactivity at later time-points. CB1 binding was significantly reduced (24-31%) in hippocampus, 4 hours after the last WIN exposure (1.5 and 5.0 mg/kg/day), but was not significantly affected in either cortex or striatum or by the low dosage in any tissue. In parallel studies, similarly-treated rats were given paraoxon (0.4 mg/kg, sc, n=4/group) 4 hours after the final WIN exposure and signs of cholinergic toxicity (SLUD signs, involuntary movements, motor activity) were monitored at 1, 2, 4 and 16 hours after dosing. With involuntary movements, greater toxicity was noted in all WIN-pretreated rats from 1-4 hours. In contrast, SLUD signs were less prominent in WIN-pretreated rats at these time-points. No cholinergic signs were noted at the last time-point in either group. In general, there was reduced ambulation and rearing within the first hour after paraoxon exposure in WIN-pretreated rats, but relative hyperactivity at later time-points. These results suggest that co-exposure to cannabinoids may modulate the expression of cholinergic toxicity. (Supported by OSU Board of Regents).

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#### TEMPORAL PARAMETERS OF ENVIRONMENTAL ENRICHMENT-INDUCED COGNITIVE ENHANCEMENT IN A RODENT MODEL OF LEAD NEUROTOXICITY.

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Environmental enrichment (EE), a non-pharmacological therapy that combines social interaction with a complex living environment, reverses molecular and cognitive deficits observed in a rodent model of lead intoxication (Guilarte et al, Ann. Neurol., 53:50, 2003). In order to translate this finding to the human condition, it is important to further understand the temporal parameters of this therapy. We tested whether the benefit of EE on cognitive function persists in adult rats after EE was removed and if a critical window exists for the application of this therapy. Rats were exposed to 0 or 1500 ppm lead acetate from conception until postnatal day (PN) 21 and then housed singly in standard rat cages (isolated) or in groups of 8 (enriched) in multi-level cages that contained toys until PN79. To test if the benefits of EE are long lasting, rats were raised in enrichment cages from PN21 until PN50 and then transferred to isolated cages until PN79 (permanence). To determine if a critical window existed for the benefit of the intervention, animals were placed in EE from PN50 to PN79. In all studies, spatial learning was assessed at PN79. Blood and hippocampal lead levels were elevated in lead exposed rats, however, no significant lead exposure effect was observed on the acquisition of the task. A significant housing effect was observed on the acquisition, probe and cue tests with rats currently receiving EE (enriched and critical window) performing significantly better on all three tasks. Nearly twice as many rats in the isolated and permanence groups exhibited a place strategy in the cue test which contributed to the significantly elevated latency in the cue test. In summary, these studies could not detect a significant cognitive deficit in lead exposed rats at PN79, which could be due to the degree of difficulty of the task. Further, EE is effective in enhancing cognitive performance but this benefit is lost after cessation of EE. [Supported by NIEHS grant # ES006189 to TRG]

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#### PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR $\alpha$ IS REGULATED BY GSK3.

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Peroxisome proliferators-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear receptor family. PPARs can be activated by a large group of chemicals called peroxisome proliferators that include industrial pollutants and hypolipidemic drugs. PPAR $\alpha$ , similar to other nuclear receptors including the estrogen receptor and the glucocorticoid receptor, has been demonstrated to be a phosphoprotein through *in vivo* phosphorylation studies. However, the kinases and phosphorylation sites within PPAR $\alpha$  are not known. Many consensus phosphorylation sites for PPAR $\alpha$  are predicted. Within these sites, there are five conserved glycogen synthase kinase 3 (GSK3) sites in the A/B and C domains of PPAR $\alpha$ . GSK3 is involved in cell cycle regulation and may help explain the role PPAR $\alpha$  plays in cell cycle regulation. In this study, the effects of GSK3 on PPAR $\alpha$  phosphorylation and activity have been explored. Co-transfection of GSK3 with

PPAR $\alpha$  resulted in an increase in ligand-inducible reporter activity that was blocked by LiCl, a GSK3 specific inhibitor. *In vivo* phosphorylation studies were done to show the role of GSK3 in the phosphorylation of PPAR $\alpha$ . These studies suggest a role for GSK3 in the phosphorylation and regulation of PPAR $\alpha$ . (Supported by NIH ES07799)

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#### PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ALPHA- AND P53-RESPONSIVE GENE REGULATION BY RIBOSOMAL PROTEIN L11.

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Peroxisome Proliferator-Activated Receptor Alpha (PPAR  $\alpha$ ) is a member of the nuclear receptor superfamily. PPAR  $\alpha$  activation with peroxisome proliferators induces a mitogenic response in hepatocytes and a tumorigenic response in rodents. Upon activation, PPAR  $\alpha$  forms a heterodimer with Retinoid X Receptor alpha (RXR  $\alpha$ ) and the complex binds to response elements in the 5 regulatory regions of target genes. Our studies have demonstrated that PPAR  $\alpha$  associates with the ribosomal protein L11 (rpL11), a component of the large 60S subunit. Recent studies have shown that rpL11 suppresses the activity of MDM2, a negative regulator of p53, leading to the stabilization and activation of p53. We show that rpL11 inhibited the transcriptional activity of PPAR  $\alpha$ . This was associated with decreased heterodimerization of PPAR  $\alpha$  and RXR  $\alpha$  and decreased binding to the PPAR-response element (PPRE). In addition to inhibition of PPAR  $\alpha$  activity, we investigated if the formation of the PPAR  $\alpha$ -rpL11 complex also affected p53 signaling. Manipulation of rpL11 expression via ribosomal stress, serum deprivation and small inhibitor RNA (RNAi) was used to study the regulation of both p53 and PPAR  $\alpha$ -responsive genes by peroxisome proliferators. The convergence of PPAR  $\alpha$  and p53 signaling pathways may play an important role in the ability of peroxisome proliferators to regulate key growth regulatory genes. Supported by NIH DK49009.

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#### THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR- $\beta$ (PPAR $\beta$ ) AGONIST GW0742 INHIBITS COLON CARCINOGENESIS.

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Recent data has shown that PPAR $\beta$  may be a moderating factor involved in the etiology of colon cancer. PPAR $\beta$  expression is reported to be elevated in human colon tumors containing an inactivated APC gene, as well as in human and azoxymethane-induced rodent tumors. Previously, the functional role of PPAR $\beta$  in colon carcinogenesis was investigated using azoxymethane (AOM)-treated PPAR $\beta$ -null mice. These studies showed that when treated with AOM, PPAR $\beta$ -null mice exhibit a dose-dependent increase in colon polyp formation as compared to AOM-treated wild-type mice. In the present studies, wild-type and PPAR $\beta$ -null mice were treated with AOM along with the PPAR $\beta$  ligand GW0742. Wild-type mice treated with AOM and ligand had a dose-dependent decrease of colon polyp formation as compared to wild-type controls treated with AOM alone. In contrast, PPAR $\beta$ -null mice treated with AOM and ligand showed no change in polyp formation as compared to PPAR $\beta$ -null control mice treated with AOM alone. Consistent with previous studies, colon polyp formation was exacerbated in PPAR $\beta$ -null mice when compared to their wild-type counterparts. These results support the hypothesis that PPAR $\beta$  attenuates colon carcinogenesis, and that the protective role is enhanced through activation of PPAR $\beta$  with the synthetic ligand GW0742. Supported by National Cancer Institute (CA 97999)

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#### UNDERSTANDING GENE EXPRESSION CHANGES CONTROLLED BY THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR $\alpha$ IN RATS.

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Peroxisome proliferator-activated receptors (PPAR's) are a group of nuclear receptors whose ligands include fatty acids, eicosanoids and the fibrate class of drugs. In humans, fibrates are used to treat dyslipidemias. In rodents, fibrates cause peroxisome proliferation, a change that might explain the observed hepatomegaly. In one

study, rats were treated with multiple dose levels of six fibrin acid analogues (including fenofibrate) for up to two weeks. Pathological analysis identified hepatocellular hypertrophy as the only sign of hepatotoxicity, and only one compound at the highest dose caused any significant increase in serum ALT or AST activity. RNA profiling revealed that the expression of 1288 genes was related to dose or length of treatment and correlated with hepatocellular hypertrophy. Based upon the biochemical pathways represented in this gene set, it is likely that these changes are linked to many of the clinical benefits of fibrate drugs. In light of the fact that all six compounds stimulated similar or identical changes in the expression of this set of 1288 genes, these results indicate that hepatomegaly, in addition to the clinical benefit, is due to PPAR $\alpha$  activation. However, the use of a PPAR $\alpha$  signature alone to identify receptor activation by drugs can be misleading. In another study, we examined the effects of MrkA (a CCR5 antagonist) and found that it generated a PPAR $\alpha$  signature comparable to that of fibrates. A small but significant additional signature was shared with known mitochondrial inhibitors, leading to our conclusion that for this compound the PPAR $\alpha$  signature was not a proximal effect but was likely due to the accumulation of endogenous ligands.

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### OVERLAPPING TRANSCRIPTIONAL PROGRAMS REGULATED BY THE NUCLEAR RECEPTORS PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ALPHA, RETINOID X RECEPTOR AND LIVER X RECEPTOR IN MOUSE LIVER.

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Lipid homeostasis is controlled in part by the nuclear receptors peroxisome proliferator (PP)-activated receptor alpha (PPARalpha) and liver X receptor (LXR) through regulation of genes involved in fatty acid and cholesterol metabolism. Exposure to agonists of retinoid X receptor (RXR), the obligate heterodimer partner of PPARalpha and LXR results in responses that partially overlap with those of PP. To better understand the gene networks regulated by these NR, transcript profiles were generated from the livers of wild-type and PPARalpha-null mice exposed to the RXR pan-agonist AGN194, 204 or the PPAR pan-agonist, WY-14, 643 (WY) and compared to the profiles from the livers of wild-type and LXRApha/LXRbeta-null mice after exposure to the LXR agonist T0901317. All 218 WY-regulated genes altered in wild-type mice required PPARalpha. Remarkably, ~80% of genes regulated by AGN194, 204 required PPARalpha including cell-cycle genes, consistent with AGN-induced hepatocyte proliferation having both PPARalpha-dependent and -independent components. Overlaps of ~31-62% in the transcript profiles of WY, AGN194, 204 and T0901317 required PPARalpha and LXRApha/LXRbeta for statistical significance. Out of the 50 overlapping genes regulated by T0901317 and WY, all but one were regulated in a similar direction. These results 1) identify new transcriptional targets of PPARalpha and RXR important in regulating lipid metabolism and liver homeostasis, 2) illustrate the importance of PPARalpha in regulation of gene expression by a prototypical PP and by an RXR agonist and 3) provide support for an axis of PPARalpha-RXR-LXR in which agonists for each nuclear receptor regulate an overlapping set of genes in the mouse liver.

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### THE TRANSCRIPTIONAL RESPONSE TO A PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ALPHA AGONIST INCLUDES INCREASED EXPRESSION OF PROTEOME MAINTENANCE GENES.

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The nuclear receptor peroxisome proliferator-activated receptor alpha (PPARalpha), in addition to regulating lipid homeostasis, controls the level of tissue damage after chemical or physical stress. To determine the role of PPARalpha in oxidative stress responses, we examined damage after exposure to chemicals that increase oxidative stress in wild-type or PPARalpha-null mice. Primary hepatocytes from wild-type but not PPARalpha-null mice pretreated with the PPAR pan-agonist WY-14, 643 (WY) were protected from damage to cadmium and paraquat. The livers from intact PPARalpha-null mice were more sensitive to damage after carbon tetrachloride treatment compared to wild-type mice. To determine the mo-

lecular basis of the protection by PPARalpha, we identified genes by transcript profiling whose expression was altered by a 7-day exposure to WY in wild-type and PPARalpha-null mice. Out of the 815 genes regulated by WY in wild-type mice ( $p < 0.001$ ;  $>1.5$ -fold or  $<-1.5$ -fold), only 2 genes were regulated similarly by WY in PPARalpha-null mice. WY increased expression of stress-modifier genes that maintain the health of the proteome including those that prevent protein aggregation (heat stress-inducible chaperones) and eliminate damaged proteins (proteasome components). Although the induction of proteasomal genes significantly overlapped with those regulated by 1, 2-dithiole-3-thione, an activator of oxidant-inducible Nrf2, WY increased expression of proteasomal genes independently of Nrf2. Thus, PPARalpha controls the vast majority of gene expression changes after exposure to WY in the mouse liver and protects the liver from oxidant-induced damage, possibly through regulation of a distinct set of proteome maintenance genes.

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### ROLE OF PPARALPHA IN CALORIC RESTRICTION EFFECTS IN THE MOUSE LIVER.

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The obesity epidemic in industrialized countries is associated with increases in cardiovascular disease (CVD) and certain types of cancer. In animal models, caloric restriction (CR) suppresses these diseases as well as chemical-induced tissue damage. These beneficial effects of CR overlap with those altered by agonists of nuclear receptors (NR) under control of the fasting-responsive transcriptional co-activator, peroxisome proliferator-activated co-activator 1alpha (PGC-1alpha). In a screen for compounds that mimic CR effects in the liver, we found statistically significant overlaps between the CR transcript profile in wild-type mice and the profiles altered by agonists of lipid-activated NR including peroxisome proliferator-activated receptor alpha (PPAR), liver X receptor and their obligate heterodimer partner, retinoid X receptor. The overlapping genes included those involved in CVD (lipid metabolism, inflammation) and cancer (cell fate). Based on this overlap, we hypothesized that some effects of CR are mediated by PPAR. As determined by transcript profiling, 19% of all gene expression changes in wild-type mice were dependent on PPAR including Cyp4a10 and Cyp4a14, involved in fatty acid omega-oxidation, acute phase response genes and epidermal growth factor receptor but not increases in PGC-1alpha. CR protected the livers of wild-type mice from damage induced by thioacetamide, a liver toxicant and hepatocarcinogen. CR protection was lost in PPAR-null mice due to inadequate tissue repair. These results demonstrate that PPAR mediates some of the effects of CR and indicate that a pharmacological approach to mimicking many of the beneficial effects of CR may be possible.

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### TOXICOGENETIC ANALYSIS OF SUSCEPTIBILITY TO ACETAMINOPHEN-INDUCED LIVER INJURY.

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The mouse is an invaluable model for the study of linkages between the genome and phenotype, facilitating an extensive knowledge base for genetic diversity, gene-environment interactions and toxicological endpoints. The integration of mouse genetics, toxicogenomics and conventional toxicology allows for a systems biology approach for discovery of the mechanisms of liver injury due to toxicant exposure. The multidimensional datasets that include detailed genotyping data, gene expression profiles and an array of phenotypic measures of liver injury could help to define a toxicity susceptibility state and aid in a discovery of biomarkers. Acetaminophen (APAP) was selected as a model hepatotoxicant because much information exists regarding the mechanisms of toxicity and conventional clinical markers largely fail to connect toxicity and the clinical outcome. A panel of 40 inbred strains that are used in the Mouse Phenome Project was selected for this study. Male mice were fasted for 18 hrs, administered an acute dose of APAP (300 mg/kg) or vehicle (0.5% methylcellulose), and sacrificed over a time course for up to 24

hrs. Liver tissue was harvested and sectioned for histology scoring and microarray analysis. In addition, blood was taken for serum analysis of classical markers of liver injury. A gradient of APAP-induced toxicity was detected across the 40 mouse strain panel. While some strains exhibited virtually no toxicity, remarkable levels of injury were detected in others. Our data indicate that strain-specific liver injury research may hold important clues to deciphering the mechanisms of a toxicity susceptibility state. Furthermore, the systems biology approach that includes recent advances in mouse genetics may aid in the prediction of individual susceptibility to a toxic agent. Supported by grants from NIEHS ES11391, ES11660, ES10126 and ES07126.

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### PARACETAMOL TOXICITY IN RATS PRETREATED WITH CYTOCHROME P450 OR GLUTATHIONE INHIBITORS USING AN INTEGRATED GENOMICS APPROACH.

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Overdosed paracetamol (APAP) has been identified as a major cause of acute liver failure, classically linked to profound glutathione (GSH) depletion associated with oxidative cell stress and/or covalent binding of cellular proteins to the APAP intermediate metabolites. However, overdose of APAP induces kidney toxicity only in rare cases which can be reversible and isolated. Literature data indicate that diverse environmental, genetic and immunological factors may influence APAP toxicity both in the liver and kidney. In this study, the toxic signature of APAP in the liver and kidney was evaluated using a genomics approach in rats pre-treated with piperonyl butoxide (PBO), an inhibitor of cytochrome P450-dependent monooxygenase activity or with L-buthionine sulfoximine (L-BSO), a selective inhibitor of GSH synthesis. Liver and kidney transcript profiles of each animal were analyzed on rat DNA microarrays (RAE230 2.0+, Affymetrix). Clinical biochemistry, urinalysis, hematology, and histopathology were also investigated. Preliminary results suggest that the inhibition of cytochrome P450-dependent monooxygenase activity by PBO showed a moderate effect on the expression of genes involved in pathways of toxicity. However, inhibition of glutathione synthesis by L-BSO exhibited significant effects on the expression of genes involved in several pathways of toxicity. This data is consistent with the histopathologic evaluation as pre-treatment with L-BSO resulted in an increase in the severity of liver and renal toxicities. The concentration of the reactive metabolite was also increased together with decreased plasma GSH levels. These data provide additional insight into the mechanism of APAP induced liver and kidney toxicity and the use of a pre-sensitized animal model.

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### DECREASED ACETAMINOPHEN AND BROMOBENZENE-INDUCED HEPATOTOXICITY AND LETHALITY IN TYPE 2 DIABETIC MICE.

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Our previous studies showed that type 2 diabetic (DB) rats are highly sensitive to diverse hepatotoxicants (allyl alcohol, CCl<sub>4</sub> & bromobenzene). The present objective was to test whether marked species difference exists in the sensitivity of type 2 diabetes to model hepatotoxicants. Type 2 diabetes was induced in male Swiss Webster mice (25-30 g) by feeding high fat diet (20% fat, 24% protein, 54% carbohydrate) for 2 months followed by injection of streptozotocin (STZ, 120 mg/kg, ip, in citrate buffer). Ten days after STZ injection, the DB mice exhibited type 2 diabetes as evidenced by normoinsulinemia, hyperglycemia, impaired fasting glucose, and oral glucose intolerance. Exposure of DB mice to normally LD<sub>50</sub> doses of acetaminophen (APAP, 600 mg/kg, ip) and bromobenzene (0.5 mg/kg, ip) resulted in only 20% mortality indicating protection. To investigate the mechanism of this protection, a time course study (0 to 96 h) was conducted after injection of APAP. Liver injury progressed in the non-DB mice resulting in 80% mortality between 6 to 24 h after APAP administration. In contrast, even though the liver injury progressed in DB mice till 12 h, it was substantially lower (as indicated by plasma ALT, AST and histopathology) compared to the non-DB mice and furthermore regressed to normal by 96 h. CYP2E1 protein (Western blot) and enzyme activity (p-NP hydroxylation assay) as well as covalent binding of <sup>14</sup>C-APAP were unaltered in the DB mice indicating that the markedly lower hepatotoxicity cannot be explained by decreased bioactivation of APAP. Expression of CYP4A1 protein (Western blot), a well-established marker of PPAR- $\alpha$  activation was same in the DB mice as com-

pared to the non-DB mice, suggesting that PPAR- $\alpha$  activation may not have a role to play in the resiliency of the DB mice. The results indicate that type 2 DB mice are resilient to the hepatotoxicity of APAP and BB and point to a marked species difference in the sensitivity of type 2 diabetes to hepatotoxicity.

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### ROLE OF POLY(ADP-RIBOSE) POLYMERASE (PARP) ACTIVATION IN ACETAMINOPHEN-INDUCED LIVER CELL NECROSIS IN MICE.

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DNA strandbreaks can induce the excessive activation of poly(ADP-ribose) polymerases (PARP), which may lead to oncotic necrosis. Based on controversial findings with chemical PARP inhibitors, the role of PARP activation in AAP hepatotoxicity remains unclear. To assess the role of PARP in the pathophysiology in more detail, we assessed PARP activation by immunostaining for PAR and used both the PARP inhibitor 3-aminobenzamide (3-AB) and PARP gene knock-out mice (PARP-/-). Treatment of C3HeB/FeJ mice with 300 mg/kg AAP resulted in liver injury as indicated by the progressive increase in DNA fragmentation and plasma ALT activities between 3 and 12 h after AAP. PAR staining was detectable in most centrilobular hepatocytes at 6 h after AAP with a further increase in staining intensity at 12 h. Treatment with 500 mg/kg 3-AB 0.5 h before AAP completely eliminated liver injury, DNA fragmentation and PAR staining. However, 3-AB pretreatment attenuated the initial depletion of hepatic glutathione suggesting that 3-AB pretreatment inhibited metabolic activation of AAP. On the other hand, treatment with 3-AB at 1.5 or 2.5 h after AAP showed a partial protective effect as indicated by reduced ALT activities, DNA fragmentation and PAR staining. Treatment of SV129 wildtype and PARP-/- mice with AAP resulted in similar liver injury. However, there was no PAR staining in PARP-/- mice. To evaluate if 3-AB may protect independently of PARP, WT and PARP-/- mice were treated with 3-AB 1.5 h after AAP. 3-AB treatment significantly reduced the increase in plasma ALT activities by 95% in WT and 71% in PARP-/. Conclusion: AAP caused DNA fragmentation followed by PARP activation during the early injury phase after AAP overdose. However, PARP activation does not play a significant role in AAP-induced hepatocyte necrosis. The beneficial effect of the PARP inhibitor 3-AB against AAP hepatotoxicity was due to reduced metabolic activation or other, unknown mechanisms independent of PARP inhibition.

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### PROTEOMIC ANALYSIS OF WHOLE LIVER AND SUBCELLULAR FRACTIONS FOLLOWING ACETAMINOPHEN-INDUCED HEPATOTOXICITY IN MALE RATS.

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While acetaminophen (APAP) is a safe analgesic when taken therapeutically, overdose results in a severe and sometimes fatal centrilobular hepatic necrosis. CYP450-mediated generation of a quinone imine intermediate and binding to critical macromolecules after glutathione (GSH) depletion causes toxicity, although additional mechanisms have been implicated. Proteomic analysis of whole liver homogenates and subcellular fractions using 2D gel electrophoresis and mass spectrometry (2D-MS) was conducted to gain further insight into APAP hepatotoxicity. Male F344 rats treated with 0, a subtoxic (150 mg/kg) and toxic (1500 mg/kg) dose of APAP were sacrificed at pre-toxic (6 hr), toxic (24 hr), and recovery (48 hr) periods of acute APAP hepatotoxicity to identify treatment-related protein changes. Image analysis of the 2D gels revealed that APAP protein expression was most altered at 24 and 48 hr after the toxic dose (1500 mg/kg) of APAP with nearly 100 versus 70 proteins changed, respectively, in whole liver separations. Only minor protein changes were noted at the subtoxic dose. The nuclear fraction from the 48 hr subtoxic dose of APAP also yielded several changes, suggesting that an adaptive nuclear response was initiated. Up- and down-regulated proteins of note included glyceraldehyde 3-phosphate dehydrogenase (upregulated 20-fold), estrogen sulfotransferases 3 and 6, GST-omega, heme scavenger hemopexin, hepatic injury marker purine nucleoside phosphorylase, and Ca<sup>++</sup> binding protein regucalcin. This proteomic study has revealed APAP-related changes in whole liver and nuclear proteins that may prove helpful in understanding the pathophysiology of APAP hepatotoxicity and may eventually lead to prognostic indicators for survival following APAP overdose.

## ROLE OF THE XENOBIOTIC RECEPTOR PXR IN ACETAMINOPHEN HEPATOTOXICITY.

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The pregnane X receptor (PXR) is a key regulator of xenobiotic metabolizing enzymes and transporters at the transcriptional level. To investigate if PXR has a role in acetaminophen (APAP) toxicity, we examined APAP hepatotoxicity and metabolism in male wild-type and PXR(-/-) mice. PXR(-/-) mice were less sensitive to APAP hepatotoxicity than wild-type mice as indicated by plasma levels of ALT. Cytochrome P450 (CYP) 2E1 levels were identical in the two mouse lines. Hepatic levels of CYP3A protein were elevated 2.5-fold in PXR(-/-) mice. In contrast, hepatic levels of CYP1A2 were decreased 3-fold in PXR(-/-) mice. These findings suggest that CYP1A2 has an important role in the development of APAP hepatotoxicity. No significant differences in APAP metabolism were observed between wild-type and PXR(-/-) mice, as measured by the individual levels of glucuronide, sulfate, and glutathione-conjugated metabolites over time. APAP uptake, measured as the level of APAP in the serum over time, appeared to be greater in wild-type mice compared to PXR(-/-) mice, but APAP was not a substrate of the transport protein MDR1. Our overall findings suggest, that in untreated mice, PXR is a positive regulator of CYP1A2 expression. Our finding that APAP hepatotoxicity is decreased in PXR(-/-) mice, shows that PXR is an important modulator of APAP hepatotoxicity, possibly through up-regulation of CYP1A2 expression.

## THE EFFECT OF S-ADENOSYL-L-METHIONINE (SAME) ON MITOCHONDRIAL GLUTATHIONE AND PROTEIN CARBONYLS FOLLOWING ACETAMINOPHEN (APAP) TREATMENT IN MICE.

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Acetaminophen (APAP), in excessive doses, is associated with hepatic toxicity. To date in the United States, one-third of all acute liver failure cases are attributed to APAP. We have shown the protective effects of S-Adenosyl-L-methionine (SAMe), a nutraceutical, on APAP-induced hepatotoxicity in male C57BL/6 mice. In our studies, male C57BL/6 (16-25 g) n= 5 animals/group  $\pm$  SEM were used. The mice were randomly divided into 4 groups, vehicle treated, APAP treated, SAMe treated, and SAMe pretreatment plus APAP. The animals were injected intraperitoneal (ip) with 500mg/kg APAP or water. SAMe was administered as a 500mg/kg dose just prior to administration of APAP or water. We monitored hepatic glutathione levels, changes in ALT, lipid peroxidation levels, liver weight and histology as parameters of APAP toxicity. SAMe protected the liver from APAP-induced damage by decreasing ALT and lipid peroxidation levels as well as preventing depletion of glutathione. Further studies investigated the protective effects of SAMe pretreatment against APAP-induced hepatotoxicity on liver mitochondrial glutathione, 4-hydroxy-2-nonenal (4HNE) adducted proteins and protein carbonyls. The results of this study have found that SAMe protects the liver from production of protein carbonyls and 4HNE adducts. One particular protein carbonyl that we have found has an approximate mass of 40 kDa. Interestingly, cytochrome P450 2E1, the enzyme responsible for producing the toxic APAP metabolite N-acetyl-benzoquinone imine, has an active form (Delta 2E1) with a mass of 40 kDa. The mechanism of SAMe reduction of APAP toxicity may be identified by characterizing specific cellular targets and identifying particular proteins affected by SAMe and APAP.

## EVIDENCE SUGGESTING A ROLE FOR NAD(P)H:QUINONE OXIDOREDUCTASE 1 IN THE PROTECTION AGAINST ACETAMINOPHEN HEPATOTOXICITY BY CLOFIBRATE TREATMENT.

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Mice pretreated with the peroxisome proliferator clofibrate (CFB) are highly resistant to acetaminophen (APAP) hepatotoxicity. While the mechanism of protection is not entirely clear, CFB diminishes APAP arylation of hepatic proteins and glutathione depletion without affecting its bioactivation by CYP450. These results

suggest that treatment with CFB enhances the detoxification of the reactive metabolite of APAP, N-acetyl-p-benzoquinone imine (NAPQI). Considering that the putative toxic metabolite of APAP is a quinone, we investigated the effect of CFB treatment on mRNA levels, protein expression and catalytic activity of the detoxifying enzyme NAD(P)H quinone oxidoreductase 1 (NQO1) in the liver. Enhanced reduction of NAPQI back to APAP by NQO1 could explain this hepatoprotection. Administration of CFB (500 mg/kg, i.p.) to male CD-1 mice daily for 5 or 10 days resulted in a significant induction of NQO1 enzyme activity. Branched DNA signal amplification showed an increase in mRNA levels for NQO1 in the 5-day treatment regimen only. NQO1 protein expression was upregulated 1.3- and 1.8-fold in the 5 and 10-day treatments, respectively. Additionally, inclusion of human recombinant NQO1 (hNQO1) in an *in vitro* activating system for APAP significantly decreased the amount of NAPQI measured in microsomal incubations. Supplementing microsomal reactions with hNQO1 along with the NQO1 inhibitor dicumarol prevented this reduction in NAPQI measured. Similarly, inclusion of a catalytically inactive mutant form of the enzyme also restored microsomal NAPQI content to control levels. In summary, CFB-mediated induction of NQO1 coupled with the ability of NQO1 to decrease NAPQI levels suggests its possible role in diminishing APAP hepatotoxicity.

## ROLE OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IN ACETAMINOPHEN HEPATOTOXICITY.

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Vascular endothelial growth factor (VEGF) is a known mitogen for endothelial cells. Previous data have shown that VEGF is upregulated in models of partial hepatectomy and ischemia reperfusion injury. It was also recently shown that VEGF increases hepatic mitogens *in vitro* (i.e., hepatocyte growth factor and interleukin 6). We investigated the importance of VEGF in hepatocyte regeneration following acetaminophen (APAP) toxicity. Initial immunohistochemistry analyses for VEGF showed staining in the centrilobular regions of the liver of B6C3F1 mice treated with APAP (300 mg/kg i.p.) and sacrificed at 48 and 72 hours. Further studies were conducted in mice treated with APAP and sacrificed at 1, 2, 4, 8, and 12 hours. ALT was significantly increased at 4 hours and VEGF levels in liver homogenates were significantly increased at 12 hours ( $p \leq 0.01$ ). To investigate a possible role for VEGF in hepatocyte regeneration, SU5416, an inhibitor of VEGF receptor signaling, was administered to APAP treated mice. SU5416 alters VEGF mediated signaling through the inhibition of tyrosine kinase. In initial studies, mice were dosed with APAP, followed by SU5416 or saline at 3 hours, and sacrificed at 24 hours. No difference in ALT values was apparent at 24 hours in the two groups of mice. To examine the effect of SU5416 on hepatocyte regeneration, mice were dosed with APAP, followed by SU5416 or saline at 3 hours, then sacrificed at 48 and 72 hours. SU5416 (25 mg/kg s.c.) treated mice had ALT values that were approximately 70% higher at 48 hours, compared to mice receiving APAP only ( $p \leq 0.05$ ). Subsequent immunohistochemical analysis for proliferating cell nuclear antigen (PCNA), a marker of cellular proliferation, showed markedly reduced staining at 48 and 72 hours in the mice that received SU5416 after APAP, compared to the APAP only treated mice. These findings suggest that endogenous VEGF production has significant hepatoprotective effects in APAP toxicity by increasing hepatocyte regeneration.

## LIPOPOLYSACCHARIDE POTENTIATES ACETAMINOPHEN HEPATOTOXICITY.

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The doses of acetaminophen (APAP) required to cause hepatotoxicity vary considerably in people. Previous studies in animals demonstrated that nontoxic doses of some xenobiotic agents are rendered hepatotoxic by a mild inflammatory episode, suggesting that modest inflammation may be a determinant of sensitivity to hepatotoxicity. Accordingly, we tested the hypothesis that cotreatment of mice with normally nonhepatotoxic doses of lipopolysaccharide (LPS) and APAP leads to development of liver injury. Fasted, C57BL/6 mice were treated with either saline or LPS (44 x 106 EU/kg, ip), two hours before or two hours after treatment with APAP (100-250 mg/kg, ip) or saline. Mice were killed 24 hours after APAP-treatment, and hepatotoxicity was estimated from increased serum alanine aminotransferase (ALT) activity and evaluation of liver histopathology. No elevation in serum ALT activity was observed in mice that received vehicle or LPS alone. LPS cotreatment (either pre- or post-APAP) caused a leftward shift of the dose-response curve for APAP-induced hepatotoxicity. This effect was most pronounced in mice that re-

ceived LPS 2 hours before APAP. Consistent with the increase in serum ALT activity, histopathology revealed centrilobular, oncocytic necrosis only in livers of LPS/APAP-treated mice. Hepatic fibrin deposition was observed in livers of LPS/APAP-treated mice but not in livers from mice treated with either agent alone. The results suggest that normally noninjurious doses of APAP are rendered hepatotoxic by a modest inflammatory response. Furthermore, in APAP-treated mice, inflammation was associated with alterations in the hemostatic system. (Supported by NIH grant DK061315.)

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#### POTENTIATION OF 3-(3, 5-DICHLOROPHENYL)-2, 4-THIAZOLIDINEDIONE (DCPT)-INDUCED HEPATOTOXICITY IN RATS BY DEXAMETHASONE (DEX) PRETREATMENT.

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3-(3, 5-Dichlorophenyl)-2, 4-thiazolidinedione (DCPT) is hepatotoxic in rats. This compound is structurally similar to insulin-sensitizing drugs, such as troglitazone, which have been shown to cause liver damage in some type-II diabetic patients. Data from the literature suggest that cytochromes P450 may have a role in generating hepatotoxic metabolites from troglitazone. The purpose of this investigation was to determine if the CYP3A inducer dexamethasone (DEX) could potentiate the hepatotoxic effects of a known non-toxic dose of DCPT. Male Fischer 344 rats ( $n = 4$ /group) were administered DCPT (0.2 mmol/kg) i.p. in corn oil after a three day dosing regimen of DEX (50 mg/kg/day) given i.p. in corn oil. Control rats were pre-treated with either corn oil (CO, 4 mL/kg/day) or DEX (50 mg/kg/day) for three days prior to a single dose of corn oil (4 mL/kg). All animals were euthanized 24 hours post-dosing. Our previous experiments with known hepatotoxic doses ( $> 0.2$  mmol/kg) of DCPT showed elevations in serum alanine aminotransferase (ALT) levels. ALT levels in rats in the DEX/DCPT group ( $160 \pm 16.0$  Sigma Frankel Units/mL) were significantly greater ( $p < 0.05$ ) than those in the CO/CO ( $34.3 \pm 6.9$ ), CO/DCPT ( $37.9 \pm 9.9$ ) or DEX/CO ( $74.5 \pm 8.2$ ) groups. Liver histology of CO/DCPT treated animals showed minimal change from the CO/CO treated group. In contrast, a significant hepatotoxic effect was seen in the liver histology of both the DEX/CO and DEX/DCPT treatment groups. In conclusion, these results suggest that CYP3A isozymes may be involved in DCPT-induced liver damage in rats. However, the nature of any hepatotoxic metabolites that are produced from DCPT requires further investigation. Supported by PHS grant ES012499.

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#### PROTECTIVE EFFECT OF DIBROMOACETATE IN PRIMARY HEPATOCYTE CULTURES.

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Chlorinated and brominated acetates are components of chlorinated drinking water. In addition di- and trichloroacetate (DCA and TCA) are the presumed hepatocarcinogenic metabolites of the groundwater contaminant trichloroethylene. These compounds have been shown to differentially induce peroxisome proliferation in mouse liver and in rodent hepatocyte cultures. One proposed mechanism for peroxisome proliferator-induced hepatocarcinogenesis is that suppression of normal hepatocyte apoptosis may promote survival of tumorigenic cells. DCA has been shown by others to inhibit spontaneous apoptosis in mouse liver. Previous studies in our lab have suggested that addition of DCA (1 mM) to sub-confluent hepatocyte cultures can ameliorate the normal cell loss that occurs in hepatocyte cultures. In the present studies we determined whether addition of various dihalogenated acetates to primary rat hepatocyte cultures could prevent the normal loss of cells from the cultures. Isolated rat hepatocytes were plated at confluence and treated, beginning 20 hrs after attachment, with 1 mM dibromo-, dichloro-, or difluoroacetate (DBA, DCA or DFA) for 48 hrs. Cell number was determined by measuring the reduction of 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). DBA treatment resulted in a significant decrease in cell loss over 48 hrs. In contrast, DCA and DFA treatment had no effect on loss of cells from the cultures. We also determined the effect of these acetates on TGF $\beta$ 1-induced hepatocyte cell death. TGF $\beta$ 1 is presumed to induce cell death in primary hepatocyte cultures via apoptosis. DBA (1 mM) treatment significantly decreased TGF $\beta$ 1 induced cell death, as determined by the MTS assay after 48 hrs. Treatment with 1 mM DCA or 1 mM DFA had no effect. Further studies are required to determine whether DBA is acting to prevent apoptosis or is acting to maintain the cell cultures and prevent toxicity through another mechanism. Supported by DOE Cooperative Agreement DE-FC09-02CH11109.

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#### NICOTINAMIDE ALTERATION OF BROMOBENZENE-INDUCED HEPATOTOXICITY.

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Previous studies have shown extensive cellular damage can activate poly(ADP-ribose) polymerase-1 (PARP-1) and cause a rapid decrease in the levels of NAD<sup>+</sup> and ATP, thereby preventing apoptosis and promoting necrosis and inflammation. The purpose of this study was to extend previous observations that inhibitors of PARP-1 could alter acetaminopen and carbon tetrachloride-induced hepatotoxicity. Bromobenzene (BB) a glutathione dependent hepatotoxicant was tested. Groups of male mice were treated with a single dosage of 112mg/kg (0.075ml/kg) BB by the interperitoneal (ip) route. Each treatment group had at least ten mice per comparison. All animals were maintained in a controlled environment and provided food and water ad libitum. This dosage of BB resulted in hepatotoxicity as measured by an increase in serum alanine transferase (ALT). BB treatment resulted in a 5 fold increase in ALT. Moderate hepatotoxicity was detected with this treatment regimen. Subsequently, another group of mice was treated with BB and three treatments of nicotinamide at 1/2, 1, and 2 hours following BB treatment. The nicotinamide treatment significantly reduced both morbidity and mortality. Serum ALT elevations were reduced by 90% at 24 hours following BB and nicotinamide treatments. BB-induced liver pathology was also blocked by nicotinamide. Mortality among BB treated animals was also significantly reduced by nicotinamide treatment. Mortality among mice treated with BB and nicotinamide was near control. Inhibition of PARP-1 appears to alter chemical-induced hepatotoxicity that has either a glutathione dependent or independent mechanism. PARP-1 inhibitors may have pharmacological application for modifying chemical-induced liver injury.

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#### COLCHICINE ANTIMITOSIS ABOLISHES SUBCHRONIC CHLOROFORM-INDUCED PROTECTION AGAINST LETHAL DOSE OF CHLOROFORM.

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Previously we showed that subchronic exposure to CHCl<sub>3</sub> (150 and 300 mg/kg/day, po) offered complete protection against a LD<sub>90</sub> dose of CHCl<sub>3</sub> (750 mg/kg, po) in male Swiss Webster mice. The hepatic and renal tissue repair was robust in CHCl<sub>3</sub> pretreated mice, which disallowed progression of tissue injury, thereby leading to recovery and survival. The control group of mice received same volume of vehicle (Alkamuls EL-620, 10 ml/kg) for 30 days prior to challenge with a LD<sub>90</sub> dose of CHCl<sub>3</sub>. Ninety percent mortality was observed in control mice due to inhibited hepatic and renal tissue repair, which permitted progression of liver and kidney injury. Objective of the present investigation was to test the hypothesis that selective ablation of tissue repair by antimitotic colchicine (CLC, 1.5 mg/kg, ip) abolishes the adaptive protection against a lethal dose of CHCl<sub>3</sub>. Male mice (25-29 g) were pretreated with 150 mg CHCl<sub>3</sub>/kg/day via aqueous gavage for 30 days prior to challenge with a single LD<sub>90</sub> dose of CHCl<sub>3</sub>. Subchronically primed mice either received CLC or saline at 42 h after the lethal dose of CHCl<sub>3</sub>. Liver and kidney injuries were measured by plasma ALT and BUN, respectively, and by histopathological examination of H & E stained tissue sections. Tissue repair was assessed by <sup>3</sup>H-thymidine incorporation and proliferating cell nuclear antigen immunohistochemistry. In mice not receiving CLC, liver injury regressed after 48 h leading to 100 % survival. CLC intervention led to inhibited cell division and tissue repair in the primed mice receiving lethal challenge with CHCl<sub>3</sub>. Consequently, liver injury progressed leading to 40 % mortality by 72 h. Renal injury of CHCl<sub>3</sub> was unaffected by CLC intervention. These findings suggest that compensatory tissue repair plays central role in the subchronic CHCl<sub>3</sub>-induced protection against lethal dose of chloroform (Supported by ATSDR U61/ATD 681482).

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#### UNDERNUTRITION FAILS TO EXACERBATE ALCOHOL-INDUCED LIVER DAMAGE DESPITE EVIDENCE OF INCREASED OXIDATIVE STRESS.

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Mechanisms and risk factors leading to alcohol-induced liver damage (ALD) remain obscure although it is widely accepted that nutrition plays important roles in modulating liver damage. Undernutrition is often present in alcoholics. To investigate the effects of undernutrition in ALD, female Sprague-Dawley rats (200g) were

infused by Total Enteral Nutrition (TEN) with or without EtOH (11g/kg/d). Diets were infused for 14 h using the overnight infusion model (1800 to 800h). Twenty-four hour urine EtOH concentrations were measured. Rats were sacrificed on day 50 and serum samples for assessment of alanine aminotransferase (ALT) and livers were collected. A combination of undernutrition and EtOH increased induction of both cytochrome P450 (CYP) 2E1 and CYP4A1 mRNA ( $p<0.05$ ). Western blotting showed that CYP2E1 apoprotein was elevated 3 and 6 fold in the 187 kcal EtOH and 154 kcal EtOH groups, respectively ( $p<0.05$ ). This was accompanied by a significantly enhanced activation of carbon tetrachloride dependent lipid peroxidation, a CYP2E1 activity marker, accompanied by an increase of thiobarbituric acid-reactive substances in liver homogenates only in the 154 kcal EtOH group ( $p<0.05$ ), indicating oxidative stress. In addition, EtOH clearance was impaired ( $p<0.05$ ), but was not accompanied by changes in ADH mRNA. Despite evidence of P450-dependent increases in reactive oxygen species production and significantly impaired EtOH clearance in the 154kcal EtOH group, alcoholic liver pathology showed comparable steatosis, macrophage infiltration and focal necrosis in both EtOH groups. ALT levels were elevated, but not significantly different between the two EtOH groups. This suggests that undernutrition does not exacerbate ALD, but appears to provide a protective response to enhanced oxidative stress. (This work was supported by RO1 AA12819 M.R.)

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#### RESILIENCY OF AGED FISCHER 344 RATS TO CHLORDECONE-AMPLIFIED CARBON TETRACHLORIDE TOXICITY.

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Objective was to investigate the effects of chlordecone (CD) +  $CCl_4$  in 3 (adult), 14 (middle aged), 24 month old (old aged) male F344 rats. After pretreatment with either 10 ppm dietary CD or normal diet, rats were challenged with a single non-toxic dose of  $CCl_4$  (100  $\mu$ l/kg, ip) or corn oil vehicle on day 16. Liver injury was assessed by plasma ALT, AST, and histopathology during a time course of 0 to 96 h. Liver tissue repair was measured by [ $^3H$ ]-thymidine incorporation into hepatic nuclear DNA, and PCNA immunohistochemistry. CYP2E1 protein, enzyme activity, *in vivo* metabolism of  $^{14}CCl_4$ ,  $^{14}CCl_4$ -derived covalent binding (2 & 6 h), and hepatic lipid peroxidation were estimated in all the age groups with or without CD diet. Exposure to  $CCl_4$  alone did not cause any mortality. The combination led to 100% mortality of young adult rats by 72 h, whereas no mortality occurred in the aged rats. Adult and middle aged rats exposed to CD +  $CCl_4$  had identical liver injury up to 36 h. Thereafter, liver injury escalated only in adults while it declined in middle aged rats. In the 24-month-old rats initial liver injury never developed to the level of the other two age groups but declined after 36 h as in the 14-month-old rats. Regardless of exposure to CD diet neither hepatomicrosomal CYP2E1 protein, the enzyme activity, nor covalent binding of  $^{14}CCl_4$ -derived radiolabel differed between the age groups. *In vivo*  $^{14}CCl_4$  metabolism was similar in all the age groups. Lipid peroxidation at 6 h was higher in the older rats, and CD pretreatment did not affect lipid peroxidation. Plasma glucose did not change in any age group. Hepatic glycogen was depleted only in adult rats exposed to CD +  $CCl_4$ . Liver tissue repair ( $^3H$ -T, PCNA) was inhibited in adults in contrast to prompt and robust response in the aged rats. Consequently, liver injury progressed only in the young adult rats. In conclusion, the remarkable resiliency of aged rats to CD +  $CCl_4$  toxicity appears to be due to prompt and robust stimulation of compensatory liver tissue repair response (NIH/AG19058).

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#### PENTOXIFYLLINE AND ANTI-TUMOR NECROSIS FACTOR $\alpha$ ANTIBODIES ARE UNABLE TO PROTECT MICE FROM FUMONISIN HEPATOTOXICITY.

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Fumonisin B<sub>1</sub> (FB<sub>1</sub>), the most abundant and toxic fumonisins produced by *Fusarium verticillioides* detected in corn and corn-based foods, causes species- and organ-specific toxicity such as equine leukoencephalomalacia, porcine pulmonary edema, and liver and kidney damage in most animal species. Fumonisin B<sub>1</sub> disrupts sphingolipid metabolism by inhibiting ceramide synthase and induces expression of many cytokines including tumor necrosis factor (TNF)  $\alpha$ . This study was designed to investigate the role of TNF $\alpha$  signaling in FB<sub>1</sub> hepatotoxicity. Male C57BL/6N mice were injected with pentoxifylline at 150 mg/kg twice a day for 5 days to inhibit TNF $\alpha$  production or anti-TNF $\alpha$  antibodies to block TNF $\alpha$  signal transduction. Mice were injected subcutaneously with 2.25 mg FB<sub>1</sub>/kg daily for 5 days, and euthanized one day after the last treatment. Blood and tissues were sampled. Results showed that both pentoxifylline and anti-TNF $\alpha$  antibodies augmented FB<sub>1</sub>-induced hepatotoxicity indicated by increases in plasma alanine aminotransferase and aspartate aminotransferase, and number of apoptotic hepatocytes. Pentoxifylline significantly reduced accumulation of free sphinganine and expression of TNF $\alpha$ . Anti-TNF $\alpha$  antibodies did not alter FB<sub>1</sub>-induced accumulation of free sphingoid bases and expression of TNF $\alpha$  in liver following FB<sub>1</sub> treatment. Neither pentoxi-

fyline nor anti-TNF $\alpha$  antibodies further increased FB<sub>1</sub>-induced expression of interleukin-12, interferon  $\gamma$ , and TNF $\beta$ , which are important inflammatory cytokines. Treatments did not alter expression of Fas and Fas ligand. Results suggest that the increased hepatotoxicity may be due to disruption of TNF $\alpha$  cellular repair mechanisms following inhibition of TNF $\alpha$  signaling. (Supported in part by USPHS ES 09403).

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#### CALPASTATIN OVER-EXPRESSION PROTECTS AGAINST TOXICANT-INDUCED PROGRESSION OF INJURY ASSOCIATED WITH ACUTE LIVER FAILURE IN MICE.

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We have recently reported that calpain released from the necrotic hepatocytes is the principal mediator of progression of acute liver injury (Limaye et al. *Toxicol. Appl. Pharmacol.* 191: 211-226, 2003). Timely onset of compensatory tissue repair is known to obtund the progression of injury and prevent acute liver failure (ALF) due to resistance of dividing/newly divided cells. Objective of the present work was to test the hypothesis that over-expression of calpastatin (CAST), an endogenous calpain inhibitor, in the dividing/newly divided hepatocytes is the underlying mechanism of their resistance against progression of injury. CAST expression was monitored over a time course in  $CCl_4$ -damaged (2 ml  $CCl_4$ /kg, ip, male SD rats) livers undergoing compensatory tissue repair and in two other models of liver cell division - early postnatal development (20 day old rats) and 70% partial hepatectomy in adult male SD rats. In each model of dividing/newly divided hepatocytes, CAST over-expression was confirmed by real-time RTPCR, Western blot, and immunohistochemical staining of liver sections. Over-expression of CAST declined to normal low levels with the cessation of cell proliferation. Cessation of cell division is known to restore the sensitivity to hepatotoxicants. These findings suggest that over-expression of CAST in the dividing/newly divided hepatocytes might underlie the resistance against progression of injury. To test this possibility, hepatic over-expression of CAST was induced in adult SW mice livers via *in vivo* adenovirus transfection, and challenged with a normally lethal dose of APAP (600 mg/kg, ip). Progression of APAP-induced liver injury was markedly attenuated in CAST over-expressing mice leading to 50% survival. These findings provide strong evidence for calpastatin's role in the resistance of dividing/newly divided cells against progression of injury initiated by APAP, culminating in ALF and animal death.

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#### IMPAIRED HEPATIC REGENERATION IN METALLOTHIONEIN KNOCKOUT MICE AFTER PARTIAL HEPATECTOMY.

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Although the translocation of metallothionein (MT) from cytoplasm to nucleus has been demonstrated in liver during times of high requirement for zinc (fetal development and the neonatal period), the role of MT in cellular growth is not well understood. In the present study, a potential role of MT in liver regeneration was investigated in wild type (WT) and MT I and II gene knockout (MT-null) mice after 35% partial hepatectomy (PH) or sham laparotomy. Hepatic MT levels and proliferation index were measured at 0, 5, 15, 24, 36, 48, and 60 hours after PH and 48 hours after sham laparotomy (control). MT levels were increased in WT mice (peak at 24 hours after PH) and declined to normal levels by 60 hours after PH. Immunohistochemical staining for MT in WT mice indicated the presence of MT in both nucleus and cytoplasm of hepatocytes at 24 hours after PH, whereas MT was present mainly in the cytoplasm at 36-60 hours after PH and 48 hours after sham laparotomy. Hepatic proliferation index in both WT and MT-null mice, as determined by argyrophilic nucleolar organizing region (AgNOR) staining and proliferating cell nuclear antigen (PCNA) immunohistochemical staining, reached a peak at 48 hours and declined by 60 hours after PH. Cell proliferation was significantly less in MT-null mice as compared to WT mice during liver regeneration after PH. These results suggest that MT may play a positive role in hepatic regeneration after PH. This research was supported by Canadian Institute of Health Research (CIHR) grants.

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#### THE ROLE OF GLUTATHIONE DEPLETION AND OXIDATIVE STRESS ON CYTOTOXICITY OF CHROMIUM (VI) IN ISOLATED RAT HEPATOCYTES.

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Chromium (VI) is an environmental and occupational carcinogen. Although it has been investigated widely, the mechanism(s) of its action is/are not fully understood. The present study was designed to evaluate the role of Glutathione (GSH) modula-

tion and oxidative stress on cytotoxicity of Cr(VI) in isolated rat hepatocytes. Hepatocytes were isolated by collagenase perfusion technique and were exposed to different concentrations of Cr VI (dichromate) (1 and 5 microM) in a time-course experiment for up to 2 hr. Cr (VI) exposure induced a significant decrease in cell viability and a significant increase in the leakage of hepatic enzymes {alanine transaminase (ALT), asparagine transaminase (AST) and lactate dehydrogenase (LDH)} in a concentration and time-related manner. In the same experiment, GSH content and thiobarbituric acid reactive substances (TBARS) generation were determined as indices of oxidative stress and lipid peroxidation respectively. Cr (VI) exposure resulted in significant depletion of GSH and accumulation of TBARS in a dose and time-related manner. The obtained results suggested that 2 h-exposure of hepatocytes to 5 microM Cr (VI) was accompanied by submaximal responses. Therefore, a subsequent dose-response experiment was designed to evaluate the role of GSH depletion and oxidative stress in Cr (VI) toxicity in hepatocytes at 2 hr. LDH release and TBARS generation were used as indicators in this experiment.

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ANTI-HYPERCHOLESTEROLEMIA DRUGS IN THE STATIN CLASS REDUCE GLUTATHIONE LEVELS AND INCREASE CASPASE 3 ACTIVATION IN AN *IN VITRO* CELL MODEL.

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The ability to identify and understand the potential adverse effects of new drugs early in the development process can help manage patient risk versus clinical benefit. Cerivastatin (CRV), simvastatin (SMV), lovastatin (LOV), atorvastatin (ATV), fluvastatin (FLV) and pravastatin (PRV) are in the statin class of cholesterol lowering drugs used in the treatment of hypercholesterolemia. All have been associated with hepatotoxicity and the skeletal muscle disorder rhabdomyolysis. CRV was recently withdrawn from United States markets because of reports of fatal rhabdomyolysis and high incidence of liver toxicity. It is believed that the mechanisms underlying these adverse effects are linked to energy depletion and apoptosis. The purpose of the present study was to screen the statin drugs in a battery of *in vitro* assays designed to evaluate cytotoxic, apoptotic, and oxidative stress potential. Rat hepatoma (H4IE) cells were seeded into 96-well plates (10, 000/well). Following a 48 hr equilibration period the cells were treated with compounds at concentrations of 0, 0.1, 1, 5, 10, 50, 100, and 300  $\mu$ M for 24 hr. Cytotoxicity was evaluated by measuring membrane leakage, mitochondrial function, and cell number. Oxidative stress was assessed by measuring total glutathione (GSH) and 8-isoprostanate. Apoptosis was determined via caspase 3 activity. The hydrophobic statins (FLV, CRV, SMV, LOV, and ATV) produced a modest decrease in ATP, cell number, and mitochondrial function, but had pronounced effects on the reduction of total GSH pools and induction of caspase 3 activity. In contrast, the hydrophilic statin (PRV) had no measurable effects in this system. These data are consistent with published studies in which the mechanism(s) associated with statin toxicity are energy depletion and induction of apoptosis. Moreover, these data indicate that early screening with a cell-based system that evaluates several biochemical processes can provide important information on the relative safety of new drugs prior to entering animal studies or beginning clinical trials.

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EFFECT OF THIAZOLIDINEDIONE (TZD) RING MODIFICATION ON 3-(3, 5-DICHLOROPHENYL)-2, 4-THIAZOLIDINEDIONE (DCPT) INDUCED HEPATOTOXICITY IN RATS.

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Previously, we observed that DCPT caused hepatotoxicity in F344 rats. DCPT has a TZD ring which is also present in insulin-sensitizing drugs used for the treatment of diabetes. There have been reports of liver toxicity in some patients taking these drugs. Metabolism in the TZD ring might contribute to the hepatic damage. DCPT may be useful for investigating the potential toxicity of TZD ring-containing compounds. 3-(3, 5-Dichlorophenyl)-4-thiazolidinone (4-DCTD), a structural analogue of DCPT, was synthesized and tested to determine if DCPT-induced hepatotoxicity is sensitive to the modifications in the TZD ring. Male, F344 rats were administered DCPT or 4-DCTD (0.4, 0.6 and 1.0 mmol/kg) ip in corn oil. Control animals received corn oil (4 ml/kg) only. Liver and kidney function and morphology were assessed at 24 hr. Serum alanine aminotransferase (ALT) levels were unaltered in the 4-DCTD-treated animals, whereas in the DCPT-treated rats, ALT levels were significantly elevated compared to the controls. In contrast, blood urea nitrogen levels were not altered by any of the compounds. Blood glucose, urine protein and glucose levels were unaffected. There was no change in liver or kidney weights. Histological results showed pockets of liver necrosis in the DCPT-treated rats at 1.0 mmol/kg dose. At lower doses, hepatocellular damage was seen near the centrilobular vein; the cytoplasm in the cells was condensed and irregularly stained.

Sections from the 4-DCTD-treated rats showed no significant changes at any doses compared to the corn oil controls. Except for minor cellular swelling in some proximal tubules, there was no evidence of kidney damage in any of the treatment groups. Our results show that DCPT-induced hepatotoxicity is dependent on the presence of a carbonyl group in the 2 position of the TZD ring. This suggests that an intact TZD ring is critical for toxicity; however, this will require further investigation. Supported by PHS grant ES012499.

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ENDOGENOUS FATTY ACID METHYL ESTERS AS A POTENTIAL MARKER OF HUMAN HEPATOCELLULAR CARCINOMA.

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Hypomethylation of DNA has been considered a major cause/effect of hepatocellular carcinoma. Due to the known higher rate of methylation of lipids compared to DNA and protein, fatty acid methyl esters (FAMEs) can serve as a surrogate marker of DNA methylation. In the present study, we determined the levels of FAMEs and determined corresponding synthetic and the hydrolytic activities in normal human liver and hepatoma tissues to comprehend altered homeostasis of FAMEs in hepatocellular carcinoma. Lipids were extracted from normal human liver and hepatoma tissues, separated by thin layer chromatography (TLC) and analyzed by gas chromatography. Hydrolytic and synthetic activities towards FAMEs were determined in the post nuclear fractions using [ $^3$ H-9, 10] methyl palmitate and [ $1-^{14}$ C] oleic acid as respective substrates. The resultant product(s) was separated by TLC and the radioactivity corresponding to the relative flow of fatty acid and ester was determined. The FAMEs levels were found to be drastically lower in hepatoma (4.5  $\mu$ g/g) than in the normal liver tissue (31.4  $\mu$ g/g). Both hydrolytic and synthetic activities were also decreased to 1724 vs. 554 nmol/hr/mg protein and 169 vs. 26 nmole/hr/mg protein in normal liver and hepatoma tissue, respectively. These results indicate that global hypomethylation in hepatocellular carcinoma can be monitored by the levels of FAMEs and related hydrolytic and synthetic activities.

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AN INVESTIGATION INTO THE RELATIONSHIP OF VITAMIN A LEVELS AND HEPATITIS, HYPERPLASIA, BENIGN OR MALIGNANT LESIONS IN RODENT LIVERS.

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This poster presents the results of an investigation into the relationship between vitamin A levels in different liver lesions, and its correlation with the immunohistochemical staining pattern of retinol-binding protein. Vitamin A serves a number of functions in the body, it is known to be required for the maintenance of healthy epithelial tissue. Vitamin A-deficient animals have been shown to be more susceptible to both infections and cancer. The decreased resistance to infections is thought to be due to the keratinisation of the mucosal cell lining the respiratory, gastrointestinal, and genitourinary tract. It also has been suggested that vitamin A-deficiency may impair the immune system. The protective effect of vitamin A against many forms of cancer might be due to the antioxidant potential of beta-carotene (source of vitamin A) and the effects of retinol and retinoic acid in regulating cell growth. Retinol represents the most important transport and storage form of vitamin A. More than 90% of the body's vitamin A reserves are stored in the liver, where it is bound to a cellular retinol-binding protein (RBP). In this study retrospective samples of liver were obtained from in-house rat carcinogenicity studies. The liver samples were to include normal tissue, hepatitis, hyperplastic, benign, and malignant lesions. Which have been previously diagnosed/observed by H&E histopathology. Immunohistochemical staining patterns were observed using an antibody against RBP in each of these tissues. RBP demonstrated a cytoplasmic staining pattern and the results indicated a partial correlation between vitamin A and the different liver lesions.

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CHARACTERIZATION OF A HIGH-POTENCY ENDOGENOUS LIGAND FOR THE ARYL HYDROCARBON RECEPTOR.

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A putative endogenous ligand for the aryl hydrocarbon receptor (AhR) was recently isolated from pig lung and identified as 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) [PNAS 99:14694]. We have synthesized this compound and further examined its activity in several *in vitro* and *in vivo* assays compared with the toxic ligand, TCDD. Electrophoretic mobility shift assays of AhR in

Hepa1c17 cell cytosol showed similar potency of activation by ITE and TCDD and similar levels of binding to the dioxin response element (DRE). In both Hepa (mouse) and human HepG2 cells stably transfected with a DRE-dependent reporter gene construct, ITE was less potent than TCDD, but elicited strong transactivation of luciferase activity which followed a similar time-course as in TCDD-treated cells. Immunocytochemistry showed rapid and strong nuclear localization of the AhR following ITE treatment of Hepa cells at 10nM and above. CYP1A1 protein induction by ITE was dose- and time-dependent; maximum levels reached were lower than maximum levels induced by TCDD. ITE (20 $\mu$ g/kg) was also administered i.p. to pregnant DRE-LacZ transgenic mice. The pattern of X-gal staining in the fetuses, indicative of AhR activation, was the same as that produced by TCDD [most prominent in genital tubercle, ear, paws]. Thus, despite the likelihood that it is readily metabolized, ITE (or an active metabolite) was able to cross the placenta and activate AhR in fetal tissues. Based on all these observations, we conclude that although ITE may differ from TCDD with respect to its cellular kinetics, it interacts with AhR very similarly to TCDD, and is a good candidate endogenous AhR ligand. (Supported by NIEHS Grant ES01247, ES09430, ES07026, and ES09702.)

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#### NATURALLY OCCURRING ANTAGONISTS OF AHR PATHWAY: A NOVEL APPROACH OF CANCER CHEMOPREVENTION?

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The aryl hydrocarbon receptor (AHR), is a cytosolic receptor which upon activation by its agonists, translocates into the nucleus and forms a dimer with the ARNT (Aryl Hydrocarbon nuclear translocator). AHR/ARNT dimer alters the regulation of AHR battery genes by binding to the dioxin responsive elements (DRE). Many AHR agonists, like the polycyclic aromatic hydrocarbons and polyhalogenated hydrocarbons are human carcinogens. For example, dioxin or TCDD (2, 3, 7, 8 tetrachlorodibenzo-p-dioxin), is the best characterized and most potent AHR agonist and is an ubiquitous environmental contaminant class-I carcinogen. In addition, many dietary constituents that have chemopreventative properties are antagonists of the AHR pathway. Thus, by using naturally occurring antagonists of the AHR pathway one can propose a chemopreventative approach towards inhibiting many human cancers. In our quest to find a potent, naturally occurring antagonist of the AHR pathway, we have assayed the impact of 15 flavonoid compounds to inhibit ligand binding, DNA binding and transactivation of the AHR. Our results indicate that of these compounds, the most potent AHR antagonists are apigenin, emodin, kaempferol and resveratrol.

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#### IMPACT OF NH-TERMINAL TAGS ON AH RECEPTOR LOCALIZATION AND DEGRADATION IN CELL CULTURE.

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The expression of a peptide/protein tag on the NH or COOH terminus of a target protein has become an important aspect of studying protein function in cell culture and transgenic animals. Since the expressed tag may impact the function of the target protein, studies were carried out to evaluate the impact of 3 distinct tags on AHR function in cell culture models. The AHR was tagged at the NH terminus with the FLAG epitope (8 amino acids), HIS/XPRESS tag (35 aa) or EGFP (238 aa). The tagged AHR was ligated into the pSI eukaryotic expression vector that drives expression with an SV40 promoter. In transient expression assays, all tagged AHRs were expressed at high levels and showed reduced levels of ligand-mediated degradation as well as both cytoplasmic and nuclear location as compared to control AHR that was not tagged. Since the high level of protein expression in transient assays are generally not physiological and may produce aberrant protein location and interactions, the tagged AHRs were ligated into retroviral expression vectors and used to generate stable cell lines. The advantage to this approach is that there are a low numbers of integration events, the cellular population is homogeneous and the level of protein expression is more physiologic. Several cell lines were selected for each tagged AHR as well as for control AHR with no tag. Western blot analysis revealed that the level of expression of the AHR in each cell was consistent with the level of AHR in wild type Hepa-1 cells and the subcellular location of the tagged AHRs was generally cytoplasmic. Importantly, exposure of the cells to TCDD resulted in nuclear accumulation of the AHR and degradation. However, the overall magnitude of AHR degradation was reduced when a tag was present on the NH-terminus. These studies suggest that the tagging of the AHR may impact ligand-mediated degradation and that the NH-terminus may be important in recognition of the degradation machinery. Supported by ES 10991.

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#### COMPUTATIONAL APPROACH TOWARDS IDENTIFYING GENES WITH DIOXIN RESPONSE ELEMENTS IN HUMAN, MOUSE, AND RAT GENOMES.

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Computational Approach Towards Identifying Genes with Dioxin Response Elements in Human, Mouse, and Rat Genomes. H.M. Theobald, C.M. Vezina, and R.E. Peterson. University of Wisconsin-Madison, School of Pharmacy. The aryl hydrocarbon receptor mediates 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) toxicity by modifying transcription of genes containing promoter-based dioxin response elements (DREs). The current study employed a computational approach to identify putative DRE-driven genes in the mouse, rat, and human genomes. Genetic promoter regions (-5000 to +1 bp, relative to the transcription initiation site) were obtained from human, mouse, and rat Golden Path and Ensembl genome assemblies and subjected to DNA pattern matching to identify TNGCGTG consensus DRE heptamers. Sequence alignment of DRE-flanking regions in human, mouse, and rat gene homologs was employed to identify genes harboring DREs that were conserved among multiple species. This analysis yielded 34 gene promoters that contained DREs whose position relative to the transcription initiation site was similar (within 500bp) and exhibited significant sequence homology between mouse, rat, and human DRE-flanking sequences. An additional 255, 120, and 27 gene promoters exhibited one or more DREs with position and sequence conservation between rat and mouse, human and mouse, and human and rat, respectively. RT-PCR of liver, lung, kidney, and brain from vehicle and dioxin exposed (25  $\mu$ g/kg po, 24 hrs) adult male C57Bl/6J mice and urogenital sinus tissues from male mice exposed as fetuses (5  $\mu$ g/kg maternal dose, gestation day 13-16) was employed to confirm the dioxin responsiveness of selected genes containing these putative DRE sequences. (NIH grant ES01332).

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#### IDENTIFICATION OF ZEBRAFISH ARNT1 HOMOLOGS: TCDD DEVELOPMENTAL TOXICITY IN ZEBRAFISH REQUIRES ARNT1.

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In order to use the zebrafish as a model to study the effects of TCDD on early development, it is essential to know which proteins are involved in mediating toxicity. Previous work has identified zfAHR2 as the receptor which binds TCDD leading to downstream effects. One previously identified form of zfARNT2, zfARNT2b, can form a functional heterodimer with zfAHR2 *in vitro*. However, zfarn2 null mutants show no protection against endpoints of TCDD developmental toxicity demonstrating that zfARNT2b cannot be the physiological dimerization partner for zfAHR2 mediating several responses to TCDD in zebrafish embryos. The purpose of the current study was to identify an alternate dimerization partner(s) for zfAHR2 that may function to mediate TCDD developmental toxicity. The zebrafish genome was searched and a novel partial transcript was identified as a likely zebrafish isoform of ARNT1. The PCR-based RACE technique was used to obtain the 5' and 3' ends of the transcript. Two forms of cDNA that appear to be alternate mRNA splice variants were detected. Analysis of the zfARNT1 proteins *in vitro* demonstrated that one form of zfARNT1, zfARNT1a, can form a functional heterodimer with zfAHR2 that specifically binds xenobiotic response elements (XREs) in gel shift experiments and induces XRE-driven transcription in COS-7 cells exposed to TCDD. However, the other form, zfARNT1b, appeared nonfunctional with zfAHR2. A morpholino targeted against the 5' end of zfARNT1 (zfarn1-MO) was used to demonstrate that a zfARNT1 protein is a functional dimerization partner for zfAHR2 *in vivo*. Injection of the zfarn1-MO prior to TCDD treatment significantly decreased the induction of zfcYP1A mRNA and protein. In addition, the zfarn1 morphants were completely protected against TCDD-induced pericardial edema and showed partial protection against reduced peripheral blood flow and craniofacial malformations caused by TCDD. UW Sea Grant.

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#### 5, 6-LEUKOTRIENE A4 A IS POTENT ACTIVATOR OF THE AH RECEPTOR.

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The aryl hydrocarbon receptor (AhR) is a ligand activated transcription factor that can be activated by a broad range of structurally diverse chemicals ranging from environmental carcinogens to dietary metabolites. While the most potent agonists thus far identified are of synthetic origin, an increasing number of natural com-

pounds have been shown to activate the Ah receptor. Evidence supporting an endogenous role for the AhR in normal biology has been established; however no high affinity endogenous ligand has been identified. We now report the ability of 5, 6-Leukotriene A4 (LTA4), a lipoxygenase metabolite of arachidonic acid, as a potent activator of the Ah receptor pathway in HepG2 cells. Electrophoretic mobility shift assays (EMSA) have confirmed the ability of this eicosanoid to directly activate the AhR inducing heterodimerization and subsequent DNA binding. Due to the instability of 5, 6-LTA4 it was imperative to test the subsequent metabolites of this compound for Ah receptor activity. DRE-driven luciferase reporter assays along with gel shift assays have revealed that numerous 5, 12-DiHETEs including the enzymatically formed LTB4 along with 5, 6-DiHETE isomers of both enzymatic and non-enzymatic origin have failed to activate the Ah receptor. This result was anticipated based on data generated via molecular modeling programs. The shape of the DiHETE molecules differs significantly from that of LTA4 due to hydrolysis of the oxido ring. These results taken together indicate that 5, 6-Leukotriene A4, an unstable epoxide intermediate of leukotriene metabolism formed on the nuclear membrane, can directly serve as an AhR agonist.

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ER- $\alpha$  REPRESSES TCDD-INDUCIBLE CYP1A1 GENE TRANSCRIPTION THROUGH A DIRECT PROTEIN-PROTEIN INTERACTION WITH AHR/ARNT.

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The aryl hydrocarbon receptor (AHR) and the aryl hydrocarbon receptor nuclear translocator (ARNT) form a heterodimeric transcription factor that binds a wide variety of environmental pollutants, including 2, 3, 7, 8 tetrachlorodibenzo-p-dioxin (TCDD). The activation of this transcription factor mediates an organism's adaptive responses to environmental contaminants and plays an important role in development and physiological homeostasis. AHR target gene activation can be repressed by estrogen, and estrogen-like compounds. In this study, we demonstrate that a significant component of TCDD-inducible CYP1A1 transcription is the result of recruitment of ER $\alpha$  by AHR/ARNT as a transcriptional co-repressor. TCDD-activated CYP1A1 and CYP1B1 transcription was repressed approximately 50% by 17 $\beta$ -estradiol in MCF-7 cells in the presence of cycloheximide, as determined by reverse transcription/real-time PCR. In the absence of cycloheximide only CYP1A1 transcription was repressed, while that of CYP1B1 was enhanced. Furthermore, we have shown by ChIP that ER $\alpha$  is present at the CYP1A1 enhancer only after co-treatment with E2 and TCDD, in MCF-7 cells. Furthermore, sequential two-step ChIP assays were performed which demonstrate that AHR and ER $\alpha$  are present together at the same time on the CYP1A1 enhancer during transrepression. Taken together these data support a role for ER-mediated transrepression of AHR-dependent gene regulation.

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ROLE OF ARNT2 IN NORMAL DEVELOPMENT BUT NOT TCDD DEVELOPMENTAL TOXICITY IN ZEBRAFISH.

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As a dimeric partner with the aryl hydrocarbon receptor (AHR), aryl hydrocarbon receptor nuclear translocators (ARNTs) play a pivotal role in mediating responses to environmental contaminants such as dioxins through the AhR signaling pathway. zfARNT2b, as a heterodimer with zfAHR2, induced xenobiotic response element- (XRE-) driven transcription in COS-7 cells when treated with 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD). In addition, zfahr2 and zfarn2a, b, c mRNAs were co-localized in the same tissues as the XRE-driven gene, zfcyp1a, after TCDD exposure. Although these results suggest that zfARNT2 may function with zfAHR2 in response to TCDD, two findings demonstrate that zfARNT2 is not the key AHR dimerization partner involved in mediating TCDD developmental toxicity. First, a morpholino oligonucleotide targeted against zfARNT2 and injected at the 1-cell stage into wild type embryos, subsequently exposed to TCDD, failed to protect against toxicity. Second, exposure of ARNT2 null mutant zebrafish embryos to TCDD also resulted in TCDD toxicity. Thus, neither knockdown of ARNT2 expression nor null mutation of ARNT2 rescued embryos from TCDD toxicity. To elucidate the role of ARNT2 in embryonic development (in the absence of TCDD), both ARNT2 morphants and ARNT2 null mutants were assessed. They were found to exhibit no gross malformations during development. However, reduced swimming motility in response to touch and startle stimuli was found in ARNT2 null mutants. Bradycardia and an arrhythmic event were found to characterize heart function and histological assessments revealed abnormal development

of the heart, brain and liver. This study suggests that ARNT1 (rather than ARNT2) may be the key dimerization partner for AHR2 in zebrafish, and although ARNT2 is not essential for TCDD developmental toxicity, it is required for normal brain, liver and heart development. (UW Sea Grant).

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ALTERED CELL CYCLE REGULATION IN AH RECEPTOR-NULL MOUSE FIBROBLASTS.

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2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD) is a ubiquitous environmental contaminant and causes diverse toxic effects. Most of the TCDD toxicity is mediated by the aryl hydrocarbon receptor (AHR). Conflicting evidence from various experimental systems indicates that the activated AHR promotes both cell cycle progression as well as cell cycle arrest. In the present study, we further investigate the role of AHR in cell cycle regulation by expressing the high-affinity mouse wild type AHR and a truncation variant that has a deletion of amino acids 323 to 494 in the PAS B domain with a tetracycline receptor-regulated system in AHR-null mouse fibroblasts. The mutant lacks the retinoblastoma protein (RB) interacting motif LXCE and is constitutively activated. In both AHR- and mutant-expressing cell lines, AHR expression were completely inhibited after 96 hours treatment of 5 $\mu$ g/ml doxycycline, while the expression of ARNT, the AHR nuclear translocator partner was not affected. TCDD treatment for 90 minutes induced the nuclear translocation of the wild type AHR protein. Consistently, Cyp1a1 induction by TCDD treatment in AHR expressing cells was comparable to expression in AHR+/+ fibroblasts, whereas Cyp1a1 mRNA levels were constitutively high in mutant-expressing cells and doxycycline treatment inhibited the induction of Cyp1a1 by TCDD. In both cell lines, doxycycline treatment significantly decreased the cell proliferation rate while TCDD had no significant effects on cell proliferation, suggesting that the presence of AHR, but not its activation by ligand is required for normal cell cycle progression. By flow cytometry, it was shown that the delay of cell cycle progression in the absence of AHR is due to a slow down of G1 and S phases. Gene expression microarray hybridization showed that the expression of a group of cell cycle negative regulators including E2F5, Cullin 5, CyclinG2 in AHR expressing cells were down regulated compared to AHR-null cell lines, providing a molecular basis for the cellular consequences of AHR function. —Supported by NIH grant ES06273

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COMPARISONS OF RAT LIVER GENE EXPRESSION PROFILES FOR TCDD, D3T AND I3C: IMPLICATIONS FOR THE TOXICITY EQUIVALENCE FACTOR (TEF) APPROACH.

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Indole-3-carbinol (I3C) is a weak Ah receptor (AhR) ligand but it is not included in risk assessments of dioxin-like compounds because it is rapidly eliminated. I3C is also a cancer chemopreventive agent in animals, and is believed to act through multiple mechanisms to affect pre- and post-initiation steps in carcinogenesis. Some of this activity is attributed to its stimulation of metabolic enzymes, favoring detoxification. Although the estimated dioxin TEF for I3C is only 0.0001, its prevalence in the diet indicates that the daily dioxin-equivalent of this compound would exceed the daily intake of all other PCDD/PCDFs by more than 100 times. To better understand the effects of I3C, rats were administered 3 daily dosages by gavage of either control vehicle or 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), a prototypic AhR ligand at 3 mcg/kg bw; 1, 2-dithiole-3-thione, a prototypic activator of the Nrf2 transcription factor at 0.3 mmol/kg bw; or, I3C at 100 mg/kg bw. Using Affymetrix RG U34 arrays, liver RNA levels of three biomarkers of AhR response (CYP1A1, CYP1A2 and CYP1B1) and two biomarkers of Nrf2 mediated response (AFAR and GSTpi) showed the expected responses for TCDD and D3T. TCDD treatment increased the expression of CYP1A1 (45-fold), CYP1A2 (5-fold) and CYP1B1 (8-fold), but not AFAR and GSTpi, while D3T treatment had effects on only AFAR (10-fold) and GSTpi (15-fold). Of interest, I3C showed an AhR like response, increasing CYP1A1 (11-fold) and CYP1A2 (4-fold), and not AFAR and GSTpi. However, CYP1B1 was not elevated, indicating that not all AhR ligands affect the same sets of genes equally. Further analyses of the sets of genes responsive to these chemicals and their associated biological networks should provide additional insight into the action of I3C as an AhR agonist and improve the rationale for its TEF categorization.

NATURALLY OCCURRING MARINE COMPOUNDS  
SHOW A SPECIES-SPECIFIC DIFFERENCE IN AHR  
DRIVEN REPORTER GENE EXPRESSION.

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The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that mediates a variety of toxicological and biological effects, including gene expression in a diverse range of species and tissues. While the best characterized ligands for the AhR are halogenated aromatic hydrocarbons such as 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) and polycyclic aromatic hydrocarbons, recent evidence has demonstrated that the AhR can bind and be activated by structurally diverse synthetic and naturally occurring chemicals. As part of a larger effort to identify the spectrum of chemicals that can bind to and activate the AhR, we have examined a library of pure and closely related mixtures of compounds isolated from a variety of marine organisms. Using AhR-responsive recombinant cell bioassay systems generated in mouse, rat and guinea pig cell lines (Hepa1c1c7, H4IIE and GPC16, respectively), a library of 151 compounds were screened at a concentration of 2  $\mu$ M and those inducing AhR-dependent luciferase reporter gene expression identified. These studies have revealed a variety of novel structurally diverse chemicals that can activate AhR-dependent gene expression. The most striking result was the large species-specific difference in the number, type and relative potency of chemicals inducing AhR-reporter gene expression, with 13, 39, and 18 marine products identified as activators of the AhR mouse, guinea pig and rat cell lines, respectively. In addition, while some compounds were active in all three species others were species-specific. The diversity in response may result from differences in the AhR and/or components of the AhR signaling pathway as well as variation between the cell lines themselves. Our studies suggest that the guinea pig AhR is significantly more promiscuous than that of the rat or the mouse. (ES07685, ES04699, ES05707, WHOI Sea Grant)

PERSISTENT BINDING OF LIGANDS TO THE HEPATIC AH RECEPTOR FROM VARIOUS SPECIES.

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The aryl hydrocarbon receptor (AhR) is a ligand activated transcription factor that mediates many biological and toxic effects of halogenated aromatic hydrocarbons (HAHs; such as 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD)), polycyclic aromatic hydrocarbons (PAHs; such as  $\beta$ -naphthoflavone (BNF)), and a wide variety of structurally diverse ligands. While the HAHs are several orders of magnitude more potent in producing biochemical effects than PAHs or other AhR agonists, only the HAHs have been observed to produce AhR-dependent toxicity *in vivo*. Here we have characterized the binding and dissociation of ligands from the AhR in order to determine whether these aspects play a role in the divergent potency/toxicity of these chemicals at the level of the AhR. Dissociation of [<sup>3</sup>H]TCDD from the hepatic cytosolic AhR of Hartley guinea pigs, Sprague-Dawley rats, C57BL/6 mice and Golden Syrian hamsters was measured over time at 25°C using hydroxyapatite. At 48 hours, the amount of [<sup>3</sup>H]TCDD specifically bound to the AhR was still 86%, 56%, 73% and 72% of time zero for the above species, respectively. This persistent occupancy was also observed for guinea pig hepatic cytosol incubated at 37°C, with 89% [<sup>3</sup>H]TCDD specific binding remaining at 44 hours. Dissociation experiments at 25°C with [<sup>3</sup>H]BNF revealed that at 48 hours, 86%, 32%, 66% of [<sup>3</sup>H]BNF was still specifically bound to guinea pig, rat, and hamster AhR, respectively. Our results demonstrate that both [<sup>3</sup>H]TCDD and [<sup>3</sup>H]BNF do not readily dissociate from the AhR of these species. The persistence of binding of both TCDD and BNF to the AhR suggests that their differences in potency/toxicity are more likely attributable to factors other than simply binding affinity/occupancy (i.e. metabolic stability). Our data also supports reassessment of previous AhR ligand binding affinity calculations and competitive binding analysis, since AhR ligand binding at these times are clearly not in equilibrium, a requirement for use of traditional receptor-ligand equilibrium binding kinetics equations. (NIEHS ES07685 and ES05707)

TCDD EXPOSURES ACTIVATE PKC, MAPK AND NFkB SIGNALING PATHWAYS IN HUMAN CANCER CELLS.

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An anthropogenic compound - 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) is one of 75 possible chlorinated dibenzo-p-dioxin. It has no industrial or commercial use and was produced as inadvertent trace contaminants in the synthesis of pesticides, chlorine-bleaching processes or incineration. One of the toxic potency for

TCDD is recognized as its interaction with cytosolic high-affinity aryl hydrocarbon receptor (AhR), followed by heterodimerization with an Ah receptor nuclear translocator and a subsequent binding on the cis-acting dioxin responsive element. Other possible pathways included the modulation of signaling molecules, like PKC, Raf-1, MAP kinase and PKA have been reported. In this study, we determined the effects of TCDD on the phosphorylation of some selected protein substrates (i.e. MARCKS, p44/42 MAPK, I $\kappa$ B) in two human cancer cell-lines (i.e. HepG2 & CaCo2). TCDD induced EROD dose-dependent curves were determined whereas the EC50 for HepG2 and CaCo-2 were 100 pg/ml and 30 pg/ml respectively. Using the EC50 doses for the cells, we conducted Western blotting to determine the phosphorylation of the protein substrates. The levels of pMARCKS, phospho-p44/p42 MAPK and pI $\kappa$ B were significantly induced at 4 hrs after TCDD exposure and remained in high levels at 24 hrs of post-treatment. The results indicated that in addition to the AhR pathway, TCDD can activate various signaling pathways in the cells. The activation of these pathways might affect some basic cell functions, such as cell differentiation and proliferation. Taken together our study indicate that TCDD mediated biological effects are pleiotropic and can be the consequences of crosstalk among different signaling molecules.

THE ARYL HYDROCARBON RECEPTOR (AHR)  
ANTAGONIST EGCG, INHIBITS AHR ACTIVATION  
THROUGH AN INDIRECT MECHANISM INVOLVING  
BINDING TO HSP90: A NOVEL MECHANISM FOR A  
REPORTED ANTI-CANCER AGENT.

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The AhR is a ligand-dependent transcription factor known to mediate the toxic effects of numerous environmental contaminants including the polycyclic aromatic hydrocarbons. Although a tremendous amount of work has emphasized activation of the AhR signaling pathway, a key question remaining is how ligands might function to turn this signaling pathway off. Previous investigations in our laboratory have demonstrated that the green tea polyphenol, epigallocatechin gallate (EGCG), is capable of antagonizing AhR-mediated gene transcription. This line of investigation was directed at elucidating the molecular mechanism of this antagonism. Competitive binding assays under numerous conditions optimal for low affinity ligands demonstrated that EGCG does not bind to the AhR ligand binding site, indicating this compound functions through a mechanism unlike typical AhR antagonists. Affinity chromatography using immobilized EGCG implicates an indirect mechanism of action involving direct binding of EGCG to the AhR chaperone protein, hsp90. This binding to hsp90 influences the AhR complex in the absence and presence of TCDD. Alone, EGCG induces an AhR conformation capable of nuclear localization but incapable of binding DNA. These altered signaling events correlate with the formation of a multimeric complex with a much higher sedimentation rate than the latent or TCDD-activated receptor. In the presence of TCDD, EGCG prevents the formation of an AhR conformation capable of binding DNA, yet it is incapable of preventing TCDD-mediated nuclear localization or degradation of the receptor. These data suggest a model in which EGCG binding to hsp90 stabilizes the hsp90-AhR complex in an intermediary state associated with XAP2. This is the first time EGCG has been demonstrated to directly bind hsp90 and the first indication that EGCG may exert many of its biological effects through targeting of this common chaperone.

A NOVEL ARNT-INTERACTING PEPTIDE AINP2  
ENHANCES THE ARYL HYDROCARBON RECEPTOR  
SIGNALING.

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Aryl hydrocarbon receptor nuclear translocator (Arnt) belongs to the basic helix-loop-helix-PER-ARNT-SIM family. It plays a vital role in diverse biological functions mediated by the aryl hydrocarbon receptor (AhR) and hypoxia inducible factor-1 signaling pathways. In both pathways, Arnt acts as the dimerization partner for the formation of the heterodimeric complex, leading to the activation of transcription of the target genes. We are interested in identifying Arnt-interacting proteins that are essential for the Arnt-dependent signaling mechanisms. To do that, we employed phage display to isolate Arnt-interacting peptides from a human liver cDNA library. A thioredoxin fusion of an Arnt construct Cdelta418 (aa1-371, lacking the C-terminal 418 amino acids) was used as the bait. Co-precipitation assay using TALON resin was used for biopanning. After five rounds of biopanning, the phage titer was enriched to more than 100-fold (from 8 to 850 plaques) whereas the negative control (thioredoxin) gave only a 15-fold (from 2 to 30 plaques) enrichment. Plaque lift assay using 32P-labelled thioredoxin-Arnt Cdelta418 protein was performed to confirm the interaction between Arnt and the positive phages. Arnt-interacting peptide 2 (Ainp2), which contains 58 amino acids, was subse-

quently isolated and identified. Northern blot analysis revealed that Ainp2 is selectively expressed in human liver. BLAST search of the GenBank database did not reveal the identity of this peptide. Ainp2 interacts with Arnt *in vitro* and *in vivo* using GST pull-down assay and mammalian two-hybrid studies, respectively. RACE PCR was performed and an open reading frame of 231 nucleotides was obtained. Transient transfection studies showed that Ainp2 increases the 3-methylcholanthrene-induced luciferase activity by 1.73-fold +/- 0.06 (n=6), suggesting that Ainp2 play a role in the AhR signaling pathway.

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### THE ROLE OF THE JUN N-TERMINAL KINASE PATHWAYS IN THE TISSUE-SPECIFIC TCDD TOXICITY.

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Exposure to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) causes diverse and tissue-specific toxic effects both in animals and humans, most of which are mediated through its binding to and activation of the aryl hydrocarbon receptor (AhR). Upon activation, AhR translocates to the nucleus and complexes with its partner, the Ah receptor nuclear translocator (ARNT), and other proteins to regulate the transcription of target genes, such as CYP1A1, responsible for most of the toxic effects of TCDD. Our previous studies suggest that TCDD activates the Mitogen-Activated Protein (MAP) kinase pathways and that the MAPKs in turn contribute to AhR activation and CYP1A1 expression. In this study, we have used genetically deficient mice that lack components of the Jun N-terminal kinase (JNK) pathway, including JNK1 and JNK2, to investigate the role of the JNK MAPK pathway in TCDD-induced CYP1A1 expression in liver, thymus and spleen. We found that TCDD treatment of mice caused a robust CYP1A1 induction in the liver, at both RNA and protein levels, which was unaffected by JNK ablation. Comparing to that in the wild type mice, the CYP1A1 induction in thymus was suppressed up to 50% in *Jnk2*, but not in *Jnk1* knockout mice, while the induction in spleen was suppressed up to 45% and 25% in *Jnk1*- and *Jnk2* knockouts, respectively. Because MEK kinase 1 is an upstream regulator of the JNK pathway, we generated compound knockout mice, *Jnk1*-/-*Mekk1* +/- and *Jnk2*-/-*Mekk1* +/-, to test the gene dose effects of the JNK pathway on CYP1A1 induction. We found that deletion of one *Mekk1* allele caused further reduction in CYP1A1 expression in both *Jnk1*-/- and *Jnk2*-/- backgrounds and this reduction took place only in thymus and spleen, but not in liver. These results suggest that the JNK pathway is involved in the modulation of AhR signaling in thymus and spleen and that JNK1 and JNK2 may make unique contributions to the tissue-specific toxicity of TCDD. (Supported by NIH R01-ES11798)

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### ISOLATION AND CHARACTERIZATION OF THE CYP1A1 PROMOTER REGION FROM ZEBRAFISH.

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Danio rerio (zebrafish) has become an important model system in the study of development and signal transduction pathways. Recent studies indicate that zebrafish show sensitivity to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) during development and the biological endpoints to TCDD exposure correlate well with those from mammalian systems. To gain an understanding of the molecular mechanism of TCDD action, it is necessary to gain insight into the binding of the AhR at the promoters of induced genes. Thus, studies were initiated to isolate and characterize the zfCYP1A1 promoter. Since the zfCYP1A1 sequence was not available through the zebrafish genome project, several oligonucleotide primer sets were designed to the most 5' portion of the zfCYP1A1 open reading frame. These primer sets were then used to PCR screen pools of PAC clones containing 100-120kB fragments of zebrafish genomic DNA. Two pools generated a positive signal and the PACs within the pool were then screened with zfCYP1A1 cDNA. 2 independent PACs were identified and these were analyzed by restriction digestion and Southern blotting. Both PACs produced identical signals. A 2.5kB fragment was isolated and sequenced. The fragment contained the putative ATG start site and first 140 nucleotides of the ORF and this sequence was 100% identical to the zfCYP1A1 mRNA from GeneBank. Similar to other CYP1A1 genes the 5' UTR contained an intron. Importantly, several xenobiotic response elements (5'-ANGCGTG-3') were identified in the upstream sequence. PCR was used to obtain various sized fragments from the promoter and these were ligated into the pGL2 vector to generate reporter constructs. The various constructs were transfected into either mouse Hepa-1 cells or zebrafish ZFL cells and treated with TCDD. Luciferase activity was detected in each cell line and compared to several constructs in which luciferase was driven by various mammalian promoters. The results show that the zfCYP1A1 promoter contains several functional XREs and can drive luciferase in both fish and mammalian cell lines. Supported by ES 08980

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### MICROARRAY EXAMINATION OF TCDD MEDIATED CHANGES IN GENE EXPRESSION IN HEPA1C1C7 MURINE HEPATOMA CELLS.

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In an effort to characterize Ah receptor (AhR)-mediated gene expression, comprehensive temporal microarray assays were performed using murine Hepa1c1c7 wild-type (WT) and aryl hydrocarbon nuclear translocator (ARNT) defective C4 mutant hepatoma cells treated with 10 nM TCDD or vehicle (DMSO) for 1, 2, 4, 8, 12, 24 or 48 hrs. Gene expression profiles were monitored using custom cDNA microarrays containing 13, 361 cDNA clones representing 7, 810 unique genes. Statistical analysis using an empirical Bayes approach identified 407 significant changes in gene expression in treated WT cells at one or more time points that grouped into 5 distinct K-means clusters. In contrast, TCDD treatment had little effect in C4 cells beyond 1 hr. TCDD affected functional categories in WT cells included regulation of cell cycle, growth, apoptosis and oxidative stress. Computational examination within -1500 and +1500 of the transcriptional start site, the region with the highest occurrence of dioxin response elements (DREs), identified at least one putative DRE in 294 of the temporally responsive genes. Comparison of *in vitro* gene expression changes to responses observed in hepatic tissue of immature ovariectomized C57BL/6 mice treated with 30 µg/kg TCDD, identified 53 significant genes in common between the two data sets with the vast majority of these involved in xenobiotic metabolism. Expression profiles for most overlapping genes exhibited a comparable pattern in response to TCDD. However, there were contradictory profiles which may be a result of differing basal levels of expression in the two systems. The similarities and differences in gene expression patterns of these genes illustrate the advantages and caveats of using cultured cell lines as models of toxicity for whole tissue responses. Supported by grant ES11271.

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### 2, 3, 7, 8-TETRACHLORODIBENZO-P-DIOXIN ALTERS EXPRESSION OF RETINOIC ACID RECEPTORS IN NORMAL HUMAN KERATINOCYTES.

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Exposure to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) results in a variety of pathological abnormalities in skin, including chloracne, enhanced invasiveness, hyperkeratinization, and keratinocyte immortalization. Most of TCDD's biological effects are mediated through activation of the aryl hydrocarbon receptor (AhR) pathway, however, data from knockout mice indicate that the AhR pathway is involved in retinoid signaling and homeostasis. Further, our data indicate that the AhR and retinoid signaling pathways interact in normal human keratinocytes (NHKs) where cotreatment alters matrix metabolism through activation of matrix metalloproteinases (MMPs). We propose that TCDD-induced skin lesions result from altered retinoid signaling. Retinoids, vitamin A derivatives, are important mediators of skin proliferation and differentiation. Ligand-bound retinoic acid receptors (RAR) and retinoid X receptors (RXR) regulate transcription directly, through binding to retinoic acid response elements (RAREs), or indirectly, through inhibiting binding of other transcription factors (AP-1). In addition, recent findings show that unliganded RAR/RXRs can lead to gene silencing through chromatin remodeling. Our data using quantitative RT-PCR demonstrate that expression of RAR $\gamma$  and RXR $\alpha$  is increased following TCDD exposure of NHKs. RXR $\alpha$  expression is induced by 2.5 fold 6h post treatment and is maintained up to 24 h; RAR $\gamma$  expression is induced by 6h post treatment as well, but returns to basal levels by 12 h. Experiments using the protein synthesis inhibitor cycloheximide demonstrate that de novo protein synthesis is necessary for TCDD-induced RAR $\gamma$  and RXR $\alpha$  expression. TCDD-induced alterations of retinoid receptor expression levels in normal human keratinocytes may lead to increased sensitivity to retinoid compounds, as evident from our data on the MMPs. Furthermore, TCDD-induced increases in RAR $\gamma$  and RXR $\alpha$  may also contribute to unliganded receptor-mediated gene silencing.

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### HEPATIC GENE EXPRESSION IN AHR-/- AND C57BL/6J MICE TREATED WITH 7H-DIBENZO[C, G]CARBAZOLE.

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7H-dibenzo[c, g]carbazole (DBC) is a rodent carcinogen and an environmental pollutant found in combustion by-products. We examined changes in gene expression in the liver of two different genotypes of mice [C57BL/6J (B6) and *Abr*-/-, with the latter on a B6 background] following a single topical application of DBC (8 mg/kg

dissolved in acetone). Mice were sacrificed 1 day later and three target tissues for DBC carcinogenicity (liver, lung, and treated skin) were isolated. Total RNA was prepared from livers and used for microarray analysis (Operon 3.0). We determined genes that were significantly different in expression in untreated *Ahr*-/- mice compared to B6, and in treated vs. untreated mice of each genotype. Basal differences in gene expression were observed in untreated B6 vs. *Ahr*-/- mice, with underexpression by >2-fold of *Fmo3*, *Cyp17a1*, and *Cyp39a1* observed in *Ahr*-/- mice compared to B6 mice. In contrast, untreated *Ahr*-/- mice expressed a number of genes at a ≥2-fold higher level than B6 mice, including *Rbp1* and *Lpl*. DBC treatment did not cause an increase in expression of any of the AHR-regulated genes except for *Ephx1*. Cell cycle-related genes *p21* (indicative of DNA-damage-induced cell cycle arrest) and *Bax* (pro-apoptotic) were upregulated to a greater extent in B6 than in *Ahr*-/- mice following DBC treatment. *Thrsp* was dramatically downregulated in DBC-treated B6 mice, but not in treated *Ahr*-/- mice. The latter observation is suggestive of toxicity in the liver of B6 mice. Western blot analysis confirmed increased levels of p21 protein in both strains of DBC-treated mice. Approximately one-third of the 300 genes that were regulated by DBC in either *Ahr*-/- or B6 mice, or both, are of unknown function, but those of known function suggest that DNA damage, hepatotoxicity, and apoptosis are early events in DBC carcinogenicity. Supported by ES-04203-16A1 (Warshawsky PI).

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#### ARYL HYDROCARBON RECEPTOR ACTIVATION INHIBITS E2F1-INDUCED APOPTOSIS.

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2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a ubiquitous environmental pollutant and confirmed carcinogen. The molecular basis for the biological effects of TCDD is largely unknown. TCDD mediates the transcription of the CYP1 family of P450 monooxygenases via activation of the AHR (aryl hydrocarbon receptor), but this does not adequately explain the diversity of TCDD effects. Transactivation by the AHR can explain gene induction, but not gene repression, which is a major effect of dioxin exposure that has not been characterized at the molecular level. We have shown that complexes containing AHR, the retinoblastoma protein RB, and the transcription factor E2F assemble at E2F-regulated promoters, resulting in repression of S-phase-specific genes and cell cycle arrest. Based on preliminary evidence, we hypothesized that AHR can also cause gene repression by forming complexes with E2F independently of RB. To test this hypothesis, we analyzed protein interactions between AHR and E2F in multiple assays. Pull-down assays using GST-E2F1 fusions as bait indicate that AHR binds directly to GST-E2F1. Additionally, immunoprecipitation experiments in RB-negative fibroblasts show that AHR and E2F1 interact *in vivo*. Using gel retardation assays, we show that AHR can specifically displace RB from its interaction with E2F at E2F binding sites occupied by RB(p105)/E2F/DP1 complexes, generating a novel complex containing E2F and AHR. We also used transient expression of a reporter gene driven by an E2F-dependent promoter in mouse hepatoma cells to show that direct AHR-E2F interactions lead to gene repression. In addition to controlling gene expression for cell cycle progression, several E2F family members, in particular E2F1, are able to activate apoptosis. Transient transfection and subsequent cell cycle analysis by flow cytometry indicate that AHR expression can inhibit E2F1-induced apoptosis. These data suggest that AHR establishes active repression interactions with E2F1 to down-regulate gene expression, thereby possibly inhibiting the apoptotic elimination of pre-neoplastic cells. (Supported by NIH R01 ES06273)

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#### ARNT1 AND ARNT2 ARE EXPRESSED IN HUMAN KERATINOCYTES *IN VITRO*.

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The basic-helix-loop-helix PAS (bHLH-PAS) family member, Aryl Hydrocarbon Nuclear Translocator (ARNT) is a ubiquitous transcription factor that is involved in cellular sensing of the environment. Both ARNT1 and ARNT2 have been shown to dimerize with the Aryl hydrocarbon receptor (AhR), the hypoxia inducible factor -1(HIF-1α) and the Drosophila single-minded protein (Sim) in response to environmental contaminants, low oxygen levels and developmental cues respectively. ARNT1 knock out mice die at embryonic day 10.5 exhibiting defective angiogenesis and embryo wasting. Skin-specific targeted disruption of ARNT1 in mice results in early neonatal death due to compromised barrier function of the skin leading to severe dehydration. The role of ARNT2 in skin is not known. Rodent skin and human skin, although similar in protecting against dehydration and maintaining body temperature, exhibit numerous differences such as density of epidermal appendages, vascularity, overall thickness, and barrier function. We re-

port that ARNT2 as well as ARNT1 proteins are expressed in human keratinocytes. To access the importance of ARNTs in the growth of human skin, ARNT1 was specifically knocked down in monolayer cultures of the human keratinocytes using RNAi. Cells were tested for viability using the MTT assay and cell growth was assayed by cell counting and Ki67 staining. Results show that knock down of ARNT1 does not affect keratinocyte viability or growth in monolayer cultures. These studies suggest that ARNT1 and ARNT2 are functionally redundant in human keratinocytes. Studies are ongoing to further investigate the role of ARNTs in the growth and differentiation of keratinocytes using an organotypic model of human skin.

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#### DETECTION OF AH RECEPTOR LIGANDS IN EXTRACTS FROM COMMON COMMERCIAL AND CONSUMER PRODUCTS USING AHR-BASED BIOASSAYS.

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2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related aromatic hydrocarbons produce a variety of toxic and biological effects, the majority of which are mediated by the Ah receptor (AhR) and AhR-dependent gene expression. While all AhR ligands were presumed to meet a defined set of criteria, recent studies have revealed that the AhR can be bound and activated by structurally diverse synthetic and natural chemicals. However, the spectrum of chemicals that can bind to and activate/inhibit the AhR remains to be established. We previously demonstrated the presence of AhR agonists in DMSO extracts of commercial and consumer products and here we have carried out a more detailed analysis of the ability of crude DMSO, ethanol and water extracts of plastic, rubber and paper products to interact with and activate the AhR signaling pathway in cells from several species. DMSO and ethanol extracts of plastic, rubber and paper products induced AhR-dependent luciferase expression in all cell lines, but induction was observed on with water extracts of newspapers. We found a good correlation between the ability of an extract (DMSO and ethanol) to competitively bind to the AhR using [<sup>3</sup>H]TCDD binding assays, to stimulate AhR DNA binding *in vitro* and to induce AhR-dependent reporter gene expression. Reductions in reporter gene induction by extracts at later time points suggests that they contain inducing chemicals are metabolically labile. Interestingly, ethanol extracts of newspapers exhibited high AhR agonist activity, suggesting the presence of relatively water-soluble agonists and bioassay-directed fractionation approaches are being used to identify the responsible chemical(s). While the toxicological and biological significance of these AhR agonists are unknown, our data reveal the widespread distribution of chemicals in everyday products that can specifically activate the AhR and AhR-dependent gene expression. (ES04699, ES07685)

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#### INTERACTION BETWEEN THE AH RECEPTOR AND NF-KB PATHWAYS: ANTAGONISM AT THE LEVEL OF HISTONE MODIFICATIONS.

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Recent studies have shown that residue-specific covalent modifications at the N-terminal tails of histone proteins are important regulatory mechanisms for transcriptional control of gene expression. These modifications, which include acetylation, methylation, ubiquitination, and phosphorylation are a result of ordered recruitment of histone modifying enzymes to the regulatory regions. The modifications give rise to a marking system (histone code) that determines the outcomes of the gene expression. In our early studies, we found a mutually antagonistic interaction between the Ah receptor and NF-κB pathways. Specifically we found that activation of NF-κB inhibits the acetylation of the histone H4 at the regulatory region of the *cyp1a1*. Here, we report that Ah receptor/NF-κB antagonism is also at the level of histone methylation. We found that: (1) In Hepa1c1c7 cells (mouse hepatoma cells) TNF-alpha treatment led to increases of methylation of arginine 3 on histone H4 (R3-H4) which is known to be the major histone substrate for PRMT1, while treatment with TCDD inhibited the methylation of the R3-H4; (2) TCDD treatment resulted in the strong acetylation of lysine 5 on H4, while activation of NF-κB by TNF-alpha abolished the acetylation of this residue; (3) In Hepa1c1c7 cells, transient cotransfection of PRMT1 suppressed AhR-driven luciferase reporter gene expression. Taken as a whole, our results suggest that histone modification is an important aspect of the interaction between the NF-κB and the AhR pathways. (Supported in part by NIH 453321, NSF 462421, NIHES 09859, American Heart Association Grant 0355131Y)

INTEGRATED ANALYSIS OF GENE EXPRESSION DATA, LITERATURE, AND BIOLOGICAL PATHWAYS OF MOUSE EMPHYSEMA STUDY.

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Gene expression data from a mouse emphysema study was used to identify genes with altered expression levels that may be associated with the tissue damage observed in lungs of mice with elastase-induced emphysema. This was made possible through integrated analysis of the expression data along with thematic analysis of the scientific literature and direct linkage to biological pathway information. Male C57BL/6 mice were treated with 37.5 U/Kg of porcine pancreatic elastase by intratracheal instillation, confirmed by pathology and lung morphometry to induce emphysema. Control and treated mice were sacrificed 1 or 4 weeks after dosing, and total RNA was used for gene expression studies. Data was imported into the OmniViz software, normalized, clustered using hierarchical methods, and analyzed for genes with altered expression. Outliers could be easily determined, and correlations among groups evaluated. Using the SAM algorithm integrated into the software, a number of genes were determined to exhibit altered expression levels for treated vs control animals. Using the gene description information, the available MEDLINE literature was retrieved and linked to the gene expression analysis in order to further characterize the genes of interest. Genes pertaining to inflammatory processes and apoptosis were found to be particularly interesting. To understand the biological pathways involved in elastase-induced emphysema, genes were then mapped to existing pathways using the OmniViz Pathway Enterprise solution, and objects colored by the expression values. By this means, changes between control and treated levels could immediately be correlated to the different pathway processes, and co-regulation events identified. In summary, by integrating the analyses of all the available data (expression, literature, and pathways) in a common framework, the genes and processes of interest can be quickly and easily identified and evaluated. (Study Sponsored by Battelle BSTI IR&D)

TRANSCRIPTIONPATH, A NOVEL METHOD THAT IDENTIFIES AND QUANTIFIES GENE TRANSCRIPTION, DETECTS CHANGES ASSOCIATED WITH TOXICOLOGY.

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Recent reports have shown that changes in gene activity can provide valuable information about toxicological endpoints. To date, RNA profiling has typically been the method of choice to identify differential gene expression, but these methods have limitations. A new and novel platform technology called TranscriptionPath, which is based on chromatin immunoprecipitation of DNA-transcription machinery complexes, identifies genes undergoing active transcription inside cells and quantifies their levels of transcription. Sequences are analyzed by either QueryMode, which determines the transcription level of any genomic region of interest using real-time PCR, or DiscoveryMode, which identifies transcribed sequences across the genome without the need for an a priori list. TranscriptionPath has been used successfully in multiple cell and tissue systems, utilizes DNA (which is more stable than RNA), is highly quantitative and reproducible, and is especially effective at detecting early responses to drug treatments. A toxicology feasibility experiment involving i.p. administration of  $\beta$ -Naphthoflavone (BNF) or Ciprofibrate (CPF) to mice (both at 200 mg/kg) tested several cytochrome P450 genes in the liver at early time points. TranscriptionPath results showed that BNF induced transcription of Cyp1a2 within 1 hr of exposure and the transcription level remained high through the 8 hr time point. Cyp4a10 and Cyp4a14 were induced at relatively low levels only at the 1 hr time point. After treatment with CPF, Cyp1a2 did not change in transcription, but both Cyp4a10 and Cyp4a14 were highly induced (up to 25-fold induction at 4 hr). These results agreed with published RNA levels, although the latter changes were less dramatic. Thus, TranscriptionPath is informative in measuring early gene expression changes in tissues tested for toxicology, should help identify the primary targets of drugs at the beginnings of metabolic pathways, and will reduce the time and cost required for preclinical safety assessment.

CELL BASED ASSAYS REVEAL INDIVIDUAL AND COOPERATIVE ROLES FOR THE GLI TRANSCRIPTION FACTORS IN MEDIATING THE HEDGEHOG SIGNAL.

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The Gli family of transcription factors (Gli1, 2 and 3) mediate the morphogenetic signal of secreted Hedgehog (Hh) proteins by regulating the expression of Hh target genes. Aberrations in Hedgehog signaling seriously affect vertebrate develop-

ment and mutations in Gli2 and Gli3 are associated with distinct classes of developmental defects. Postnatally, inappropriate activation of Hh signaling has been associated with several types of cancers. To characterize individual and cooperative roles of the three Gli transcription factors in regulating the expression of Hh target genes, we have generated embryonic fibroblast cells from Gli mutant mice. We first examined how loss of function of one Gli gene affected expression of the Hh target genes Patched1 (Ptcl) and Hedgehog interacting protein1 (Hip1) in the presence or absence of Sonic Hedgehog (Shh) peptide. Loss of Gli1 (Gli1<sup>-/-</sup>) had no effect on target gene induction. Gli2<sup>-/-</sup> cells showed markedly reduced induction of target genes, which was further reduced with the additional loss of Gli1 (Gli1<sup>-/-</sup>Gli2<sup>-/-</sup>). Loss of Gli3 (Gli3<sup>-/-</sup>) elevated basal and Shh-induced target gene expression, whereas cells lacking both Gli3 and Gli1 (Gli1<sup>-/-</sup>Gli3<sup>-/-</sup>) exhibited increased basal but not induced target gene expression. Gli2<sup>-/-</sup>Gli3<sup>-/-</sup> cells showed higher basal target gene expression but were not capable of Shh-stimulated target gene induction. These results demonstrate that Gli2 and Gli3 modulate Shh target gene expression directly and independently. They further show that Gli2 and Gli3 modulate Gli1 expression, which coordinately contributes to target gene regulation. These observations are consistent with genetic studies of Gli gene function and provide a comprehensive and tractable system by which to examine the mechanisms underlying the complex regulation of Hh target genes.

TOXICOGENOMIC ANALYSIS OF INTESTINAL RESPONSES TO NORMAL OR IMBALANCED HUMAN MICROFLORA IN GERM-FREE MICE OR HFA MICE EXPOSED TO ANTIMICROBIALS.

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The human intestinal microflora is a balanced ecosystem that is very important in maintaining an individual's health as salvage of energy and absorption of nutrients by metabolic activities, control of intestinal epithelial cell proliferation and differentiation, development and homeostasis of the immune system and protection against pathogens. The gastrointestinal tract represents the first barrier met by exogenous compounds of food or orally delivered drugs. Although intestinal microflora system is generally stable, therapeutic doses of antimicrobials or low levels of antimicrobials that reside in livestock products may change the balance of microflora and impact human health. We colonized germ-free mice with normal human flora and global transcriptional responses in colon crypt cells were observed with mouse DNA microarray chip. The change of gene expression in colon crypt cell of human flora associated (HFA) mice exposed orally to tetracycline (200mg/kg) and ciprofloxacin (200mg/kg) for 4 days was also investigated to understand the response of colon crypt cells by the imbalanced microflora induced by antimicrobials. As a whole, the gene expression patterns were similar between germ-free mice and antimicrobials-treated mice compared to HFA mice. 92 genes including Tegt, Rpa1, Ccnd and etc were expressed in germ-free and antimicrobials-treated mice significantly different from HFA mice. These genes were related to apoptosis, cell cycling, immune response and signal transduction. Real-time RT-PCR analysis largely confirmed these findings by the comparison study between RT-PCR and DNA microarray chip on the expression of Tegt, Casp14, Sprr2a, Il6, Eif2ak3, Mpp1, Slc26a1, Saa3, Mt1 and Ang genes in germ-free, HFA and antimicrobials-treated HFA mice. Some of these 92 significant genes could play a role in understanding the interactions between host human and microflora and in assessment of the impact of exogenous compounds on microflora and human health using HFA mice.

INDUCTION OF CYCLOOXYGENASE-2 BY CORTICOSTERONE IN CARDIOMYOCYTES.

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Mental stress is an unavoidable event in life. It increases the level of glucocorticoids (GCs) in our circulating system. However, the biological effect of elevated GCs on the heart has not been well studied. Cyclooxygenase-2 (COX-2) is a rate-limiting enzyme of prostaglandin biosynthesis and a key mediator of inflammation. In lymphocytes, GCs suppress the expression of COX-2 and the immune response. In cardiomyocytes, we found increases of COX-2 mRNA and protein following corticosterone (CT) treatment. Elevation of COX-2 mRNA or protein was first detected in 2 hrs or 4 hours, respectively, and was sustained for at least 4 days. The GC receptor antagonist, mifepristone, blocked the induction of COX-2 mRNA and protein. COX-2 promoter analysis showed transcriptional activation of the gene by CT, and further analysis revealed that the cis-elements CRE and NF-IL6 contributed to COX-2 promoter activation. CT also induced the activation of Phosphoinositol-3 Kinase (PI3K) and phosphorylation of AKT. PI3K inhibitors, LY and Wortmannin, and an AKT inhibitor prevented CT from inducing COX-2 mRNA or protein, suggesting the involvement of the PI3K/AKT signaling pathway. This pathway is known to phosphorylate and deactivate GSK3beta, resulting in decreased phosphorylation of the transcription factor CREB at Ser129 and therefore activation of

CREB. Our data showed that inhibition of GSK3beta by LiCl or SB 216763 increased COX-2 promoter activity and gene expression. Finally, we tested the functional consequence of COX-2 induction using NS398 and found that this COX-2 specific inhibitor abrogated doxorubicin-induced apoptosis. Therefore CT induces COX-2 expression via a PI3K mediated CREB pathway. However, COX-2 induction may be detrimental to cardiomyocytes.

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### DEVELOPING AN *IN VITRO* GENE EXPRESSION ASSAY FOR PREDICTING HEPATOTOXICITY.

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A crucial step in the development of new pharmaceutical compounds is assessing the potential for toxicity. Hepatotoxicity is of primary concern because the liver is the principle organ involved with drug metabolism. Most toxicology studies are conducted in 1 or 2 week animal studies, requiring a significant quantity of time and drug. An assay that, requires a minimal amount of compound, can identify potential toxic liabilities, and be used in the earlier stage of drug discovery, would be of great benefit. Microarray technology is a potential tool that can be used for this type of analysis. The purpose of this study was to evaluate if gene expression profiles from rat hepatocytes could be predictive of the toxic mechanism and classification of known hepatotoxins based on animal studies. To begin this study, *in-vitro* conditions were evaluated using quantitative PCR for albumin and select cytochrome p450 gene expression. Treatment time points were then determined using full microarray gene expression. Finally, hepatocytes were treated with three classes of compounds: AhR Ligands, Peroxisome Proliferators, and non-hepatotoxins. Gene expression profiles were measured for each class of compound. Results show that 1) the conditions used to grow cells in culture are important in maintaining consistent gene expression in naïve hepatocytes, 2) duration of treatment affects gene expression, and 3) using *in vitro* conditions, compounds with distinct mechanisms of toxicity yield similar expression profiles.

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### ELUCIDATION OF THE MECHANISM OF KINASE INHIBITOR-INDUCED KIDNEY AND LIVER TOXICITY USING THE "OMICS" TECHNOLOGIES.

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A kinase inhibitor (compound X) in development for the treatment of asthma/rheumatoid arthritis, was terminated due to hepatic and renal toxicity in rats. The objective of the present study was to elucidate the mechanism(s) of toxicity using biochemical and molecular approaches and to develop a back-up selection strategy. Male rats were treated with compound X (0, 10, or 200 mg/kg/day; 1 or 14 days). Kidney and liver samples were collected for histopathology, toxicogenomics and -proteomics. Urine samples were collected for metabolomic analysis. Mechanism-based inactivation of CYP450 was assessed both *in vitro* and *in vivo*. Compound-related histopathology findings consisted of renal tubular dilatation/degeneration and hepatocellular hypertrophy. Toxicogenomics and -proteomics identified significant alterations of genes and proteins involved in xenobiotic metabolism, lipid and protein metabolism, and redox balance. Metabolomics identified methylguanidine, a product of creatinine formed via oxidation by reactive oxygen species, in the urine of rats treated with compound X. The results from these studies are consistent with free radical generation and oxidative stress as primary mechanisms of renal and hepatic toxicity for compound X. In addition, mechanism-based inhibition of CYP 3A was detected both *in vivo* and *in vitro*. The reactive moiety on compound X was identified via glutathione conjugates using LC/MS/MS in incubations with human liver microsomes. These findings correlated with cytotoxicity of a series of compounds in rat primary hepatocytes. In conclusion, the emerging technologies were successfully combined with biochemical approaches to elucidate the mechanism of toxicity induced by compound X. Results obtained from such an integrated approach helped to identify a compound from a new lead series that progressed as an early development candidate.

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### CHARACTERIZATION OF FUNCTIONAL VARIATION IN HUMAN GSTM3 IN GLIOMA CELLS USING RNAI.

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Glutathione S-transferase M3 (GSTM3) is expressed in testis, lung, lymphocytes, brain, and in many tumor types. While GSTM3 can metabolize a number of xenobiotic chemicals, relatively little is known about the endogenous role of this, the an-

cestral, GSTmu gene. Some reports suggest that GSTM3 expression level may be associated with bronchogenic carcinoma and that GSTM3 protein may have prostaglandin E2 synthase (PGES) activity in human brain. We have observed that GSTM3 expression varies widely among individuals in both normal and tumor samples, and have characterized a common promoter polymorphism (A-63C, rs1332018) in humans that dramatically reduces GSTM3 expression (at least 9-fold). To explore the biological impact of phenotypic variation in GSTM3 on growth and metabolism, we created a congenic cell line system by stably transfecting constructs containing GSTM3 cDNA or a knock-down RNAi vector into glioma cell lines. These cell lines express highly different levels of GSTM3 and this was verified by Taqman and western blot analysis. To measure impact on growth we have compared growth curves and proliferation rates (measured by MTT assay) in these lines and observe that over-expression of GSTM3 was associated with higher rates of cell proliferation while lack of GSTM3 expression, and RNAi knockdown of expression, inhibited cell growth. Evaluation of impact on PGE synthesis and growth factor pathways are in progress. RNA from these cell lines was applied to Agilent human 1Av2 microarrays in order to identify candidate genes whose expression levels varied across the cell lines in parallel or contrary to GSTM3 overexpression and downregulation ( $p < 0.001$ ). We used real-time PCR to confirm differential expression of four candidate genes from the microarray analysis. These candidate genes are in cell cycle and growth factor pathways and play roles in growth stimulation, cell cycle arrest, tumor suppression, and inhibition of metastasis. Current effort focuses on determining if GSTM3 expression mediates growth through perturbation of candidate gene expression.

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### DIFFERENTIAL GENE EXPRESSION IN F344 RATS FOLLOWING EXPOSURE TO EITHER POLYBROMINATED DIPHENYL ETHERS (PBDES) OR PCBs.

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PBDEs, widely used as flame retardants, are found as environmental contaminants and are present in mammalian tissues. 2, 2', 4, 4'-Tetrabromodiphenyl ether (BDE-47) and 2, 2', 4, 4', 5-pentabromodiphenyl ether (BDE-99), two major PBDEs in the environment, are components of the commercial mixture Great Lakes DE-71® (DE-71), used mostly in polyurethane foam. PBDEs are structurally similar to dioxins and PCBs, and may share mechanisms of toxicity with those chemicals. The goal of the present work was to determine if the DE-71 mixture and congener components influence expression of genes regulated through biological pathways affected by either coplanar or non-coplanar PCBs. Male F344 rats were dosed daily for three days by gavage, with up to 100  $\mu$ mol/kg of BDE-47 or -99, up to 150 mg/kg DE-71 (containing 100  $\mu$ mol/kg of BDE-47), 0.01  $\mu$ mol/kg of coplanar PCB126, or 10  $\mu$ mol/kg of non-coplanar PCB153. Rats were sacrificed 24 hr following the last dose, RNA was isolated from liver, converted to cDNA, and analyzed by real-time PCR for differential gene expression. Target genes were for isozymes of the CYP1A, 2B and 3A families. BDE-47, -99, and PCB153 had little or no effect on Cyp1a1 gene regulation. In contrast, PCB126 and 150 mg/kg of DE-71 increased Cyp1a1 mRNA ca. 2000-fold over controls. DE-71 at 15 mg/kg gave an approximate 80-fold increase. PCB153, but not PCB126, up-regulated both Cyp2b and 3a genes. These genes were also up-regulated by BDE-47, and -99 at doses of 100  $\mu$ mol/kg and by DE-71 at an equivalent dose. Results indicate that DE-71 may be a mixed-type inducer in rat. Up-regulation of Cyp1a1 gene expression by DE-71 appears to be due to components other than BDE-47 and -99. Differential gene regulation by PBDEs is demonstrated here in the rat at much higher doses than estimated for human exposure (ng range/person/day). Ongoing work will characterize differential gene expression in rodents exposed to more relevant lower doses of DE-71 and its congener components.

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### EXTRACTION OF GENES WITH STABLE EXPRESSION IN RAT LIVER TREATED WITH VARIOUS COMPOUNDS - ANALYSIS OF THE DATA IN THE TOXICOGENOMICS PROJECT IN JAPAN -.

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National Institute of Health Sciences and 17 pharmaceutical companies have started a five-year project, the Toxicogenomics Project in Japan (TGPJ) in 2002. The objective of TGPJ is to construct a large-scale database of over 150 drugs on rats and develop a system which will forecast the toxicity of new chemical in the early stage of drug development. In TGPJ, we are constructing a comprehensive gene expression database of rat liver and kidney (*in vivo*), rat primary cultured hepatocytes and human primary cultured hepatocytes (*in vitro*) treated with over 150

chemicals using Affymetrix GeneChip. In order to establish a useful forecasting system, quality of the data stored in the database should be as high as possible. As one of the ways to assure the quality of the data, we check the microarray data by Real Time PCR of internal controls of which expression is stable throughout the experiments. We evaluated all the gene expression data from male SD rats treated with 42 chemicals stored in the present database (3, 6, 9, 24 hours after single dose with three dose levels and 3, 7, 14, 28 days of repeated daily dose with three dose levels, using Affymetrix rat RAE 230A chip). We have extracted 12 stable genes including peroxisomal 2-enoyl-CoA reductase, integral membrane protein Timp21-1 (p23), etc., based on their expression level and variation. We also evaluated so-called "housekeeping genes", such as beta-actin, GAPDH, etc., which are commonly used as internal control genes for Real Time PCR, or other genes reported to be showing invariant expression. Among the 42 chemicals, 15 were selected as representatives of typical pharmacological action and gene expression profile. Expression of all these genes was confirmed by Real Time PCR in the samples from the control and the high dose group received repeated administration for 28 days. It was concluded that our new set of genes is superior to other genes in terms of the stability of their expression at least in our database.

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CHARACTERIZATION OF AN ACUTE MOLECULAR MARKER OF NON-GENOTOXIC HEPATOCARCINOGENESIS IN RODENTS BY GENE EXPRESSION PROFILING IN A LONG-TERM CLOFIBRIC ACID STUDY.

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The evaluation of non-genotoxic potential, early during the development of drugs, is one of the current challenges in toxicology. In a short-term *in vivo* toxicity study, the NAD(P)H cytochrome P450 oxidoreductase (CYP-R) and the transforming growth factor- $\beta$  stimulated clone 22 (TSC-22) were identified as potential candidates as molecular markers of rodent hepatic chemical carcinogenesis (1). We investigated their modulation during a long-term non-genotoxic study in the rat, to evaluate their potential as acute markers of carcinogenesis. Clofibric acid (CLO), which belongs to the broad class of chemicals known as peroxisome proliferators was chosen as the non-genotoxic hepatocarcinogen. Male F344 rats were given a single non-necrogenic, but initiating dose of diethylnitrosamine (0 or 30 mg/kg) coupled with a diet containing none or 5000ppm CLO for up to 18 months. Necropsies of 5 rats per groups were performed at 18, 46, 102, 264, 377, 447 (for the Control, DEN and DEN+CLO rats), 524, and 608 days (for the CLO and Control rats). Gene expression modulation measured using Affymetrix microarrays did not confirm CYP-R gene expression alteration. TSC-22 gene expression, however, was found to be strongly down-regulated in peritumoral and tumoral liver tissue (validated by RT-PCR), already at the early stages of the carcinogenic process. In addition, we showed under-expression of TSC-22 was maintained throughout this long-term study, but disappeared after CLO withdrawal. TSC-22 has been previously linked to induction of apoptosis and its down-regulation upon CLO dosing could result in suppression of the apoptosis pathway. Taken together, our results showed that TSC-22, but not CYP-R, could be a potential acute early molecular marker for non-genotoxic hepatocarcinogens in rodents. (1) Kramer JA et al. *Chem Res Toxicol*. 2004, 17: 463-70.

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PROFILES OF GLOBAL GENE EXPRESSION IN IONIZING RADIATION-DAMAGED HUMAN DIPLOID FIBROBLASTS REVEAL SYNCHRONIZATION BEHIND THE G1 CHECKPOINT.

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Cell cycle checkpoint function and transcriptional responses to ionizing radiation (IR)-induced DNA damage were quantified in human diploid fibroblasts. Assays of clonal expansion determined 1.5 Gy IR as the D0 dose in three telomerase fibroblast lines. Fibroblasts exhibited >90% arrest of progression from G2 to M at 2 h post-IR and from G1 to S 6-12 h after 1.5 Gy. Normal rates of DNA synthesis and mitosis 6-12 h after irradiation caused the S and M compartments to empty by over 70% at 24 h. Microarray technology monitored global gene expression in IR-treated cells and a new microarray analysis algorithm, EPIG, identified nine IR-responding gene expression patterns including a dominant p53-dependent G1 checkpoint response. Many p53 target genes, like CDKN1A, GADD45, BTG2 and PLK3, were significantly up-regulated at 2 h post-IR while numerous genes whose

expression is regulated by E2F family transcription factors were significantly down-regulated at 24 h post-IR, including CDK2, CCNE1, CDC6, CDC2, MCM2 and many other cell cycle regulators. Numerous genes that participate in DNA metabolism were also markedly repressed in checkpoint-arrested fibroblasts as a result of cell synchronization behind the G1 checkpoint. However, cluster and principal component analyses of gene expression revealed a profile of gene expression 24 h after IR with greatest similarity to that of G0 growth quiescence. These results demonstrate a highly stereotypic pattern of response to IR that reflects primarily synchronization behind the G1 checkpoint but with prominent induction of additional markers of G0 quiescence such as GAS1.

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EXPRESSION OF THE HELIX-LOOP-HELIX INHIBITOR OF DNA BINDING-1 (ID-1) GENE IS REGULATED BY RETINOIC ACID IN NORMAL HUMAN KERATINOCYTES.

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The Id proteins (Inhibitor of DNA Binding) are a class of helix-loop-helix (HLH) proteins that lack a DNA binding domain and function by forming inactive heterodimers with other HLH proteins. Ectopic overexpression of Id-1 results in immortalization or delayed senescence of normal human keratinocytes (NHKs), demonstrating that Id-1 is an important regulator of keratinocyte differentiation. Retinoids, vitamin A analogues, are powerful regulators of cell growth and differentiation and is necessary for the maintenance of epithelial differentiation. We have found that exposure of NHKs to all-trans retinoic (atRA) acid results in a significant increase in Id-1 mRNA and protein. Our hypothesis is that atRA-induced expression of Id-1 is a critical step in retinoic acid mediated effects in NHKs. Our data show that atRA stimulation of Id-1 is specific; no change was observed in Id-2 and -3 expression following atRA exposure. Retinoic acid stimulation of Id-1 does not involve mRNA stability, suggesting that it is regulated at the level of transcription. To investigate this possibility, we isolated 2.2 kb of the Id-1 promoter and linked it to a reporter gene (luciferase; pGL3) for transfection into NHKs, and our data demonstrate that atRA-induced Id-1 expression is mediated through sequences in the 2.2 kb 5' of the gene. Data from other laboratories suggest that the bone morphogenic proteins (BMPs) are involved in activation of Id-1 expression in other cell types. Furthermore, atRA exposure of chondrocytes results in increased expression of BMP-2 and -4. The goal of these experiments are to determine the mechanism of atRA-induced Id-1 expression, focusing on the role of the BMP proteins. Furthermore, these data will also elucidate the role of Id-1 in mediating atRA effects on skin differentiation. This will provide a better understanding of the mechanism of action of retinoic acid in skin, as well as to define the role of Id-1 in this pathway.

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APPLICATION OF A CROSS-PLATFORM RNA STANDARD FOR ASSESSING MICROARRAY DATA COMPARABILITY.

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Microarray data comparability remains a challenge for the integrated use of genomic data generated across sites and platform formats. Tools needed to address this issue include an RNA standard that performs similarly on multiple microarray formats and serves a function analogous to a standard reference material in single analyte assays. We have designed and tested a reagent that can provide a basis for performance assessments on Affymetrix RAE230A, CodeLink UniSet Rat I, and Agilent G4130A rat oligonucleotide arrays. The reagent is a set of two mixed tissue RNA samples formulated to contain different ratios of RNA for each of four rat tissues (brain, liver, kidney, and testis). The analytes in this assay bind to a subset of gene probes that were matched across platforms to the same UniGene cluster or GenBank accession number, were determined to be tissue-selective on each of the 3 platforms using average baseline expression levels in single tissue samples from control animals, and have signal intensities that span the dynamic range of each platform. This reagent forms the basis of a ratiometric assay in a complex biological background that can be used to objectively assess the effect of different array processing methods and conditions on microarray data comparability within and across platforms, and could be a paradigm for similar performance standards for mouse and human arrays.

## GENE EXPRESSION MODIFICATIONS IN A MOUSE EMPHYSEMA MODEL INDUCED BY ELASTASE.

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The purpose of this study was to characterize gene expression changes in the lung associated with elastase-induced emphysema in mice. Male C57BL/6 mice were administered porcine pancreatic elastase (0 or 37.5 U/Kg BW) by intra-tracheal instillation. Groups of control or treated mice (n = 6) were sacrificed at 1 or 4 weeks after dosing. Anatomical pathology and lung morphometry were used to confirm the induction of emphysema. Left lung lobes were snap frozen and total RNA was used for Cy3/Cy5 fluorescently labeled cRNA target amplification using Agilent's Low RNA Input Kit. The data were normalized with loess curve fit in Bioconductor and using an Aquantile method for between array variability. The normalized data were then examined to identify genes associated with tissue damage observed in elastase-instilled lung. Several genes associated with inflammatory diseases, such as Sjogren's syndrome antigen B and uteroglobin, were up-regulated at week 1 and remained up-regulated through week 4. Other genes, such as E-selectin and Dyrk1b, were up-regulated at week 1 but returned to normal by week 4. E-selectin initiates leukocyte recruitment and adhesion to lung endothelial cells, and Dyrk1B overexpression in lung epithelia destabilizes cyclin D1 to block cells in G0/G1. Zic2 expression was down-regulated at week 1 but returned to normal by week 4. Zic2 is a transcription factor repressor of D1A dopamine receptors, whose stimulation increases lung edema clearance by regulating Na<sup>+</sup> K-ATPase function in the alveolar epithelium. Calpastatin, an intracellular inhibitor of calpain, was down-regulated at week 1 and week 4. Activation of calpain triggers the cleavage of Bcl-2 proteins and induces the intrinsic apoptotic pathway in lung carcinoma cells. Further analysis of this data set, including future integration with proteomics and metabolomics data from the same study, will lead to a better understanding of pathogenesis of the elastase-induced emphysema model in mice. (Sponsored by Battelle BSTI IR&D)

## TUMOR SUBTYPE CHEMOTHERAPEUTIC RESPONSES IN THE BREAST.

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Microarray studies have identified distinct and clinically relevant subtypes of breast tumors that arise from different cell types in the breast. To characterize the differences in response to therapy between two of these breast subtypes, we treated basal-like epithelium derived cell lines and luminal epithelium derived cell lines with doxorubicin (DOX) and 5-fluorouracil (5FU) and assayed for gene expression responses by DNA microarrays. Using Significance Analysis of Microarrays, we determined that the dominant gene expression response was a general stress response, common to both treatments yet specific to each cell type. A cluster of genes involved in DNA damage response was upregulated in both subtypes; however, they were more significantly induced in the luminal subtype. Similar analyses were conducted on breast tumors sampled before and after neoadjuvant chemotherapy with either DOX or 5FU/mitomycin C. We determined that the dominant *in vivo* response was again a general stress response and found a small set of induced genes. However, when we split the basal-like and luminal subtype tumors and did separate analyses, we saw independent lists with little overlap. We next compared the *in vitro* and *in vivo* data and identified a shared response within each subtype but with little similarity between subtypes. These studies illustrate that the cell type of origin plays a major role in response to treatment and further suggest that subtype specific therapies are needed.

## USE OF GENE EXPRESSION CHANGES IN CIRCULATING BLOOD CELLS TO MONITOR XENOESTROGEN EXPOSURE IN NEONATAL MICE.

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Many molecular alterations in blood cells and serum have been associated with toxicant-induced tissue damage, inflammation and repair. The analysis of molecular biomarkers in circulating blood is emerging as a powerful approach for monitoring toxicant exposure. We have used transcript profiling of circulating blood cells to investigate the utility of gene expression biomarkers for monitoring xenoestrogen exposure in a rodent model experimental system. Neonatal mice were given five daily subcutaneous doses (Post Natal Day (PND) 1-5) of an estrogenic chemical carcinogen

(diethylstilbestrol; DES) at a dose that induces increased uterine weight and that has previously been established to result in a high incidence of uterine tumours after 18 months, or vehicle control (arachis oil). Blood samples were obtained from treated mice by cardiac puncture at PND17, 30 and 50, and RNA was isolated using the PAXgene blood RNA System. Uterine weight, transcript profiling and histological data were collected in order to provide a reference bioassay for xenoestrogen exposure. Gene expression profiles of circulating blood cell RNA, measured using Affymetrix Genechip mouse genome 430 2.0 microarrays, reveal that DES treatment induced more than 100 gene expression changes at PND17. A large proportion of these changes showed a dose-dependent response to DES. RT-PCR analysis is currently being used to examine the temporal expression patterns and the inter-animal variability of these DES-responsive genes. We conclude that analysis of gene expression changes in circulating blood cells can be used to monitor exposure to xenoestrogens in our animal model. Moreover, our data suggest that the measurement of gene expression changes in circulating blood cells may lead to the identification of novel biomarkers for toxicant exposure.

## COMPARISON OF WHOLE BLOOD GENE EXPRESSION PROFILES FROM THREE NON-HUMAN PRIMATE SPECIES AND HUMANS.

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Non-human primates (NHP) are often used to evaluate the potential safety and efficacy of medical countermeasures against chemical exposure. This is because rodents frequently do not respond to chemical insult as humans do, and because human clinical trials cannot be performed to determine efficacy of potential countermeasures. Three species of NHP have been used in the development of chemical warfare agent (CWA) medical countermeasures: *Macaca mulatta* (rhesus macaque), *Macaca fascicularis* (cynomolgus macaque) and *Cercopithecus aethiops* (African green monkey). We utilized a gene expression profiling approach to identify potential genomic biomarkers that may be used to develop methods for extrapolating safety and efficacy data obtained in NHP to humans. Whole blood was obtained from each species of primate. RNA isolated from whole blood was used to generate oligonucleotide microarray probes. A comparable number of genes were detected in each species after hybridization to human gene chips (Affymetrix Human U133 Plus 2.0 arrays). Principal component analysis of these gene expression profiles revealed that each NHP species partitioned away from the human samples in a similar manner. A Boolean analysis of the expression profiles revealed that 1, 079 genes were detected in all species and in all biological replicates within each species. These genes are distributed among 154 molecular functions and 156 biological processes. This set of genes provides the foundation for identifying potential biomarkers for use in determining safety and efficacy of CWA medical countermeasures and for developing methods to extrapolate results obtained in NHP to humans.

## THE BIOLOGY OF DRUG SIGNATURES.

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We have developed a large library of Drug Signatures<sup>TM</sup>, biomarkers for toxicologic, pathologic, and pharmacologic mechanisms of action, to improve compound selection during drug discovery. These biomarkers are derived from a database integrating ~15, 000 gene expression microarray results from rats treated with >600 compounds with traditional measurements of toxicity, pathology, and pharmacology. Currently in our database we have over 350 unique signatures, 180 derived from liver data alone. As an example of the utility of signatures, two glucocorticoid compounds, fluocinolone acetonide and fludrocortisone acetate, which have known cardio-, immuno- and hepatotoxicities, were tested in rats at their respective maximum tolerated doses in 5-day studies. While hierarchical clustering of gene expression data from the liver revealed that both compounds were similar to other glucocorticoid agonists in the database, no information on toxicity was gleaned from this analysis. Both compounds, however, matched signatures that correlated with both their pharmacological mechanism of action and known toxicities. These signatures include, among others, the Glucocorticoid-Mineralocorticoid Receptor Agonist, ALT Increase, and Lymphocyte Decrease signatures. Fludrocortisone's lower scores on pathology signatures relative to fluocinolone's correlated directly with the lesser degree of toxicity induced by fludrocortisone relative to that induced by fluocinolone. Genes in the signatures that these compounds match provide useful information on the biology behind the pharmacology and toxicity of these compounds. In particular, genes in the Lymphocyte Decrease signature were primarily those that are highly expressed in lymphocytes. This observation indicates that toxicities in other organs can be readily detectable in the liver. The advantage of a large

database of signatures is that compounds can be characterized pharmacologically and toxicologically within minutes by scoring gene expression data against all of our signatures, hastening decision making during drug discovery.

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#### GENE EXPRESSION ANALYSIS OFFERS UNIQUE ADVANTAGES TO HISTOPATHOLOGY IN PREDICTION OF OVERALL LIVER CONDITION.

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Histopathological analysis of liver biopsy samples has poor predictive value for Acetaminophen intoxication. One reason might lie in the great differences in the degree of liver damage observed in different regions of the liver. This study examines whether gene expression analysis provides a more representative picture of the overall condition of the liver or if it is closely linked to the liver phenotype at the origin of the mRNA sample. Rats were treated with a single dose of APAP (1500 mg/kg) and sacrificed 24 hours after exposure. Left, median and right anterior liver lobes were harvested for RNA extraction and histopathological analysis. Livers of sham-treated control animals were also collected and examined. Histopathological evaluation revealed major differences in the degree of necrosis observed in the three liver lobes. The most severe damage was observed in the right anterior liver lobe, followed by the median lobe, while the left liver lobe showed the least amount of necrosis. Gene expression analysis was performed comparing mRNA abundance in the three different liver lobes of individual treated animals with lobe specific pooled controls. Surprisingly, gene expression patterns of all three lobes were in strong agreement and elucidated alterations in the same biological processes, independent of the phenotypic appearance of the examined liver lobe. We concluded from our study, that in case of clear toxicity induced by acetaminophen, each liver sample provides similar gene expression information, independent of location and phenotype of the tissue. This has major implications for the clinical setting. Histopathology performed on human liver biopsy samples often has very poor predictive power, mainly due to marked regional differences in the degree of liver damage. In these cases, a small sample is not necessarily representative of the whole organ after intoxication with APAP. Our results suggest that genomic analysis of the same sample might have the potential to give a more precise indication of hepatotoxicity and overall prognosis for the patient.

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#### CHANGES IN GENE EXPRESSION INDUCED BY TECHNICAL CHLORDANE, CIS-NONACHLOR, TRANS-NONACHLOR AND OXYCHLORDANE IN RAT LIVERS MONITORED BY OLIGONUCLEOTIDE MICRO-ARRAYS. CURRAN, I.H.A., HIERLIHY, A., AND BONDY, G., TOXICOLOGY RESEARCH DIVISION, FOOD DIRECTORATE, HPFB, HEALTH CANADA, OTTAWA, ONTARIO, CANADA.

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Technical chlordane is a mixture of structurally related chlorinated compounds which have been utilized as a pesticide from the late 1940's until the late 1980's. The pesticide is stable in the environment and has been shown to induce hepatocellular carcinomas in male and female mice. This study examines the effects of chlordane, the major components cis-nonachlor and trans-nonachlor and the metabolite oxychlordane on gene expression in rat livers using micro-arrays and RT-PCR. In this study, female and male Sprague-Dawley rats were administered chlordane, cis-nonachlor, trans-nonachlor, oxychlordane or corn oil vehicle control by gavage for 28 days at a dose of 2.5mg/kg body weight. Livers were then extracted for total RNA and transcript profiles were compared between treatment and control groups using oligonucleotide micro-arrays (1152 genes). Semi-quantitative RT-PCR was performed on micro-array identified genes using individual male and female liver samples to verify results. Micro-array analysis in female rat livers found the following number of genes were up or down regulated two-fold in three trials for the following treatments: Chlordane, 3 genes; cis-nonachlor, 8 genes; trans-nonachlor, 15 genes; and oxychlordane, 31 genes. Classification of the genes identified gene products primarily involved in xenobiotic metabolism and ion/solute transport. Two of the metabolism genes affected were CYP2b19 and CYP3A, as expected from compounds classified as phenobarbital-type inducers. Verification of changes in gene expression via RT-PCR found the genes SDCT-1, gamma-ATPase, PEPT2 and TWIK were up-regulated in oxychlordane treated female rat livers and down-regulated in oxychlordane treated male rat livers. These compounds alter gene expression of solute/ion transporters, membrane channels and other membrane proteins and have sex related effects in rat models.

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#### EFFECT OF REPRODUCTIVE TOXICANTS ON GENE EXPRESSION IN THE RAT TESTES.

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During research and development of drugs, several compounds were failure due to testicular toxicity. Therefore, biomarkers of testicular toxicity that can be used in early phase of drug development are needed. In the present study, 4 reproductive toxicants, 2, 5-hexanedione (2, 5-HD), ethylene glycol monomethyl ether (EGME), cyclophosphamide (CP) and sulfasalazine (SASP), were administered to male rats for one day and gene expression in the testes 6 hours post dosing was monitored by cDNA microarray and the testes were histopathologically examined. No histopathologically abnormal findings were detected in the 2, 5-HD, CP and SASP. EGME produced slight degeneration of spermatocytes as a primary change. cDNA microarray analysis revealed that the number of affected Sertoli cells-related genes was the highest in the 2, 5-HD group. Many germ cells-related genes and a few Sertoli or Leydig cells-related genes were altered in the EGME group. In the CP group, many spermatogenesis-related genes were affected and additionally, the number of changes in the expression of DNA repair-related genes was the highest among the treatment groups. SASP also affected spermatogenesis-related genes but the number of the affected genes was the lowest among the treatment groups. These showed that there were compound related features in the affected genes and it is considered that there are different toxicological pathways in each compound. Interestingly, however, it was found that the same 3 genes were affected by the all tested compounds. They were heat shock protein 70-2, insulin growth factor binding protein 3 and glutathione S transferase pi genes. The changes in genes expression were detected prior to histopathological changes or at the time primary pathological changes were found. Therefore, the 3 genes were considered as useful biomarkers for evaluating the testicular toxicity that has different mechanism and for applying to evaluate toxicity in early development of the drug.

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#### GENE EXPRESSION CHANGES IN FETAL THYMUS EXPOSED TO ORGANIC COMPOUNDS EXTRACTED FROM DIESEL EXHAUST PARTICLES.

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Diesel exhaust particles (DEP) consist of a variety of toxic compounds, including polycyclic aromatic hydrocarbons (PAHs) and their derivatives (e.g., dioxins and quinones), on a carbon core. Although this feature seems to cause toxicity via multiple pathways, the mechanism of action of DEP has not been fully elucidated. In this study we investigated how DEP affect thymocyte cellularity via changes in gene expression in fetal thymus organ culture. Fetal thymuses excised from C57BL/6 mice were exposed to DEP extracts (DEPe) for 24 hr, and changes in gene expression were analyzed with an Affymetrix GeneChip. DEPe up-regulated 8 genes, and the most highly up-regulated gene was CYP1A1, which is well known as an arylhydrocarbon receptor (AhR) target gene. Other known AhR target genes (CYP1B1, Tiparp, and adseverin) were also up-regulated. Next, we compared changes in gene expression in response to AhR ligands and DEPe. Of the 8 genes up-regulated by DEPe, 6 genes were up-regulated by 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), and 7 were up-regulated by benzo[a]pyrene (B[a]P). These results suggests that AhR activation is the main pathway in the regulation of gene expression by DEPe. We also tested the possibility of involvement of reactive oxygen species from quinones in gene regulation. 1, 2-Naphthoquinone (NQ) up-regulated AhR target genes and other genes, whereas 9, 10-phenanthraquinone (PQ) exhibited some similarities to DEPe in gene expression profile. However, we did not identify any genes regulated by quinones among the genes changed by DEPe. Examination of effects on thymocyte cellularity revealed that DEPe decreased the numbers of thymocytes and skewed differentiation toward CD8 T cells. The same effects were observed when exposed to TCDD, B[a]P, or NQ, but not to PQ. Our results suggested that organic compounds adsorbed on to DEP regulate gene expression and then affect thymocyte development mainly through AhR activation.

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#### MOLECULAR CLONING AND CHARACTERIZATION OF HUMAN CYT19, AN S-ADENOSYL-L-METHIONINE:AS-METHYLTRANSFERASE FROM HEPG2 CELLS.

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Chronic arsenic exposure poses a threat to millions of people throughout the world due to arsenic in drinking water. Methylation has been considered the primary detoxification pathway of inorganic arsenic in many species but there is evidence that

methylation may increase arsenic toxicity. It has been shown that methylated arsenicals that contain AsIII are more cytotoxic and genotoxic than either arsenate or arsenite. Rat liver S-adenosyl-L-methionine: arsenicIII-methyltransferase has been identified and is homologous to human Cyt19, but there are species specific differences in arsenic biotransformation and toxicity. Additionally, there is considerable variation among humans in the rate of methylation of inorganic arsenic leading to measurable differences in toxicity. Therefore, it is important to better understand the enzymes that catalyze the methylation of arsenic in humans. In this study, we PCR amplified and cloned cyt19, a putative arsenic methyltransferase from human HepG2 hepatoma cells. The PCR product was ligated into an *E. coli* pET expression vector with a polyhistidine tag at the amino-terminal residue. The recombinant human cyt19 was successfully expressed in BL21 (DE3) and purified using a nickel-nitrilotriacetic acid metal-affinity chromatography. The recombinant protein catalyzes the methylation of arsenite as well as monomethylarsonic acid (MMA). The specific activity of arsenite methylation was 28 pmol/mg protein/min in a reaction mixture containing 5mM GSH, 1 mM DTT, 1 mM MgCl<sub>2</sub>, 100  $\mu$ M S-adenosyl-L-methionine, 50 $\mu$ M sodium m-arsenite, and 5  $\mu$ g of S-adenosyl-L-methionine: arsenic methyltransferase in 100mM tris/100mM sodium phosphate buffer pH 7.4 at 37 °C for 30 minutes. The results suggest that the human cyt19 gene, in fact is translated to an S-adenosyl-L-methionine: arsenic methyltransferase which methylates both arsenite and MMA.

**132** GLOBAL CHANGES IN POSTTRANSLATIONAL COVALENT HISTONE MODIFICATIONS MEDIATED BY LOW-DOSE ARSENIC.

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*In vitro*, arsenic mediates induction of a large number of genes including the oxidative stress-response battery, a hallmark of arsenic exposure. In microarray experiments, arsenic also down-regulates at least as many genes as it induces, suggesting that arsenic triggers substantial alterations to chromatin structure through mechanisms that have yet to be elucidated. Gene promoter elements, when packaged as nucleosomes, exclude DNA-interacting proteins thereby preventing transcription, while active gene transcription necessitates relaxation of chromatin structure to permit protein access. Covalent modifications of promoter-region histones, spanning only a few nucleosomes, occur concomitantly with gene induction or repression. Here we investigate the global effects of low-dose arsenic treatment on covalent histone modifications. In general, acetylation relaxes histone structure permitting access of transcriptional proteins; conversely, deacetylation closes down chromatin structure, impairing transcription. Histones were isolated from human keratinocyte-derived HaCaT cells by acid extraction following exposure to 5  $\mu$ M arsenic. Reverse-phase high-pressure liquid chromatography (HPLC) was used to separate raw histone extracts into each major histone constituent, namely histones H2A, H2B, H3 and H4. Covalent modifications on each core histone were analyzed by matrix-assisted-laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). In some instances, these modifications were confirmed using TAU-PAGE separation and immunoblot identification of specific modifications. The experiments presented here demonstrate that 1) at toxicologically relevant concentrations, arsenic modifies the pattern of covalent histone modification; 2) particular core histones are targets for arsenic-mediated covalent modification; 3) specific amino acid residues in each histone are targets for modification by arsenic. (Supported by NIEHS (2 T32 ES 07250 11 A 15 and P42 ES04908) and NIH (R01 ES10807))

**133** ARSENIC CAUSES DOSE-DEPENDENT ENHANCEMENT AND SUPPRESSION OF RETINOIC ACID RECEPTOR (RAR)-MEDIATED GENE ACTIVATION.

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Chronic exposure to arsenic is a significant human health threat and is associated with increased risks of various cancers, diabetes, heart disease, reproductive and developmental problems and other diseases. We previously demonstrated that arsenic acts as a potent endocrine disruptor at very low levels, altering steroid signaling at the level of receptor-mediated gene regulation for all five steroid receptors (i.e., ER, PR, GR, MR, AR). The goal of these studies was to investigate the effects of arsenic on other members of the nuclear receptor superfamily: in particular, the Retinoic Acid Receptor which is a type II receptor that is normally a heterodimer of the ligand-activated Retinoic Acid Receptor (RAR) and the common binding partner, Retinoic Acid X Receptor (RXR). Human NT2 cells were treated with 0.01 to 5  $\mu$ M sodium arsenite (As) for 24 hr, with or without the RAR ligand, all-trans retinoic acid (ATRA). Gene expression was measured in a transiently transfected RARE-luciferase construct or in the endogenous retinoid-inducible CYP26A1

gene. ATRA at 10 nM (EC50) induced RARE-luc expression by 20-fold and CYP26A1 by 10-fold. Arsenic co-treatment increased ATRA induction of RARE-luc by approximately 1.5-fold at the lowest As concentrations of 0.01 to 0.25  $\mu$ M; whereas As above 1  $\mu$ M caused a 40% decrease in ATRA-inducible expression. Similar results were seen with the CYP26A1 gene. Arsenic alone at 2  $\mu$ M suppressed basal expression by approximately 50%. These results are essentially identical to those previously obtained for steroid receptor-mediated gene expression, indicating that arsenic has widespread effects on at least two classes of nuclear hormone receptors. These endocrine disrupting effects, particularly at the very low doses used, may be important for understanding the wide array of pathophysiological processes linked with chronic low level inorganic arsenic exposure. In addition, these results suggest there may be different patterns of biological responses at different exposure concentrations. (NIH-NIEHS P42 ES07373)

**134** ARSENITE INHIBITS CELL CYCLE PROGRESSION OF U937 MYELOID LEUKEMIA CELLS THROUGH INDUCTION OF CDC25A DEGRADATION.

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Arsenic acts as a toxicant, a carcinogen and an effective chemotherapeutic in the treatment of acute promyelocytic leukemia. Its effects on humans are well-documented, but arsenic's mechanism of action is not well-understood. Arsenic has been shown to inhibit cancer cell cycle progression, but the targeted cell cycle phase has been debated. We monitored the cell cycle progression of cell cycle phase-enriched populations of U937 cells in the presence and absence of 5 $\mu$ M NaAsO<sub>2</sub> and determined that arsenite inhibits cell cycle progression through each cell cycle phase. 5-Bromo-2'-deoxyuridine pulse chase experiments show that S-phase transit time is markedly increased by arsenite (15h with treatment vs. 11h without). G2/M appears to be the phase most sensitive to arsenite-induced apoptosis, but given the large percentage of S phase cells in an asynchronous population, arsenite's inhibition of S phase progression may contribute significantly to overall growth inhibition. Cell cycle progression is controlled by the activities of cyclin dependent kinases (cdks), which are in turn regulated by cyclin binding and by activating and inhibitory phosphorylations. Cdc25 dual specificity phosphatases dephosphorylate sites of inhibitory phosphorylation as the last step before cdk triggers progression into the next cell cycle phase. Treatment of U937 cells with 5 $\mu$ M NaAsO<sub>2</sub> for 2h results in a dramatic decrease in protein levels of cdc25A, which controls entry into and progression through S phase by dephosphorylating Tyr15 and Thr14 on cdk2. Cdc25A is regulated by ubiquitin-dependent proteasomal degradation, triggered by its phosphorylation. A time course of concurrent treatment of cells with cycloheximide and arsenite reveals that cdc25A is degraded more quickly in arsenite-treated cells. Arsenite-dependent degradation of cdc25A could contribute to inhibition of S phase entry and progression. Studies are ongoing to establish the dependence of arsenite-induced cdc25A degradation on ubiquitination and proteasome activity and the involvement of cdc25A in arsenite-induced growth inhibition.

**135** MOLECULAR MECHANISMS UNDERLYING INHIBITION OF INSULIN-STIMULATED GLUCOSE UPTAKE IN ADIPOCYTES EXPOSED TO TRIVALENT ARSENICALS.

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Chronic environmental or occupational exposures to inorganic arsenic (iAs) have been associated with increased prevalences of non-insulin-dependent (type-2) diabetes mellitus. The clinical symptoms of the disease indicate that iAs or its metabolites interfere with insulin signaling and/or suppress insulin-stimulated glucose metabolism in tissues. We have previously shown that exposures to subtoxic concentrations of arsenite (iAs<sup>III</sup>), methylarsine oxide (MAs<sup>III</sup>O) or iododimethylarsine (DMA<sup>III</sup>I) inhibit glucose uptake in murine 3T3-L1 adipocytes by interfering with insulin-stimulated GLUT4 translocation from the perinuclear compartment to the plasma membrane. The present work examined molecular mechanisms underlying these effects. We found that treatments of cultured 3T3-L1 adipocytes with iAs<sup>III</sup> or MAs<sup>III</sup>O resulted in suppression of insulin-dependent phosphorylation of protein kinase B (PKB/Akt) and inhibition of PKB/Akt activity. Neither iAs<sup>III</sup> nor MAs<sup>III</sup>O interfered with activation of the components of the insulin-activated signal transduction pathway upstream from PKB/Akt. Notably, in an *in vitro* assay system, both arsenicals inhibited activity of PKB/Akt isolated by immunoprecipitation from untreated insulin-stimulated adipocytes. Unlike iAs<sup>III</sup> and MAs<sup>III</sup>O, DMA<sup>III</sup>I did not suppress PKB/Akt phosphorylation or activity in intact adipocytes. However, DMA<sup>III</sup>I inhibited *in vitro* activity of the immunoprecipitated enzyme isolated from untreated cells. Expression of constitutively active PKB/Akt restored the normal pattern of insulin-dependent PKB/Akt phosphorylation and glucose uptake in cells treated with iAs<sup>III</sup> or MAs<sup>III</sup>O, but had no effect on inhibition of glucose uptake in cells exposed to DMA<sup>III</sup>I. These results suggest that

iAs<sup>III</sup> and MA<sup>III</sup>O interfere with insulin-stimulated glucose uptake in 3T3-L1 adipocytes by inhibiting PKB/Akt phosphorylation and the activity of the phosphorylated enzyme. The inhibition of glucose uptake by DMA<sup>III</sup>I may involve mechanisms independent of Akt.

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### FURTHER STUDIES ON ABERRANT GENE EXPRESSION IN ARSENIC-INDUCED MALIGNANT TRANSFORMATION OF LIVER EPITHELIAL CELLS.

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Inorganic arsenic induces liver tumors in humans and rodents. In prior research we found chronic exposure (>18 wk) of rat liver epithelial TRL1215 cells to low level arsenite (500 nM) induces malignant transformation. Initial microarray studies revealed changes in gene expression in these chronic arsenic exposed (CAsE) cells that warrant further study. Real-time RT-PCR showed large increases in CAsE cell stress-response genes like glutathione S-transferase-pi (GST- $\pi$ , 3-fold), heme oxygenase-1 (4-fold), superoxide dismutase-1 (5-fold) and metallothionein-1 (MT-1; 4-fold) compared to control. The positive cell-cycle regulatory genes cyclin D1 (10-fold) and PCNA (3-fold) were overexpressed in CAsE cells, while negative regulatory genes p21 (30% of control) and p16 (1%) were markedly depressed. In CAsE cells c-jun, c-met, c-myc, Wilms tumor protein-1 (WT-1) and  $\alpha$ -fetoprotein (AFP) expression increased from 3 to 13-fold, while expression of insulin-like growth factor II (IGF-II) and fibroblast growth factor (FGF) were abolished. Western-blot analysis confirmed increases in GST- $\pi$ , WT-1, AFP and decreases in p16 and p21 protein. MT overexpression was not due to DNA methylation as bisulfite sequencing showed the MT-1 promoter region was slightly more methylated in CAsE cells than controls. Treatment of CAsE cells with the DNA demethylating agent 5-aza-deoxycytidine (Aza; 2.5  $\mu$ M, 72 hrs) greatly increased MT-1 (19-fold) compared to control cells (5-fold), indicating CAsE cells are more sensitive to DNA demethylation stress. Conversely, Aza did not restore expression of p16, IGF-II, or FGF to control levels in CAsE cells, indicating suppression was due to factors other than DNA methylation changes. Thus, an intricate variety of gene expression changes occur in arsenic-induced malignant transformation, including oncogene activation and increased and decreased expression of genes critical to growth regulation.

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### ARSENITE DECREASES CYP3A INDUCTION IN CULTURED RAT HEPATOCYTES BY DUAL MECHANISMS.

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We previously reported that 24 h treatment with 5  $\mu$ M arsenite decreased induction of CYP3A protein in dexamethasone (DEX)-treated rat hepatocyte cultures, with minor decreases in CYP3A23 mRNA, as measured by solution hybridization. Here, we used a more sensitive assay to investigate earlier effects of arsenite on CYP3A23 mRNA. In rat hepatocyte cultures exposed to DEX and arsenite for 6 h, CYP3A protein was decreased greater than 90% compared to DEX treatment alone. At the same time, arsenite caused a 30-40% decrease in CYP3A23 mRNA in DEX-treated cultures, yet remained 5-fold higher than in untreated cultures. These results suggest arsenite acts through post-transcriptional as well as a transcriptional mechanism to decrease CYP3A. Since arsenite did not decrease intracellular glutathione or increase lipid peroxidation, we conclude these decreases do not involve oxidative stress. Exogenous heme did not prevent the arsenite-mediated decrease in CYP3A protein induction. Arsenite did not increase degradation of CYP3A holoprotein. We are currently investigating whether arsenite negatively regulates mRNA translation by interacting with the untranslated regions of CYP3A23 to result in decreased CYP3A protein levels. We examined whether arsenite effects CYP3A induction by the nuclear receptor PXR, the major mediator of CYP3A induction by steroids. Arsenite caused a 30% decrease in expression of a reporter construct containing the PXR-sensitive, CYP3A23 upstream regulatory region. Thus, one action of arsenite may be to inhibit transcription of CYP3A23 by interfering with PXR-mediated induction. We conclude that arsenite utilizes dual mechanisms to decrease CYP3A induction in primary cultures of rat hepatocytes.

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### ANALYSIS OF GLOBAL AND GENE SPECIFIC DNA METHYLATION IN LIVERS OF NEWBORN MICE TRANSPLACENTALLY EXPOSED TO A CARCINOGENIC DOSE OF ARSENIC.

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Arsenic is a human carcinogen that can target the liver. Our prior work has shown that exposure of pregnant mice to inorganic arsenic induces a high incidence of liver tumors in male offspring when they reach adulthood. Initial analysis of newborn mice exposed to a hepatocarcinogenic dose of arsenic *in utero* revealed remarkable gene expression changes. Some evidence indicates arsenic can alter gene expression by altering DNA methylation. Thus, this study examined early DNA methylation changes in arsenic-induced transplacental hepatocarcinogenesis. Pregnant mice received drinking water containing 85 ppm arsenic (as sodium arsenite) or unaltered water (control) from gestation day 8 to 18. Liver samples were taken from newborn males, and global genomic DNA methylation was determined by the methyl acceptance assay. Arsenic exposure *in utero* did not alter hepatic global DNA methylation in the newborn. Methylation in GC-rich regions was assessed using amplification of genomic DNA digested with methyl-sensitive restriction enzymes. Arsenic exposure resulted in 5 distinct regions of reduced methylation and 2 regions of increased methylation in GC-rich sites. PCR analysis of hepatic Ha-ras following methylation-sensitive enzyme digestion of DNA showed reduced methylation of a key CpG island after arsenic exposure. Although arsenic induced metallothionein-I (MT-I) overexpression, both methylation specific PCR and DNA sequencing following sodium bisulfite indicated the promoter region of MT-I gene was un-methylated in control and arsenic-treated mice, suggesting mechanisms other than methylation regulate arsenic-induced MT overexpression. Overall, hepatic global genomic DNA methylation status was not altered by arsenic, but GC rich methylation appeared reduced. Changes in gene specific methylation depended on the gene in question.

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### ARSENITE INDUCES CYTOKERATIN EXPRESSION IN MICE LIVER.

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Increased levels of cytokeratins are observed in human liver disease such as primary biliary cirrhosis and in alcoholic liver disease related to Mallory bodies formation as a consequence of protein accumulation, essentially cytokeratins. Modified synthesis and abnormal organization of these intermediate filaments are considered indicators of liver damage. The liver is also the main organ where many carcinogens such as arsenic are metabolized. Studies in humans exposed to inorganic arsenic by the oral route have noted signs or symptoms of liver injury. Lipid vacuolation and fibrosis have been reported in rats exposed to arsenic in drinking water. Increased synthesis and disruption of cytokeratin 18 cellular organization, have been induced in WRL-68 human fetal hepatic cell line by sodium arsenite. In this work, male BALB/c mice were given orally 2.5, 5 and 7.5 mg/kg/day of sodium arsenite. Cytokeratin expression was monitored in liver after 2 and 9 days. Significant induction of CK18 mRNA and protein synthesis were observed in those animals exposed to the lower doses when liver samples were examined by RT-PCR and Western blotting respectively. We did not observe significant induction at the highest dose employed, suggesting that arsenite is inhibiting the synthesis of this important protein to hepatocyte architecture. The induction of cytokeratin 18 could be considered as an early indicator of liver damage by arsenic. This work was partially supported by PAPIIT and CONACYT.

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### REDUCTION OF ARSENATE BY HUMAN ERYTHROCYTE (RBC) LYSATE AND RAT LIVER CYTOSOL (CS) IS LINKED TO GLYCOLYSIS.

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Reduction of arsenate (AsV) to the more toxic arsenite (AsIII) is carried out *in vivo* by hitherto unidentified enzymes. Purine nucleoside phosphorylase (PNP) is an efficient AsV reductase (AsVR) *in vitro*, though it seems to be irrelevant *in vivo* (Nemeti et al., Toxicol. Sciences, 74, 22, 2003). Intact human RBC possess an AsVR activity, which appears dependent on glutathione (GSH) and NAD supply but independent of PNP (Nemeti and Gregus, Toxicol. Sciences, in press). Studies on AsV reduction in intact RBC have led to the hypothesis that the AsVR activity lies between glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and enolase in

the glycolytic pathway. To further characterize this PNP-independent AsVR, we examined the effects of GSH, glycolytic substrates, NAD, ATP, and related nucleotides on AsV reduction in lysed RBC and rat liver cytosol in the presence of BCX-1777, a PNP inhibitor. AsIII formed in the assays from AsV was quantified with HPLC-HG-AFS analysis. In both hemolysate and CS, GSH enhanced AsV reduction in a concentration dependent manner. Glycolytic substrates, especially fructose-1, 6-bisphosphate and phosphoglyceric acids (PGA), improved AsVR activity. NAD, especially together with these substrates, markedly increased AsIII formation, whereas NADH was a strong, ATP and ADP were moderate inhibitors. In CS but not in hemolysate, NADP enhanced AsV reduction moderately. In hemolysate but not in CS, 2-phosphoglycollate, which increases the breakdown of the RBC-specific compound 2, 3-bisphosphoglycerate to 3-PGA, doubled AsVR activity. Collectively, hemolysate and rat liver cytosol possess a PNP-independent AsV reductase activity that depends on the availability of GSH, NAD, and glycolytic substrates. Our data suggest that this activity might be ascribed to two functionally linked glycolytic enzymes, GAPDH and phosphoglycerate kinase (PGK), because GAPDH uses NAD while PGK uses 3-PGA as substrate. Testing of the AsVR activity of these two enzymes is presented in the accompanying poster (Gregus and Nemeti). (Supported by OTKA and the Hungarian Ministry of Health)

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THE ROLE OF THE RAS SIGNALING PATHWAY IN ANDROGEN-INDEPENDENCE ACQUIRED DURING ARSENIC-INDUCED MALIGNANT TRANSFORMATION OF HUMAN PROSTATE EPITHELIAL CELLS.

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In humans progression of prostate cancer to androgen-independence (AI) signals a marked worsening of prognosis, and is linked to autocrine or paracrine growth. Evidence indicates that the androgen receptor (AR) and Ras can be involved in acquired AI in prostate cancer. Inorganic arsenic is a potential human prostate carcinogen but its role in prostate tumor progression is undefined. Thus, we studied AI in CAsE-PE cells, a malignant transformant of the non-tumorigenic human prostate epithelial cell line RWPE-1 induced by chronic arsenic exposure. The transformant grew at a much faster rate in complete medium than control cells and also showed sustained growth in steroid-reduced medium. Although similar levels of AR occurred in control and CAsE-PE cells, androgens were less effective in stimulating cell proliferation and AR-related gene expression in CAsE-PE cells. For instance, dihydrotestosterone increased PSA transcript much more in control (4.5-fold) than CAsE-PE (1.5-fold) cells. CAsE-PE cells also showed relatively low levels of growth stimulation by non-androgen steroids, such as estradiol. These data indicate that arsenic did not induce AR over-expression or loss of AR ligand specificity, both of which can occur with acquired AI in prostate cancer. CAsE-PE cells did, however, display constitutively increased expression of unmutated K-Ras (13-fold). Ras is a common component of many signaling pathways activated in advanced prostate cancer. Indeed, events downstream of K-Ras, including A-Raf and B-Raf (both serine-threonine MAP kinases) showed increased constitutive expression in CAsE-PE cells (2.2-fold and 3.2-fold, respectively) compared to control. Thus, arsenic induced malignant transformation is associated with acquired AI in human prostate cells and Ras activation and up-regulation of down-stream pathways appear to correlate with this tumor progression.

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ARSENITE BINDING TO SUBSETS OF THE HUMAN ESTROGEN RECEPTOR- $\alpha$ .

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Enzyme inhibition by arsenicals has been described many times, but the underlying binding of trivalent arsenicals to peptides and proteins has received little attention. The purpose of this study was to determine Kd and Bmax values for arsenite binding to nine synthetic peptides (up to 25 amino acids long) which contained between 0 and 4 sulfhydryls. We selected the human estrogen receptor- $\alpha$  protein for study because arsenite is a potential nonsteroidal environmental estrogen and several interactions between arsenic exposure, estrogenicity and carcinogenicity are known. We utilized radioactive <sup>73</sup>As labeled arsenite and vacuum filtration methodology to determine the binding affinity of arsenite to synthetic peptides based on a zinc finger region containing up to 4 free sulfhydryls and the estrogen binding region containing up to 3 free sulfhydryls. In our studies, amino acids other than cysteine (including methionine and histidine) did not bind arsenite. Peptides modeled on the estrogen receptor with two or more nearby free sulfhydryls (2 or 5 intervening amino acids) had Kd values in the 1-4  $\mu$ Molar range. Peptides containing a single sulfhydryl or two sulfhydryls spaced 17 amino acids apart had higher Kd values in the 100-200  $\mu$ Molar range, demonstrating lower affinity. With the exception of peptide 24 which had an unusually high Bmax value of 234

nmol/mg, the binding capacity of the studied peptides was proportional to the number of free cysteines. Based on our experimental Kd values and published literature values for tissue sulfhydryls, 99% or more of arsenite *in vivo* should be bound to tissue sulfhydryls and not free. The binding of trivalent arsenicals to protein sulfhydryl groups and the ensuing enzyme inhibition and altered biological function can initiate at least five proposed modes of arsenic's carcinogenic action - induced chromosomal abnormalities, altered DNA repair, altered DNA methylation patterns, altered growth factors and enhanced cell proliferation. (Disclaimer: This is an abstract of a proposed presentation and does not necessarily reflect EPA policy.)

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ALTERED PROTEIN EXPRESSION FOLLOWING *IN UTERO* EXPOSURE TO ARSENIC.

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Inorganic arsenic is a known human carcinogen and common drinking water contaminant affecting millions of people worldwide. Though the effects of maternal arsenic exposure on the developing lung are unknown, this metalloid is known to cross the placenta. Using an *in utero* exposure model, we have previously identified genes that are altered by exposure to arsenic in the maternal drinking. A more complete understanding of the *in utero* effects of arsenic requires analysis of alterations in protein expression. We hypothesize that *in utero* exposure to inorganic arsenic through maternal drinking water causes altered protein expression in the developing lung, indicative of downstream molecular and functional changes. From conception to embryonic day eighteen, we exposed pregnant Sprague-Dawley rats to 500 parts per billion arsenic (as arsenite) via the drinking water. Protein was isolated from a single pup from 3 separate arsenic exposed litters. Protein was also isolated from three control pups (each pup from a different litter). Samples were pooled and arsenic-induced alterations in protein expression were determined using BD Power Blot analysis. This technique can analyze over 1000 separate proteins. Blots were run in triplicate and protein content was measured using densitometry. Each of the treated blots was compared against each of the control blots. Only those proteins in which expression was 1.25 up or down regulated by arsenic in each sample were considered as being altered. Thirty five proteins or posttranslationally modified proteins were identified as being altered (21 up- and 14 down-regulated). Analysis of potential protein function indicated that nucleus/nuclear transport proteins, cancer related proteins, tyrosine kinase substrates and cytoskeleton related proteins were altered by arsenic. The present study shows that arsenic induces alterations in protein levels in the developing lung. These data may be useful in the elucidation of molecular targets and biomarkers of inorganic arsenic exposure during lung development. Supported by NIH grants ES-04940 and ES-06694.

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ABERRANT GENE EXPRESSION IN THE NEONATAL MOUSE LUNG FOLLOWING *IN UTERO* EXPOSURE TO INORGANIC ARSENIC.

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Inorganic arsenic is a lung carcinogen in humans. Our prior work showed *in utero* exposure to arsenic in mice can induce lung cancers in female offspring after they become adults. To define early molecular changes in the lung after *in utero* arsenic exposure, pregnant C3H mice were given carcinogenic doses of arsenic in the drinking water (42.5 and 85 ppm arsenic as sodium arsenite) from day 8 to 18 of gestation and aberrant gene expression was examined in the lungs of female offspring as newborns. Real-time RT-PCR analysis was used to determine expression of thirty-six selected genes associated with carcinogenesis or arsenic exposure. After *in utero* arsenic exposure, estrogen receptor- $\alpha$  (ER- $\alpha$ ) showed marked, dose-related increases in expression in newborn female lung. Compared to control, pulmonary ER- $\alpha$  increased over 9-fold after 42.5 ppm arsenic and over 14-fold after 85 ppm arsenic. Recent clinical evidence indicates ER- $\alpha$  overexpression is common in lung tumor specimens from women, and may contribute to gender linked difference in lung cancer risk. Another gene highly overexpressed in female lung after *in utero* arsenic exposure was  $\alpha$ -fetoprotein (AFP) which showed a maximum increase in expression of over 11-fold compared to control. AFP is normally a fetal protein that is overexpressed in many tumors, including aggressive lung cancers. AFP is also considered a growth enhancing protein. Other gene expression changes in the lungs of arsenic-treated female newborn mice included increased expression of myc (2-fold), metallothionein-1 (3-fold), glutathione peroxidase (2-fold), betaine-homocysteine S-methyltransferase (10-fold), insulin-like growth factor-1 (IGF-1; 2-fold) and IGF-2 (2-fold). Overexpression of IGF-1 and IGF-2 is common in tumors. Thus, transplacental exposure to arsenic at doses that can be carcinogenic to the female lung produced rapid gene expression changes relevant to pulmonary carcinogenesis, such as increased AFP and ER- $\alpha$ .

ARSENIC INDUCES CHROMOSOMAL AND CENTROMERIC ABNORMALITIES IN HUMAN LUNG CELLS.

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Arsenic is a known human carcinogen, causing lung and bladder cancer. Studies have shown humans chronically exposed through drinking water, present increased micronuclei in oral mucosal cells, urothelial cells and lymphocytes. However, the genotoxicity of arsenic in human lung cells has not been investigated. Because lung is a major target for arsenic-induced carcinogenicity, we investigated its cytotoxicity, clastogenicity and centromeric effects in human lung fibroblasts. We found that arsenic is cytotoxic to human lung cells in a concentration-dependent manner, with 1, 5 and 10  $\mu$ M inducing 65, 45 and 25% relative survival, respectively. Arsenic is also clastogenic to human lung cells in a concentration-dependent manner, with 1, 5 and 10  $\mu$ M damaging 3, 7 and 15% of metaphases, respectively. Arsenic also caused concentration-dependent increases in centromere spreading, c-anaphase chromosomes and endoreduplication in human lung cells. Specifically evaluating cells treated with 5  $\mu$ M arsenic, we found 16% of metaphases with at least one spread centromere or c-anaphase chromosome. When cells treated with 5  $\mu$ M arsenic for 24 h were allowed to recover in arsenic free media, 20% fewer centromeric aberrations were noted and a 5% increase in endoreduplicated chromosomes when compared to those exposed to 5  $\mu$ M arsenic for 24 h. These data suggest centromeric abnormalities and endoreduplication may be causative mechanisms in arsenic-induced lung cancer. This work was supported by a grant from the Maine Cancer Foundation.

CHARACTERIZATION OF HUMAN BLADDER UROTHELIAL CELLS MALIGNANTLY TRANSFORMED BY ARSENITE.

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Arsenic is implicated in development of human bladder cancer. Chronic arsenite [As(III)] exposure can cause the malignant transformation of human urothelial cells (UROtsa). Since monomethylarsonous acid [MMA(III)] is reported to be more toxic than As(III) in normal UROtsa cells, studies were undertaken to compare the toxicity of MMA(III) and As(III) between the UROtsa and As(III)-transformed UROtsa cells. MMA(III) and As(III) were equally cytotoxic to normal and transformed cell lines with MMA(III) being 12-fold more toxic. The ability of transformed cells to biotransform As (III) was determined. While the UROtsa cells were found to oxidize and methylate As(III) to As(V), MMA(III), and MMA(V), biotransformation of As(III) by the transformed cells was limited to redox cycling to As(V). Gene expression studies comparing transformed UROtsa to control UROtsa were conducted with a two-fold change/repetition (N=3) for significance( $p>0.05$ ). Increased expression was seen with genes associated with cell signaling (Ca++ binding protein A8), metabolism/biotransformation (GSH-S-transferase M3), and cell receptors (glypican 3 mRNA). Down regulation was seen in genes related to signal transduction (TTK protein kinase), cell cycle (cyclin A1 and D2), tumor suppressor genes (p53 activated fragment-1), structural and cellular matrix associated proteins (annexin A3 and actin protein 2/3 complex B1), and cell adhesion molecules (metalloprotease 1 and 2 inhibitor). Studies are underway to transform UROtsa cells with MMA(III) to determine if similar effects will be seen. By characterizing the morphological and genetic differences between the normal and the transformed UROtsa, biomarkers of exposure or injury to the bladder may be identified that can be used in future epidemiological studies. (Supported by NIH grants ES04940, CA023074, ES06694, ES07091).

ARSENITE AND MONOMETHYL ARSONOUS ACID-INDUCED TOXICITY IN HUMAN UROTHELIAL CELLS.

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Arsenic is a known bladder carcinogen. However, the mechanism of arsenic-induced bladder injury or carcinogenesis is unknown. Studies investigating gene expression caused by arsenite [As(III)] and monomethylarsonous acid [MMA(III)]

exposure in human urothelial (UROtsa) cells revealed the presence of both oxidative stress and protein damage. These results prompted the study of reactive oxygen species generation in As(III) and MMA(III)-induced cytotoxicity in UROtsa cells. Reduction of intracellular glutathione levels via administration of bathionine S-R-sulfoximine (25  $\mu$ M) increased cytotoxicity of MMA(III) and As(III) by 4 and 7 fold, respectively, indicating the critical role of cellular antioxidant status. However, concurrent treatment of UROtsa cells with radical scavengers [DPPD (10  $\mu$ M and 100  $\mu$ M) and DMSO (0.1% and 0.5%)] only ameliorated the cytotoxicity of higher concentrations of As(III) and MMA(III). Thus efforts to block the production of As(III) and MMA(III)-induced reactive oxygen species via adenoviral-mediated infection of both superoxide dismutase and catalase are underway. In addition, to demonstrate that As(III) and MMA(III) produce reactive oxygen species and induce oxidative DNA damage, the fluorescent probe CM-H2DCFDA is being used to detect oxyradical production using confocal microscopy and increases in 8-hydroxy-2-deoxyguanosine (8-OHdG) are being detected via HPLC to illustrate oxidative DNA damage. These studies will improve the understanding of the role reactive oxygen species play in As(III) and MMA(III) toxicity in a target organ cell line (Supported by NIH grants ES04940, ES07091, ES06694).

METABOLISM AND TOXICITY OF ARSENIC IN HUMAN URINARY BLADDER EPITHELIAL CELLS EXPRESSING RAT ARSENIC (+3)-METHYLTRANSFERASE.

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The enzymatic methylation of inorganic arsenic (iAs) is catalyzed by arsenic (+3)-methyltransferase (AS3MT). AS3MT is expressed in rat liver and in human hepatocytes but not in a human urothelial cell line (UROtsa), which does not methylate iAs. Thus, UROtsa cells are an ideal null background in which the role of iAs methylation in the modulation of toxic and cancer promoting effects of this metalloid can be examined. A retroviral gene delivery system was used in this study to create a clonal UROtsa cell line (UROtsa/F35) that expresses rat AS3MT. The metabolism and cytotoxicity of arsenite (iAs<sup>III</sup>) and methylated trivalent arsenicals were characterized in both UROtsa and UROtsa/F35 cells. In contrast to parental cells, UROtsa/F35 cells methylated iAs<sup>III</sup>, yielding methylarsenic (MAs) and dimethylarsenic (DMAs) that contained either As<sup>III</sup> or As<sup>V</sup>. When exposed to MAs<sup>III</sup> UROtsa/F35 cells produced DMAs<sup>III</sup> and DMAs<sup>V</sup>. MAs<sup>III</sup> and DMAs<sup>III</sup> were more cytotoxic than iAs<sup>III</sup> in both UROtsa and UROtsa/F35 cells. The greater cytotoxicities of MAs<sup>III</sup> and DMAs<sup>III</sup> as compared to iAs<sup>III</sup> were associated with greater cellular retention of both methylated trivalent arsenicals. Notably, UROtsa/F35 cells were more sensitive than UROtsa cells to the cytotoxic effects of iAs<sup>III</sup>, but were more resistant to the cytotoxicity of MAs<sup>III</sup>. Increased toxicity of iAs<sup>III</sup> in UROtsa/F35 cells was associated with inhibition of DMAs production and intracellular accumulation of MAs at high exposure levels. The resistance of UROtsa/F35 cells to moderate concentrations of MAs<sup>III</sup> was linked to a rapid conversion of MAs<sup>III</sup> to DMAs and efflux of DMAs. However, concentrations of MAs<sup>III</sup> that inhibited DMAs production by UROtsa/F35 cells were equally toxic for both cell lines. Thus, the extent of the production and accumulation of MAs<sup>III</sup> is a key factor that determines the toxicity of iAs in methylating cells. (This abstract does not necessarily reflect EPA policy.)

GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE (GAPDH) AS AN ARSENATE REDUCTASE IN HUMAN RED BLOOD CELLS (HRBC) AND RAT LIVER CYTOSOL (RLC).

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The mechanism for reduction of arsenate (AsV) to the more toxic arsenite (AsIII) is unknown. Purine nucleoside phosphorylase (PNP) works as an arsenate reductase (AsVR) *in vitro*, however, it appears irrelevant *in vivo* (Nemeti et al., Toxicol. Sciences. 74: 22, 2003). The accompanying poster (Nemeti and Gregus) demonstrated that hRBC lysate and rLC contain a PNP-independent AsVR activity that is supported by glutathione (GSH), NAD and glycolytic substrates, especially phosphoglyceric acids (PGA). Because NAD is substrate for GAPDH, whereas 3-PGA is substrate for phosphoglycerate kinase (PGK), the roles of these two functionally linked glycolytic enzymes in AsV reduction have been tested. AsIII formed in the assays from AsV was quantified by HPLC-hydride generation-atomic fluorescence spectrometry. A mix of purified GAPDH and PGK catalyzed GSH-dependent reduction of AsV, provided NAD was present and GAPDH was supplied with a glycolytic substrate, either with glyceraldehyde 3-phosphate (Ga3P) directly or indirectly by PGK (from 3-PGA and ATP). It was also shown that GAPDH (purified

from rabbit muscle or hRBC) alone exhibited AsVR activity that depended on the enzyme concentration, was enhanced in a concentration-dependent manner by its substrates (Ga3P, NAD) as well as GSH, and was strongly inhibited by NADH. Konigic acid, a selective GAPDH inhibitor, decreased both the classical enzymatic activity and the AsVR activity of rabbit muscle GAPDH in a concentration-dependent manner, abolishing both activities at 25 mM. Konigic acid also decreased both the GAPDH and the AsVR activities of intact hRBC, hRBC lysate and rLC, at high concentration abolishing AsIII formation in intact hRBC, almost completely inhibiting it in hRBC lysate and partially inhibiting it in rLC. Thus, GAPDH works as a GSH-dependent AsVR; it appears largely responsible for the PNP-independent AsVR activity in hRBC and partly responsible for such activity in rLC. The *in vivo* relevance of GAPDH as an AsV reductase remains to be tested. (Supported by OTKA and the Hungarian Ministry of Health)

## 150 ARSENITE INDUCES PROCOAGULANT ACTIVITY IN HUMAN PLATELETS BY PHOSPHATIDYL SERINE EXPOSURE AND MICROPARTICLE GENERATION.

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Many epidemiological studies reported that there are strong correlations between arsenic in drinking water and occurrences of cardiovascular disease as well as human cancer, but the exact mechanism is not clearly known. We investigated if arsenic can affect procoagulant activity of platelet, which is essential in blood clotting, thrombus formation and cardiovascular disease development. Effects of arsenic on platelet procoagulant activities are examined *in vitro* using human platelets and *in vivo* in a rat animal model. Arsenite significantly enhanced platelet procoagulant activity induced by thrombin in a concentration- and time-dependent manner as observed in prothrombinase assay. It is generally considered that the increase of procoagulant activity is mediated by exposure of anionic phospholipids (phosphatidylserine; PS) and microparticle (MP) generation. In flow cytometry analysis, we found that arsenite enhanced PS exposure and MP formation induced by thrombin. Generated MPs were also confirmed in confocal microscopy and found to have procoagulant activity like PS-exposed platelets. These effects of arsenic appeared to be mediated by enhanced calcium increase as investigated with Fluo-3. Pretreatment of calpeptin, a calpain inhibitor, abolished arsenic effects on platelets showing that calpain is involved in these processes. In addition, arsenite inhibited aminophospholipid translocase, suggesting exposed PS could be maintained in outer membranes. Consistent with arsenite-enhanced platelet procoagulant activity observed *in vitro*, arsenite infusion actually led to the increase of thrombus formation *in vivo* in rat venous thrombosis model. In conclusion, arsenite induces enhancement of procoagulant activity with the increase of PS exposure and MP formation in human platelets and resultant venous thrombosis as observed *in vivo*. These enhanced platelet procoagulant activity may be a major contributing factor in cardiovascular disease associated with chronic intake of arsenic in drinking water through enhanced blood clotting and thrombus formation.

## 151 ARSENIC METHYLTRANSFERASE CYT19 EXPRESSION AND ACTIVITY IN RAT TISSUES.

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Inorganic arsenicals such as arsenite (iAs<sup>III</sup>) and arsenate (iAs<sup>V</sup>) are well-known worldwide environmental contaminants and human carcinogen. Arsenic is known to be metabolized by repetitive reduction and methylation, and excreted mainly in urine as methylated arsenicals such as monomethylarsonic acid (MMA<sup>V</sup>) and dimethylarsinic acid (DMA<sup>V</sup>). Although it has been known that the arsenic methylation is catalyzed by arsenic methyltransferase, Cyt 19, very little is known about characteristics of the enzyme. In the present study we report gene expression and enzyme activity of Cyt19 in rat tissues. We investigated mRNA and protein levels of Cyt19 in rat tissues, such as heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis, by Northern and Western analysis, respectively. The rat tissues cytosol was incubated at 37 °C for 3 h with 10 μM iAs<sup>III</sup>, 5 mM GSH and 1 mM S-adenosyl-L-methionine in 75 mM phosphate buffer (pH 7.0). The reaction was stopped by boiling the reaction mixture for 5 min, and then treated with H<sub>2</sub>O<sub>2</sub> to oxidize all arsenicals to pentavalency. The metabolites, MMA<sup>V</sup> and DMA<sup>V</sup>, were analyzed on a reversed phase column by high-performance liquid chromatography-inductively coupled argon plasma mass spectrometry (HPLC-ICP MS). Both mRNA and protein levels were highest in the liver. The intermediate expression of Cyt19 mRNA was observed in heart and testis. However, arsenic methylating activity was detected only in the liver cytosol using the current HPLC-ICP MS method.

## 152 THE ROLE OF VALENCE AND METHYLATION STATE ON THE ACTIVITY OF ARSENIC DURING MITOSIS.

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Trivalent methylated arsenicals are much more potent DNA damaging agents, clastogens, and large deletion mutagens than are their inorganic and pentavalent counterparts. Previously, we had noticed that many of the arsenicals induced "c-type" anaphases characteristic of spindle poisons. In the present study, we exposed human lymphoblasts for 6 h to six arsenicals: sodium arsenite (NaAs5), sodium arsenite (NaAs3), monomethylarsonic acid (MMA5), monomethylarsonic acid (MMA3), dimethylarsinic acid (DMA5), and dimethylarsinic acid (DMA3). Slides were then prepared, and the mitotic indices (MI) were calculated. NaAs5 caused a small but significant increase in MI. MMA5 also caused only a slight increase in MI that just reached statistical significance. In contrast, DMA5 caused a highly significant increase in MI producing ~75% the MI of demecolcine, the positive control. NaAs3 had no significant effect on MI and was quite toxic. MMA3 induced more than a twofold increase in MI compared to the control. DMA3 gave inconsistent results. We also exposed each arsenical directly to tubulin and spectrophotometrically measured the effect on polymerization. None of the pentavalent arsenicals had a substantial effect on polymerization of tubulin. In contrast, NaAs3 inhibited polymerization at 1 mM and above, and MMA3 and DMA3 at 10 μM and above. Taken together, these results give a complex picture of how arsenicals may affect cells. Some are cytotoxic, which may prevent the cells from cycling, thereby reducing the MI. Simultaneously, at lower concentrations, these same arsenicals may react with the spindle and cause an apparent increase in MI by arresting the cells at metaphase or anaphase. Thus, the metabolites of arsenic are active not only as chromosome breaking and DNA damaging agents but can also interfere with cell division. They can effect this through interaction with the spindle potentially leading to aneuploidy, a common driving force in genomic instability and ultimately in tumor formation. [This abstract does not necessarily reflect EPA policy].

## 153 INORGANIC ARSENIC BIOTRANSFORMATION AND MMA(V) REDUCTASE/GSTO1-1 KNOCKOUT MICE.

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The hypothesis that GSTO1-1 knockout mice biotransformed inorganic arsenic differently than wild mice was tested. In the biotransformation of inorganic arsenic, MMA(V) reductase catalyzes the reduction of arsenate, MMA(V), and DMA(V) to the more toxic arsenic species. MMA(V) reductase and human GST-Omega (hGSTO1-1) are identical proteins. Methods: Male knockout mice, in which Exon 3, of the gene for GSTO1-1 has been eliminated, were injected i.m. with Na arsenate (4.16 mg As per Kg of body weight) and the arsenic species in tissues measured at 0.5, 1, 2, 4, 8, and 12 hours by HPLC-ICP-MS. The wild mice (W) used as controls were DBA/1LacJ. Results: The concentration of arsenate was greatest in the bladder and the kidneys and peaked in these tissues at 0.5 hour. The highest concentration of arsenite was in the kidneys and liver. MMA(V) concentrations were the lowest when compared with the other arsenic species. The highest concentration of MMA (III) was in the kidneys of both KO and W mice. DMA(V) accumulated more in the lungs and bladder, but the concentration of DMA(III) was highest in the bladder for both the KO and W mice. Conclusion: Our results suggest that there were only minor differences in the concentrations of arsenic metabolites in the tissues of KO and W mice. However, the MMA reductase/GSTO1-1 activity in the liver of KO mice was only 20% of that in wild mice. There appears to be another enzyme(s) able to reduce arsenic(V) species but to a small extent. (Supported by NIEHS Grant No. ES-04940).

## 154 DETERMINATION OF TRANSCRIPTION FACTORS IMPORTANT IN ARSENIC-INDUCED ALTERATIONS IN GENE EXPRESSION.

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Chronic arsenic ingestion has been linked with an increased incidence of cardiovascular and vascular disease and cancer in multiple organs, including the lung. What remains unclear are the disease risks and biological effects associated with chronic ingestion of arsenic at lower levels commonly found in the water supplies of the United States. To investigate these effects, C57BL/6 mice ingested drinking water with or without 50 ppb arsenic for five or eight weeks. RNA from three control and three arsenic exposed animals was labeled and hybridized on six independent Affymetrix mouse 430(A) arrays, each containing over 14 k full length genes.

Signals were normalized and statistical analysis for differential gene expression between the control and arsenic exposed groups was assessed. The genes with changing expression levels are involved in a variety of functional groups including: structural regulation, chaperones, receptors, signal transduction and immunological pathway members, cytokines, and enzymes. These data indicate that significant biological effects occur at drinking water arsenic concentrations routinely found throughout the US. To investigate regulation of these genes we used TRANSFAC® analysis tool, which provides comprehensive information on gene regulation at the level of transcription, which is generally considered the most important step in controlling gene expression. We examined transcription factors that were exclusively associated with either arsenic-induced up regulated genes or with arsenic-induced down regulated genes. Of interest were the zinc fingers, MAP kinase, and stress-induced transcription factors that were associated with arsenic-induced down regulated genes. These included: KR, GATA-1, GBF, Ttk69K, CBF1, SRF, TGA1A, and v-Maf. These data indicate that arsenic may down regulate gene expression by affecting transcriptional activation. Supported by NIEHS SBRP grants ES07373 and ES04940.

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#### ARSENIC IN SEAFOOD: WHAT PERCENTAGE OF THE TOTAL IS INORGANIC ARSENIC?

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One of the most significant sources of human exposure to arsenic is food, with the highest arsenic concentrations found in seafood. In most seafood, organic forms of arsenic, mainly arsenobetaine, predominate (80–99%); however, other arsenicals, both inorganic and organic, may also be present. Arsenobetaine is not considered toxic, but inorganic arsenic is toxic and carcinogenic. Consequently, the speciation of arsenic in seafood is a critical consideration for estimating human health risk from seafood consumption. Commonly, only the total arsenic concentrations are available. For estimating human cancer and non-cancer risks, an approximation is needed for the percent of the total arsenic that is inorganic. A survey and evaluation of the data on arsenic speciation in various types of seafood was performed in order to determine published concentrations of total arsenic and inorganic arsenic in finfish, aquatic invertebrates, and seaweed. Sources of data included the published literature and EPA documents, and covered seafood worldwide, as well as seafood from specific US sites. A goal of this survey was to determine if default values could be derived for the percent inorganic arsenic in various types of seafood. While average percent inorganic arsenic values typically are <10% for fish and shellfish, the percent inorganic arsenic values for individual samples of a particular type of fish or shellfish can vary widely from not detected to nearly 30%. Frequently, the quality of the data was not documented and the number of samples limited. In seaweed samples, the percent inorganic arsenic values vary even more widely, from <1% to 60%. Data were not adequate to determine whether the percent inorganic arsenic differs in seafood from contaminated versus uncontaminated areas. This literature review indicates that a nation-wide default that accurately characterizes the percent inorganic arsenic in fish, shellfish, or seaweed is not supported by the available published data.

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#### ASSESSMENT OF EXPOSURE TO ARSENIC AND LIFE STYLE IN KOREAN.

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Arsenic(As) is widely distributed in environment and has been known as a human carcinogen. The main source of As in general population is a contaminated food or drinking water. We observed in previous study that seafood consumption increased urinary As metabolites, dimethylarsinic acid(DMA), in volunteers. In this study, we investigated the exposure level of As and life style/diet habit in Korean. We recruited 336 adults (137 males and 199 females) of over 30 years old from 3 districts (urban, rural and coastal areas). The subjects were interviewed for occupation, residence, smoking, drinking, drug, drinking water and past medical history, and were analyzed for diet habit by 24 hour recall method. Blood and urine sampled from subjects. Exposure level to As was evaluated with urinary total and inorganic As metabolites, and some biological indices, such as GSH, GPX, lipid peroxidation and uric acid, were analyzed. Urinary total As was analyzed by hydride generation method, and arsenic speciation was performed by using HPLC with ICP-mass spectrometry. The exposure level of As was age-dependent and affected by seafood consumption, while was negative correlated with animal protein, animal oil and meat consumption. The DMA was major arsenic species in urine. This result suggests that life style/diet habit could be role as one of factors to determine the exposure level to As in general population.

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#### URANYL ACETATE INDUCED OXIDATIVE STRESS IN CHINESE HAMSTER OVARY AA8 CELLS: EFFECT ON DNA OXIDATION.

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Environmental and occupational exposures to uranium as a result of uranium mining, residual mine tailings, uranium in drinking water and the use of depleted uranium in the military may lead to adverse health effects. Previous work has shown that uranyl acetate produced DNA strand breaks in the presence of vitamin C. The purpose of the current study was to determine whether uranyl acetate can cause DNA damage through production of reactive oxygen in mammalian cells. The ability of uranyl acetate to produce oxygen radicals was determined by loading CHO AA8 cells with the oxygen radical sensitive dye CM-H<sub>2</sub>DCFDA (Molecular Probes). Cells were exposed to uranyl acetate at concentrations up to 300  $\mu$ M and production of oxygen radical was determined using flow cytometry. Slight increases in radical production were seen immediately after addition of uranyl acetate to the cell suspension. Intracellular thiol status, as measured by the dye cell tracker green, was unchanged by the uranium exposure. In order to determine if the slight increase in radical production could lead to DNA oxidation, CHO AA8 cells were exposed to uranyl acetate at concentrations ranging from 50 to 300  $\mu$ M for 40 minutes or 24 hrs. Single cell gel electrophoresis (comet assay) was used to determine DNA strand breaks. No differences in total or oxidation dependent DNA strand breaks was seen between control and uranyl acetate exposed cells. These data indicate that uranium exposure alone may lead to minimal oxidative stress. However, the stress does not appear to be sufficient to cause significant DNA oxidation. Supported by NIH grants CA96320 and CA096281.

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#### THE HPRT SPECTRA OF SPONTANEOUS, URANYL-ACETATE- AND HYDROGEN-PEROXIDE-INDUCED HPRT MUTANTS IN CHINESE HAMSTER OVARY EM9 CELLS.

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Naturally-occurring uranium and depleted uranium (DU) are believed to be health hazards by virtue of both their chemical and radiological properties. The mechanism behind uranium's chemotoxic effects has yet to be elucidated. Previous work has shown that DU, as uranyl acetate (UA), was mutagenic at the hypoxanthine (guanine) phosphoribosyltransferase (hprt) locus in XRCC1-deficient CHO EM9 cells. The current study compared the mutations in these cells at the hprt locus brought about spontaneously and by exposures to UA and H<sub>2</sub>O<sub>2</sub> to suggest the molecular mechanism(s) that may be responsible for these mutations. Independent cultures of CHO EM9 cells were grown for 6 weeks to accumulate spontaneous mutations, or were exposed to 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> or 200  $\mu$ M UA for 24 hours to induce mutations. Mutants at the hprt locus were selected with 6-thioguanine. The mutants' hprt mRNA was copied and amplified by RT-PCR and these cDNAs were sequenced. The genomic DNA of mutants with one or more exons absent in their cDNA sequences was isolated and the relevant hprt exons amplified by PCR. A total of 44 UA-induced, 33 spontaneous, and 17 H<sub>2</sub>O<sub>2</sub>-induced mutants were characterized; putative splicing mutants were not fully characterized. Base substitutions comprised 30%, 39%, and 12% of UA, spontaneous, and H<sub>2</sub>O<sub>2</sub> mutants, respectively. Insertions in cDNA sequences were 6 times more frequent in UA than spontaneous mutants, and no insertions were found in the H<sub>2</sub>O<sub>2</sub> mutants. Of the 52%, 58%, and 88% genomic deletions found in UA, spontaneous, and H<sub>2</sub>O<sub>2</sub> mutants, respectively, the frequency of spontaneous deletions that included more than one exon was 35% and 23% that of UA or H<sub>2</sub>O<sub>2</sub> mutants, respectively. This abundance of insertions and multixon deletions suggests that UA may generate mutations in ways that are distinct from both spontaneous and free-radical mechanisms, and points to strand breaks or crosslinks as being possible relevant DNA lesions. Supported by NIH grant CA096320.

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#### CYTOTOXICITY, GENOTOXICITY AND MUTAGENICITY OF COMBINED EXPOSURES OF URANYL ACETATE AND SODIUM ARSENITE IN CHO CELLS.

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High concentrations of uranium contaminate water sources in the Colorado plateau, the West Central Platform, and the Rocky Mountains. Arsenic levels have also been shown to be high in areas of uranium mining and processing, which are common in the Southwestern United States. This creates a potential for human co-exposures to arsenic and uranium. Previous work from our labs has shown that uranium as uranyl acetate (UA) caused DNA strand breaks *in vitro*, and was more cytotoxic and mutagenic in the XRCC1-deficient CHO EM9 cell line, than the

parental CHO AA8 line. Arsenic is known to inhibit DNA repair, to affect signal transduction, and to induce oxidative stress and apoptosis. The current hypothesis being tested is that if UA causes DNA damage and As(III) inhibits DNA repair or induces oxidative stress, then combinations of uranium and As(III) could be more toxic than either metal alone. This hypothesis was tested in CHO EM9 cells exposed to combinations of UA and sodium arsenite by measuring cytotoxicity through colony forming ability, mutations at the hypoxanthine (guanine) phosphoribosyltransferase (hprt) locus, and DNA damage using the comet assay. Cell survival data indicated a slight synergistic effect for combined exposures; however, 24 hr co-exposures to sodium arsenite at a range of 1-20 uM did not increase hprt mutations induced by 200 uM UA, and no increases in tail moment were observed for combined exposures by the comet assay. Thus under these conditions combined exposures of arsenic and uranium were not more genotoxic than individual exposures. Results from these studies could ultimately contribute to the interpretation of epidemiological studies in populations exposed to uranium by identifying confounding variables of human exposures. Supported by NIH grants CA096320 and CA096281.

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#### GENOTOXICITY OF PARTICULATE URANIUM AND CHROMIUM.

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Depleted uranium (DU) and hexavalent chromate (Cr(VI)) are commonly used by the military and thus exposure of soldiers and non-combatants to these compounds is potentially frequent and widespread. Particulate forms of these metals are a concern because of their ability to impact and persist at bifurcation sites in the lung. Cr(VI) is a known human lung carcinogen and DU is considered a probable human carcinogen, affecting the bronchial cells of the lung. However, the mechanisms of Cr(VI)-induced carcinogenesis are unknown and only one investigation has studied particulate DU and only two have studied soluble DU in human bronchial cells. Thus the primary goal of this study was to characterize and compare the genotoxicity of Cr(VI) and DU in human bronchial cells. We found that lead chromate, was less toxic to human lung cells than similar concentrations of uranium oxide. Specifically, at concentrations of 0.5, 1, 5 and 10 ug/cm<sup>2</sup>, lead chromate induced 71, 43, 15, and 1 percent survival (relative to the control), respectively, while uranium oxide induced 57, 32, 1 and 0 percent survival respectively at these same concentrations. By contrast, we found that lead chromate was more clastogenic than uranium oxide in human lung cells. Uranium oxide induced 6, 9, 15 and 26 percent damage at concentrations of 0.5, 1, 5 and 10 ug/cm<sup>2</sup> while lead chromate induced 27 and 37 percent damage at the lower concentrations of 0.5 and 1 ug/cm<sup>2</sup>. At 5 and 10 ug/cm<sup>2</sup>, lead chromate induced cell cycle arrest and no metaphases were observed. Future work will consider potential effects of co-exposure to these cells and their ability to induce neoplastic transformation. This work is supported by a grant from the Army Research Office Department of Defense, and Grant # ES010838 from the National Institute of Environmental Health Sciences, National Institutes of Health.

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#### NEPHROTOXIC EFFECTS OF DEPLETED URANIUM (DU) IN THE RAT. INTERACTIONS WITH STRESS.

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Military use of DU has renewed interest in its toxicology. Data here are part of a larger study to assess neurotoxicology and toxicokinetics of DU and alteration by stress. Specifically we address the issue of the nephrotoxicity of soluble DU in the presence of stress. Adult male Sprague-Dawley rats were administered a single im dose of 0, 0.1, 0.3 or 1.0 mg/kg DU (as uranyl acetate). Just before day 0 dosing 1/2 were exposed to swim stress resulting in plasma corticosterone levels of 7644 +/- 131 (n=206) and 190 +/- 91 (n=200) ng/ml, mean +/- SD, for stressed and unstressed rats, respectively. Sacrifice was on days 1, 3, 7 and 30 (n=4/group/day). DU exposure in all rats resulted in dose-related elevation of blood serum urea nitrogen and creatinine (evident at day 1, further elevated by day 7, elevated with a downward trend by day 30). Serum albumin and hematocrit declined with time in high dose DU rats. Dose dependent increases in serum uranium values peaked at approximately 3 days. Renal lesions included prominent acute tubular necrosis at all treatment levels, primarily noted in the inner cortex and outer medullary stripe, with extension along the medullary rays in mid and high dose treatment groups. Tubular regeneration occurred rapidly in low dose (0.1 mg/kg) rats and was almost complete by day 30. High dose rats had more extensive tubular necrosis and a relatively

delayed regenerative response, with regions of multifocal chronic interstitial nephritis and cortical scarring on day 30. Rats receiving the mid dose (0.3 mg/kg) displayed lesions of intermediate severity. Stress had no effect on the nephrotoxicity. These data demonstrate that even very low doses of soluble DU are nephrotoxic, and that stress has no effect on this process. Supported by DAMD17-1-01-0775, US Army Medical Research and Materiel Command

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#### BIOLOGIC EFFECTS AFTER BRIEF INHALATION EXPOSURE TO URANIUM OXIDE OR DEPLETED URANIUM OXIDE AEROSOLS.

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To study of the transport of inhaled uranium oxides to the brain, rats were exposed to aerosols of UO<sub>2</sub>, UO<sub>3</sub>, UO<sub>2</sub> + UO<sub>3</sub> or depleted UO<sub>x</sub> (DUO<sub>x</sub>). Control groups were exposed to TaO<sub>2</sub> or air only. The uranium compounds were purchased as 99.8% purity and the DUO<sub>x</sub> was prepared by heating DU 0.75% titanium metal to a 30 mesh powder and then ball-milling. Groups of 30 rats each were exposed for 15 min. to nominal aerosol concentrations of 500mg/m<sup>3</sup> and scheduled for sacrifice at 2 hr., 30, 180 and 365 days after end of exposure. The achieved aerosol concentrations were UO<sub>2</sub>, 572 mg/m<sup>3</sup>; UO<sub>3</sub>, 329 mg/m<sup>3</sup>; UO<sub>2</sub> + UO<sub>3</sub>, 302 mg/m<sup>3</sup> and DUO<sub>x</sub>, 609 mg/m<sup>3</sup>. The aerosol particle sizes were all respirable, ranging from 1.6 to 2.4 microm MMAD. Fifteen rats exposed to UO<sub>3</sub>, the most soluble of the compounds used, died 2 to 13 days post inhalation exposure (d.P.E.). All but one died of acute renal tubular necrosis and uremic pneumonia, characteristic of acute uranium-induced toxicity. Rats sacrificed 2 hr. P.E. had minimal nephropathy and minimal histologic changes in the lungs, regardless of exposure. In rats sacrificed 30 d.P.E., focal septal pulmonary fibrosis was present in 80-100% of those exposed to UO<sub>3</sub> or DUO<sub>x</sub>. None of those exposed to UO<sub>3</sub> and 17% of exposed to UO<sub>2</sub> + UO<sub>3</sub> had pulmonary fibrosis. The incidence of nephropathy was increased in all rats exposed to UO compounds relative to the 2 hr. sacrifice. In rats sacrificed 180 or 360 d.P.E. the pattern of increased incidence of nephropathy and pulmonary fibrosis continued in those exposed to the UO compounds. These results show that more soluble UO<sub>3</sub> can induce acute renal effects in rats after a brief inhalation exposure. In addition, pulmonary effects can be seen after brief inhalation of any of the UO compounds. These effects are most likely secondary to the acute renal toxicity. Research supported by US Army Medical Research Development Command under DAMD 17-01-1-0794

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#### RENAL BIOMARKER RESULTS IN DEPLETED URANIUM (DU) EXPOSED GULF WAR VETERANS.

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In the Exposure-Effect Pathway of toxicant insult (National Research Council, Committee on Biological Markers, 1987), intermediate perturbation steps are described in the continuum of insult that pre-date the ultimate health effect. Biomarkers of these intermediate steps (biologically effective dose, early biologic effect) allow qualitative and semi-quantitative estimates of these early, pre-clinical effects. Ideally, biomarker measures may serve as indicators of needed health interventions prior to progression to a clinically apparent and untoward outcome. In occupational cohorts exposed to renal toxicants, primarily heavy metals and solvents, a European Cooperative Study (*Diseases of the Kidney and Urinary Tract*, 2001 Schrier R.W. (Ed.), p. 1256), comprising a suite of renal biomarkers is underway. The aim of this collaboration is to determine if patterns of biomarker excursions are predictive of the specific toxicant of exposure. A cohort of Gulf War I soldiers who were victims of "friendly fire" involving depleted uranium (DU) munitions have been followed biennially over the last 12 years (McDiarmid, et al., 2000, *Env Res* 82:168-180; McDiarmid, et al., 2001, *J Occup Env Med*, 43:991-1000). Although no frank indicators of renal disease have been observed, during the last three surveillance visits (1999, 2001, 2003) spot urine samples have been submitted to the European Cooperative Study Group for biomarker analysis. Measures of B2 microglobulin, retinol binding protein (RBP), N-acetyl-B-D glucosaminidase (NAG), alpha<sub>1</sub>-microglobulin, microalbumin and total protein have been determined. Results from 2001 urine samples showed statistically significant increases in urine microalbumin and RBP concentrations standardized per gram creatinine in the group of soldiers excreting high urinary U and RBP mean concentrations were again elevated in 2003. These indicators of tubular effects are consistent with known renal targets of uranium and support the need for continued surveillance of this DU-exposed cohort.

## ASSESSING THE RENAL TOXICITY OF DEPLETED URANIUM AND OTHER URANIUM COMPOUNDS.

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The primary target for uranium toxicity is the kidney. The most frequently used guideline for uranium kidney burdens is the International Commission on Radiation Protection (ICRP) value of 3 µg U/g kidney, a value that is based largely upon chronic studies in animals. In the present effort, we have developed a risk model equation to assess potential outcomes of acute uranium exposure. Twenty-seven previously published case studies in which workers were acutely exposed to soluble compounds of uranium (as a result of workplace accidents) were analyzed. Kidney burdens of uranium for these individuals were determined based on uranium in the urine, and correlated with health effects seen over a period of up to 38 years. Based upon the severity of health effects, each individual was assigned a score (- to +++) and then placed into an Effect Group. A discriminant analysis was used to build a model equation to predict the Effect Group based on the amount of uranium in the kidneys. The model equation was able to predict Effect Group with 85% accuracy. The risk model was used to predict the Effect Group for soldiers exposed to DU as a result of friendly-fire incidents during the 1st Gulf War. This model equation can also be used to predict the Effect Group of new cases in which acute exposures to uranium have occurred.

## UTILITY OF PATIENT MAILED URINE SPECIMENS FOR RENAL BIOMARKER ANALYSIS IN DEPLETED URANIUM (DU) EXPOSED 1991 GULF WAR VETERANS.

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Surveillance of health effects of chronic depleted uranium (DU) exposure in a cohort of 1991 Gulf War veterans is being conducted through the Baltimore VA DU Follow-up Program. Slow release of DU from metal fragments embedded in muscle tissue has resulted in ongoing exposure evidenced by elevated urine U concentrations. World-wide distribution of the affected cohort and logistic constraints of travel for participation in biennial surveillance assessments at the Baltimore VA have made it necessary to conduct urine U excretion surveillance by mail. More recently, concern about the development of renal insults as duration of exposure lengthens (now greater than 12 years) created the need to determine the feasibility of assessing renal proximal tubular function using mailed urine specimens. Tubes containing an aliquot of a stabilization buffer (1M imidazole, 2% Triton X-100, 20 mM benzamidine, 2000 U/ml Aprotinin, 1% sodium azide, pH 7.0) were mailed to each patient with instructions on how to collect, freeze and ship a specimen for overnight delivery. Specimens were analyzed for microalbumin, β2-microglobulin, retinol binding protein (RBP), α1-microglobulin, N-acetyl-β-D-glucosaminidase (NAG), intestinal alkaline phosphatase (IAP) and total protein by standard techniques. Results were consistent with samples collected at the Baltimore VA hospital during an in-patient admission within the last six months of obtaining the mailed-in specimen, indicating the utility of this method as a means of enhancing surveillance of renal proximal tubule function in this and other cohorts with chronic exposure to renal toxicants.

NK CELL CYTOTOXICITY EVALUATION: TECHNICAL AND ANALYTICAL ALTERNATIVES TO CONVENTIONAL <sup>51</sup>CHROMIUM RELEASE ASSAY.

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Natural killer (NK) cell cytotoxicity is commonly used to detect and/or characterize immunotoxic potential of new medicines and environmental agents. Moreover, European regulatory guidelines (CPMP/SWP/1042/99) specify use of NK cell cytotoxicity in the initial screening of new chemicals for immunotoxicity. The conventional and most widely used assay to measure NK cell cytotoxicity is based on <sup>51</sup>chromium release; however, hazardous and technically burdensome procedures associated with radioisotopes have prompted investigations into alternative methods. A fluorescent-based approach utilizing the lanthanide chelate, Eu<sup>+3</sup>-DTPA, was compared to <sup>51</sup>chromium release assay. Cynomolgus monkey peripheral blood mononuclear cells (PBMCs), pre-stimulated with rHuIL-2 (50 – 5000 U/mL), were cultured with NK target cells, K-562s, labeled with either <sup>51</sup>Cr or Eu<sup>+3</sup>-DTPA. A comparison of percent specific lysis indicated significant correlation between the two methods ( $r = 0.98$ ;  $p < 0.05$ ). Further evaluation of NK cell activity by Eu<sup>+3</sup>-DTPA release assay was conducted with human and non-human primate PBMCs pre-treated for 48 hours with culture medium or rHu-IL-2 (50 U/mL). Percent

lysis of K-562 target cells was converted to mean lytic units by statistical methods utilizing non-linear mixed effects model and Von Krogh's equation. Results from the analysis indicate that spontaneous killing of K-562 target cells by human NK cells is significantly greater than that of cynomolgus and rhesus monkey ( $p < 0.0001$ ). However, treatment with rHu-IL2 caused significant enhancement ( $p < 0.0001$ ) of NK cytotoxicity among all three species groups. These findings support utility of a fluorescent-based approach in the measurement of NK cell activity.

## ANTI-NKP46 IMPROVES DETECTION OF NATURAL KILLER CELLS IN MACAQUE BLOOD.

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Immunophenotyping of lymphocyte subpopulations by flow cytometry has become an important and well established tool for detection of immunomodulatory actions of pharmaceuticals in toxicology. In humans, natural killer (NK) cells are identified by a combination of antibodies against CD16 and CD56 (defined as CD3 negative, CD16 and/or CD56 positive lymphocytes). It was observed that this CD combination is not well suited for macaques, since CD56 recognizes predominantly monocytes and is not strongly expressed on NK cells. Therefore NK cells are normally defined as CD3 negative and CD16 positive lymphocytes in macaque samples. In order to optimize the identification of NK cell in macaques, we tested another NK-marker, NKP46, which is highly specific for NK cells. Blood samples from six adult rhesus monkeys (Macaca mulatta), 9 adult and 25 juvenile cynomolgus monkeys (Macaca fascicularis, aged 5 to 13 years and 12 to 18 month, respectively) were tested using CD16 and NKP46 antibodies alone or in combination. In rhesus monkey, 97% of CD16 and/or NKP46 positive cells were detected by CD16 alone, but only 62% by NKP46 alone. The recognition pattern in cynomolgus monkey was different with CD16 recognizing 88 or 91% and NKP46 identifying 84 or 87% of the CD16 and/or NKP46 positive cells. These data indicate that a combination of these markers provides a better estimation of NK cell numbers as compared to CD16 alone. For rhesus monkeys, this difference is rather small with only 3 % of NK cells being not recognized whereas in cynomolgus monkeys approximately 10 % of NK-cells are not detected. It was also observed, that the CD4/CD8 ratio and the relative amount of NK-cells was significantly lower in juvenile versus adult/elderly cynomolgus monkeys. In conclusion, NKP46 can be used for optimized detection of NK-cells in macaque blood.

## IMMUNE SYSTEM DEVELOPMENT IN THE CYNOMOLGUS MONKEY: LYMPH NODES AND SPLEEN.

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Immunosuppression and immunomodulation are highly relevant parameters during toxicological evaluation of biologics. Given the broad medical spectrum of biologics, information on the prenatal and ontogenetic development of the immune organs is becoming pivotal. Lymph nodes and spleen are peripheral sources of numerous immune cell types. Hence these organs are important targets for drugs and drug-related side effects. Organ development in the cynomolgus monkey was studied from day 35 of gestation (gd 35) until adulthood. Special emphasis was placed on immune cell generation during development. Histological standard stains and immunocytochemical methods (e.g. CD-antibodies) were used to distinguish between different immune cell subpopulations. At gd 35, spaces in mesenchyme were visible indicators of lymph node development in the cervical position cranial to the apertura thoracica. They were filled with a mesenchymal reticulum at gd 50 which was settled with first lymphocyte-like cells on gd 70. At gd 60 there was an initial immunoreactivity with anti-HLA-DR antibody (Human Leucocyte Antigen D Region) followed by CD3 immunoreactivity indicative for T-lymphocytes at gd 70. Until perinatally, lymph node development and maturation was followed by a cascade of immunoreactivities indicating lodging of natural killer cells (M1014), T-cells (CD2), B-lymphocytes (CD20) and B- and T-cell precursors (CD117), whereas T-helper (CD4) and T-suppressor cells (CD8) were first apparent in postnatal animals. This development pattern is similar to that reported for lymph nodes in humans. In conclusion, the cynomolgus monkey represents a suitable model for evaluation of the immune system in developmental toxicity studies.

## ANALYTICAL VALIDATION OF PERIPHERAL BLOOD IMMUNOPHENOTYPING FOR CYNOMOLGUS MONKEY.

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A study was conducted to evaluate the precision, linearity, antibody titer optimization, and stability of blood samples for a peripheral blood immunophenotyping assay in cynomolgus monkeys. Validation included quantification of total T lymphocytes, helper and cytotoxic T lymphocytes, B lymphocytes, and natural killer

(NK) cells using flow cytometry. Immunophenotyping was conducted using a dual-platform methodology and heterogeneous lymphocyte gating strategy. Potassium EDTA was used as the standard anticoagulant in order to facilitate the dual platform methodology. The intrasample precision was assessed by taking ten measurements of one immunophenotyping set from one peripheral blood sample per animal. Intersample precision was evaluated using ten immunophenotyping sets from one peripheral blood sample per animal. The linearity was determined by preparing replicate immunophenotyping samples with decreasing blood volumes. To assess antibody titer optimization, replicate immunophenotyping samples were prepared with decreasing antibody volumes. To assess stability, lymphocyte values for whole blood samples and fixed immunophenotyping samples were compared at multiple timepoints over a 72-hour period. Stability testing also included a comparison of lymphocyte values for blood samples shipped via overnight courier. The assay was determined to be accurate, precise and linear over a wide range of blood volumes. In addition, the antibody cocktail can be used at many dilutions. Both fresh whole blood and fixed immunophenotyping samples were stable over extended periods, and when shipped via overnight courier. During validation, it was discovered that to correctly delineate natural killer cells, serum must be removed from the peripheral blood sample prior to staining with the antibody cocktails. The validation has shown that the assay generates precise and reproducible data for detection of lymphocyte populations in peripheral blood samples from cynomolgus monkeys.

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IMMUNOGENICITY AND IMMUNOTOXICITY ASSESSMENTS OF TWO DRUG AFFINITY COMPLEX COMPOUNDS IN CYNOLOGUS MONKEYS.

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The Drug Affinity Complex (DAC) is a technology that enables covalent binding (conjugation) of a drug to albumin, thus extending the half-life ( $t_{1/2}$ ) of the pharmacophore to a duration comparable to that of albumin. The immunogenic and immunotoxic potential of two DAC compounds, CJC-1131, a glucagon-like peptide (GLP-1) analogue, and CJC-1295, a growth hormone releasing factor (GRF<sub>1-29</sub>) analogue, were evaluated in Cynomolgus monkeys. Four monkeys/sex/group were administered multiple subcutaneous (SC) injections of a vehicle control and pharmacologically active doses of CJC-1295 (50 µg/kg), CJC-1131 (2 µg/kg), as well as native GRF<sub>1-29</sub> (50 µg/kg) and GLP-1 (2 µg/kg) as controls over a 6-month treatment period. Dosing occurred daily during Months 1 and 4, and weekly during Months 3 and 6. The animals were not dosed during Months 2 and 5, and for 28-day recovery period following the last dose. All animals were observed for clinical signs daily during the treatment periods and assessed for immunogenicity and immunotoxicity prior to dosing, and at the end of each month. Evaluation of the pharmacological activity of the test articles was after the first dose, and at the end of Months 3 and 6. There were no treatment-related effects on survival, clinical signs, peripheral blood immunophenotyping, leukocyte function (natural killer cell activity) or peripheral lymphocyte activation in this study. There was no clear indication of presence of neutralizing antibodies, as measured by pharmacological endpoints, i.e., oral glucose tolerance test (OGTT) for CJC-1131 and GLP-1. In conclusion, the two DAC compounds produced no evidence of immunogenic or immunotoxic effects in Cynomolgus monkeys.

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DEVELOPMENT AND VALIDATION OF SEMI-QUANTITATIVE ASSAYS FOR TOTAL AND NEUTRALIZING PRIMATE ANTIBODIES AGAINST A HUMAN THERAPEUTIC MONOCLONAL ANTIBODY.

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A therapeutic human monoclonal antibody (Compound X) to a receptor that has been associated with the growth of certain tumors is being developed. Compound X binding to receptors results in receptor internalization and degradation, thus decreasing receptor expression. Preclinical toxicity studies in cynomolgus monkeys included the assessment of the anti-Compound X (primate anti-human antibody, PAHA) response. Semi-quantitative assays for total and neutralizing PAHA antibodies were developed and validated. The total PAHA assay is an ELISA-based assay and has demonstrated good intra- and inter-assay precision (4.1% and 1.6% respectively) and sensitivity. Antibody levels of approximately 0.624 µg/ml and greater are detectable in the assay. A bioassay was developed to determine the neutralizing activity of the PAHA. This involved using an ELISA to measure receptor levels in the MCF-7 tumor cell line after exposure to Compound X either with or without test serum. The bioassay is able to detect changes in receptor levels at 0.1-5ng/ml of Compound X. The Compound X-induced decreases in receptor levels

were attenuated with positive control anti-Compound X antibodies. The intra- and inter-precision are 9.2% and 15.95% respectively. The sensitivity of the bioassay is 1.25 µg/ml. Both validated assays were used to support regulatory toxicity studies for the compound. Preclinical toxicity studies demonstrated rapid development of PAHA that altered the pharmacokinetics of Compound X.

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TEMPORAL STABILITY OF WHOLE BLOOD AND MONONUCLEAR CELL CULTURES TO SUPPORT LYMPHOCYTE PROLIFERATION FOLLOWING MITOGEN AND RECALL ANTIGEN STIMULATION.

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Immunology endpoints are increasingly being applied to preclinical and clinical studies to evaluate drug immunotoxicity and immunopharmacology. Because of the logistical delay which may occur between sample withdrawal and immune function analysis, it is necessary to evaluate the impact of this delay on the suitability of the test system. We have compared the stability of lymphocyte proliferation assays using unseparated whole blood with mononuclear cell cultures. Heparin blood was obtained from healthy volunteers and stored at room temperature. After 3-48 hrs, mononuclear cells were purified, then cultured in RPMI+10% human serum with PHA (phytohemagglutinin) or PPD (tuberculin purified protein derivative) for up to 6 days. Whole blood was diluted to 10% v/v with RPMI and cultured as above. The proliferating cells in both cultures were labelled with tritiated thymidine for the last 18 hrs of the assay, then incorporated radioactivity measured by liquid scintillation counting. Both cultures supported PPD and PHA responses after storage of blood at ambient temperature for at least 24 hrs. Peak stimulation for PHA and PPD was observed after 4 and 6 days respectively in both cultures, consistent with the known kinetics of a mitogen versus a recall antigen response. Importantly the hierarchy of responsiveness observed between the individuals was seen in both cultures using blood which had been stored for up to 24 hours. The results from whole blood cultures using 48 hr old blood indicated that the rank order for some individuals had started to change as the proliferative capacity of these cultures started to significantly decline. These results indicate that it is possible store blood for up to 24 hrs prior to performing a lymphocyte proliferation assay. Since there was no obvious difference between either culture, the inclusion of all cell types in the whole blood assay and the ease of set up offers an advantage over the traditional mononuclear cell culture.

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PERIPHERAL LYMPH NODES: ISSUES WITH THE COLLECTION AND ANALYSIS FOR IMMUNOTOXICITY STUDIES.

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There is great interest in identifying reliable, sensitive indicators of immunotoxicity in safety assessment studies following the finalization of regulatory guidances in the USA and Europe. Because evaluation of peripheral lymph node weights and histopathology from routine safety assessment studies has been proposed as standard procedure for identifying immunotoxic potential of new chemical entities, we elected to evaluate: 1) variability of weights for specific peripheral nodes, and 2) histomorphologic variability of specific peripheral lymph nodes, in normal rats. Popliteal lymph nodes (n= 87), spleen (n=196) and thymus (n=132) from control rats in ten studies conducted at three different laboratories were collected, weighed and microscopically examined. Control rat popliteal lymph node weights ranged\* as follows: AZ (4 studies) CTBR (1 study) GSK (2 studies) Across All ABS 1.5-5.5X 3.3-3.9X 4.4-5.7X 1.5-5.7X %BW 1.5-5.0X 3.3-4.7X 4.0-5.0X 1.5-5.0X Control rat spleen weights ranged as follows: AZ (2 studies) CTBR(4 studies) GSK (2 studies) Across All ABS 1.6-2.0X 1.5-2.0X 1.3X 1.3-2.0X %BW 1.3-1.6X 1.5-1.7X 1.3X 1.3-1.7X Control rat thymus weights ranged as follows: AZ (0 studies) CTBR(4 studies) GSK (2 studies) Across All ABS 1.7-2.6X 2.0-2.3X 1.7-2.6X %BW 1.3-2.9X 1.8-2.2X 1.3-2.9X \* Weight ranges expressed as ratio of highest to lowest weight (g). Popliteal nodes demonstrated considerable histologic variability for all lymph node compartments due to sectioning differences, marked variation of normal tissue architecture and variability in background immunostimulation. Histologic variation was less in control spleen and thymus. These data suggest that organ weights are more variable for popliteal node than spleen and/or thymus, and that peripheral lymph nodes histology from normal rats can be highly variable. Consequently, evaluation of popliteal lymph nodes is subject to variable interpretation.

VALIDATION OF A METHOD FOR THE DETECTION OF ANTI-PROTEIN-X IgG ANTIBODIES IN RAT AND DOG SERUM BY ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA).

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The undesired host immune response against biopharmaceutical therapeutics have become a serious concern for manufacturers, regulatory agencies and clinicians, as the biopharmaceutical's immunogenicity could lead to serious side effects or loss of product efficacy. A qualitative ELISA for the detection of anti-Protein-X IgG antibodies was developed and validated at CTBR. The anti-Protein-X antibodies present in rat or dog serum are captured on a Protein-X coated plate and detected using secondary antibodies conjugated to peroxidase. Parameters validated included cut-off determination, specificity and recovery, precision and stability. The inhibition of the positive control antibody binding to the plate in the presence of Protein-X in excess (immunodepletion) or in dosing amounts (drug interference) was also assessed. High, medium, low and species (rat or dog immunoglobulin) positive controls were used in the validation. The cut-off was established as the mean value of individual lots of normal serum (rat or dog) plus 1.645 SD. The inter- and intra-assay precision, expressed as the coefficient of variation (CV), met the acceptance criteria of  $\pm 20\%$  for high and medium positive controls, and  $\pm 25\%$  for the low positive control. The minimum required serum samples dilution was established at 1/10. No prozone effect was evident for positive control antibody concentrations up to 100  $\mu\text{g}/\text{mL}$ . Stability of spiked serum samples at room temperature for approximately 6 hours, at approximately 40°C for approximately 24 hours, and after three freeze-thaw cycles at approximately -80°C met the acceptance criteria, with the difference of the stability samples being within  $\pm 25\%$  when compared to the control samples. The longterm stability at approximately -80°C is ongoing.

THE REAL TIME MONITOR OF ANTIBODY-CELL BINDING BY BIOSENSOR ASSAY.

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The binding of antibody(Ab) to cell with target antigen is generally detected by flow cytometry. Flow cytometry is useful for detecting whether Ab bind to cell or not. But, the association and dissociation can't be directly detected by flow cytometry. We tried direct monitor of Ab-cell binding and could monitor on real time by biosensor assay using Biacore 3000. Rituxan, human Ab drug, was immobilized on Biacore sensorchip with the level of about 10000 RU. Raji cells, Burkitt's Lymphoma cell line, at  $1 \times 10^4$ ,  $1 \times 10^5$  and  $1 \times 10^6$  cells/mL were applied on Rituxan-immobilized sensor chip, and the binding response contented association phase and dissociation phase could be directly monitored in the cell concentration dependent manner. Additionally, the binding response of Raji cells was inhibited when anti-Rituxan Ab was pre-treated. These results suggest that biosensor assay is useful for direct monitor of Ab-cell binding.

ADVANTAGE OF USING WHOLE SHEEP RED BLOOD CELLS VERSUS CELL MEMBRANE IN ELISA ASSAYS TO ASSESS IMMUNOSUPPRESSION *IN VIVO*.

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To detect a T-cell dependent antigen-induced primary antibody response, an ELISA using sheep red blood cells (SRBC) membranes is generally used. A problem of this method is that SRBC-specific antibody titers are different among lots of SRBC membrane preparations and are not consistently detected in sera from all SRBC-immunized rats. In order to resolve the problem, whole SRBC instead of SRBC membranes were used in the present experiment. However, the ELISA using whole SRBC has not been widely used because of the weak attachment of SRBC to ELISA plates. The objectives of this study are to improve attachment of whole SRBC to ELISA plates and to validate the SRBC-specific antibody response assay using whole SRBC-ELISA. Male Sprague-Dawley rats were injected with  $3 \times 10^8$  SRBC/animal iv (Day 0) and bled on Day 6. To fix and attach SRBC to the plate, a small amount of high-concentrated glutaraldehyde solution was added after SRBC suspension was inoculated into a well of the plate. As a result of cell fixation procedure with glutaraldehyde, the attachment of SRBC to ELISA plates was maintained throughout ELISA procedures. With respect to lot differences of SRBC, antibody titers measured using the same lot used for immunization was nearly identi-

cal to those measured using different lots of SRBC. As additional advantages, IgM was detected in all of sera from rats immunized with whole SRBC-ELISA, but not SRBC membrane method. The ELISA method using whole SRBC in detecting immunosuppression in rats was validated with well-known immunosuppressive agents, cyclosporin and cyclophosphamide. The immunosuppressive effects of these compounds were observed at doses used in routine toxicological evaluation. These results indicate that the improved whole SRBC-ELISA method provides reproducible and reliable results in the T-cell dependent antibody response assay.

AN ALTERNATIVE KLH-BASED PROTOCOL FOR THE SRBC PLAQUE-FORMING CELL ASSAY: DOSE RANGE FINDER STUDY.

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To assess potential effects of pharmaceuticals on the immune system, a primary antibody response to a T cell-dependent antigen is suitable to complete the initial screening phase (EMEA guidance CPMP/SWP/1042/99, and FDA Guidance for Industry, Immunotoxicology Evaluation of Investigational New Drugs Oct 2002). A standard protocol is based on immunization with the antigen sheep red blood cells (SRBC). Since there are different disadvantages related to this protocol, a new design is wanted, with a standard antigen (for example keyhole limpet haemocyanin, KLH), different time points for determination of the response (IgM as well as IgG), and less variability. This would greatly improve the assessment of T cell-dependent immunity. Therefore we assessed the response to KLH in rat during a 28 day treatment study with two known immunosuppressiva (cyclosporine A, cyclophosphamide), comparing different routes of KLH dosing (day 21). Both a single i.v. and i.p. injection appeared to be suitable in inducing a KLH-specific IgM and IgG response (measured at day 7 after injection), whereas especially cyclophosphamide induced a clear immunosuppression. In June 2004, a European group of labs has shared results and designed what they considered to be the optimal design for a validation study: the Immunotoxicity Inter Laboratory Project, to be performed in January 2005. Before this ring-study can take place, a dose range finder is currently being performed, in which the optimal dose of KLH is determined. In this study, three doses of KLH (100, 300, 900  $\mu\text{g}/\text{animal}$ ) are tested, in two strains of commonly used rats (Wistar, Sprague-Dawley), and these results will be presented as well. The final ring-study will involve different immunomodulating substances, measurement of both primary as well as secondary (IgM, IgG) responses, and a direct comparison with the traditional read-out (SRBC PFC-assay).

SENSITIVITY OF THE KEYHOLE LIMPET HEMOCYANIN (KLH) ELISA MODEL IS DIRECTLY RELATED TO DOSE USED FOR SENSITIZATION.

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The plaque assay, T-dependent antibody response to sRBC, has been reported to be a sensitive and predictive functional immune assay for detecting immunomodulatory compounds. It is one of the functional assays accepted by various regulatory agencies for evaluation of humoral immune responses. Numerous laboratories have chosen to use ELISA to the T-dependent antigen KLH as an alternative to the plaque assay. The objective of these studies was to compare the sensitivity of the KLH ELISA to plaque assay using known immunosuppressive agents. Dose response studies were conducted in female B6C3F1 mice with cyclophosphamide, CPS, (5, 10, 20, 40, 60 mg/kg); azathioprine, AZA, (25, 50, 100, 200 mg/kg); and dexamethasone, DEX, (0.1, 0.3, 1.0, 3, 5 mg/kg). Dosing began on day of sensitization and continued until sacrifice. Assays were evaluated on peak response day. In CPS treated mice, the mg/kg dose producing statistically significant suppression was 10 in the plaque assay, 60 using 100  $\mu\text{g}$  KLH (sensitizing dose), and 10 using 2 mg KLH. In AZA treated mice, 25 in the plaque assay, 200 using 100  $\mu\text{g}$  KLH and 100 using 2 mg KLH. For DEX treated mice, 3 in the plaque assay, none using 100  $\mu\text{g}$  KLH and none using 2 mg KLH. Additional studies demonstrated that a CPS dose of 20 mg/kg produced a response 9% of control in the plaque assay, 82% of control using 100  $\mu\text{g}$  KLH, 45% of control using 500  $\mu\text{g}$  KLH, 40% of control using 1 mg KLH and 9% of control using 2 mg KLH to sensitize. Ongoing studies in rats also indicate a sensitization dependent response in the KLH ELISA model. These studies demonstrate that the KLH sensitization dose dramatically affects the magnitude of suppression observed and must be considered when using the KLH ELISA for hazard identification and risk assessment. (Supported in part by the NIEHS Contract ES 05454).

## EVALUATION OF T CELL RESPONSES TO KEYHOLE LIMPET HEMOCYANIN IN THE RAT MODEL.

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The rat primary antibody response model is routinely used to identify immunomodulatory or immunotoxic potential of novel drugs. In this model, the key parameters measured are anti-KLH IgM and IgG antibody responses in rats given a single intravenous dose of keyhole limpet hemocyanin (KLH). The robust anti-KLH antibody titers indicate that antigen-specific T cell help was provided, suggesting that this model could potentially be adapted to measure direct effects on T cells as well. To characterize antigen-specific T cell responses in this model, *ex vivo* stimulation with KLH of lymphocytes in spleen obtained 14 days after KLH administration was used to evaluate cytokine production and cell proliferation. IFN- $\gamma$  and IL-2 production were measured at the single cell level by ELISPOT and a panel of cytokines (IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-10, GM-CSF, INF- $\gamma$  and TNF- $\alpha$ ) secreted into supernatants were measured by multiplex bead-based assays (Bio-Plex). In the ELISPOT assays on splenocytes from KLH-immunized rats, there were KLH concentration dependent increases in the number of spot forming cells (SFCs) secreting IL-2 or IFN- $\gamma$  of up to 4 and 5-fold, respectively, relative to unstimulated splenocytes. In the Bio-Plex assay there were KLH-induced increases in IFN- $\gamma$ , GM-CSF and IL-4 in KLH-immunized rats, and KLH-dependent proliferation was greater in splenocytes from KLH-immunized rats than non-immunized control rats. These data demonstrate that KLH-dependent T cell responses can be measured in the anti-KLH primary antibody response rat model. The parallel evaluation of antigen-specific T cell and antibody responses in this model could contribute significantly to the understanding of mechanisms of drug-induced immunotoxicity.

## COMPARISON OF ANTIBODY TITER AND CONCENTRATION IN A RAT IMMUNOTOXICITY MODEL.

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Numerous laboratories are developing assays to measure a primary T-cell dependent antibody response to evaluate the immunotoxicity potential of drug candidates. Methods evaluating antibodies specific to keyhole limpet hemocyanin (KLH) commonly measure antibody titers or quantitated concentrations ( $\mu$ g/mL); however, a direct comparison of the sensitivity of these approaches for evaluating immunosuppression has not been reported. The current study evaluated anti-KLH IgM responses in rats following treatment with cyclophosphamide (CP) by measuring endpoint titers and quantitating antibody concentrations. Male and female Sprague-Dawley rats were treated orally with CP at 3 and 6 mg/kg for 14 days. Animals were given a single intravenous injection of KLH at 0.35 mg/kg 5 days prior to sacrifice. Sera were diluted 1:100, and then serially diluted to 1:25, 600 or greater to determine anti-KLH IgM endpoint titers. To quantitate the concentration of anti-KLH IgM, a standard curve was generated using a rat anti-KLH IgM monoclonal antibody. The results of this study demonstrated a strong correlation ( $r^2 > 0.9$ ) between the endpoint titer values and the quantitated concentrations of IgM in control and drug-treated animals. Cyclophosphamide at 3 and 6 mg/kg caused statistically significant decreases in anti-KLH IgM endpoint titers and concentrations in male rats when compared to controls. The concentrations of IgM specific to KLH were decreased by 34% and 98% in males given CP at 3 and 6 mg/kg, respectively. In females, significant decreases (96%) in IgM concentrations were seen in rats dosed 6 mg/kg; however, statistical significance was not reached at 3 mg/kg. The quantitative data in females were consistent with the endpoint titer values. The findings of this study show good correlation between the quantitated antibody concentrations and endpoint titer data, and demonstrated comparable sensitivity. In addition, the magnitude of suppression seen following oral CP administration was comparable to previous studies using intraperitoneal injections.

## THE UTILITY OF HOST RESISTANCE ASSAYS IN LEAD CANDIDATE SELECTION AND PHARMACEUTICAL SAFETY ASSESSMENT.

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The successful conduct of host resistance assays is partly an art and partly science, primarily because of the complexity of the host/pathogen interactions although the traditional reliance upon morbidity or survival as the parameter of interest has also been a factor. This poster presents data generated for a host resistance to listeria infection assay in which tissue infectivity and body temperatures were measured, in addition to, survival. Balb/C mice were pretreated with 20, 40 or 80 mg/kg doses of

cyclophosphamide, or saline, for 3 days prior to experimental infection with approximately  $2 \times 10^4$  CFU of *Listeria monocytogenes* by intravenous injection. Following infection, animals were monitored for mortality, signs of ill health and/or hypothermia and were euthanized on Day 6 or Day 10. Liver infectivity was '0.67, '1.35 and '78.25 of the saline control values for animals pretreated with cyclophosphamide at doses of 20, 40 and 80 mg/kg/day, respectively. These results validated the use of the 80 mg/kg/day dose as an immunomodulatory control in this experimental model. To illustrate the versatility of host resistance models for lead candidate selection as well as safety testing, data are also presented for a host resistance to *Candida albicans* infection assay adapted to screen compounds for anti-fungal activity and for a tumoral host resistance assay adapted to screen compounds for immunostimulatory activity. Finally, this poster presents methodological considerations for host resistance assays including techniques for experimental infection in a host resistance to influenza virus assay.

## ALTERED SIGNAL TRANSDUCTION IN HUMAN KERATINOCYTES FOLLOWING EXPOSURE TO BI-FUNCTIONAL ALKYLATING AGENTS.

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Human basal keratinocytes are adversely affected by cutaneous exposure to vesicating agents. Both bis-(2-chloroethyl) sulfide [sulfur mustard; SM] and bis-(2-chloroethyl) methyamine [nitrogen mustard; NM] are vesicants and potent bi-functional alkylating agents capable of covalently modifying DNA and protein. Previous reports indicated that the NF- $\kappa$ B and p53 signaling pathways could be modulated following exposure to these agents. Here we investigate potential *in vitro* model systems to elucidate the initial effects of bi-functional alkylating agents on cellular signaling events. We hypothesized that SM and NM would rapidly induce p53 phosphorylation and I $\kappa$ B $\alpha$  degradation in keratinocytes cultured *in vitro*. Immortalized human HaCaT keratinocytes (a p53-mutated cell line) and cultured normal human epidermal keratinocytes (NHEKs) were exposed to 12.5, 50, 100 or 200  $\mu$ M SM for up to 4 hrs. SM-induced p53 phosphorylation increased in a concentration- and time-dependent manner. As determined by immunoblotting, SM exposure resulted in phosphorylation on serine 15 of p53 within 15 min in both cell lines. The total level of p53 remained unchanged in HaCaT cells and increased in NHEKs following 4 hrs of SM exposure. SM-induced phosphorylation of p53 was only partially blocked by pretreatment of the cells with caffeine, an inhibitor of cell cycle checkpoint kinases. Exposure of each cell line to NM resulted in phosphorylation of p53 similar to that seen after SM exposure. Loss of I $\kappa$ B $\alpha$  was observed only in NHEKs following exposure to 200  $\mu$ M SM or NM by 4 hrs. Treatment of keratinocytes with the mono-functional molecule 2-chloroethyl ethylsulfide or with bis-(2-hydroxyethyl) sulfide did not result in phosphorylation of p53 or I $\kappa$ B $\alpha$  loss within the 4-hr timeframe examined. These data provide a basis for understanding the initial biochemical and molecular events that are modulated in response to bi-functional alkylating agents in immortalized and normal keratinocytes *in vitro*.

## 2, 4, 6-TRINITROTOLUENE CAUSES REDUCTION OF ENOS ACTIVITY BUT PHOSPHORYLATES ENOS THROUGH ACTIVATION OF PI3K/AKT SIGNALING IN ENDOTHELIAL CELLS.

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Exposure of humans to environmental pollutants causes a cardiovascular dysfunction, but the mechanism is not well understood. 2, 4, 6-Trinitrotoluene (TNT), which is widely used in explosives, is an important occupational and environmental pollutant. We have reported that TNT has a potent inhibitory action on the enzyme activity of neuronal NOS (nNOS) by shutting electrons away from the normal catalytic pathway, resulting in decreased nitric oxide (NO) formation and concomitant generation of H<sub>2</sub>O<sub>2</sub> derived from O<sub>2</sub><sup>-</sup>. However, our epidemiological study in TNT factory, China and experiments with rats revealed that TNT exposure caused an increased systemic NO production *in vivo*. Since H<sub>2</sub>O<sub>2</sub> can activate PI3K/Akt signaling, leading to endothelial NOS (eNOS) phosphorylation, we hypothesized that such a phenomenon caused by TNT exposure may be associated with an adaptive response to the chemicals. We found that although TNT inhibited eNOS activity in a cell-free system with IC<sub>50</sub> value of 49.4  $\mu$ M, exposure of bovine aortic endothelial cells (BAEC) to TNT (100  $\mu$ M) upregulated phosphorylation of eNOS that is regulated by activation of Akt, but not ERK1/2. These phosphorylations of eNOS and Akt caused by TNT were markedly blocked by wortmannin and LY294002, inhibitors of PI3K and NAC, a free radical scavenger. These results indicate that TNT-dependent phosphorylation of eNOS is mediated by presumably H<sub>2</sub>O<sub>2</sub> through PI3 kinase/Akt signaling.

PROTEIN KINASE C $\zeta$  MEDIATES LPS ACTIVATION OF NUCLEAR FACTOR KAPPA B (NF- $\kappa$ B) IN KIDNEY EPITHELIAL CELLS.

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PKCs are known to mediate multiple signaling transduction processes in mammalian cells. Here, we report the participation of the atypical PKC isoform, PKC $\zeta$ , in the activation of the mammalian transcriptional activator, NF- $\kappa$ B, by lipopolysaccharide (LPS) in kidney epithelial (NRK52E) cells. Treatment of NRK cells with LPS (1 $\mu$ g/l) rapidly induced NF- $\kappa$ B-DNA binding, as measured by EMSA, with maximal binding intensity by 30 minutes after LPS administration. This effect was preceded by rapid phosphorylation and degradation of the NF- $\kappa$ B inhibitor protein, I $\kappa$ B $\alpha$ . Pretreatment of NRK cells with various PKC inhibitors demonstrated that a cell-permeant pseudosubstrate specific to PKC $\zeta$  completely prevented LPS-induced NF- $\kappa$ B-DNA binding and reduced cytoplasmic I $\kappa$ B $\alpha$  phosphorylation, whereas other PKC inhibitors (Go 6976, Ro-31-8220, H8, bisindolylmaleimide) were without effect. Further studies showed that LPS promoted PKC $\zeta$  translocation from cytosol to membranal fractions and induced PKC $\zeta$  activity in a manner consistent with the kinetics of NF- $\kappa$ B-DNA binding. Transfection of cells with a PKC $\zeta$ -expressing plasmid (PKC $\zeta$ -WT) synergistically increased the duration and intensity of LPS-inducible NF- $\kappa$ B-DNA binding. Conversely, transfection with a dominant negative PKC $\zeta$  mutant (PKC $\zeta$ -DN) prevented LPS-induced degradation of I $\kappa$ B $\alpha$ . These findings support the view that PKC $\zeta$  mediates signaling events involved in the activation of NF- $\kappa$ B by LPS in kidney epithelial cells. Supported by ES04696, ES07032 and ES07033.

4-HYDROXYNONENAL-MEDIATED INHIBITION OF ERK-ELK-AP-1 SIGNALING.

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Lipid peroxidation products (e.g. 4-hydroxynonenal, 4-HNE), and the extracellular-signal-regulated kinases 1 and 2 (Erk1/2) are implicated in many pathologic states (e.g. alcoholic liver disease). The mechanism by which chronic alcohol consumption inhibits Erk1/2 signaling is unknown. To establish the potential for 4-HNE to affect this kinase pathway, sequence analysis of the Erk1/2 proteins shows these enzymes to be rich in amino acids reactive toward 4-HNE (ie: His, Cys, Lys). To determine the mechanism(s) involved in hepatic Erk1/2 inhibition that correlates with lipid peroxidation, an *in vitro* system was utilized to demonstrate Erk-2 is a target for 4-HNE modifications. Additionally, tryptic digest and mass spectral analysis of the purified, adducted Erk-2 identified two specific modification sites at His178 and Cys63. Primary rat hepatocyte cultures were employed to verify Erk1/2 adduction *in situ* by 4-HNE at pathologically relevant concentrations. Using western blot analysis, it was confirmed that the Erk modification(s) resulted in a concentration-dependant inhibition of induced Erk phosphorylation, which corresponded to a decrease in kinase activity of the immunoprecipitated phospho-Erk. 4-HNE also inhibited the Erk1/2-mediated phosphorylation of Elk-1 and its substrate AP-1, in both total cell lysates and nuclear fractions. In fact, 4-HNE treatment of hepatocytes resulted in a decrease in activated AP-1, as determined by its ability to bind the TPA-responsive element (TRE) sequence. To our knowledge, these are the first data to show 4-HNE covalently modifies Erk1/2 both *in vitro* and in primary cell culture systems. The modification of His178, which lies in the phosphorylation-site of Erk-2, reveals a mechanism by which 4-HNE inhibits the Erk-Elk-AP1 survival pathway in hepatocytes. The comprehensive data presented here corroborate this hypothesis. Because Erk1/2 is central to proliferation and survival, 4HNE-mediated inhibition of the kinase may impact a multitude of disease states related by oxidative stress and lipid-peroxidation. (supported by NIH/AA09300 to D R P)

INORGANIC PHOSPHATE CONTROLS CELL GROWTH THROUGH AKT/MEK-MEDIATED ERK-DEPENDENT MNK1 TRANSLOCATION IN NORMAL HUMAN BRONCHIAL EPITHELIAL CELLS.

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Inorganic phosphate (Pi) is abundant in the diet, and the kidney where sodium-dependent Pi(Na/Pi) transport systems in the brush-border membrane mediate the rate-limiting step in the overall Pi absorptive process, is a major regulator of Pi

homeostasis. Among 3 Na/Pi co-transporters, two types (type II, III) have been identified in mammalian lung. The Pi transport into the lung cells is regulated mainly by dietary and serum Pi values. The potential importance of Pi as a novel signaling molecule and pulmonary expression of Na/Pi co-transporters with poor prognosis of lung cancer prompted us to begin to define the pathways by which phosphate regulates lung cell growth using nontumorigenic human bronchial epithelial cells (NHBE). Pi activated Akt phosphorylation on Thr308, and activated signal transmits on the MEK/ERK signalling cascades. Our data clearly indicate that Pi controls cell growth by activating the ERK cascades as well as the translocation of MnK1 into nucleus through manipulating the Akt and MEK pathways. Such Akt- and MEK-mediated signaling of Pi may provide critical clues for treatment as well as prevention of diverse lung diseases including cancer. Supported by BK21

ACTIVATION OF THE TRANSCRIPTION FACTOR AP-1 BY 2, 2', 4', 4'-TETRACHLOROBIPHENYL (TCB) INVOLVES EXTRACELLULAR SIGNAL-REGULATED KINASES (ERKs) ENHANCED EXPRESSION OF C-FOS PROTOONCOGENE AND IN RAT LIVER CELLS.

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Signal transduction pathways play a central role in cell proliferation, differentiation and many physiological processes through altered gene expression. In ongoing studies with polychlorinated biphenyls (PCBs) we observed that orthochlorine substituted congeners activated the extracellular signal-regulated kinases (ERKs) a sub-group of mitogen activated protein kinase (MAPK) family of signal transduction kinases in liver epithelial cells. The activation of ERKs may, in turn, activate nuclear transcription factors that regulate gene expression. In this study, we examined the enhanced DNA binding activity of the transcription factor AP-1 of WB-F344 rat liver epithelial cells in response to 2, 2', 4', 4'-tetrachlorobiphenyl (TCB) and the involvement of ERKs in the activation of AP-1. Using gel-shift assays and RT-PCR we examined AP-1 DNA binding activity and the induction of AP-1 components. Nuclear extracts from WB cells treated with the TCB (20.0  $\mu$ M) caused a time dependent increase in DNA binding activity. Gel supershift analysis showed that the DNA binding was due to c-jun protein and was independent of c-fos protein. In contrast, RT-PCR analysis showed that TCB increased the expression of c-fos mRNA but not that of c-jun in these cells. Pre-treatment of WB cells with an inhibitor of ERK kinase U-0126 (10.0  $\mu$ M), completely abolished AP-1 DNA binding and decreased the expression of c-fos gene. These results suggest that diorthochlorinated PCBs induce AP-1 transcription factor activity and alter cellular gene expression through ERK activation (supported by NIEHS/Superfund grant no. ES-04911 and a Philip Morris Research grant).

EFFECT OF EXTRACTION TECHNIQUE, LONG-TERM STORAGE AND GENDER ON GENE EXPRESSION PROFILES OF HUMAN WHOLE BLOOD.

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Blood gene expression profiles (GEPs) may be a good surrogate for determining whether toxicologically-induced changes have occurred in inaccessible target tissues. For blood to be used in this way, it is first necessary to elucidate how biological and technical factors can contribute to the final GEPs. Blood was obtained from 42 adult volunteers. Two whole blood RNA extraction kits (PAX and ZR) were used to extract RNA. To determine the effects of storage, GEPs from blood which had been stored for 96 days in PAX tubes at -20C were compared to GEPs from blood of the same subjects (n=5 females) which had been isolated within 24h of collection. To determine the effect of isolation technique, RNA was isolated within 24h of blood collection (n=5 males) using both PAX and ZR. All samples were hybridized to Affymetrix genechips. RNA isolated after storage showed a slight decrease in quality. Hierarchical clustering and principal component analysis showed GEPs from the paired fresh and frozen samples partitioned by individual rather than time of preparation. GEPs from samples isolated using different RNA extraction methods partitioned by method rather than individual. GEPs also partitioned between the sexes. We conclude that biological differences account for the largest degree of variation in GEPs, with intergender variation being greater than intra-gender variation. Technical variation, whilst less significant, is nevertheless a contributing factor. Most important is the method of RNA collection/extraction. Long-term frozen storage also appears to affect GEPs to some degree. This may be related to a slight overall reduction in the quality of RNA obtained from blood which has been frozen for an extended time. Funded by EPA/UNCCH Toxicology Research Program Training Agreement T 829472. This abstract does not necessarily reflect EPA policy.

## NANBIOTECHNOLOGY: DEVICES TO QUANTIFY CELLS AND THEIR FUNCTIONS.

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*In vivo*, the efficacy of an immune response is dependent on the existence of the appropriate types and numbers of immune cells and their ability to communicate. The ability of immune cells to properly communicate is dependent on their ability traffic to centers of interaction (secondary lymphoid organs) and effector sites (sites of infection or cancer) in tissues. Cell trafficking requires expression of chemokine receptors and adhesion molecules as well as cytoskeletal changes affecting cell deformability. A microelectronic device consisting of a 100  $\mu\text{m}^2$  electrode coated with antibody to capture specific cell types has been developed to quantify the absolute number of a lymphoid subset in one  $\mu\text{L}$  of blood. Three additional microdevice designs have been developed to assess cell deformability and chemotaxis. Differential deformability and chemotaxis occurs after various forms of stressors (e.g., oxidative stress and cold restraint), which cause a loss of reactive cellular thiols. Differences in deformability and chemotaxis have been observed with blood leukocytes from young (<6 mon) vs older (>18 mon) mice as well as with blood leukocytes after *in vitro* exposure to impermeant (copper:phenanthroline) or permeant (hydrogen peroxide) oxidants. In cold restraint stressed mice, the subset that appears most sensitive to loss of surface thiols is the NK population. Loss of surface thiols is shown to prevent lymphocytes from being able to flow through into 5  $\mu\text{m}$  channels or chemotaxis through 5  $\mu\text{m}$  gaps in response to SDF-1 $\alpha$  or C3a. Supported by the Nanobiotechnology Center, a STC program of NSF (ECS-9876771).

## A HEADSPACE PROCEDURE FOR THE QUANTIFICATION OF 1- AND 2-BROMOPROPANE IN HUMAN URINE.

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A test procedure was developed to detect and quantify the levels of 1- and 2-bromopropane in urine. 1-Bromopropane (1-BP) is a commonly used industrial solvent, and 2-bromopropane (2-BP) is often found as an impurity component in industrial grade 1-BP. Both are of a health concern for exposed workers because of their chronic toxicity. Central neurological and peripheral neuropathy disorders have been reported in workers exposed to 1-BP; reproductive and hematopoietic disorders has been reported for workers exposed to 2-BP. In the test procedure described, urine samples were diluted with deionized water and placed into a sealed headspace vial. A static-headspace sampler (Teledyne-Tekmar Model 7000) was used to heat each sample at 75°C for a 35 minute equilibrium time. Quantification of the two analytes was by means of a gas chromatograph equipped with a dimethylpolysiloxane capillary column and an electron capture detector. 1-Bromobutane was used as an internal standard for this test procedure. A multi-level recovery study using fortified urine samples (0.5 to 8  $\mu\text{g}/\text{ml}$ ) demonstrated full recovery; 104 to 121% recovery was obtained. Precision ranged from 5 to 17 % for the 15 to 20 spiked samples at each level analyzed over multiple experimental trial days. The limit of detection for this test procedure was approximately 2  $\text{ng}/\text{ml}$  1-BP and 7  $\text{ng}/\text{ml}$  2-BP in urine. A six week storage study was conducted to determine the most appropriate method to collect and store fortified urine samples. Glass serum vials with crimped caps and Teflon lined septa stored at 4°C demonstrated full recovery of both 1-BP and 2-BP.

## EVALUATION OF TEST PROCEDURES FOR THE QUANTIFICATION OF URINARY (2-METHOXYETHOXY)ACETIC ACID.

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(2-Methoxyethoxy)acetic acid (MEAA) is a metabolite and biomarker for exposure to 2-(2-methoxyethoxy)ethanol [diethylene glycol monomethyl ether, DEGME, or DiEGME], a glycol ether which is of concern because of general toxicity. Glycol ethers have been frequently reported to damage the hematopoietic system, the male reproductive system and have demonstrated fetal/embryonic developmental toxicity. Specifically of interest to this laboratory is the use of 2-(2-methoxyethoxy)ethanol as an anti-icing additive to the military jet fuel JP-8. 2-(2-Methoxyethoxy)ethanol is readily absorbed through the skin and of concern for human dermal exposure. Test procedures to quantify the level of MEAA in human urine were developed and compared. Gas chromatography using a mass selective detector and a 50-m X 20.20-mm (id) dimethylpolysiloxane capillary column were used in each procedure studied. Two derivatization procedures were used in this evaluation. First, MEAA was extracted from fortified urine with ethyl acetate. Esterification of MEAA to the corresponding ethyl ester was one approach, and de-

rivatization of MEAA to the corresponding t-butyldimethyl silane derivative was the second approach for gas chromatographic analysis. Recovery studies using 2, 5, 10 and 20  $\mu\text{g}/\text{ml}$  MEAA fortified human urine samples demonstrated good accuracy and precision for both procedures. Recoveries using the ethyl ester procedure varied between 95-105% with precision [measured as percent relative standard deviation (%RSD)] as high as 14.3%, and recoveries using the silylation reagent were between 94-99% with precision (%RSD) as high as 7.3%. The t-butyldimethyl silane derivative procedure was less labor intensive and demonstrated better precision. The diethyl ester derivative procedure had better chromatographic performance from the more extensive sample cleanup.

## AUTOMATIC BIOSENSOR ANALYZER FOR RAPID ASSAY OF NEUROPATHY TARGET ESTERASE (NTE) IN BLOOD.

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Previous studies showed that whole blood NTE measured with a biosensor is a reliable biomarker of exposure to neuropathic (NP) organophosphorus compounds (OPs). For rapid assessment of human exposure to NP OPs, a prototype electrochemical flow-injection device for analysis of NTE in blood was created. The device operates automatically with 96-well plates and allows 8 samples to be analyzed simultaneously. NTE is assayed by the differential inhibition method using phenyl valerate as substrate. All sampling and analysis steps are carried out and monitored automatically. A "user friendly" interface enables all functions easily to be controlled and monitored by a computer and specially developed software, so that a minimum of operator effort is required. Data processing and calculation of NTE activity are done automatically using a phenol calibration curve. A new approach to construction of the amperometric biosensors was studied using layer-by-layer technology, resulting in 4-fold enhancement of biosensor sensitivity. Validation of electrode measurements was done using hen brain NTE. NTE activities and  $I_{50}$  values for NTE inhibitors determined with the standard spectrophotometric method were statistically identical to respective values obtained by the new LBL biosensor. Using the new device, blood and brain NTE inhibition by mipafox, DFP, ethyl- and propyl-dichlorvinylphosphates, and three fluorinated 1-aminophosphonates,  $(\text{RO})_2\text{P}(\text{O})(\text{CF}_3)_2\text{NHS(O)}_2\text{C}_6\text{H}_5$ , were well correlated ( $r = 0.995$ ,  $n = 7$ ), supporting the use of blood NTE as a biochemical marker of exposure to NP OPs. The results indicate that the new device could be used for rapid assessment of human exposures to NP OPs, e.g., to support consequence management and minimize risks of chemical terrorist attacks. (Supported by CRDF RB2 2488 and ARO DAAD19-02-1-0388).

## AN IMPROVED METHOD FOR THE QUANTITATION OF 8-HYDROXY-2'-DEOXYGUANOSINE IN BIOLOGICAL SAMPLES.

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8-Hydroxy-2'-deoxyguanosine (8-oxodG) is an important biomarker of oxidative stress. Numerous methods have been established for quantitation of 8-oxodG, including HPLC-ECD, LC-MS/MS, GC/MS, ELISA and the comet assay using FPG. However, concentrations several orders of magnitude different have been reported for endogenous 8-oxodG, demonstrating the need for an improved quantitation method. Moreover, 8-oxodG is prone to artifactual formation, which must be prevented to achieve accurate quantitation. We hypothesized that coupling immunoaffinity (IA) chromatography to capillary LC-ESI<sup>+</sup>-MS/MS would provide the requisite accuracy, sensitivity, and specificity required to measure 8-oxodG in a variety of samples while using reasonable amounts of DNA. Capillary LC typically offers 10-fold greater sensitivity over micro LC; thus, we developed such a method for the analysis of 8-oxodG. The presence of the free radical scavenger 2, 2, 6, 6-tetramethylpiperidine-N-oxyl (TEMPO) during DNA isolation and enzymatic hydrolysis prevented artifactual formation of 8-oxodG from digested DNA samples and reduced the appearance of interference peaks compared to HPLC clean-up. 8-OxodG was quantified using stable isotope internal standard, [<sup>15</sup>N]<sub>5</sub>-8-oxodG. The limit of detection was determined with standards to be 0.5 fmol on column with a signal-to-noise ratio of 3, enabling the detection of 8-oxodG in as little as 5  $\mu\text{g}$  DNA. Preliminary data

demonstrated that endogenous 8-oxodG levels in freshly isolated calf thymus DNA was  $0.5 \pm 0.06$  8-oxodG/10<sup>6</sup> dG after IA clean-up, as compared to  $1.8 \pm 0.5$  after HPLC clean-up. This difference is thought to be due to lower amounts of interference, providing more accurate integration. Commercially available calf thymus DNA gave endogenous 8-oxodG levels of  $15.8 \pm 0.4$  8-oxodG/10<sup>6</sup> dG, reflecting artifact due to improper DNA isolation. Based on these preliminary data, this method appears to be suitable for the assessment of oxidative damage *in vivo*.

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#### BIOLOGICAL SAMPLE ANALYSIS METHODS DEVELOPMENT AND VALIDATION FOR A TOXICOKINETIC STUDY OF BIS(2-CHLOROETHOXY)METHANE.

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The purpose of this work was to develop and validate analytical methods supporting toxicokinetic studies of bis(2-chloroethoxy)methane (CEM) in rodents. Two methods were required for this purpose; one for CEM and a second for thiadiglycolic acid (TDGA), the metabolite of interest. For CEM the method consisted of extraction of the CEM from the plasma or tissue followed by gas chromatography coupled with mass spectrometry (MS) using selective ion monitoring (SIM). For thiadiglycolic acid the method consisted of methylation of the acid, followed by extraction of the metabolite with toluene and analysis by gas chromatography coupled with MS/SIM. CEM-d8 and ethyl-3-mercaptopropionate were used as internal standards (IS) for CEM and TDGA methods, respectively. TDGA was derivatized with boron trifluoride-methanol complex. Standards for the CEM analysis were prepared in rat plasma and acceptable for quantifying mouse plasma and rat and mouse heart and thymus. Standards for the TDGA were prepared in water because of the possibility of endogenous amounts in plasma and tissue. Quality control samples for each were prepared in both plasma and tissues from both species. Quadratic regression equations weighted 1/x were necessary to relate the response ratio of analyte to internals standard (y) to the concentration (x) of CEM and TDGA. The chromatograms from specificity samples of rat plasma and tissue (heart and thymus) had no significant interfering peaks. The analytical method met the acceptance criteria for accuracy and precision with a maximum relative error and relative standard error less than 20 %. The plasma and tissue samples of CEM and TGA were stable when stored at -20 oC. The analytical methods were determined to be acceptable for quantitation of CEM and TDGA that will be generated from a definitive toxicokinetic study of CEM in F344 rat and B6C3F1 mice. [Supported by NIEHS Contract No. N01-ES-05456.]

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#### VALIDATION OF A METHOD FOR THE DETECTION OF ANTI-KEYHOLE LIMPET HEMOCYANIN (KLH) IgM ANTIBODIES IN THE NEONATAL/JUVENILE CD-1 MOUSE SERUM BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA).

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Recent Guidance (FDA, EMEA, MHLW/JPMA) have recommended the T-cell dependent antibody response (TDAR) assay to assess the immune response. There is also growing interest in the assessment of potential immunotoxicity to the developing immune system. In this study, neonatal/juvenile CD-1 mouse were immunized intra-peritoneally with 0.5 mL of keyhole limpet hemocyanin (KLH; 0.2 mg/mL) on 2 occasions (28 and 35 days pp) and blood was collected pre-KLH, and 7 and 14 days post-KLH. In addition, one group of mice received 25 mg/kg/day of cyclophosphamide for 4 consecutive days. The results obtained 7 and 14 days post-KLH were from 0.59 to 2.20  $\mu$ g/mL and 1.75 to 5.74  $\mu$ g/mL respectively for anti-KLH IgM, and from 0.28 to 18.96  $\mu$ g/mL and above the upper limit of quantitation ( $> 10 \mu$ g/mL) respectively for anti-KLH IgG. Cyclophosphamide did not significantly inhibit the anti-KLH antibody response at the dose tested. Validation of ELISA methods for the detection of anti-KLH IgM and anti-KLH IgG antibodies, included specificity/selectivity, linearity of dilution / parallelism, intra- and inter-assay precision and accuracy, stability, and prozone effect. The validated detection ranges were from 7.81 to 1000 ng/mL and 3.13 to 400 ng/mL for anti-KLH IgM and anti-KLH IgG respectively. The intra- and inter-precision and accuracy met the acceptance criteria and no prozone effect was observed up to 10000 ng/mL. The specificity of the ELISAs was good; the results were below LLOQ. The selectivity, linearity of dilution, parallelism, and stability of study samples at room temperature for approximately 6 hours, at 40C for approximately 24 hours, after 4 freeze-thaw cycles at -80oC, and long-term storage at approximately

-80oC are ongoing. The detection of anti-KLH IgM and anti-KLH IgG using these ELISA methods will prove useful in toxicology studies. Optimization of KLH immunization and cyclophosphamide dose is presently ongoing.

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#### NANBIOTECHNOLOGY: GRATING-COUPLED SURFACE PLASMON RESONANCE (GCSPR), A CELL AND PROTEIN MICROARRAY PLATFORM FOR THE ANALYSIS OF TOXICANT EFFECTS.

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Grating-coupled surface plasmon resonance (GCSPR) technology is a novel method for molecular and cellular measurements in a microarray format. This technology uses an optical reflectance method to measure the optical properties of an aqueous medium close to a gold surface. Polarized light illuminates a gold surface coated with receptors and the angle of minimal reflectance is measured. An increase in mass, resulting from ligand or cell capture by surface-immobilized receptors, increases the GCSPR angle that is detected at multiple regions of interest (ROI) on each gold chip using a CCD camera to measure light intensity. We have previously shown that GCSPR can detect antibody, IL-2, and metallothionein. At present, the GCSPR assay approximates an ELISA in sensitivity and is amenable to improvements in sensitivity. GCSPR can also detect intact mammalian cells, bacteria, viruses and toxin analogs. GCSPR could detect Jurkat T cells captured by chip-bound anti-CD3 antibody. GCSPR was also used to detect apoptotic cells on immobilized Annexin V. *Bacillus globigii* (B.g.), a surrogate bacterial pathogen, was detectable using immobilized anti-B.g. antibody. These results indicate that complex samples can be characterized via GCSPRI according to the patterns of expressed cell surface antigens that uniquely identify each target population. Furthermore, specific and multiplexed detection was demonstrated on a single biosensor chip for B.g. and ovalbumin. We are currently developing a GCSPRI assay of polycyclic aromatic hydrocarbons, and are also developing PDMS stamps for micro-contact printing of receptors on the chip surface. Our results indicate that GCSPRI is a label-free, real-time, multiplexed technology that can be used to detect bacterial pathogens, toxins and toxicants in both environmental and clinical samples. Supported by NIEHS ES25490, NASA NAS 9-02016, and NSF DMI 0215192.

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#### RAT KIDNEY INJURY MOLECULE-1 (RKIM-1) ELISA: A SENSITIVE ASSAY FOR EARLY DETECTION OF KIDNEY TUBULAR INJURY IN PRECLINICAL TOXICITY STUDIES.

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Kidney injury molecule-1 (Kim-1), a type 1 transmembrane glycoprotein, is upregulated ~10-100 fold following renal proximal tubular damage in humans, rats, and mice. Following renal injury the ectodomain of Kim-1 is shed into the rat and human urine and it can serve as a useful biomarker for early diagnosis of renal injury. The objective of the present work was to develop an ELISA for detecting Kim-1 in rat urine that can be used in preclinical drug development studies to screen for nephrotoxicity. A fusion protein of rat Kim-1 ectodomain and human Fc was generated by standard techniques and stably transfected into Chinese Hamster Ovary (CHO) cells. Transfected CHO cells were grown in serum free media and rat Kim-1-Fc was purified using a protein A-sepharose column to yield ~200  $\mu$ g/ml. Two mouse monoclonal antibodies were made against Kim-1Fc protein by standard antibody production techniques and these were used to construct a sandwich Kim-1 ELISA. To validate this ELISA we collected daily urine samples (0 to 10 days) from male Sprague-Dawley rats (n=5; 250-275 g) kept in metabolic cages and treated with three doses of cisplatin (2.5, 5, or 7.5 mg/kg respectively). Other rats were treated with 5 or 7.5 mg cisplatin/kg respectively and sacrificed at day 1, 2, 3, 4, or 5 (n = 4 for each group). Blood and kidneys were collected to estimate the extent of renal damage. The Kim-1 ELISA was sensitive over a concentration range of 200 pg/ml to 6 ng/ml. Kim-1 levels were undetectable in vehicle-treated controls. After 5 mg/kg cisplatin, Kim-1 was detectable in the urine on day 1, increased to 400 pg/ml on day 2, 2.5 ng/ml on day 3, and peaked at 7 ng/ml on day 5 whereas other conventional biomarkers (plasma creatinine, BUN, urinary N-acetyl- $\beta$ -glucosaminidase, glycoursuria, proteinuria) had normal values until day 3. Thus, we have established a non-invasive, rapid, sensitive, reproducible, and high throughput method to detect nephrotoxicity that can serve as a valuable tool for preclinical studies and risk assessment.

ANALYSIS OF HYDROXYMETHYLVINYL KETONE DERIVED 1, *N*<sup>2</sup>-PROPANODEOXYGUANOSINE BY LC-MS/MS.

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The carcinogenic effect of 1, 3-butadiene (BD) is dependent on metabolism to several mutagenic metabolites. 1, 2,3, 4-Diepoxybutane (DEB) is hypothesized to be the main metabolite involved in mutagenicity following exposure to low levels of BD in mice, while metabolites of 3-butene-1, 2-diol (BD-diol) are thought to become involved in the mutagenic responses observed in both mice and rats at higher exposures. We have previously demonstrated that the dose-response curves for DNA and hemoglobin adducts of 3, 4-epoxy-1, 2-butanediol (EB-diol) mimic the dose-response curves for *Hprt* mutations in rodents exposed to BD-diol, suggesting that EB-diol is involved in this mutagenic response. However, because BD-diol can also be metabolized to hydroxymethylvinyl ketone (HMVK), it is necessary to evaluate the formation of this reactive metabolite. The goal of the current project was to develop a MS based method to quantify the  $\alpha$ , regioisomer of HMVK derived 1, *N*<sup>2</sup>-propanodeoxyguanosine ( $\alpha$ -HMVK-dGuo) and evaluate the formation of this adduct in exposed rodents. The method involved enzymatic hydrolysis of DNA, HPLC purification of the hydrolysate, and adduct measurement by LC-ESI<sup>+</sup>-MS/MS. Validation experiments indicated that the intra- and inter-experimental variabilities were 11% and 5-20%, respectively. The limit of detection approached 5 fmol of analyte standard injected onto the column or 5 fmol/200  $\mu$ g DNA. Using this method, DNA samples from liver of female F344 rats exposed to 0 or 36 ppm BD-diol for 4 weeks, and liver from female Sprague-Dawley rats exposed to 1000 ppm BD for 13 weeks were analyzed. Preliminary data showed that the adduct was not present in detectable amounts. While experiments must be conducted to understand the mechanism(s) behind these results, the data indicate that HMVK may be efficiently detoxified and unable to significantly contribute to BD-diol associated mutagenicity.

DEVELOPMENT AND VALIDATION OF A BIOANALYTICAL METHOD FOR TUNGSTEN.

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Tungsten (W) has been nominated for study to the National Toxicology Program (NTP) because of reported associations between concentrations of W in drinking water and childhood leukemia. As part of the NTP study on W, the chemical disposition of W in plasma, liver, kidney, uterus, femur, and intestine or rodents are being defined after exposures by oral gavage, intravenous, or drinking water. Transplacental disposition is also being characterized by conducting drinking water exposures during gestation. Prior to disposition studies, a bioanalytical method has been developed and validated for each of the aforementioned tissues. The method includes both W and molybdenum (Mo), which is being measured in liver and small intestine because W replaces Mo in Mo dependant enzyme systems. The method requires acid digestion followed by analysis by inductively coupled plasma mass spectrometry. The method has been evaluated by spiking several concentrations of W and Mo into all of the tissues. The method showed acceptable linearity (correlation coeff. > 0.99), precision (coefficient of variation < 20 % across range), accuracy (within 20 % across range), recovery (within 20 %) and stability. The range of the assay spans -50 ng to 30  $\mu$ g per sample. The assay is suitable for chemical disposition studies of W in rodents.

N-(2, 3, 4, 5, 6-PENTAFLUOROPHENYL)DITHIOCARBAMATE AS A SENSITIVE DERIVATIZING REAGENT FOR THE ESTIMATION OF EPOXIDES OF 1, 3-BUTADIENE AND OTHER PETROCHEMICAL ALKENES.

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1, 3-Butadiene (BD) is one of the 20 highest volume chemicals produced in the US. In addition to its release from automobile exhaust in urban areas with traffic congestion, it is present in areas of petrochemical production. Apart from being an environmental pollutant, BD has been shown to be a probable human carcinogen falling under group 2A as per a recent evaluation by IARC. The enzymes of the cytochrome P450 system metabolize BD forming electrophilic intermediates 1, 2-epoxy-3-butene (EB) and 1, 2,3, 4-diepoxybutane (DEB), which, in turn, mediate the toxicity of BD. Several methods have been reported for the measurement of EB

and DEB, including GC-MS, MALDI-MS/MS, and <sup>32</sup>P-postlabeling followed by HPLC. These methods are cumbersome apart being expensive. In the present work attempts were made to develop a sensitive method for the determination of low levels of epoxides of BD, with an emphasis on simplicity of adaptation. We exploited the technique of derivatization of epoxides using N-(2, 3, 4, 5, 6-pentafluorophenyl)dithiocarbamate (PFDTC). The reaction of 2, 3, 4, 5, 6-pentafluoroaniline with carbon disulfide in the presence of strong base such as potassium ethoxide facilitated the preparation of PFDTC in a highly pure form. Standards and samples were analyzed by HPLC using a ODS column (4 mm x 150 mm; Supelco) with 40% acetonitrile in water as mobile phase (flow = 1 mL/min; detection: 234 nm). Using the present method low levels of epoxides of BD and other alkenes could be determined in cell culture, plasma, and tissue homogenates. The present method is highly reproducible with recoveries of  $\geq$ 95%, and as low as 1 pmol of the analyte can be successfully detected. This cost effective method can be standardized for routine analysis of epoxides and related compounds. Further, the present approach opens up avenues for improving the sensitivity by use of gas chromatography with electron capture detection. (Funding from NIEHS ES10018 and Louisiana Biomedical Research Network is acknowledged.

A LUMINESCENT METHOD FOR MEASURING DRUG-DEPENDENT CHANGES IN P-GLYCOPROTEIN ATPASE ACTIVITY.

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P-glycoprotein, also known as P-gp or MDR1, is an ATP-dependent efflux pump for a wide range of drugs that plays an important role in multi-drug resistance and certain adverse drug-drug interactions. Drugs that are transported by P-gp can be identified as stimulators of its ATPase activity and we have developed a luminescent method to detect this effect. The method relies on the ATP-dependence of the light-generating reaction of firefly luciferase. After a pool of ATP is first exposed to the P-gp ATPase ATP consumption is detected as a decrease in luminescence from a second reaction with firefly luciferase. The quantity of ATP consumed can be interpolated from a luciferase/ATP standard curve and from this the rate of ATPase activity can be calculated. We used a stabilized mutant of the luciferase enzyme in a formulation that provided stable, glow-style signals from multi-well plates. The method detected the stimulation of recombinant human P-gp ATPase activity by various drugs with fold increases and EC50s in good agreement with published values. Stable reagents are used to produce stable luminescent signals in a method that is easily configured in high throughput mode.

COMPARISON OF CALUX BIOASSAY AND HRGC/MS CHEMICAL ANALYSIS FOR THE DIOXIN LEVEL IN ENVIRONMENTAL MATRICES.

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Two different methods, HRGC/MS chemical analysis and CALUX bioassay were used for testing environmental water and soil samples if CALUX bioassay is applicable for the measurement of dioxin-like compounds. Furthermore, REPs for 17 kinds of dioxin-like compounds were determined and compared with WHO-TEFs of corresponding compounds. In the water samples, HRGC/MS-TEQs ranged from 0 to 2.281 pg-TEQ/L, and the highest TEQ value was 2.281 pg-TEQ/L. All samples except one sample showed total-TEQ values below the 1 pg-TEQ/L. HRGC/MS-TEQ values of soil samples was in a range of 0 to 65.6 pg-TEQ/g (dry) and the highest TEQ value was found in Maseo, where the refinery used to be near. CALUX-TEQs of water samples ranged from 0.7 to 18 pg-TEQ/L and CALUX-TEQs of soil samples ranged from 0.6 to 650 pg-TEQ/g (dry). The correlation between CALUX-TEQs and HRGC/MS-TEQs was very high ( $r^2 = 0.982$ ). However, the absolute values obtained by CALUX bioassay were always higher than that by HRGC/MS. To interpret this data, the REP values for 17 kinds of dioxin-like compounds were determined by CALUX bioassay. And REP values were compared with WHO-TEF values. The correlation between estimated REP-TEQs and HRGC/MS-TEQs turned out to be very high ( $r^2 = 0.998$ ) and the absolute values obtained by the analysis of HRGC/MS and estimated based on REP EC50 were appeared to be very similar. And also, there were good correlation ( $r^2 = 0.970$ ) between estimated REP-TEQs and CALUX-TEQs. However, the absolute TEQ values obtained by CALUX bioassay were always about ten times higher than the estimated REP-TEQ values. Thus, the CALUX bioassay can be highly recommended as an early warning system for routine measurement of dioxin-like compounds in environmental matrices. [This research was supported by Grant from KOSEF.]

A METHOD FOR REPEATED JUGULAR-VEIN TOXICOGENETIC BLOOD SAMPLING IN CONSCIOUS, NON-CANNULATED RATS.

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Repeated blood sampling for toxicokinetics is a common requirement in rat toxicology studies of pharmaceuticals. Most collection techniques have limitations in terms of the degree of invasiveness (e.g. retroorbital sampling or surgical cannulation), low sample volume (e.g. caudal venipuncture) or the need for terminal sampling and/or anesthesia (e.g. cardiac puncture or abdominal aorta). Jugular vein sampling under anesthesia raises concerns over drug-anesthetic interaction and the effect of anesthesia on the animals (e.g. potential to cause peripheral vasodilatation with associated risk of hemorrhage). Our laboratory routinely employs a jugular blood sampling technique in conscious, minimally-restrained rats. Critical to the success of the technique is extensive training in the method of minimal-restraint and in identifying the location of the jugular vein. The rat is held vertically in one hand with thumb and middle finger directing its forelimbs dorsally. The index finger pulls downward the back of the head/neck, in order to cause the rat's head to tilt upwards. This maneuver results in hyper-extension of the rat's upper body, and causes the jugular veins to be more evident on both sides of the sternoclavicular area. The other hand inserts the needle/syringe into the vein and the sample is withdrawn normally. Samples of at least 1 ml can be easily collected once the technique is mastered. On completion of the sampling, pressure is applied until the bleeding ceases. This technique enables repeat samples from the same puncture site within a single day and over multiple treatment days (dependant upon the volume of blood collected at each timepoint). Evaluation of clinical signs, behavior, body weight and food intake indicate no evidence of undue stress on the animals. Once the technique has been mastered, the success rate in terms of on-time sample collection is close to 100%, even in instances of very tightly scheduled collection times on multiple animals.

ELECTROSTATIC SURFACE CHARGE OF NANOPARTICLES ACTIVATES CNS MACROPHAGES (MICROGLIA).

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Nanometer size particles carry free radical activity on their surface and can produce oxidative stress (OS)-mediated damage upon impact to target cells. The initiating event of phage cell activation (i.e., the oxidative burst) is unknown, although many proximal events have been identified (e.g., phagocytosis, increases in NADPH oxidase and NF- $\kappa$ B activity). Data generated in an immortalized microglia cell line (BV2) suggest a role for electrostatic surface charge in this activation. Nanometer size, spherical polystyrene micelle (SPM) beads, coated with either carboxyl (COOH-) or dimethyl amino ((CH<sub>3</sub>)<sub>2</sub>-N-) functional groups, measured 860 nm and 850 nm in diameter, respectively. Confluent BV2 cells were labeled with a H<sub>2</sub>HFF-based fluoroprobe that fluoresces in the presence of the reactive oxygen species, H<sub>2</sub>O<sub>2</sub>. Kinetic readings, collected on cells exposed to either COOH- or (CH<sub>3</sub>)<sub>2</sub>-N- coated SPM showed significantly higher fluorescence relative to baseline, in a concentration-response fashion. Subsequent experiments demonstrated that both COOH- and (CH<sub>3</sub>)<sub>2</sub>-N- labeled SPM stimulated significant levels of IL-1 $\beta$  and TNF $\alpha$  after 6 and 24 hr, respectively. Confocal microscopy was used to document the translocation of FITC-labeled COOH- SPM as they entered the microglia and formed large aggregate populations within the cytoplasm over a 24 hr period. Efforts are underway to determine if charge can stimulate other inflammatory changes proximal to the cytokine release in microglia and macrophages. Previous studies (Veronesi et al., 2003) reported that COOH- and (CH<sub>3</sub>)<sub>2</sub>-N- SPM could stimulate cytokines and their message in human epithelial cells, a phenomenon thought to be mediated through ASICS and VR1 receptors. It is currently unknown whether these or other charge sensitive (e.g., scavenger) receptors are involved in the electrostatic activation of phage cells. (This abstract has been reviewed by the USEPA, NHEERL and does not necessarily reflect its policy).

INCREASED REACTIVE OXYGEN SPECIES PRODUCTION AND METABOLIC ALTERATIONS IN MITOCHONDRIA OF EXPERIMENTAL DIABETIC RAT TISSUES.

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We have previously shown a tissue-specific increase in oxidative stress in streptozotocin (STZ)-induced diabetic rats. In this study, we have further investigated the production of reactive oxygen species (ROS) in different subcellular fractions of the

pancreas and other tissues in STZ-diabetes. We have demonstrated that mitochondria from diabetic rats have strong adaptive response against oxidative stress. Protein carbonylation, a marker for aldehyde-dependent protein oxidation, was increased in the cytosol and microsomes of the tissues while mitochondria exhibited only a marginal change. The maximum effect was seen in the pancreas. Nitric Oxide synthase (NOS) activity was increased in the pancreatic microsomes while mitochondrial and cytosolic NOS were inhibited. Pancreatic mitochondria showed an increased utilization of oxygen and increase in the activities of respiratory complexes II, III and IV. Expression of oxidative stress marker proteins, Hsp70, heme oxygenase 1 and GSTA4-4 was also increased in the pancreas from experimental animals. The expression of complex IV- 70 kDa and 30 kDa subunits were also increased in diabetic rats. These results may suggest an important role for mitochondria in diabetes. The increased mitochondrial metabolic adaptation may have implications in the progress of disease. (Supported by NIH Grant GM34883-18 (NGA) and FMHS/Terry Fox Research Funds (HR).

FAILURE OF DIETARY VITAMIN E TO PREVENT DOXORUBICIN-INDUCED CARDIAC MITOCHONDRIOPATHY *IN VIVO*.

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Doxorubicin (DOX) is a potent, broad-spectrum antineoplastic agent whose clinical use is limited by a cumulative and irreversible cardiomyopathy. The mechanism of toxicity is assumed to involve redox cycling of DOX by mitochondria to generate oxygen free radicals, making mitochondria both a primary source and target of oxidative injury. This investigation was designed to assess the cardioprotective potential of dietary d-alpha-tocopherol succinate (TOCO) in the prevention of mitochondrial cardiomyopathy resulting from DOX treatment. Adult male Sprague-Dawley rats received 7 weekly s.c. injections of either 2 mg/kg DOX or saline and had free access to either normal rat chow or rat chow supplemented with 2 g TOCO/kg (n=12, with 4 treatment groups). Dietary TOCO significantly enriched the d-alpha tocopherol content in serum, liver, and heart mitochondria, though not in whole heart. Heart mitochondria specifically showed a 2-fold increase in TOCO concentration. Oxidative injury in cardiac tissue due to DOX treatment was evidenced by a 3-fold increase in protein carbonyl content. TOCO was protective against this DOX-induced oxidative damage to cardiac proteins. However, dietary TOCO did not protect against mitochondrial dysfunction (defined by decreases in calcium loading capacity and respiration rates) or damage to cardiomyocytes noted by histological examination. From this we conclude that although dietary TOCO enriches cardiac mitochondrial membranes, either 1) this dietary-mediated enrichment of d-alpha tocopherol is not sufficient to protect mitochondrial membranes from oxidative injury or 2) oxidative stress alone is not responsible for the persistent mitochondrial cardiomyopathy caused by long-term DOX therapy. (Supported by HL-58016 and the 3Gasper and Irene Lazzara Foundation.

PRIMAQUINE-INDUCED HEMOLYTIC ANEMIA: ROLE OF SPLENIC MACROPHAGES, LIPID PEROXIDATION AND CYTOSKELETAL PROTEIN ALTERATIONS IN THE HEMOTOXICITY OF 5-HYDROXYPRIMAQUINE.

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We have recently demonstrated that the phenolic primaquine metabolite, 5-hydroxyprimaquine (5-HPQ), is a direct-acting hemolytic agent in rats. To investigate the fate of red cells *in vivo* after *in vitro* exposure to 5-HPQ, rat 51Cr-labeled red cells were incubated with 5-HPQ then re-administered intravenously to rats pretreated with clodronate-loaded liposomes, which have been shown to deplete splenic macrophages. Blood, spleen and liver samples were taken at various intervals and the radioactivity counted. In saline-liposome treated rats given 5-HPQ-treated red cells, an enhanced rate of removal of radioactivity from the circulation was observed as compared with vehicle-treated red cells. The loss was matched by a corresponding increase in radioactivity appearing in the spleen. In contrast, the rate of removal of radioactivity from the circulation of clodronate-liposome rats was significantly lower, and the appearance of radioactivity in the spleen was markedly delayed. Splenic removal of red cells damaged by treatment with 5-HPQ was not found to be associated with oxidative damage to membrane lipids. However, splenic removal was found to correlate with profound alterations in erythrocyte membrane skeletal proteins, principally in the form of disulfide-linked hemoglobin-skeletal protein adducts. The data indicate that splenic macrophages are responsible for removal of intact 5-HPQ-treated red cells and that cytoskeletal protein damage rather than membrane lipid oxidation may underlie the process of macrophage recognition.

OXIDATIVE STRESS CAUSED BY GLUTATHIONE  
SYNTHESIS INHIBITOR BUTHIONINE SULFOXIMINE  
RESULTS IN GENOME REARRANGEMENTS IN MICE.

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Oxidative stress has been associated with cancer and other diseases, such as neurodegenerative, bronchopulmonary and cardiovascular diseases, to name a few. A number of xenobiotics, including cigarette smoke, diesel exhaust, and certain pharmaceuticals, cause oxidative stress. L-Buthionine-S, R-sulfoximine (BSO) is used to pharmacologically induce oxidative stress in research studies on the effects of oxidative injury. BSO induces endogenous oxidative stress by irreversibly inhibiting  $\gamma$ -glutamylcysteine synthetase, an essential enzyme for the synthesis of glutathione (GSH), a major cellular thiol antioxidant. We tested whether lowering cellular antioxidant capacity by BSO can lead to genomic rearrangements that may play a role in carcinogenesis or other genetic disease. We determined the frequency of DNA deletions, the levels of oxidative DNA damage measured by 8-OH deoxyguanosine (8-OHdG), and GSH levels, in mouse embryos exposed to BSO via drinking water given to pregnant dams. A low (2 mM) BSO dose treatment resulted in elevated frequencies of DNA deletions, higher 8-OHdG levels, but no changes in GSH concentration. A 10-fold higher (20 mM) BSO dose caused a 1.6-fold increase in DNA deletion frequency, 5-fold higher levels of 8-OHdG and depleted GSH to 30% of control level. The thiol antioxidant N-acetyl-L-cysteine had a protective effect against BSO treatment-induced DNA deletions. This study showed that endogenous oxidative stress results in genomic rearrangements that may play a role in development of cancer.

INHIBITION OF HSP90 $\alpha$  CHAPERONE ACTIVITY BY  
4-HYDROXY-2-NONENAL.

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Intracellular concentrations of 4-hydroxy-2-nonenal (4-HNE), malondialdehyde (MDA) and other lipid-derived aldehydes are elevated under conditions of oxidative stress. These aldehydes have a documented ability to modify proteins, thereby disrupting function. Proteomic analysis of rat liver subcellular fractions has demonstrated consistent modification of heat shock protein 90 (Hsp90) by 4-HNE and MDA in a model of chronic alcoholic liver disease. A series of *in vitro* studies were conducted to test the hypothesis that these lipid aldehydes interfere with Hsp90 chaperone activity. Purified recombinant Hsp90 $\alpha$  was treated with increasing concentrations of 4-HNE and MDA, resulting in a corresponding increase in the severity of protein modification by these aldehydes, demonstrated by immunoblot. 4-HNE protein adducts were confirmed and located by tryptic digest and HPLC-MS/MS peptide analysis. Pretreatment of Hsp90 $\alpha$  with 10  $\mu$ M 4-HNE resulted in a 68% inhibition in chaperone activity using a firefly luciferase refolding system, while the chaperone was completely resistant to inactivation following pretreatment with 10  $\mu$ M MDA. Pretreatment with 10  $\mu$ M 4-oxononenal (4-ONE), which is highly reactive towards Cys residues, also resulted in significant inhibition of Hsp90-assisted protein refolding. Because MDA is comparatively less reactive toward Cys than 4-HNE or 4-ONE, these results suggest a Cys specific mechanism. Finally, disappearance of luciferase was measured throughout the duration of the timecourse; however, rapid substrate degradation does not account for the observed 4-HNE-mediated inhibition of protein refolding, as demonstrated experimentally. The data presented here demonstrate modification of Hsp90 by 4-HNE and MDA in the livers of rats fed a chronic high fat/ethanol diet, and inhibition of Hsp90 chaperone-like activity by Cys-reactive lipid aldehydes. This work was supported by NIH/NIAAA RO1AA09300 and NIH/NIEHS RO1ES09410 (DRP), NIH/NIEHS F32 ES11937 (JAD), and NIH/NIAAA F31 AA014308 (DLC).

HIGH FAT DIET DIFFERENTIALLY AND SITE-SPECIFICALLY ALTERS LUNG GLUTATHIONE AND ACUTE CYTOTOXICITY IN MALE AND FEMALE MICE.

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The average American consumes 37% of their calories from fat. Very little is known about the effect of high fat diet on lung cells. To evaluate the effect of diet on the lung, male and female C57Bl/6J mice were fed a high fat diet (58% kcal fat) or a matching low fat diet (10.5% kcal fat) beginning at 4 wks of age. Body weight and intra-abdominal fat pad weight (8-12 wks of age) were significantly increased by the high fat diet ( $P < 0.01$ ). Intrapulmonary and extrapulmonary airways were microdissected for analysis of glutathione (GSH; measured using HPLC) and total glutathione S-transferase activity (GST; turnover of CDNB substrate). GST activity did not vary significantly between high and low fat groups, by airway level or by

sex. However, GSH levels were significantly elevated by high fat diet in intrapulmonary airways of female mice ( $P < 0.01$ ). GSH levels in extrapulmonary airways of females and both airways in the male were unaffected by diet. Lungs from mice fed a high fat diet were more fibrous on dissection. Collagen deposition defined by picrosirius red staining was increased in mice fed a high fat diet. Additional animals were challenged with naphthalene (NA) i.p. to assess pulmonary responses to injury at 1 and 24 hrs. Female animals were more susceptible than males to NA injury. High fat diet reduced the amount of NA injury in the large intrapulmonary airways of female mice but had no effect in males. Male mice increased intrapulmonary GSH levels in response to NA injury and this was unaffected by diet. However, female mice were incapable of increasing their 1 hr GSH levels in response to NA injury in either the high or low fat diet groups. We conclude that a high fat diet changes the steady-state biology of the lung and that changes in response to diet and/or injury challenge are airway level and sex-specific. Support: California Tobacco-Related Diseases Program grant 12IT-0191, NIH ES013066, ES04311, ES05707

MOLECULAR PATHOLOGICAL ANALYSIS OF  
HEPATOCARCINOGENESIS IN MICE TREATED WITH  
DICYCLANIL.

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In order to clarify the mechanism of hepatocarcinogenesis induced by dicyclanil (DC), molecular pathological analyses in the liver of mice were performed. At Experiment I, a large-scale cDNA microarray and the validation of RT-PCR were performed using the liver of mice given the diet containing 0 and 1500 ppm of DC for 2 weeks. The gene expressions of metabolism- or oxidative stress- related genes, such as Cyp1a, Aldh1a1, and Txnrd1, were predominantly up-regulated in the liver of mice treated with DC. At Experiment II, in addition to histopathological analyses, stress pathway-specific small-scale microarray and the validation of real-time RT-PCR were performed in the liver of mice using a two-stage hepatocarcinogenesis model, in which they were given 1500 ppm of DC for 7 weeks after injection of dimethylnitrosamine (DMN) with a partial hepatectomy (PH). The number of gamma-glutamyltransferase (GGT)-positive cell and PCNA Labeling Index were significantly increased in the liver of the DMN+DC+PH group that showed fluctuations of gene expressions of Cyp1a, Txnrd1, Ogg1, and Gadd45a related to oxidative stress or DNA damage/repair. To evaluate oxidative stress and DNA damage induced by the treatment of DC, 8-hydroxy-2-deoxyguanosine (8-OHdG) was measured in the liver DNA of two-stage hepatocarcinogenesis model mice given DC for 13 weeks (Experiment III). In addition to the formation of GGT positive foci, significant increases of 8-OHdG were observed in the DMN+DC+PH groups. These results suggest that DNA damage due to oxidative stress is partially associated with the mechanism of DC-induced hepatocarcinogenesis on mice.

ROLE OF NITRIC OXIDE IN DIESEL EXHAUST  
PARTICLE-INDUCED GENOTOXIC AND MUTAGENIC  
ACTIVITIES IN THE RAT LUNG.

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Exposure of rats to diesel exhaust particle (DEP) has been shown to induce the formation of inducible nitric oxide synthase (iNOS) and elevate nitric oxide (NO) production by alveolar macrophages (AM). DEP are known to induce pulmonary inflammation and increase pulmonary susceptibility to infection through altered metabolic function and secretion of pro- and anti-inflammatory cytokines. The present study examines the role of NO in DEP-altered P450 activities which mediate genotoxic and mutagenic effects in the lung. Male Sprague-Dawley rats were intratracheally (IT) instilled with saline, DEP (35 mg/kg) or the DEP organic extract (DEPE). To illustrate the role of iNOS, another group of rats was treated with an iNOS inhibitor, aminoguanidine (AG, 100 mg/kg), by i.p. injection 30 min prior to and 3, 6 and 9 h after IT exposure. At 1 day post exposure, DEP-induced genotoxicity and lung-S9 dependent mutagenicity were monitored via comet assay and the Ames test with *S. typhimurium* YG1024, respectively. The results show that AG treatment markedly inhibited DEP-induced NO production by AM without affecting iNOS levels. DEP-exposed AM exhibited significant DNA damage compared to controls. Both DEP and DEPE induced CYP1A1 activity, which was significantly reduced by AG. However, DEP and DEPE attenuated CYP2B1 activity that was not altered by AG. CYP1A1 and CYP2B1 supersomes activated 2-aminoanthracene (2-AA) mutagenicity in the Ames test, suggesting that both CYP isoforms were involved in 2-AA activation. DEP- and DEPE-S9, although containing less CYP2B1, activated 2-AA to a similar extent as the control. AG treatment significantly lowered DEP- and DEPE-S9, but not control-S9, dependent activa-

tion of 2-AA mutagenicity. These results demonstrate that DEP exposure induces genotoxicity and mutagenicity in the lung. The organic component of the particle is responsible for DEP-induced CYP1A1, while NO plays a major role in DEP-induced mutagenic effects in the lung by regulating CYP1A1 activity.

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#### CONTRIBUTION OF REACTIVE OXYGEN SPECIES TO PARA-AMINOPHENOL-INDUCED CYTOTOXICITY.

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Para-aminophenol (PAP), a metabolite of N-acetyl-para-aminophenol (APAP), causes toxicity in the renal proximal tubule. LLC-PK1 cells show a concentration-dependent loss of viability following a 4-hour PAP treatment with an EC50 of 0.1mM. Oxidation of PAP is a key step in its cytotoxicity, however the mechanism is yet to be determined. Previously we have shown that pre-incubation and/or co-incubation with ascorbic acid or glutathione (GSH) protects cells from PAP toxicity. This suggests that PAP may be causing the formation of reactive oxygen species (ROS). The present studies were designed to determine the presence of ROS and to identify the species that may be produced. LLC-PK1 cells were pre-treated with varying concentrations of catalase (0-100 units/ml), hydroxyl radical scavengers [DMTU (0-50mM) and mannitol (0-75mM)], or metal chelators [1, 10-phenanthroline (0-0.25mM), and bathocuproine (0-10 mM)] for 30 minutes. We then added 0, 0.1mM PAP or 0.15mM PAP, incubated for an additional 4 hours and measured cell viability 20 hours later. Menadione was used as a positive control as it undergoes redox cycling leading to formation of superoxide anions. Toxicity due to PAP was attenuated by all pre-treatments. These data suggest that PAP cytotoxicity is at least partially due to generation of reactive oxygen intermediates. Supported by NIH R15GM065196.

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#### ENVIRONMENTAL BASIS OF NEURODEGENERATION (ND) AND AGING: DIBROMOACETONITRILE (DBAN) INDUCES PROTEIN OXIDATION, INHIBITS PROTEASOMAL ACTIVITY AND ALTERS CYTOSOLIC PROTEOME IN NEUROGLIAL CELLS.

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Our goal is to investigate the role of environment in fetal basis of ND/aging. Epidemiological studies indicated that *in utero* exposure to drinking water disinfectant byproducts (DBP) such as DBAN causes susceptibility to adverse pregnancy outcomes including CNS anomalies. Our previous studies indicated that DBP cross the placenta, retain in fetal brain cortex and hippocampus, where it induces oxidative stress (OS) and apoptosis. OS-mediated oxidation of amino acid side chains (protein carbonyls) are increased in ND/aging. Inefficient degradation of carbonylated proteins by proteasomes result in the accumulation of proteolysis resistant protein aggregates in ND/aging. We hypothesize that DBP-induced OS causes protein carbonylation, accumulation of oxidized proteins and proteasomal inhibition leading to neuronal loss. Neuroglial cells (Rat C-6 glioma) were exposed to DBAN (0-400 ppb) for 24 or 48 h. The results indicated that DBAN induced a concentration and time-dependent increase (128-136% of control, p < 0.05) in carbonylated proteins and a decrease (65-58% of control) in proteasomal activities. DBAN-induced changes in cytosolic proteome was evaluated by 2D gel electrophoresis (2DGE) at variable pH ranges. Replicate 2DGE from control and treated cells were scanned and analyzed using Progenesis software. Several of the expressed proteins with a  $\pm$  2 fold variation were identified using peptide mass finger printing- MALDI-TOF MS. Notable among the up-regulated proteins at 50-400 ppb DBAN was the proteasomal C-5 component [AA56702]. Identification of this protein was further validated by LC/MS/MS. Up-regulation of proteasomal C-5 protein indicates cellular stress to overcome carbonylated protein accumulation. In conclusion, increased accumulation of carbonylated protein following DBAN treatment may have a role in ND/aging. Ongoing studies using mice at various age levels will further clarify the role of DBP in fetal basis of ND/aging.

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#### AGE-RELATED ALTERATIONS IN KAINIC ACID-INDUCED NEURONAL OXIDATIVE DAMAGE.

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Aging is associated with processes that lead over time to an increase in vulnerability to cellular and tissue damage and the probability of death. Recent research findings in brain have highlighted increased excitatory stimulation as contributors to aging

as well as neurodegeneration that accompanies Alzheimer's disease, HIV-associated dementia, ischemic stroke, and some forms of epilepsy and cerebral palsy. Associative data from patients' tissue and animal models have widely supported the hypothesis that neuronal oxidative damage is a major effector contributing to neurodegeneration. Mice exposed to kainic acid (KA, 1 nmol/5  $\mu$ l, icv) showed increase in cerebral F<sub>2</sub>-isoprostanes (F<sub>2</sub>-IsoPs, 158%), F<sub>4</sub>-neuroprostanes (F<sub>4</sub>-NeuroPs, 237%) and citrulline (249%) formation 30 min after the exposure. At the same time, pyramidal neurons in the hippocampus of young and old mice had significant reduction in dendritic length (60%) and spine density (40%) compared to control (100%). Pretreatment with ibuprofen (14  $\mu$ g/ml of drinking water for 2 weeks) or  $\alpha$ -tocopherol (100 mg/kg, i.p. for 3 days) completely blocked the icv KA-induced increase in cerebral F<sub>2</sub>-IsoPs, F<sub>4</sub>-NeuroPs, citrulline and decrease in spine density of hippocampal pyramidal neurons in young mice. Importantly, the neuro-protectant effects of these agents were lost in old mice following excitotoxicity. These data strongly suggest that different mechanisms are involved in cerebral neuroprotection of aged mice compared to young mice. The existence and nature of changes with aging have important clinical implications for therapeutic strategies for neuroprophylaxis in both normal aging and neurodegenerative disease. (Supported by ADRC, P50 AG05136 to D.M.).

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#### PROTECTIVE EFFECTS OF TAURINE AGAINST REACTIVE OXYGEN SPECIES IN ARPE-19 CELLS.

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Reactive oxygen species (ROS) are generated in the retina as a result of its constant exposure to light radiation. Damaged retinal cells may result in cell death without replacement. Loss of retinal cells is a factor in many ocular diseases. Retinal pigmented epithelial cells (RPE) are responsible for supporting the rods and cones during oxidative stress and the loss of RPE cell functions have been shown to be an early indicator of retinal disease such as age-related macular degeneration. Human RPE cells contain high concentrations of a number of antioxidants including taurine (B-amino-ethane-sulfonic acid), a non-essential amino acid. Deficiency in taurine can lead to loss of RPE cells and blindness. A human RPE cell line (ARPE-19) is used in this present study to model the effects of taurine on oxidative damage. The oxidative stress was provided by ROS such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and menadione. ARPE-19 cells were routinely grown in DMEM/F12 medium plus 10% FBS and gentamicin prior to being switched to a serum free medium since FBS naturally contains taurine. Cells were seeded in 24 well plates and incubated with or without 2mM taurine for 72 hours. The cell cultures were then exposed to various concentrations of H<sub>2</sub>O<sub>2</sub> for 30 minutes. After rinsing, 10% alamarBlue in culture medium was added to each well and the fluorescence developed in 4 hours was measured in a fluorescent plate reader. The results showed that at high cell densities, the LD50 of H<sub>2</sub>O<sub>2</sub> cytotoxicity in ARPE-19 cells was raised from 7.8mM to 27.9mM in taurine treated cells. At low cell density, the LD50 was raised from 7.3mM to 65.8mM. Similar studies are being conducted with menadione cytotoxicity. We demonstrated that taurine, *in-vitro*, exhibits a partial protective effect against oxidative damage in ARPE-19 cells.

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#### INDUCTION OF INFLAMMATORY MEDIATORS AND ANTIOXIDANTS FOLLOWING EXPOSURE OF MACROPHAGES TO PARAQUAT.

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Paraquat (1, 1'-dimethyl-4, 4'-bipyridinium), a commercially important herbicide, is known to be a potent inducer of lung fibrosis in rodents and humans. It is also cytotoxic for nigral dopaminergic neurons and may be an environmental risk factor for Parkinson's disease. Macrophages are phagocytic cells known to release a variety of inflammatory mediators and are thought to participate in the cytotoxicity of paraquat. In many cell types including macrophages, paraquat undergoes redox cycling which can generate active radical species which cause modifications in cellular macromolecules and promotes lipid peroxidation. In the present studies we determined if paraquat activated macrophages to produce inflammatory mediators. Using RAW264.7 murine macrophages, we found that paraquat readily undergoes redox cycling as determined by the formation of hydroxyl radicals as measured by the hydroxylation of terephthalate. This was associated with a time- and concentration-dependent induction of heme oxygenase-1 (HO-1), a potent antioxidant. Following paraquat treatment macrophages were found to express cyclooxygenase-2 (COX-2), inducible nitric oxide synthase and tumor necrosis factor- $\alpha$  mRNA as determined by real time PCR. Maximal induction of HO-1 as well as production of inflammatory mediator gene products was observed after 24 hr and 300  $\mu$ M paraquat suggesting that they are coordinately regulated. Our data demonstrate

that paraquat is an effective inducer of a variety of pro-inflammatory and cytotoxic mediators in macrophages. Moreover, prostaglandins and nitric oxide, as well as TNF- $\alpha$  may mediate cytotoxicity in target cells. Paraquat is also an effective inducer of an antioxidant known to be important in promoting macrophage survival. Taken together, these data suggest a potential mechanism mediating the actions of paraquat. Supported by NIH grants ES05022, ES10791, CA093798 and CA100994.

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ASCORBATE INHIBITS UROPORPHYRIA IN ASCORBATE-REQUIRING MICE.

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Hepatic accumulation of uroporphyrin (URO) (uroporphyrin) is modulated by ascorbic acid (AA) in AA-requiring ODS rats. Microsomal CYP1A2-catalyzed oxidation of uroporphyrinogen is inhibited by AA. In patients with the human uroporphyrin disease, porphyria cutanea tarda (PCT), plasma AA is in the deficiency range. High hepatic iron levels enhance development of experimental uroporphyrin. Phlebotomy to remove iron is the important therapy in PCT. In this study, AA-requiring mice (*Gulo(-/-)*) in a C57BL/6 background) were used to examine the effect of AA in uroporphyrin induced by treatment with PCB126, and 5-aminolevulinate (ALA), with and without iron dextran treatment. Mice were continuously fed either low (75 ppm) or high (1000 ppm) AA in the drinking water. At low AA consumption, yielding hepatic AA in the range of 0.15-0.25 mM, hepatic URO levels after 2 weeks of ALA feeding were 4-6 fold higher than when animals were given AA sufficient to raise hepatic AA to 0.7-0.9 mM, similar to wild-type levels. When animals were administered Fe dextran (500mg Fe/kg), as well as PCB and ALA, high levels of hepatic URO accumulated, regardless of the amount of AA consumed. Hepatic AA levels were not significantly affected by the iron dextran. These data indicate that in the presence of excess hepatic iron, the protective effect of AA is greatly diminished. This may be due to a higher level of oxidative stress produced by the Fe loading. In conclusion, the results of this study further support the important role of AA in regulating development of uroporphyrin, but indicate that this effect can be overcome by excess iron in the liver. This work was supported by the Department of Veterans Affairs and NIH ES06263.

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DETERMINATION OF PARAMETERS INDICATIVE FOR OXIDATIVE STRESS IN CARBON TETRACHLORIDE TREATED RATS.

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A role of oxidative stress has been proposed in the toxicity of numerous chemicals, in the process of ageing as well as in the pathogenesis of many diseases and carcinogenesis. Oxidative stress is known to be followed by cellular and tissue damage whereby lipids and proteins are the major targets. The aim of this study was to compare the sensitivity of different markers for oxidative stress in male Wistar rats treated with carbon tetrachloride (1 ml/kg body weight). As a measure for lipid peroxidation hepatic isoprostane F2 $\alpha$  was determined by ELISA and LC-MS/MS following solid phase extraction. Hepatic malonedialdehyde was analysed following derivatization with thiobarbituric acid by HPLC with fluorescence detection. As a measure for cellular antioxidants in the liver  $\alpha$ -tocopherol and ascorbic acid were determined by HPLC, and reduced as well as oxidized glutathione by spectrophotometry following derivatization dithio-bis-nitrobenzoic acid. Two and 6 hours after treatment, hepatic 8-isoprostane F2 $\alpha$  concentrations were maximally increased by a factor of about 40 and 13, respectively. Malonedialdehyde was increased only by a factor of 1.5.  $\alpha$ -Tocopherol and ascorbic acid concentrations in the liver were increased to 160% and decreased to 40%. The glutathione status in the liver was not influenced following treatment. In conclusion, the present study demonstrates the sensitivity of hepatic 8-isoprostane F2 $\alpha$  concentration as marker for oxidative stress induced by carbon tetrachloride.

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6-HYDROXYDOPAMINE ACTIVATES THE ANTIOXIDANT RESPONSE ELEMENT THROUGH A COMBINATION OF OXIDATIVE, EXCITOTOXIC, AND STRUCTURAL FACTORS.

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Parkinson's disease (PD), a progressive neurodegenerative disorder, is characterized by loss of midbrain dopaminergic neurons. The etiology of sporadic PD is unknown; however, oxidative stress is thought to play a major role in disease patho-

genesis. The antioxidant response element (ARE) is a cis-acting enhancer element that is upstream of many phase II detoxification and antioxidant genes. Transgenic reporter mice engineered to express ARE-driven human placental alkaline phosphatase (hPAP) have been shown to be *in vivo* and *in vitro* sensors of oxidative stress. 6-hydroxydopamine (6OHDA), a mitochondrial complex I inhibitor and oxidative stressor, is a toxin used to model sporadic PD. 6OHDA-induced toxicity can be attenuated by exogenous antioxidants. Additionally, 6OHDA shares some structural similarities with hydroquinone and tert-butyl hydroquinone, two potent activators of the ARE. We hypothesized that 6OHDA would activate the ARE due to generation of ROS as well via structural activation. Primary cortical cultures prepared from ARE-hPAP mice exposed to 6OHDA demonstrate ARE activation in a dose-dependent fashion. Pretreatment with high-dose antioxidants reduced, but did not abolish ARE activation, indicating a component of ARE activation that is oxidative stress independent. Pretreatment with MK-801, an NMDA receptor antagonist, also reduced ARE-activation at low concentrations of 6OHDA suggesting that the oxidative stress caused by 6OHDA is caused, in part via an excitotoxic mechanism. When administered stereotactically to ARE-hPAP mice, 6OHDA induces ARE activity *in vivo*. The data suggest that ARE-mediated gene activity may be a mechanism to cope with oxidative stress due to 6OHDA. This work is supported by ES08089 and ES10042 from NIEHS. The authors disclose no conflicts of interest.

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ACTIVATION OF TRANSCRIPTION FACTOR AP-1 IN ANILINE-INDUCED SPELENIC TOXICITY.

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Exposure to aniline results in selective toxicity to the spleen, leading to a variety of sarcomas on chronic exposure in rats. However, the molecular mechanism(s) by which aniline leads to tumorigenic response in spleen is not known. Previous studies have shown that aniline exposure leads to iron accumulation and oxidative stress in the spleen. We hypothesize that aniline-induced oxidative stress in the spleen causes activation of redox-sensitive transcription factor AP-1, which could regulate the transcription of genes involved in fibrosis and cell growth regulatory pathways. To test this hypothesis, male SD rats were treated with 0.5 mmol/kg/day aniline via drinking water for 30 days, and AP-1 activation was determined in the nuclear extracts of splenocytes. The binding activity of AP-1, as determined by electrophoretic mobility shift assay (EMSA) using AP-1 consensus motif, showed a significant increase in AP-1 binding in the nuclear extracts of the freshly isolated splenocytes from aniline-treated rats in comparison to the controls. AP-1 binding was also determined in the splenocytes cultured for 2 h, which showed a marked increase in binding activity in the nuclear extracts of splenocytes from aniline-treated rats as compared to the controls. The specificity of AP-1 binding for relevant DNA motifs was verified by competition EMSA assays using an excess of unlabelled consensus and mutant oligonucleotides. The wild type AP-1 motif completely inhibited the AP-1 binding, whereas the mutant AP-1 consensus motif failed to block the AP-1 binding in both control and aniline-treated splenocytes. Supershift EMSA using antibodies specific to c-Jun further confirmed the specificity of AP-1 binding activity. These observations suggest that aniline-induced oxidative stress results in the activation of AP-1, which could regulate genes that lead to fibrogenic and/or tumorigenic response in spleen.

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DOW-NREGULATION OF NITRIC OXIDE AND ANTIOXIDANT SYSTEMS IN ALCOHOL-INDUCED HYPERTENSION.

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Epidemiological studies show that there is link between chronic alcohol consumption and the prevalence of hypertension in humans. We have recently reported that both dose and time of alcohol ingestion is important in causing hypertension in experimental rat model. However, the cellular and molecular mechanisms implicated with ethanol-induced hypertension are not completely understood. The aim of this study was to investigate the alterations in blood pressure and cardiac nitric oxide and antioxidant systems in chronic ethanol treated rats. Male Fisher rats were given 20% ethanol (4 g/kg, p. o.) through orogastric tube daily for 12 weeks and controls received 5% sucrose through orogastric tube daily for 12 weeks. The systolic, diastolic and mean blood pressure (BP) was recorded weekly. After 12 weeks, rats were sacrificed and heart dissected and left ventricle isolated and analyzed. Results show that ethanol ingestion caused a significant increase in systolic, diastolic and mean BP ( $p < 0.001$ ) compared to control after 12 weeks. The endothelial nitric oxide synthase and vascular endothelial growth factor (VEGF-A) expressions were down-regulated leading to depletion of cardiac NO levels in ethanol treated rats compared to control. The antioxidant enzymes: CuZn-SOD, Mn-SOD, catalase and GSH-Px activities as well as protein expressions were significantly depleted in the cardiac tissue of ethanol-treated rats compared to control. Endogenous antioxidant glutathione levels in the heart tissue significantly decreased in rats treated with ethanol

compared to control. It is concluded that chronic alcohol ingestion caused hypertension which was related to the down regulation of endothelial nitric oxide generating system and antioxidants in the heart of rats(supported by NIH RCMI grant #2 G12 RR03050-19).

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#### HO-1 INDUCTION CONTRIBUTES TO PCB-INDUCED OXIDATIVE STRESS IN DOPAMINERGIC CELLS.

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Polychlorinated biphenyls (PCBs) are persistent and widespread pollutants that have been associated with disruption of dopaminergic (DAergic) pathways, leading to neurological and cognitive impairments. Previous studies in our laboratory have shown that low-level exposure to Aroclor 1254 (A1254) elicited an oxidative stress (OS) response in MN9D DAergic cells, including sustained reactive oxygen species (ROS) production, depletion of mitochondrial MnSOD, and a robust induction of heme oxygenase-1 (HO-1). HO-1 metabolizes liberated heme groups to give rise to CO, the antioxidant biliverdin, and iron (Fe), a pro-oxidant. This study tests the hypothesis that the PCB-induced cellular stress response generates a sustained induction of HO-1, thereby releasing labile iron that can participate in the Fenton reaction to generate ROS. A1254 exposure stimulated HO-1 expression and led to an accumulation of total intracellular iron after a 24h period. Pretreatment of MN9D cells with the HO-1 inhibitors, tin and zinc protoporphyrins, or desferoxamine, an iron chelator, decreased PCB-induced ROS production and protected against cell death. Cells that were infected with an HO-1 adenoviral construct had increased ROS production and were more vulnerable to cell death than those infected with anti-sense HO-1 or GFP constructs. Additionally, HO-1 protein levels were elevated to a greater extent in HO-1 over-expressing cells compared to controls following treatment with A1254. Alterations in HO-1 protein were accompanied by increased ROS production and subsequent cell death in MN9D cells infected with the HO-1 construct, while infection with anti-sense HO-1 was able to protect against PCB-induced oxidative stress and cell death. Together, these data suggest that induction of HO-1 past a critical cellular threshold can increase neuronal susceptibility to oxidative stress and lead to further DAergic cell injury and death. Supported by NIH ES00375, P30ES01247, and T32 ES07026.

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#### REACTIVE OXYGEN/NITROGEN SPECIES IN ACETAMINOPHEN (APAP) TOXICITY IN FRESHLY ISOLATED MOUSE HEPATOCYTES.

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APAP toxicity in freshly isolated mouse hepatocytes occurs in two phases. Despite the fact that the initial phase (0 - 2 hr) involves GSH depletion and covalent binding, it occurs with little toxicity. Toxicity occurs primarily during the second phase (2 - 5 hr). We have previously shown that the latter phase occurs with mitochondrial permeability transition (MPT), a lethal event for the cell. Since MPT may occur as a result of oxidative stress and leads to oxidative stress, we evaluated the role of oxygen/nitrogen stress in APAP toxicity. Hepatocytes were incubated with APAP (1mM) for 2 hr and subsequently washed (2x) to remove APAP and reincubated with media (2 - 5 hr). This allowed for separation of metabolic events in phase 1 from toxic events in phase 2. Addition of dichlorofluorescein (DCF), a dye converted to a fluorescent product by reactive oxygen/nitrogen species, at 2 hr resulted in a large increase in fluorescence compared to control. Inclusion of the MPT inhibitor cyclosporine A and the antioxidant N-acetylcysteine eliminated both toxicity and the increase in DCF fluorescence. To determine the role of peroxynitrite, a reactive nitrating and oxidizing species formed from interaction of superoxide with nitric oxide, proteins were analyzed by Western blot for nitrated tyrosine. Nitrotyrosine residues linearly increased in the reincubation. Inclusion of the nitric oxide inhibitor, L-NMMA (1mM), at 2 hr decreased toxicity 57% at 5 hr. Since APAP is a phenol that has been reported to scavenge peroxynitrite, the effect of APAP was determined when added back to the hepatocytes at 2 hr. APAP (1, 10, and 50 mM) decreased toxicity by 11%, 24%, and 48%, respectively at 5 hr. To determine the role of iron and oxidative stress in toxicity, the iron chelators deferoxamine (1mM) and phenanthroline (0.05 mM) were added at 2 hr. Both chelators completely eliminated toxicity. Overall, the data reveal a role for both reactive oxygen and reactive nitrogen species in APAP toxicity.

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#### ACETAMINOPHEN INDUCED HEPATOXICITY IN A GCLM- NULL MOUSE MODEL.

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Recent reports suggest that a substantial fraction of acute liver failure incidence in the United States is related to acetaminophen (APAP) overdose. APAP is bioactivated to a reactive intermediate, NAPQI, which is scavenged by glutathione

(GSH), an important cellular antioxidant. The rate-limiting step in GSH biosynthesis is catalyzed by glutamate cysteine ligase (GCL). This enzyme is a heterodimer composed of catalytic (GCLC) and modifying (GCLM) subunits. GCLM functions to modify the activity of GCLC by increasing its affinity for glutamate and attenuating the GSH feedback inhibition. To more thoroughly characterize the role of GCLM in GSH biosynthesis and APAP-induced liver damage, we have utilized our GCLM-null mouse model. This mouse was generated by replacing exon 1 of the GCLM gene with a b-galactosidase/neomycin phosphotransferase (b-Geo) fusion gene. Following a 12 hour fast, mice were dosed with 300 mg/kg APAP via i.p. injection and sacrificed after six hours. GSH depletion associated with APAP exposure was similar in all three genotypes, relative to vehicle treatment. Both serum ALT activity and histologic evaluation indicated hepatic damage for all three genotypes (GCLM wild-type, heterozygous and null). However, the null mice exhibited severe damage indicated by both high histologic scores and ALT activities relative to the heterozygous and wild-type mice. GCLM promoter activity in the null mice, as measured by b-galactosidase activity, was diminished, perhaps due to the high degree of hepatic damage. Collectively, these data reassert the importance of GSH in APAP-mediated hepatotoxicity. Importantly, however, these data indicate a critical role of GCLM in preventing oxidative damage. This may be clinically relevant, as the GCLM gene is polymorphic in human populations. (Supported by NIH Grants 1P42ES04696, 1R01ES10849, 1T32ES07032, and 1P30ES07033).

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#### INDUCTION OF METALLOTHIONEIN SYNTHESIS MEDIATED THROUGH OXIDATIVE STRESS IN MITOCHONDRIA AND ITS BIOLOGICAL ROLE.

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Enhanced production of reactive oxygen species (ROS) in mitochondria may contribute a variety of chronic disease and ageing. The metallothionein (MT), a cysteine rich and metal binding protein, is induced by various stress conditions. Recent study shows that MT is present in mitochondria. We previously reported that mitochondrial inhibitors such as antimycin A or 2, 4-dinitrophenol (DNP) induced MT. However, the involvement of oxidative stress in mitochondria in the induction of MT by mitochondrial inhibitors remains obscure. Administration of DNP into mice induced expression of MT mRNA in the liver and increased the MT concentration. Another mitochondrial inhibitors, sodium malonate also induced MT synthesis in mice. Addition of DNP into culture medium resulted in expression of MT mRNA in fibroblast cells, indicating that incorporated DNP in the cells induce MT synthesis. Malonate is a competitive inhibitor on succinate dehydrogenase. Pre-injection of succinate caused the reduction of malonate-induced MT synthesis, suggesting that induction of MT synthesis is mediated through inhibition of respiratory reaction and ROS production in mitochondria. Intracellular accumulation of hydrogen peroxide caused by the actions of mitochondrial inhibitors was greater in MT-null than in wild-type fibroblast cells, suggesting that MT plays a role as a radical scavenger against intracellular ROS caused by mitochondrial stress. Furthermore, ROS production by DNP was greater in wild cells than in phospholipid hydroperoxide glutathione peroxidase-overexpressed cells, which is localized mitochondrial membrane. The data suggest that mitochondrial oxidative stress induces synthesis of MT, which may contribute to regulation of mitochondrial ROS production.

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#### HEPATOCYTE-SPECIFIC DELETION OF GCLC RESULTS IN PROGRESSIVE LOSS OF MITOCHONDRIAL FUNCTION.

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Hepatocyte-specific deletion of glutamate-cysteine ligase catalytic subunit (*Gclc*), encoding rate-limiting enzyme in glutathione (GSH) biosynthesis, results in growth retardation and early death in mice at approximately post-natal day (PND) 30. In these mice, the glutathione level in liver was 4% of the control. Ultrastructural examination of hepatocytes demonstrated the progressive transformation of mitochondria to become swollen with tubular cristae. This morphological transformation was prevented by the administration of N-acetylcysteine (NAC) which also prevented hepatic failure and death. GSH levels in liver mitochondria from knockout mice at PND 14, 21, 25, and 28 dropped to 42%, 19%, 16%, and 16% of age matched controls. In parallel with mitochondrial GSH loss, was a decline in cellular ATP and mitochondrial function, as measured by respiratory control ratio and membrane potential. Hydrogen peroxide liberated by respiring mitochondria increased with GSH loss. In NAC rescued mice, liver GSH levels increase to 9-14% of the control and mitochondrial levels normalize. Nevertheless, mitochondrial respiratory function did not fully recover until PND 120. We conclude that liver failure in hepatocyte *Gclc* knockout mice parallels the loss of mitochondrial function. NAC administration rescues mitochondrial function albeit with some delay. Supported in part by NIH grant R01ES012463.

A NOVEL HALOENOL LACTONE DERIVATIVE INDUCES OXIDATIVE STRESS IN HUMAN TUMOR CELLS THROUGH A GLUTATHIONE S-TRANSFERASE MEDIATED MECHANISM.

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Reduced glutathione (GSH) is the most prevalent non-protein thiol in animal cells. It maintains a reduced cellular environment and also acts as a nucleophile in reactions with both exogenous and endogenous electrophilic species. As a consequence, reactive oxygen species (ROS) are frequently targeted by GSH in both spontaneous and catalytic reactions. Since ROS have defined roles in cell signaling events as well as in human disease pathologies, an imbalance in expression of GSH and associated enzymes has been implicated in a variety of circumstances. A novel haloenol lactone (HEL), which we synthesized as an isozyme-selective GST-Pi inactivator, was found to deplete intracellular GSH to potentiate cytotoxicity induced by cisplatin *in vitro*. We also found HEL causes dose-dependent, time-dependent increase of intracellular ROS level at low concentrations *in vitro*. This increase of ROS was correlated to the cell viability in MCF-7 cell line. GST-Pi over-expressed transgenic MCF-7 cells showed more resistance to H2O2-induced injury than its wild type. HEL compound reverses this resistance through selective GST-Pi inhibition. GST-Pi is known as a direct inhibitor of c-Jun N-terminal kinase (JNK) *in vivo*. These results reveal that HEL may not only induce oxidative stress by depletion of GSH but also induce ROS-related apoptosis through a GST-mediated mechanism.

IDENTIFICATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM TOBACCO AS INHIBITORS OF NEURONAL NITRIC OXIDE SYNTHASE.

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Neuronal nitric oxide synthase (nNOS) plays a major role in cell damage and death during stroke. Specific inhibitors of nNOS would help to decrease free radical damage. Tobacco contains thousands of bioactive compounds, many of which may selectively inhibit nNOS. Our aim is to identify and isolate selective nNOS inhibitors from tobacco which do not inhibit endothelial NOS as it plays a vital role in maintaining tissue perfusion and decreasing ischemic/hypoxic damage. Several varieties of tobacco (T1565, TW109, TI464, TW88, TW152, TW87) were differentially extracted and lyophilized, ensuring for both stable storage as well as for ensuring ample supply of compound for further characterization with NOS isoforms. Increasing amounts of dried extracts from the different tobaccos (hexane, 125-755?g; methanol, 125-625?g; aqueous, 100-500?g) were included in our NOS activity assays to establish dose-response relationships for potency as nNOS inhibitors. Hexane extracts were found to be the most potent inhibitors of nNOS (65% inhibition) followed by the methanol extracts (active only at higher amounts) and lastly by the aqueous extracts. Further analysis, by analytical high performance liquid chromatography, of the hexane extracts displaying either high (TW1565), moderate (TI465), or low (TW109) nNOS inhibitory activity have revealed unique and distinct chromatographic profiles indicating differences in amounts of the chemical constituents present in different extracts and it is postulated that these differences may account for the observed differential inhibition of nNOS activity. Presently, subfractions of hexane extracts from the more potent nNOS inhibitory tobaccos are being collected to conduct both more in-depth nNOS inhibition studies, and to continue chemical characterization and identification of the active constituents by both mass-, and nuclear magnetic resonance-spectroscopy (Supported by KSEF-475-RDE-005)

DEVELOPMENT AND VALIDATION OF AN ASSAY FOR IODIDE IN SERUM USING ION CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION.

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An ion chromatography method was developed and validated to assay iodide in serum sampled from rats and rabbits that had been exposed to iodomethane. Serum was prepared from whole blood collected at the time of sacrifice on wet ice and kept frozen at < -65° C. For analysis, serum samples were thawed unassisted at ambient temperature. Proteins were separated from the serum samples by ultrafiltration. A 0.100 mL filtered serum sample was then injected into the ion chromatograph without additional sample preparation. Iodide was separated in < 20 min by anion-exchange chromatography using a 25 mM nitric acid eluent. The analyte of interest was detected by pulsed amperometry using a silver working electrode. The method showed linear response over the concentration range of 100 to 5,000 ng/mL iodide

(r2 > 0.9950) with a lower limit of quantitation of 100 ng/mL iodide. The accuracy of the procedure, determined by spiked recovery measurements at 100 ng/mL iodide, was within 90-110%. The analytical procedure was applied to detect trace iodide down to 20 ng/mL in serum samples using the method of standard additions.

METHYLATION OF HEMOGLOBIN CYSTEINE AS A BIOMARKER OF EXPOSURE TO IODOMETHANE.

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Iodomethane (MeI) is a water-soluble monohalomethane under review for US registration as a non-ozone depleting alternative to methylbromide in the pre-plant soil fumigation market. As part of the toxicological assessment of MeI, hemoglobin adducts were investigated as a measure of internal dose following MeI exposure. In this study, methylation of hemoglobin cysteine residues to form S-methyl-L-cysteine (SMC) was investigated. SMC formation has been used previously to estimate internal dose of methylating agents, including methylbromide. Male and female rats, and female rabbits were exposed to various concentrations of MeI for 6 hours. Whole blood was obtained 48 hrs post exposure, and globin was isolated from RBCs by acid-acetone precipitation and hydrolyzed in 6N HCl under vacuum. Deuterated SMC was added as an internal standard to account for incomplete recovery of SMC. Both liquid phase and vapor phase acid hydrolysis were examined. Hydrolysates were dried under vacuum, silylated and analyzed by GC/MS with selected ion monitoring. Preliminary data indicated that SMC was present in control rat and rabbit globin at concentrations of approximately 100 and 20 nmol/g, respectively, and that inhalation exposure to MeI produced dose-dependent increases in SMC in both species. Because of the variable recovery of SMC in hydrolysates, an alternative procedure was developed in which globin was oxidized with performic acid prior to acid hydrolysis. This procedure resulted in quantitative conversion of SMC to SMC sulfone (SMCO2), which is stable to the hydrolytic conditions used. Our preliminary results indicate that SMC formation in Hb is a useful biomarker for exposure to MeI. Data from these studies will facilitate understanding of the relationships between exposure concentration, exposure duration and internal dose for MeI.

UPTAKE OF METHYL IODIDE IN THE NASAL CAVITY OF RATS AND RABBITS.

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Methyl iodide (MeI) is a water soluble monohalomethane under review for US registration as a non-ozone depleting alternative to methyl bromide for the pre-plant soil fumigation market. Both acute and subchronic inhalation exposures to MeI in rats have resulted in lesions of the nasal olfactory epithelium. Efforts are underway to develop a hybrid computational fluid dynamics-physiologically based pharmacokinetic model for MeI to better describe interspecies nasal dosimetry differences, and to potentially reduce RfC uncertainty factors. Validation of this model, however, will require unique experimental data to describe the nasal absorption of MeI. To this end, a method for evaluating the uptake of MeI in the nasal cavity of the intact animal was developed. The procedure involved insertion of a small-diameter air-sampling probe in the nasal cavity to the nasopharynx region of anesthetized animals. The exterior portion of the probe was connected directly to a mass spectrometer to provide a continual real-time analysis of concentrations of MeI in the nasal cavity. A plethysmography system was used to individually monitor breathing parameters, including frequency and tidal volume for each animal. Animals were placed in a sealed glass chamber and exposed to MeI at initial chamber concentrations ranging from 1 to 50 ppm. Studies were conducted on n=3 rabbits per exposure concentration for a total of 9 animals. For rats, studies were conducted on n=6 animals at a single exposure concentration. In the rabbit, the percent of MeI absorbed in the nasal cavity ranged from 53 to 89% (average 68 ± 11) regardless of exposure concentration. Similarly, the percent of MeI absorbed in the nasal cavity of the rat ranged from 52 to 75% (average 66 ± 9). Furthermore, plethysmography results indicate that breathing parameters were unaffected by initiation of the MeI exposure for these anesthetized animals. (Supported by Arvesta Corp.).

SPECIES AND TISSUE COMPARISON OF THE GLUTATHIONE CONJUGATION OF METHYL IODIDE IN RAT, RABBIT, AND HUMAN CYTOSOL.

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Methyl iodide (MeI) is an intermediate in the manufacture of some pesticides and pharmaceuticals and it is under review for US registration as a non-ozone depleting alternative for methyl bromide in the pre-plant soil fumigation market. MeI is me-

abolized via conjugation with glutathione (GSH) and further metabolism to S-methyl cysteine and methanethiol, or via a minor CYP450 oxidation to formaldehyde. A potential mode of action for any adverse effects due to MeI exposures may involve iodide release or GSH depletion. The GSH-dependent loss of MeI from the headspace of sealed vials containing cytosol prepared from the liver and kidney of rats, human donors, female rabbits or rabbit fetuses and from rabbit olfactory and respiratory epithelium was compared. A mathematical model was developed and metabolic rate constants were determined using Simusolv (Dow Chemical Co., Midland, MI). MeI was well metabolized in most of the tissue cytosol samples, but not in blood or fetal rabbit kidney. In rabbit tissues, the relative order of the pseudo-first order rate constant (Vmax/Km) was olfactory epithelia > liver > respiratory epithelia > kidney. The metabolism in olfactory and respiratory epithelial cytosol had a Km values that were several times higher than for any other tissue (on the order of 25 mM for olfactory and 2.5 mM for respiratory), suggesting an essentially first order rate of metabolism in the nasal area. Vmax rates were similar in liver and kidney cytosol from rats and human donors (40 and 48 in liver and 68 and 55 nmol/min/mg in kidney for rat and human tissue, respectively), but were lower in rabbit tissues (10 and 4.4 in liver and kidney, respectively). The metabolism of MeI in human liver cytosol prepared from 5 individual human donors indicated a single outlier, with much less metabolism than the other human liver samples. Typically, hepatic metabolism was greater than renal and adult rabbit metabolism was greater than fetal. (Sponsored by Arvesta Corp)

## 234 IODOMETHANE: 2-DAY MECHANISTIC INHALATION EXPOSURE STUDY IN THE RAT.

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A previous study showed that iodomethane (MeI) caused hyperplasia of the nasal olfactory epithelium after subchronic inhalation exposure. This study investigated the mechanism of toxicity and measurement of exposure biomarker data in male Sprague-Dawley rats. MeI undergoes rapid metabolism to free iodide from the methyl group; therefore, effects on thyroid hormones, UDP-glucuronyltransferase (UDPGT), clinical pathology, and blood for S-methyl-L-cysteine (SMC) hemoglobin adducts were determined the morning after two days of 6 h/day whole body exposures to 0, 25 or 100 ppm MeI (n=10 rats/group). Additional exposure groups (n=3 rats per exposure concentration) were sampled at 0, 1, 3, 6, 9, 24, 25, 27, 30, 33, and 48 hours for quantification of inorganic serum iodide and reduced glutathione [GSH, mM] in various tissues. Results for SMC will be reported separately. GSH was analyzed in nasal respiratory and olfactory tissues, blood, liver, and kidney. Time course and exposure dependent reductions in GSH were observed in all tissues. For example, at peak exposures GSH was reduced to 17 to 40% of control concentration in nasal respiratory and olfactory tissues, and 60 to 80% of control in blood, liver and kidney. Serum iodide concentrations increased with duration and magnitude of exposure and ranged from 1, 200 to 87, 000 ng/mL, compared with 20 ng/mL in control rats. At the 48 h collection time, T<sub>3</sub> and T<sub>4</sub> were significantly reduced by 62 to 90% of control with a concomitant increase in TSH of 186-360% (p < 0.05). rT<sub>3</sub> and UDPGT were not affected. Adverse serum chemistry changes included increased serum cholesterol concentrations and decreased serum triglyceride concentrations. Clinical pathology and thyroid hormone changes suggest effects on liver enzyme and thyroid function. The results for serum iodide and GSH will be used for validation of a computational fluid dynamic physiologically based pharmacokinetic model.

## 235 EVALUATION OF RESPIRATORY PARAMETERS IN RATS AND RABBITS EXPOSED TO IODOMETHANE.

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Laboratory animals exposed via inhalation to iodomethane (MeI) exhibited adverse systemic CNS effects and lesions of the nasal olfactory epithelium following acute and subchronic exposures. Interactions of MeI in the nasal passage may alter systemic toxicokinetics. We used unrestrained plethysmographs to determine the MeI effect on the breathing frequency and minute volume (MV) in exposed rats and rabbits. Groups of 4 rats each were exposed to 0, 25 or 100 ppm and groups of 4 rabbits each were exposed to 0 and 20 ppm MeI for 6hrs. Breathing frequency and MV were measured, recorded and averaged on 1 minute periods over the course of the exposure. Both rats and rabbits demonstrated an approximate 35% drop in breathing frequency in the first 45 and 90 minutes of exposure, respectively, at all exposure concentrations followed by a plateau in respiratory rate. The MV measurements over the 6-hour period were summated in order to determine the total

volume of air inhaled by the animals during the course of the exposure. Rats exposed to 0 ppm inhaled 80.5 ±17.2 L (mean ± SD), while the 25 and 100 ppm groups were within 8% of the control value. In contrast, rabbits exposed to 20 ppm MeI demonstrated a 30% increase in total inhaled air volume when compared to 144 ± 54 L for the 0 ppm rabbits. Rats and rabbits exposed to MeI concentrations of up to 100 ppm, did not demonstrate a sensory irritant effect or a decreased inhalation uptake of test atmosphere. The results of this study will be integrated into a computational fluid dynamics-physiologically based pharmacokinetic model for both rats and rabbits.

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### IODOMETHANE PARTITION COEFFICIENTS IN RAT AND RABBIT TISSUES AND HUMAN BLOOD.

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Iodomethane (MeI) is a proposed non-ozone depleting alternative for methyl bromide in the pre-plant soil fumigant market. In support of physiologically based pharmacokinetic (PBPK) modeling, MeI tissue-to-air partition coefficients (PCs) were determined in rat tissues (blood, brain, fat, kidney, liver, muscle, thyroid, nasal tissue), rabbit tissues (fetal blood, maternal blood, brain, fat, kidney, liver, muscle, thyroid, placenta, nasal tissue), human blood (male and female), and saline. Weighed tissue aliquots were placed in septum-sealed vials, MeI (10,000 ppm) was added, and vials were incubated at 37°C or 30°C for nasal tissue and saline. A reference vial was prepared identically, but without tissue. Headspace MeI concentrations were analyzed at 1, 1.5, 2, 2.5 and 3 h by GC/FID. MeI headspace concentrations continuously decrease when in the presence of blood and liver, therefore, PCs were estimated by log-linear regression extrapolation to 0 h [TAP, 128:92-96, 1994]. The respective PCs for brain, fat, kidney, muscle, and nasal tissue were similar across animal species. The rabbit thyroid PC was 3 times higher than the rat thyroid PC (rabbit: 39, rat: 11). The rat liver PC was 2 times higher than the rabbit liver PC (rat: 24, rabbit 13). The rat blood PC was 2.5 times higher than the rabbit blood PC (rat: 39, rabbit: 16). The human blood PC (18) was more similar to the rabbit blood PC than the rat blood PC. There was no significant difference between male and female human blood PCs. The rabbit fetal blood PC (12) was similar to rabbit maternal blood PC (16). The measured PCs were compared with predictions derived from algorithms based on MeI solubility in oil and water. Measured PCs for rat and human blood were generally higher than the predicted PCs. The measured and predicted PCs for rat muscle and fat were approximately equivalent. Measured PCs are favored for incorporation into the PBPK models being developed to aid in MeI risk assessment activities.

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### DERIVATION OF HUMAN TOXICITY REFERENCE VALUES FOR METHYL IODIDE USING PBPK MODELING.

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Methyl iodide (MeI) has been proposed as a replacement for methyl bromide as a pre-plant soil fumigant that does not deplete stratospheric ozone. In toxicity studies performed as part of the registration process, the three effects have been identified in animals that warrant consideration in developing human-equivalent toxicity reference values: nasal lesions (rat), acute neurotoxicity (rat), and late-term fetal resorptions (rabbit). Uncertainties in the risk assessment process can be reduced using an internal measure of target tissue dose that is linked to the likely mode of action (MOA) for the toxicity of methyl iodide when determining appropriate regulatory criteria. Physiologically based pharmacokinetic (PBPK) models have been developed for methyl iodide for this purpose. The major components of the PBPK model are submodels for MeI and iodide for adult/maternal and fetal animals, sub-compartmentalized descriptions of the nasal cavity, stomach, and thyroid anatomy, and synthesis and turnover of the metabolic co-factor glutathione (GSH) in blood and tissues. To the extent possible, species and life-stage specific parameter values (physiological, thermodynamic, and biochemical/metabolic) were identified in the literature or developed via experimentation. When parameter values were unavailable for a given species and lifestage, values from other species and lifestages were used. Model predictions were compared to a variety of experimental data from MeI-exposed rats and rabbits, including nasal uptake of MeI, whole body closed-chamber uptake of MeI, blood and tissue GSH depletion, and plasma iodide concentrations. The model was used to reduce uncertainties in the risk assessment extrapolations involved in developing human toxicity reference values (e.g. interspecies, high to low dose, exposure scenario).

## THE DISPOSITION AND PHARMACOKINETICS OF RADIOIODIDE IN PREGNANT RABBITS AND FETUSES.

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Methyl iodide (MeI) is a water soluble monohalomethane under review for US registration as a non-ozone depleting alternative for methyl bromide in the pre-plant soil fumigation market. Iodide is a metabolic byproduct of MeI metabolism and reacts with blood and tissue macromolecules. A physiologically based pharmacokinetic model exists for iodine in adult rats, pregnant rats and fetuses, and lactating rats and neonates, but not for pregnant rabbits and fetuses which have been a subject of extensive toxicity testing. Thus, a study was conducted to determine the distribution (primarily in blood and tissues containing the sodium iodide symporter) and kinetics of radioiodide in pregnant rabbits and fetuses. Time-pregnant New Zealand White rabbits received a single IV injection of 131-sodium iodide (131NaI) at either a high (10 mg/kg body weight) or low (0.75 mg/kg body weight) dose on gestation day 25. At various intervals ranging from 0.5 to 24 hrs post injection, blood and tissues (thyroid, stomach contents and skin) were collected from each doe, and blood, stomach content, thyroid and amniotic fluid were collected from a random sampling of 3 fetuses per doe per time point. Radioiodide accumulated as expected in the maternal thyroid, where concentrations were the highest tissue measured in both dose groups. Likewise, fetal samples had measurable radioiodide concentrations at all time intervals, although concentrations in amniotic fluid were low at early (0.5 and 1 hr) time points and peaked in concentration at 6 hrs post dosing. In contrast to that observed for the doe, fetal stomach contents showed the highest accumulation of radioiodide for both dose groups, followed by the thyroid. (Supported by Arvesta Corp.).

## PRENATAL DEVELOPMENTAL TOXICITY STUDIES IN RABBITS REVEAL A SUSCEPTIBLE WINDOW OF METHYL IODIDE-INDUCED FETOTOXICITY LATE IN GESTATION.

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Methyl iodide (MeI), an intermediate used in the manufacture of some insecticides and pharmaceuticals, is under review for US registration as a non-ozone depleting alternative to methyl bromide in the pre-plant soil fumigation market. Previous OPPTS 870.3700 guideline developmental toxicity studies conducted at WIL showed dose-dependent increases in the mean litter proportions of late fetal deaths and postimplantation loss following inhalation exposure of pregnant rabbits to MeI concentrations ranging from 10 to 75 ppm during gestation days (GD) 6-28. The objective of the current study was to pinpoint the critical period of exposure during gestation that produced the embryo-fetotoxicity. Artificially inseminated New Zealand White female rabbits were exposed to MeI by whole-body inhalation (6hrs/day) at a concentration of 20 ppm during different windows of exposure varying from 2-23 days in duration throughout gestation (GD 6-28), early gestation (GD 6-14), mid gestation (GD 15-22) or during two-day specific intervals late in gestation (GD 23-24, 25-26 or 27-28). No maternal or developmental toxicity resulted from maternal exposure during GD 6-14, 15-22 or 27-28. However, MeI-related developmental effects, including increased mean litter percentages of late fetal deaths with or without a corresponding decrease in mean numbers of viable fetuses, were observed for females exposed during GD 6-28 ( $p < 0.01$ ), 23-24 and 25-26. Although the incidence of developmental effects observed on GD 23-24 or GD 25-26 was not statistically significant, as noted for GD 6-28, it can be deduced that the gestational window of GD 23-26 was the most susceptible time of exposure for eliciting developmental toxicity.

## METHYL IODIDE-INDUCED HYPOTHYROIDISM IN FETAL RABBITS.

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Methyl iodide (MeI) is a water soluble monohalomethane under review for US registration as a non-ozone depleting alternative to methyl bromide in the pre-plant soil fumigation market. A previous developmental toxicity study identified a window of susceptibility for MeI-induced fetotoxicity late in rabbit gestation, i.e., gestation days (GD) 23-26. To investigate a possible mode of action, the ontogeny of fetal serum chemistry, hematology, serum iodide, glutathione, hemoglobin adducts as well as thyroid structure and function was characterized in unexposed rabbits during GD21-27. Furthermore, 10 time-mated New Zealand White rabbits per group were exposed to MeI at 25 ppm by whole-body inhalation for 6 hrs/day fol-

lowing either a 2-day (GD23-24) or 4-day (GD23-26) exposure regimen. The mean incidence of late fetal deaths was 5-6% across exposure groups, compared to 1-2% in the baseline groups. T4 and T3 levels in fetal serum were 64% and 47% of baseline values, respectively, following the 2-day exposure and 21% and 105% of baseline following the 4-day exposure. TSH was 87% of baseline values following 2 days of exposure and 711% of baseline following 4 days of exposure. Increased TSH and diminished T3 and T4 serum concentrations were consistent with microscopic changes present in the thyroids of 56% and 99% of fetuses following the 2- and 4-day exposure regimens, respectively. MeI-induced changes in fetal thyroids were characterized by decreased amounts of colloid in the follicular lumen, a hypertrophic follicular epithelium and vacuolation of the epithelial cytoplasm. This window of fetal susceptibility to MeI exposure corresponded directly with the timing of critical developmental events observed in the fetal thyroids of the baseline groups, i.e., the appearance of colloid in the follicular lumen and T3 production beginning on GD22. These data implicate MeI-induced fetal hypothyroidism as a possible mode of action for late stage fetal deaths in rabbits resulting from exposure on GD23-26.

## QUANTITATIVE ASSESSMENT OF DERMAL SENSITIZATION RISK FROM TREATED ARTICLES: HEXAVALENT CHROMIUM (CRVI) AS A CASE STUDY.

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Reporting of the potential for a pesticide to cause allergic contact dermatitis (ACD) within EPA's Office of Pesticide Programs (OPP) is typically communicated through precautionary labeling language for residential exposures, and through appropriate engineering controls or personal protective equipment in the occupational setting. In contrast to labeled pesticides, the marketing of treated articles (in which a registered pesticide is incorporated into the article to protect the integrity of the article itself) bear no such labels, and thus the public may unknowingly be exposed to potential dermal sensitizers without a means of communication of risk. Recently, the OPP, through scientific peer review and discussion, developed the foundation of a scientifically sound approach to quantitative assessment of dermal sensitization risk using hexavalent chromium in treated wood as a case study. A level of concern of 0.009 µg/cm<sup>2</sup> for hexavalent chromium was determined by the OPP to be protective against induction of ACD in non-sensitized persons as well as protective against elicitation of ACD in previously sensitized persons. This case study provides a scientifically sound basis for future risk assessments involving quantitation of risk for ACD.

## THE USE OF RISK ASSESSMENT IN EVALUATING FOOD BIOSECURITY OPTIONS.

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For nearly a century, the US Department of Agriculture (USDA) has been protecting the nation's food supply. Food Safety and Inspection Service (FSIS) program personnel in meat, poultry, and egg product plants at ports-of-entry and at various food distribution points, work to prevent, detect, and respond to food safety emergencies. Recent world events have heightened awareness for the need to safeguard the food supply and supporting infrastructure from biological or chemical attack. In biosecurity evaluations, risk assessment helps to focus limited resources on foods, agents, and areas of greatest concern and which would likely yield the largest benefits in averting or minimizing the effects of an attack. United States regulatory agencies currently use numerous methods to evaluate the risk of a chemical or biological attack on the nation's food supply, including: Operational Risk Management Method (ORM); Risk Ranking Method (RRM); and the CARVER Method. The ORM process allows systematic risk decision-making that manages risk as part of a whole operation. There are six steps to implement ORM: Hazard Identification, Risk Assessment, Risk Control Measures, Control Decision, Risk Control Implementation and Supervision and Review. RRM is applied in four steps: Hazard Identification, Hazard Characterization, Exposure Assessment, and Risk Characterization. The CARVER Methodology considers seven factors that affect the attractiveness of a target: Criticality; Accessibility; Recoverability; Vulnerability, Effect, recognizability and shock. The purpose of this report is to provide basic information on the current procedures for evaluating the risk associated with food contaminated with chemical or biological agents.

## MYCOTOXIN RISK ASSESSMENT FOR THE PURPOSE OF SETTING INTERNATIONAL REGULATORY STANDARDS.

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The 2003 Council for Agricultural Science and Technology Mycotoxin report states that one 21st century goal is development of uniform regulations worldwide for foodborne mycotoxin contamination. This study informs that endeavor by a

risk assessment and economic analysis of two important mycotoxins: fumonisins and aflatoxins. The goals are to identify the nations that would be most heavily impacted by tighter mycotoxin regulations, examine costs and benefits as a function of regulatory stringency, and address risk-risk tradeoffs between health benefits and economic losses from compliance with those regulations. Among industrial nations, the United States would experience the heaviest economic losses from more precautionary mycotoxin standards. Environmental conditions in the developing world, however, are more conducive to mycotoxin accumulation in crops. Contrary to concerns expressed among policymakers, the less developed countries that would likely experience the greatest loss from tighter mycotoxin standards are not sub-Saharan African nations, but China and Argentina. If a fumonisin standard of 0.5 mg/kg were adopted worldwide, total export losses from fumonisins in corn may exceed \$300 million annually: threefold higher than if the less stringent US standard of 2 mg/kg were adopted. Likewise, export losses from aflatoxins in peanuts may exceed \$450 million under the current EU regulatory standard of 4 µg/kg; almost fivefold higher than if the US standard of 20 µg/kg were adopted. Stricter standards are unlikely to improve health significantly. In less developed countries such as China where hepatitis B and C are prevalent, tighter aflatoxin standards may increase health risks until improved control methods for aflatoxins are found, as high-quality crops are exported instead of being consumed domestically.

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#### ASSESSMENT OF THE TOXICOLOGICAL RISK OF CHEMICAL MIXTURES AT WORKPLACE.

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Workers are commonly simultaneously exposed to multiple chemical agents. The regulation in Quebec, similarly to the ACGIH approach prescribes that where two or more substances are present in the work location and where they have similar effects on the same organs of the human body, the effects of these substances are considered to be additive, unless it is established otherwise. This project was undertaken to develop a toxicological database allowing the identification of possible additive or other interactive effects of mixtures present in the work environment. In the first phase of the project, standard general literature references were used to compile critical data such as target organs, effects in target organs, mechanisms of action, and toxicokinetic characteristics for each of the 668 chemical substances of the Regulation. The results of the first phase allow the prediction of potential additivity among components of a mixture. In the second phase of this project, the types of interaction for mixtures most likely to be found in workplaces and for which primary literature data are available were specified. The toxicological data were evaluated only for realistic exposure concentrations up to the STEL or ceiling value or five times the 8-h TWA PEL in humans and up to 100 times the 8-h TWA PEL or ceiling value in animals. In total, 675 studies were evaluated for 209 binary mixtures of substances. For the majority of cases where potential additivity was identified in phase 1, there is a lack of supporting toxicological data in the primary literature. In these cases, the results of the first phase will be useful for prevention purposes. The resulting database integrates the results from both phases of project. A Web-based computer tool allows the user to easily find if there is potential additivity or interaction among components of a mixture. Supported by IRSST.

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#### SCIENTIFIC PEER REVIEW TO INFORM REGULATORY DECISION-MAKING: LEADERSHIP RESPONSIBILITIES AND CAUTIONS.

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Congress, the courts, and the public are giving increasing attention to peer review practices in federal agencies. Heightened interest carries heightened expectations for effective peer review policies and practices and, as a result, enhanced reliability and credibility for scientific and technical reports used to inform regulatory decisions. To meet these expectations, decision-makers and leaders in the federal agencies and other organizations need to know if the scientific and technical foundations of their decisions can be expected to withstand scrutiny as rulemaking products wend their way through interagency reviews, public comment and stakeholder processes, congressional oversight and judicial review. Effective peer review can provide information for this assessment. A recent ILSI Risk Science Institute report, entitled "Scientific Peer Review to Inform Regulatory Decision-Making: Leadership Responsibilities and Cautions," offers useful pointers on peer review, and potential pitfalls, with a "heads up" rather than "how to" emphasis. Without delving into the myriad technical and administrative details surrounding the peer review process, this poster highlights nine fundamental "leadership responsibilities" that determine the nature and course of peer review for any report or project. (Supported by EPA Cooperative Agreement CR-827695-01 and the ILSI Risk Science Institute)

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#### A STRATEGY FOR THE SELECTION OF THE NON-RODENT IN REGULATORY PHARMACEUTICAL TOXICITY TESTING.

J. Burnett and J. Gardner. *Covance Laboratories Ltd., Harrogate, United Kingdom*. Sponsor: D. Everett.

Current regulatory practice requires safety testing to be carried out in a rodent and non-rodent mammalian species. Selection of the non-rodent species involves a strategy whereby consideration is given to the following: Similarities to human metabolic, pharmacokinetic and/or biochemical processes Similarities to human toxicity or ADME profile based on *in vivo* data Justification for numbers and species Availability of background data Previous experience with similar compounds within the same pharmacological class This poster describes how and where the use of *in vitro* and radiolabelled ADME profiling provided the basis for selection of the non-rodent species in a regulatory pharmaceutical toxicity testing programme.

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#### CIRCADIAN VARIATION IN FEEDING BEHAVIOUR OF RODENTS RECEIVING POWDERED DIET ON REGULATORY TOXICITY STUDIES: 1) THE MOUSE.

D. Everett, R. Jones and F. Hallema. *Covance Laboratories Ltd., Harrogate, United Kingdom*.

During regulatory toxicity testing studies using rodents, test article is commonly administered admixed with powdered diet in order to ensure a stable plasma level. Circadian variation in feeding behaviour may result in alteration of exposure to test article. This poster reports characterisation of the circadian variation of food intake in mice maintained under typical toxicity laboratory husbandry conditions. The results are of particular use in interpreting data from investigations such as measurements of systemic exposure to test article, and helping determine the timing of such investigations during the working day.

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#### ADDRESSING TOXICOLOGICAL CHALLENGES TO COMMUNITY WATER FLUORIDATION IN WASHINGTON STATE.

D. Dodge, R. C. Pleus and M. K. Peterson. *Intertox, Seattle, WA*.

Fluoride is added to thousands of public water systems in the US as a public health measure. Its primary beneficial effect is the reduction of dental caries, a health problem for nearly 80% of the world's population. The percentage of the population receiving fluoridated water in Washington State is lower than the national average; by several measures, children in Washington State lag behind other states in oral health. In order to improve the oral health of the state, the Washington Dental Service Foundation (WDSF) provides partial funding for and actively promotes water fluoridation in state communities. Despite endorsement by nearly every major public health, dental, and medical association in the nation and world, there is strong and vocal opposition to fluoridation that has made local government and the public wary of passing fluoridation measures. Recently we have assisted WDSF in addressing claims of toxicity put forth by opponents of fluoridation in several Washington communities where fluoridation measures are currently in debate. Allegations include the following: 1) health effects from fluoride itself, including skeletal fluorosis, bone fractures, cancer, and effects on the central nervous system; 2) health effects due to contaminants (e.g., arsenic, mercury) in the concentrated fluoride additive; and 3) health effects due to the leaching of chemicals (e.g., lead, aluminum) from water systems. Many of these allegations fail to recognize dose-response principles. For instance, fluoride is known to cause adverse effects at sufficient doses, but not at concentrations found in optimally treated drinking water. Risks from chemical contaminants are purported as significant simply due to their detection in concentrated solution, despite being present at levels far below toxicity and regulatory thresholds upon dilution. Still other allegations are based on poorly designed studies. We describe our experiences defending the safety of fluoride in Washington communities in the face of opposition challenges.

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#### HOW STAFF GROW IN A CRO.

L. K. Earl. *Toxicology Consultancy, Huntingdon Life Sciences, Cambridgeshire, United Kingdom*. Sponsor: C. Hardy.

The benefits of providing continuous support, learning and opportunities for further development within the industrial environment are well known. Investment in staff professional development potentially leads to improved morale, feelings of self-esteem and career development opportunities. In return, highly trained, capable and motivated staff provide quality output both in respect of science and customer satisfaction. A further tangible benefit should be good staff retention. At

Huntingdon Life Sciences there is a career-long approach to learning and development supported by structured teaching programmes and appropriate support for all toxicologists within the Company. The Toxicology Education Steering Group oversees the content, direction and organisation of the programmes in place. Development of new recruits begins with an intensive induction programme and shadowing of experienced toxicologists. A foundation in the toxicological sciences is then provided and staff members are examined as a prerequisite to further promotion. Those staff meeting the criteria for taking professional examinations such as the International Diploma in Toxicology and Diploma of the American Board of Toxicology are encouraged to participate in the structured learning programme designed to prepare delegates for the examinations. In addition to in-house support, Huntingdon Life Sciences collaborates with other well established UK based industrial organisations and university toxicology departments to provide continuing education for all staff toxicologists. This holistic approach has resulted in the intellectual and professional development of staff, excellent staff retention (approximately 3% staff turnover per annum) and a high level of customer satisfaction (97% of our sponsors said performance met or exceeded their expectations).

## 250 PERCEPTION OF METHYLMERCURY RISK INFORMATION.

D. D. Petersen<sup>1,2</sup>. <sup>1</sup>ORD, USEPA, Cincinnati, OH and <sup>2</sup>Biology, University of Cincinnati, Cincinnati, OH.

Approximately 8% of American women have blood Mercury levels exceeding the EPA reference dose (a dose below which symptoms would be unlikely). The children of these women (over 300, 000 per year) are at risk of neurological deficits (lower IQ scores) primarily because of the mother's consumption of contaminated fish. Even affluent health conscious high-end fish consumers that eat swordfish or other predatory species several times a week are exhibiting symptoms of Mercury toxicity. These health risks of Persistent Bioaccumulative Toxicants (PBT) such as methyl-mercury are often underestimated because their amplification in the food chain results in toxicity, even though ambient levels of these PBTs are within seemingly acceptable limits in the water itself. Two concerns have developed from this situation (1) some of the affected groups have not been identified, and (2) means of effectively communicating the complicated risk messages to the affected groups does not readily exist. We have conducted a series of focus groups throughout the United States and have identified the primary issues surrounding how fish consumers perceive the risks. Three different formats for communicating methylmercury risk information were tested in subpopulations differing age, sex, education, risk group, occupation and locality. Written materials were preferred increasingly with age, while multimedia formats were preferred inversely with age. Web-based materials were preferred by groups seeking knowledge-level information, but not for awareness-level information. The primary factors identified, and the regional, age group and risk group differences can be used to make Mercury risk communication messages more effective by targeting the right information to the right people.

## 251 TOXICOLOGY EDUCATION - TO LEARN OR TO TEACH?

T. Malmfors. Malmfors Consulting AB, Johanneshov, Stockholm, Sweden.

A Lehman said: You too can be a toxicologist in two easy lessons, each of ten years, but he did not say how these two lessons should be spent. During the last decades many toxicology education programmes have been developed but many have also been closed down. On the other hand it is probably still a valid view that toxicologists are in great demand. How does this fit together? There are three possibilities: 1. The students recruited for the toxicology programmes do not have a suitable background. 2. The education is not conducted in an optimal way. 3. The education programmes do not deliver the right product. It is possible that the need of toxicological expertise is satisfied in other ways, e.g. by professionals with a suitable background acquiring the necessary knowledge by other means or by themselves. Furthermore, there is a need of toxicologists in developing countries. The need of toxicology knowledge in these countries is different from what the present education in the developed countries aim for. While senior toxicologist are the outermost goal in the developed countries, it is more important in the developing countries to train various experts, e.g. engineers, chemists, biologists, veterinarian and physicians, in toxicology. Thus, it would be interesting to evaluate how education programmes could serve the need of toxicologists both in developed and developing countries better by offering education to various professional groups and using training methods which are well suited to the students and what they are supposed to learn. Development of electronic communication offers possibilities for new interactive training methods as distance on-line teaching and modelling. Furthermore the facilitated access to facts makes evaluation skills even more important and has to be practised accordingly. Various interactive methods have been used at RASS and in a toxicology education programme in Estonia, Latvia and Lithuania, countries in transition. Group discussions of study cases written by the participants themselves have turned out to be a good method to train evaluation skills. In this way it is possible to cut down on the ten year lesson time.

## 252 BLOOD LEAD, ANEMIA, AND SHORT STATURE ARE INDEPENDENTLY ASSOCIATED WITH COGNITIVE PERFORMANCE IN MEXICAN SCHOOL CHILDREN.

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Lead exposure and nutritional factors are both associated with cognitive performance. Lead toxicity and nutritional status are also associated with each other. We examined whether nutritional status variables account for part or all of the association between cognitive performance and lead exposure. First-grade children (n = 724) ages 6–8 y, attending Mexican public schools located in the vicinity of a metal foundry were asked to participate and 602 enrolled in the study. Blood lead, iron status, anemia, anthropometry, and cognitive function were assessed. Results from 7 standardized tests are presented here. The mean blood lead concentration was  $11.5 \pm 6.1 \mu\text{g}/\text{dL}$  ( $0.56 \pm 0.30 \mu\text{mol}/\text{L}$ ) and 50% of the children had concentrations  $>10 \mu\text{g}/\text{dL}$  ( $0.48 \mu\text{mol}/\text{L}$ ). The prevalence of mild anemia ( $<124 \text{ g/L}$ ) was low (10%) and stunting ( $<2 \text{ SD}$ ) was nonexistent (2.3%). In bivariate analyses, lead was negatively associated with 4 cognitive tests and was also inversely correlated with iron status, height-for-age Z scores, and head circumference. In multivariate models, the association between lead and cognitive performance was not strongly affected by nutritional variables, suggesting that the relation of lead to cognition is not explained by lead's relation to iron deficiency anemia or growth retardation. In multivariate models, hemoglobin concentration was also positively associated with Peabody Picture Vocabulary Test and Number Sequencing performance, whereas serum ferritin was negatively related to the Coding subscale of the Wechsler Intelligence Scales for Children-Revised Mexican Version (WISC-RM). Funded by the Spencer Foundation, Chicago, IL.

## 253 LEAD EXPOSURE AS RISK FACTOR BY ALLERGY DISEASES IN MEXICAN CHILDHOOD. A PILOT STUDY.

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A number of studies have suggested the prevalence of allergic disorders is attributable to the exposure to different environmental factors able to elicit Th2 mediated immunological responses. Lead exposure has been associated with positive modulation of Th2 responses and allergy, in human and animal models. In Torreon Coahuila, in region Lagunera-Mexico, allergy diseases are the most frequent cause of school absenteeism in children population, and exposure to lead also represents an important health problem. To evaluate lead exposure as a risk factor to allergy development in children, a pilot case-control study (28 cases and 23 controls) was performed. The clinical diagnosis of allergy, allergens contact and genetic predisposition were done through medical evaluation, skin-prick test for common allergens and a questionnaire. Total IgE level, eosinophiles were tested. Th1/Th2 cytokines profile were also evaluated in PBMC cells stimulated with ionomicine and PMA. IFN-gamma, IL-13, IL-2 and IL-4 into CD4+ cells were determined by flow cytometry. Results showed allergic rhinitis was the most frequent allergy. Allergy was positive associated to female gender, body mass index, IgE level, eosinophiles percentage and CD3+ and CD4+ cells percentage, before and after activation, respectively. Preliminary results showed Th1/Th2 cytokines response were similar in both groups; blood lead levels were under  $10 \mu\text{g}/\text{dL}$ , whereas urinary total arsenic levels (evaluated as a possible confounding factor) was under  $55 \mu\text{g}/\text{L}$ . The interaction arsenic-lead effects on allergy in children of the studied region is discussed. Sponsored by CONACYT 137610M.

## 254 DEVELOPMENT OF CHILD-SPECIFIC REFERENCE VALUES FOR SCHOOL SITE RISK ASSESSMENT.

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California law requires the Office of Environmental Health Hazard Assessment (OEHHA) in the California Environmental Protection Agency (Cal/EPA) to identify chemical contaminants at school sites that may be of concern because of child-specific exposure and child-specific physiological sensitivities. OEHHA identified 78 candidate chemicals that are known to be found, or likely to be found, at school

sites and have the potential for affecting children at lower doses than they do adults. This report is available at <http://www.oehha.ca.gov>. In order to make child-specific numerical health guidance values available, another requirement, OEHHA conducted extensive searches in the peer-reviewed literature on selected chemicals to locate quantitative studies in young animals or data from young humans. OEHHA concluded that the potential for children's sensitivity is chemical-specific. It may be due to pharmacokinetic differences in the way children's bodies absorb, distribute, metabolize, or excrete specific chemicals, or to pharmacodynamic differences in chemical/target tissue interactions, such as in the endocrine, immune, nervous, respiratory, and reproductive systems when developmental changes are occurring. When quantitative data on a critical effect specific to children can be used to determine a LOAEL, OEHHA has created a child-specific reference dose (chRD) or child-specific reference concentration (chRC) that will be used in conjunction with the "Guidance for Assessing Exposures and Health Risks at Existing and Proposed School Sites," also available at <http://www.oehha.ca.gov>. OEHHA previously reported chRDs for cadmium, chlordane, heptachlor, heptachlor epoxide, methoxychlor, and nickel. OEHHA will present chRDs for endosulfan, manganese, and pentachlorophenol, and chRCs for lead and toluene. The critical effect, citations for important studies, scientific rationale, and relationship to other existing health guidance values for each chRD or chRC will be presented.

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ENVIRONMENTAL FACTORS AND PUBERTY TIMING: SUMMARY OF AN EXPERT PANEL WORKSHOP.

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The children's health question of whether environmental factors affect puberty timing is debated in the scientific community. The Role of Environmental Factors on the Timing and Progression of Puberty-Expert Panel Workshop was convened by Serono Symposia International to address this issue. The workshop goal was to review the data and come to consensus on three questions: 1) Are there sufficient data to suggest a secular trend in the timing of puberty markers in boys or girls from 1940 to present? 2) What are the priority research needs for environmental factors and puberty timing (for human and animal studies)? 3) What are the implications of the findings for children's public health protection? The data review, issues, and conclusions from the workshop will be presented. Major conclusions included: The majority of the panelists concluded that data for girls are sufficient to suggest a secular trend toward an earlier breast development onset and/or menarche but that data for other female pubertal markers were less reliable. Data for boys' puberty timing were considered insufficient to make a conclusion. Research recommendations included longitudinal epidemiology studies (e.g., the National Children's Study) to examine puberty markers and hormonal measurements, and the relationships between exposure to endocrine disrupting chemicals and puberty timing, and animal and human studies investigating whether precocious puberty, delayed puberty, or isolated precocious breast development are associated with outcomes later in life. Altered puberty timing was considered an adverse outcome although no agreement was reached on the magnitude of change in timing considered adverse. (Disclaimer: The views expressed are those of the author and do not necessarily reflect the views or policies of the USEPA.)

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BISPHENOL A EXPOSURE AND ENDOCRINE DISORDERS IN CHILDREN.

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To clarify end points of bisphenol A (BPA)-induced health risk, we studied association between BPA exposure levels and endocrine disorders in children. To estimate BPA exposure, we analyzed urinary BPA, which was excreted as conjugated forms, with HPLC/FD. Study subjects were 84 Koreans, who answered informed consents (age, 4-16 yrs: mean, 9.2 (std, 2.1 yrs); girls, 76 %; boys, 24%): Cases (N=17) included patients who had hyperthyroid (N=7), early puberty (N=8), cryptorchidism (N=1), and varicocele (N=1); Others were hospital controls (N=67). As results, urinary BPA was detected in 76 % of subjects (range, non detectable - 311 ug/L; median, 0.31 ug/L, detection limit, 0.12 ug/L). The urinary BPA levels in cases was approx. 4 fold higher than controls (14.1 (std, 28.8 ug/L) vs. 3.79 (std, 9.51 ug/L); p=0.051). In a case of girl-endocrine disorders, such as hyperthyroid and early puberty, the above trend was more obvious (case vs. control, 14.4 (std, 33.0 ug/L) vs. 2.60 (std, 6.19 ug/L); p=0.04). Therefore, hyperthyroid and early puberty in girls are suspected as end points of BPA-induced health risk. This study was supported by NITR (National Institute of Toxicological Research)/ Korean Food & Drug Administration.

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AN AGE-DEPENDENT HALF-LIFE MODEL FOR ESTIMATING CHILDHOOD BODY BURDENS OF DIBENZODIOXINS AND DIBENZOFURANS.

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This study utilized an age-dependent half life model to examine the range of childhood (ages 0-7) body burdens that correspond to selected exposure scenarios involving background dietary and environmental doses of dioxins. The scenarios examined included breast-fed and non-breast-fed infants feeding for 6 months, other dioxin uptake from foods through age 7, and exposures to urban residential soils; 50th and 95th percentile dose estimates were assessed. A simple toxicokinetic model was used which assumes that the volume of distribution for dioxins is total adipose volume and that an equilibrium exists between ingested/excreted dietary fats and the body burden of dioxins. Congener-specific half life values were estimated for the dioxin congeners that comprise the vast majority of TCDD toxic equivalents (TEQ) in human breast milk and adipose tissues, and dietary dioxin uptake estimates based on a 2001 report of the Joint FAO/WHO Committee on Food Additives were used. The model illustrates that much lower dioxin body burdens for infants and young children are generated by taking growth and fat excretion into account, resulting in lower and age-dependent effective half life estimates (0.4 to 2 years for TCDD, ages 0-7). In conjunction with observed patterns of actual body burden TEQ measurements in children, the age-dependent half life model suggests that the current tolerable daily intake estimates of 1 to 4 pg TEQ/kg-day are not likely to lead to body burdens in children that exceed background levels for adults. Due to the shorter effective half life values in young children, intake limits based on correlation of adult body burdens to animal body burdens in reproductive toxicity studies probably overstate the dioxin body burdens and associated risks per daily dose in children.

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ARE THERE AGE RELATED DIFFERENCES IN CHILDRENS SUSCEPTIBILITY FOR DEVELOPING SECONDARY ACUTE MYELOGENOUS LEUKEMIA?

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Secondary Acute Myelogenous Leukemia (s-AML) is a well recognized clinical entity, often the unfortunate consequence of treatment with certain classes of cytotoxic chemotherapy. Drugs known to cause AML following therapy for a primary malignancy are usually alkylating agents and/or topoisomerase II inhibitors. A variety of other potential risk factors for AML have been studied; however, only ionizing radiation, cigarette smoking and chronic high dose exposure to benzene have a sufficiently solid scientific backing to make a meaningful association. Currently, there is considerable attention being placed on childrens exposure to potential leukemogenic agents, particularly regarding benzene exposure from environmental sources. In this study, a known etiological agent for secondary acute myelogenous leukemia in children was evaluated. Specific data which allowed an evaluation of the effect of age on a childs risk of developing secondary leukemia was found in the cytotoxic chemotherapy literature. Several studies reported treatment of different aged children for the same disease with the same treatment protocols. Hodgkins disease was used to evaluate the effect of age on risk following exposure to alkylating agents, while Acute Lymphoblastic Leukemia studies reported the age specific risk of s-AML following treatment primarily with topoisomerase reactive drugs. These two classes of drugs are known to act via different mechanisms and whether or not they represent an appropriate surrogate for benzene induced AML is subject to debate. Nonetheless, the age of the child did not appear to be an independent variable for risk following treatment with either class of drug. Although the number of studies and cases is very small, the published literature on chemotherapy induced AML in children does not support the hypothesis that children will necessarily have increased susceptibility.

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PBPK MODELING OF INTER-CHILD DIFFERENCES IN PHARMACOKINETICS ON THE BASIS OF SUBJECT-SPECIFIC DATA ON HEPATIC CYP2E1 LEVELS.

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An interindividual variability factor of 10 is used in non-cancer risk assessments to account for pharmacokinetic (PK) and pharmacodynamic (PD) differences among individuals. There has not been any effort to evaluate the inter-child variability in PK or PD of environmental agents. The objective of the present study was to incorporate experimental data on age-specific hepatic CYP2E1 content in children

(autopsy samples; 41 males and 74 females; birth to 17 years) within PBPK models for evaluating the inter-child differences in PK of toluene. The methodology involved expressing the maximal velocity for hepatic metabolism (Vmax) in terms of the CYP2E1 content in a previously validated adult PBPK model for toluene. The adult toluene PBPK model, with enzyme content-normalized Vmax, facilitated the calculation of individual-specific Vmax with knowledge of hepatic CYP2E1 protein level. The child-specific physiological parameters, except liver volume, were computed with knowledge of age and body weight, whereas physicochemical parameters were kept age-invariant based on available data. The individual-specific liver volume was included in the model. The resulting model simulated the internal dose measures for various exposure scenarios. For example, continuous exposure (12 hr) to 1 ppm resulted in an area under the arterial blood concentration vs time curve (AUC) of 0.34 to 0.41 mg/L·hr in infants under the age of a month with a low CYP2E1 concentration (<3.69 pmol/mg protein). The simulations indicate that infants under the age of one month with higher levels of CYP2E1 (4.33 to 55.93 pmol/mg protein) as well as children over a month old would have lower AUC (0.08 to 0.21 mg/L·hr). The latter values were closer to those simulated for adults (AUC 0.09 mg/L·hr). This study demonstrates the integration of data on hepatic CYP2E1 levels within PBPK models to assess the population variability of dose metrics relevant for risk assessment.

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DEVELOPMENTAL EXPOSURE TO ENVIRONMENTAL ESTROGENS IS ASSOCIATED WITH OBESITY LATER IN LIFE.

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Obesity is a significant human health problem that has reached epidemic proportions in the last 2-3 decades. Obesity and overweight are associated with numerous diseases including increases in cancer rates. Commonly held causes of obesity are overeating and sedentary lifestyle imposed on a background of genetic predisposition for the disease. Although much interest has focused on these factors, the etiology of obesity remains uncertain. An emerging hypothesis is that early developmental exposure to environmental chemicals plays a role in the development of obesity later in life. Research in our laboratory has focused on effects of estrogenic compounds on differentiation. Our working premise is the developing organism is extremely sensitive to perturbation by estrogenic chemicals, and that exposure to these chemicals during critical stages of differentiation may have permanent long lasting consequences. Using diethylstilbestrol (DES) as a model chemical, we examined effects of DES on body wt.; low doses (prenatal or neonatal) caused increased body wt. and % body fat. Using PIXImus™ mouse densitometry, % fat mass was determined in 16 wk. old neonatal DES (0.001 mg/kg/day) treated mice; DES caused a significant increase over controls. Neonatal exposure to 2-OH estradiol (20 mg/kg/day) or 4-OH estradiol (0.1 mg/kg/day), doses that are approx. equal in estrogenicity to DES (0.001 mg/kg/day), caused increased body wt. showing DES is not unique. Neonatal exposure to genistein (50 mg/kg/day) caused a significant increase in body wt. Mice exposed to various estrogens are being compared to determine alterations in fat depot wt as well as hormone levels (leptin, adiponectin, etc.). Activity and feed consumption are also being measured. Our data supports the idea that brief exposure to low levels of environmental estrogens early in life is associated with increased body wt. as mice age.

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PHYSIOLOGICAL PARAMETERS FOR EARLY LIFE STAGES.

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At an ILSI Risk Science Institute (RSI) workshop on assessing risks for children, the need for a comprehensive and authoritative source of physiological parameter values for early developmental life stages was noted. However, these data in humans are scattered in many different publications in the literatures of diverse disciplines and specialties, making them difficult and time-consuming to find. Over the past year an RSI expert working group has been compiling, reviewing and evaluating the available data on key physiological parameters for life stages (stages of functional maturation) from the perinatal period through adolescence, to develop a consistent and credible data set for PBPK modeling. These data have been integrated with rodent data in a pre-existent database. In this poster the database will be described, including types of data included, criteria for data selection, and approaches for summarizing data. To explore the issues associated with trying to populate the database, several case studies were conducted on selected parameters (liver weight, liver blood

flow, renal clearance, and specific enzyme systems), characterizing the nature of available data, as well as data quality issues and specific data deficiencies. For example, there is a fairly rich database on liver volume/weight over most of life, including the perinatal period, whereas data on liver blood flow is primarily flow velocity rather than flow volume. Extreme inter-individual variability of the levels of some enzymes in the perinatal period appears to result from variation in the extent of maturation. A common conclusion of the case studies was that, for the database to be useful, the data that are being incorporated must be critically evaluated, with careful consideration given to data quality and representativeness. (Supported by EPA Cooperative Agreement CR830343-01, Health Canada Contract No. H4045-03-EXSD015/4500063129 and the ILSI Risk Science Institute)

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NON-CLINICAL RODENT PEDIATRIC STUDIES AT CTBR: ASSESSMENT OF MORTALITY AND GROWTH.

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The use of pharmaceutical and biotechnology products to treat pediatric patients requires that the conduct of pediatric animal studies be considered before clinical trials. Neonatal, weanling and juvenile rats often represent suitable models for these non-clinical studies. The most commonly used dose route in these studies is oral gavage, CTBR has conducted such studies since the 1980's with a growing number being performed since 1998. In each study, litters of rats (Sprague-Dawley or Wistar Hannover) were administered dose formulations prepared as either solutions in water or as suspensions in methyl cellulose type vehicles. Dose volumes of 4 to 10 mL/Kg/day were administered once or twice per day starting as early as Day 4 post partum (pp) for between 1 and 90 days. Typically, a standard battery of parameters (clinical observation, mortality, frequent body weight measurements, physical development, food intake post weaning, laboratory investigations, ophthalmoscopic examination, and gross and histopathological examinations) was assessed. In some studies, blood samples were obtained as early as Day 4 pp from subsets of pups for toxicokinetic profiling. Other investigations also conducted on subsets of pups were dependent upon the expected toxicology and class of compound tested and included behavioral or immunological assessments. Gradual improvement of both the pup handling procedures and modification of the dosing cannulae have contributed to mortality rates that have been comparable to those in un-dosed litters of pups from pre and postnatal studies (0 to 1% for Days 4 to 21 pp) for a number of years. Growth patterns for litters of pups dosed by gavage are similarly comparable to those of un-dosed litters. In conclusion, oral gavage dosing of rat pups does not adversely effect pup survival or growth.

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AGE-RELATED DIFFERENCES IN SUSCEPTIBILITY TO TOXIC EFFECTS OF VALPROIC ACID IN RATS.

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Prediction of adverse drug effects in children is a timely issue driven by recent legislation specifically mandating safe and effective drugs for children. Doses for children are often adult doses normalized to appropriate pediatric parameters in the clinic. They generally do not take cognizance of very special developmental differences that may produce significant vulnerabilities in vital target organs of children that can result in serious toxicities that are atypical in adults. Valproic acid (VPA) has been reported to cause several cases of fatal hepatotoxicity in infants and temporary increases in serum transaminases (~40% of patients). In this study, different age groups of male Sprague-Dawley (SD) rats (25, 40, 80 days old) corresponding to human toddlers, young and mature adults were treated with VPA at doses of 120, 360, 500, or 650 mg/kg i.p. injection for 4 days to determine pediatric vs. adult susceptibility. The order of sensitivity to the toxicity of VPA observed in this study was 25>80>40 days. The most dramatic toxic effects were observed in 25 day old rats at 650 mg/kg VPA: 57% survival rate, early elevations in ALT, serious G.I. disturbances, and white blood cell deficiencies. In addition, histopathology at this dose showed focal necrosis and apoptosis in the liver. Decreased serum platelet counts and diminished rate of growth were observed at all doses. In comparison, no lethality occurred in the 40 and 80 day old rat groups, and a decreased growth rate was detected in 40 day rats with all treatments. There was also an absolute weight loss in the highest dose groups at 40 and 80 days as well as the middle dose group at 80 days. This correlated with lowering of serum total protein and white blood cell counts in these age groups. Focal necrotic lesions and apoptotic cell death were observed in the liver of 80 day old rats treated with the two highest dose levels. These data indicate that the pattern of toxicity of VPA in different age groups of male SD rats is quite dissimilar. Further studies will explore these findings and its implications for pediatric drug safety.

ASSESSMENT OF HAZARD POTENTIAL AND RISK FOR ASTHMATIC RESPONSES FOLLOWING RESIDENTIAL EXPOSURE TO PHTHALATES.

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It has been hypothesized that exposure to phthalates via indoor dust inhalation may produce airway inflammation contributing to childhood asthma. Accordingly, an assessment of hazard and risk was undertaken to understand the potential for airways effects from indoor air exposure to phthalates. Phthalate esters most commonly used for indoor applications are BBP, DEHP and DINP, but lower molecular weight esters, e.g., DEP and DBP may also be present. Absent specific study designs, hazard potential was evaluated in several ways: (1) Repeated exposure studies in rats assessed the potential for airway inflammation using histologic evaluation of the respiratory tract, emphasizing the airway epithelial lining. Repeated exposure to DBP at 500 mg/m<sup>3</sup> (aerosol) produced hyperplasia and hypertrophy in nasal tissue and metaplasia in the trachea, but there were no pulmonary effects or inflammation. In previous studies of DEHP and DIDP, slight, non-specific irritation of respiratory epithelium was produced at levels > 100 mg/m<sup>3</sup>. (2) The potential for sensory irritation was obtained from studies of C6 and C10 monoesters. Exposure levels associated with 50% decreases in respiratory rate (RD50) were 525 and 187 mg/m<sup>3</sup> for the C6 and C10 monoesters respectively. (3) The potential for BBP, DEHP and DINP to influence IgE or cytokine levels was assessed in a mouse dermal application model. Total serum IgE and levels of IL-6, a cytokine associated with inflammatory responses in the lung, were not elevated. Based on measurements of phthalate levels in dust, exposure to DEHP from indoor dust inhalation would be approximately 50 ngm/m<sup>3</sup>. Thus, margins between human exposure and minimal effect levels in rodents were in the range of 100, 000 to 1, 000, 000.

BENZYL ALCOHOL: SAFETY ASSESSMENT IN JUVENILE RATS.

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Benzyl alcohol is used as an excipient in intravenous formulations of some pharmaceutical products, because of its antibacterial and preservative actions. It is now established that premature and newborn infants are particularly susceptible to the toxic actions of benzyl alcohol and that the use of solutions and diluents containing benzyl alcohol in newborn infants can result in "gasping syndrome" accompanied by metabolic acidosis and neurologic toxicity in newborn infants. This susceptibility probably results from differences in the metabolic and pharmacokinetic handling of benzoic acid in neonates. Regulatory measures have been taken to avoid the use of such products in newborn infants. Information is less readily available about the susceptibility of older pediatric groups (toddlers, children and adolescents) to benzyl alcohol. In order to address this question, in the present study we have examined the susceptibility of rat pups to the toxic actions of benzyl alcohol during the period from weaning to sexual maturity. Benzyl alcohol was administered by oral gavage daily to groups of 10 male and 10 female rat pups from post natal day 22 for a period of 6 weeks. Dose-levels of 100, 300 and 600 mg/kg/day were selected on the basis of published data. Control animals received vehicle only (distilled water). In addition to the evaluation of standard toxicology parameters (body weight, clinical observations, ophthalmology, haematology, blood biochemistry, urinalysis and histopathology) the study included evaluation of reactivity, learning and memory, motor activity, sexual development and respiratory function. Treatment with benzoic acid resulted in "gasping syndrome" at the higher dose-levels. A NOAEL was established for benzyl alcohol in juvenile rats.

LIPID PEROXIDATION PRODUCT INDUCED GASTRIC GROWTH IN POSTNATAL RATS BY MODULATION OF SELECTED PROTEIN KINASES.

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Many food and food products contain the lipid oxidation product 2, 4-hexadienal (HX). Low level of exposure to HX is widespread. Long term feeding of HX led to abnormal growth of forestomach in mice and F344 rats. Our study showed that short term feeding of HX to Sprague-Dawley rats up-regulated aldehyde dehydrogenase only in their forestomachs suggesting that HX targets this tissue specifically. We examined whether short term HX feeding to young rats would affect gastric growth and whether the growth induction is accompanied by changes in specific protein kinases (PKs). Feeding rat pups for 5 days increased the mass of the fore- and glandular stomach but decreased that of the liver. The esophagus and kidney were not affected. Screening for PKs was done by Western blots using specific antibody to each of 75 selected PKs. HX feeding resulted in ~1.5 fold increase in PKC $\mu$ , CDK7 and CK2 $\alpha$ ; 2.3 fold increase in CDK9 and 1.35 fold increase in CK2 $\alpha$ . Our preliminary data indicated the trophic effect of short term HX feeding on the rat stomach is specific as it promotes growth in this organ only.

Concomitant increases in several key PKs that have important roles in cell cycle regulations were evident. Therefore, we propose that HX acts through selected PK signaling pathways in the target tissue in its growth promoting action.

GENE EXPRESSION PROFILES OF THE ARTICULAR CARTILAGE IN JUVENILE RATS TREATED WITH THE QUINOLONE ANTIBACTERIAL AGENT OFLOXACIN.

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To investigate the molecular mechanism of chondrotoxicity induced by quinolone antibacterial agents, ofloxacin (OFLX) was orally administered once at 2700 mg/kg to male juvenile (3-week-old) Sprague-Dawley rats, and then gene expression analyses in the articular cartilage of the distal femur were performed 2, 6 and 24 h after administration by using Affymetrix GeneChip Rat Toxicology U34 microarray, in conjunction with histological examination. Histopathologically, OFLX induced degeneration and/or necrosis of chondrocytes with edematous matrix and a decrease in Safranin-O staining intensity 6 h later, and cavitations 24 h later. In microarray analyses, OFLX caused up-regulation of metallothionein (MT, 2, 6 and 24 h), c-fos (6 and 24 h) and heme oxygenase-1 (HO-1, 24 h). In contrast, OFLX showed down-regulation of alpha-tubulin (Tuba1, 6 and 24 h), NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 5 (Ndufa5, 6 and 24 h) and immunoglobulin heavy chain binding protein (BiP, 6 h). To further assess the expressions of these genes, quantitative real-time RT-PCR assay was conducted in the articular cartilage of juvenile rats receiving a single oral administration of OFLX at 300, 900 and 2700 mg/kg. Significantly time- and dose-dependent increases in MT-1 and -2, c-fos, HO-1 and BiP, and decreases in Tuba1 and Ndufa5 genes were observed. MT-1 and -2, c-fos and HO-1 have been reported as antioxidant response genes; Tuba1 to be related to cell proliferation and cytoskeletal organization; Ndufa5 as a component of mitochondrial respiratory chain. The above results demonstrate that the mechanisms of ofloxacin chondrotoxicity may be due to oxidative stress, followed by inhibitions of the microtubule-based process and dysfunctions of the mitochondria.

A NOVEL APPROACH TO GENERATE JUVENILE ANIMAL DATA DURING THE PRE/POSTNATAL RANGE FINDING STUDY IN THE RAT.

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In response to the changing regulatory environment, there has been considerable discussion on testing strategies and design of juvenile animal studies. In a desire to obtain early data on exposure and toxicity in juvenile rats, to better anticipate potential safety issues and to support prompt changes in clinical paediatric development plans, a combined range finding study has been developed. The aim of this poster is to detail the study design which is currently used in our laboratories to generate sufficient scientific data for the dose selection for both pre- and postnatal developmental toxicity studies and juvenile toxicity studies. Briefly, the combo study design can be summarized as follows: During the pregnancy/lactation study phase, 5 groups (including 2 control groups) of pregnant females are dosed from Day 6 of gestation till Day 7 of lactation. The females are allowed to litter and pups are then selected at Day 7 for the subsequent juvenile study phase. After an appropriate wash-out period, 4 groups of pups, including one of the control groups (naive animals), are dosed directly with the test material from Day 12 till Day 25 of age. Toxicokinetic data are collected from unselected pups at Day 7 (exposure via the maternal milk) and from directly dosed animals at Day 25 post-partum. The study design offers significant advantages in animal usage, cost and timing in the collection of data both from indirectly or directly dosed pups. This enables early assessment of potential safety concerns and the possibility of advancing any definitive juvenile animal study should results indicate a necessity. Further, these data may add confidence prior to initial entry into paediatric populations. In summary, the proposed combo range finding study design may play a valuable role in the pre-clinical paediatric testing strategy for some classes of pharmaceuticals.

IMPACT OF DOSE RATE ON NEUROTOXICITY OUTCOME IN CD RAT PUPS GIVEN THE N-METHYL-D-ASPARTATE (NMDA) RECEPTOR ANTAGONIST, MK-801.

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This study tested the hypothesis that a slower dose rate (small episodic doses vs. large bolus doses) would lessen the severity of MK-801 neurotoxicity in rat pups. CD rat pups (20/group) were dosed by gavage with 0, 0.2 or 0.5 mg MK-801/kg/d

from postnatal day (PND) 7-20. MK-801 was given as bolus (1x/day, 7AM) or divided doses (3x/day at 7 AM, 12 and 5 PM). Bolus pups were gavaged with water at 12 and 5 PM to control for gavage stress. Pups were monitored for clinical observations, body weight, motor activity (MA; 10/group), pre-pulse inhibition of the startle reflex (PPI; 10/group), and gross brain measurements (10/group). Clinical signs in the high-dose groups included increased activity, splayed hindlimbs, incoordinated gait, tremors (bolus only), and increased reactivity and tonic-clonic convulsions (divided only). Low-dose pups had incoordinated gait. Clinical signs were more prominent prior to PND16, but had resolved by PND20. Body weights were decreased significantly in all dose groups by PND10 with decreases of 22 and 33% in the high-dose divided and bolus groups by PND20. On PND21, MA in the low-dose divided group was decreased significantly. On PND22, PPI was decreased significantly in the high-dose bolus and divided groups (43 and 25%, respectively vs. 63% in controls) without a significant decrease in startle amplitude. This indicates a greater effect on sensorimotor gating in the divided group. Brain weights were decreased on PND22 by 11 and 8% in the high-dose bolus and divided groups, respectively. Small changes (3-5%) in cerebellar length and width were detected. These results were partially explained by kinetics data, which showed that MK-801 has a longer half-life in pups than adults, such that divided and bolus doses yielded similar MK-801 blood concentrations. Thus, dose rate impacted neurotoxicity outcome in an endpoint-dependent manner. Test material kinetics and potential exposure scenarios should be considered when designing juvenile toxicity studies.

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#### COMPARATIVE DATA OF HAND-REARED AND MATERNALLY REARED INFANT CYNOLOGUS MONKEYS FOR TOXICITY STUDIES.

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Preclinical juvenile nonhuman primate toxicity studies are expected to require experimentation in a closely controlled environment so that various biochemical, behavioral and physiological parameters could be monitored as indicators of toxicity. Based on the condition that juvenile nonhuman primates were purchased from commercial breeders and separated early from their natural mothers, this prospective study was conducted to document the developmental changes according to the measurable parameters of growth, neuromotor and behavioral assessment, hematology and biochemistry measurements of twelve maternally-reared or hand-reared male and female infant cynomolgus monkeys (*Macaca fascicularis*) examined repeatedly across the first four months of life. Six (6) hand-reared infants, which were separated from their mothers within 24 hours after birth and six (6) infant macaques that continued to be nursed by their respective mothers within 2 months after birth, upon transport to the testing facility were included in the study. The available background data on these juvenile monkeys as early as two weeks of age were presented. Based on the parameters measured, our results showed that the data from the artificially reared juvenile monkeys, which experienced early maternal weaning, were comparable with those obtained from the maternally reared infants. Where toxicological research activities required that juvenile nonhuman primates be maintained early under strictly regulated conditions that would require artificial rearing, our specialized nursing and husbandry procedures were effective to produce normal growth, satisfactory health and adaptive neurobehavioral skills in these hand-reared monkeys.

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#### INFLUENCE OF MATERNAL TOXICITY IN STUDIES ON DEVELOPMENTAL TOXICITY: A WORKSHOP REPORT.

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This Workshop, prepared by an Organizing Committee, was convened to explore whether the criteria to assess the influence of toxic effects induced in the maternal organism on the development of the embryo/foetus could be improved for classification purposes. Studies following OECD 414 guidelines often cause maternal toxicity because of high doses that secondarily can produce indirect effects on the progeny. Four topical presentations, followed by breakout group and plenary discussions, evaluated criteria for the interpretation of developmental effects occurring concurrent with maternal toxicity. Research needs were defined to provide better insight into the incidence of secondary effects and more precise distinctions from primary developmental toxicity. The workshop led to the following conclusions/recommendations: 1) Hazard-based classifications overlook exposure conditions. Primary developmental toxicity and secondary effects caused by chemically-induced perturbations of maternal homeostasis are not differentiated. Anticipated human exposure should be considered, and risk assessment should be entered into regulatory classifications. 2) Route and mode of dosing, dose-response data, as well as toxicokinetics/ toxicodynamics should guide study design since

toxic effects depend on how a chemical enters into the body. 3) The use of more sensitive end points (acute phase response, e.g., serum proteins; serum zinc and hepatic metallothionein concentrations; hematology; clinical chemistry/pathology) to detect the onset of maternal toxicity received solid endorsement. An experimental way forward with pilot test chemicals emerged. Such new knowledge could enter into future study design. The new data might improve the interpretation of OECD 414 study results and provide an improved scientific basis for regulatory decisions. 4) The Long-Range Research Initiative of the chemical industry that had sponsored the Workshop should consider supporting an experimental examination of the added value of expanded end points that would characterize the onset of maternal toxicity more accurately.

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#### TIME-MATED RABBITS WITH VASCULAR ACCESS PORT (VAP): A NOVEL APPROACH TO THE VASCULAR SYSTEM FOR SERIAL INFUSION OR REPETITIVE BLOOD COLLECTION.

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Vascular access ports (VAPs) are currently available in a multitude of animals: rodents, lagomorphs, canines, ferrets, non-human primates and farm animals. For more than 3 years, time-mated rabbits equipped with VAPs have been available to the reproductive toxicology community. The fasted rabbit was anesthetized with a combination of Xylazine and Ketamine. Buprenorphine was administered 10 minutes prior to anesthesia, in order to reduce pain during recovery. Isoflurane was available if needed. A VAP (PMID port attached to 5F polyurethane catheter with rounded tip, devices fabricated from medical grade materials under stringent controls) was surgically implanted into one of the rabbit's jugular vein and secured caudal to the scapula. Post surgery analgesic, Buprenorphine, was administered for 2 days during recovery. The port/catheter was flushed and locked weekly prior to shipment. 380 New Zealand White (NZW) rabbits were surgically implanted with jugular VAPs by Covance Research Products (CRP), allowed 2 weeks to recover, then time-mated prior to shipment. All VAPs were patent prior to shipment and 370 remained patent after being placed on study. 360 of the 380 rabbits had viable fetus on necropsy. Animals on study were repetitively infused daily for 2 weeks. Internal and external data has provided evidence of success rates of more than 90% (i.e., animal with litter after mating, evidence of pregnancy during necropsy, patent device and no evidence of fetal anomalies in control animals). The time-mated VAP technology is an asset to researchers who investigate the effects a specific compound has on the fetus.

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#### EFFECTS OF FEED RESTRICTION ON EMBRYO-FETAL DEVELOPMENT IN RATS AND RABBITS.

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Pharmaceuticals intended to reduce appetite and promote weight loss will generate safety data that will be challenging to interpret. To aid with this, the effects of feed restriction on rat and rabbit embryo-fetal development were investigated. Groups of 20 pregnant Sprague-Dawley rats were offered ad lib, 20, 15, 10 and 7.5 g/d from GD 6-17 and groups of 15 pregnant New Zealand White rabbits were offered 150 (control), 110, 75, 55, 35 and 15 g/d from GD 7-19. Cesarean sections were performed on GD 21 (rats) or 29 (rabbits) and fetuses were examined for external, visceral and skeletal development. Maternal BW in rats at the end of the feed restriction period, GD 18, was reduced 0.88 and 0.80x control in the 20 and 15 g/d groups, respectively. Mean BW loss occurred at 10 and 7.5 g/d (0.95 and 0.85x GD 6 BW, respectively). Fetal BW was reduced 0.95, 0.93, 0.90 and 0.76x control at 20, 15, 10 and 7.5 g/d, respectively, resulting in a reduction in gravid uterine weight at 10 and 7.5 g/d. There was no increase in external or visceral anomalies. Skeletally, there was an increase in wavy ribs and a decrease in ossification only at 7.5 g/d. In rabbits at the end of the feed restriction period, GD 20, BW was 0.97, 0.98, 0.93, 0.94 and 0.86x control for the 110, 75, 55, 35 and 15 g/d groups, respectively. Only at 15 g/d was there a net BW loss (GD 20 BW was 0.93x GD 6 BW). Six does aborted in the 5 g/d group; there were no other abortions associated with feed restriction. Fetal BW was significantly reduced at 75, 55, 35 and 15 g/d (0.95, 0.90, 0.86 and 0.84x control, respectively). There was no increase in developmental anomalies. These data demonstrate that in rats, feed restriction-induced reductions in maternal BW of ~10% caused a reduction in fetal BW and up to a 15% maternal BW loss had no effect on embryo viability, but reduced fetal BW enough to cause developmental delays. Feed restriction-induced maternal BW loss in rabbits of ~7% was associated with abortion, but mild maternal BW reductions resulted in reduced fetal BW at feed levels  $\leq$  75 g/d.

## MICROARRAY-BASED ANALYSIS OF NUTRITION-ETHANOL INTERACTIONS DURING GESTATION.

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Undernutrition markedly potentiated fetotoxicity of ethanol (EtOH) during pregnancy. To understand the mechanisms of this interaction, time-impregnated rats were fed either adequate (NRC requirements, 220 kcal/ kg<sup>3/4</sup>/d) or undernourished diets (30% less calories, 160 kcal/ kg<sup>3/4</sup>/d) via intragastric infusion containing either 12 g/kg/d EtOH or isocaloric amount of carbohydrates from gestation days (GD) 6-15. Undernourished dams had 1.6 fold greater mean urine EtOH concentrations (p = 0.007), higher complete litter resorptions (33% vs. 0%), lower litter weight and lower numbers of pups/litter (p <0.05) compared to control rats given the same dose of EtOH but fed adequate diets. Undernutrition in the absence of EtOH did not increase any parameters of fetal toxicity. Affymetrix RU34A GeneChip microarray-based comparisons were performed on maternal hepatic gene expression on GD 15. Expression of 101 genes was altered by the combination of EtOH and undernutrition as compared to only 30 genes in the rats fed an adequate diet in the presence of EtOH (p <0.05). Hierarchical clustering of gene expression data resolved the affected genes into 8 clusters. Gene ontology analysis revealed that genes associated with stress and external stimulus responses, transcriptional regulation, cellular homeostasis and protein metabolism were affected uniquely in the EtOH-nutrition group, but not by EtOH alone. Microarray data were confirmed using real-time RT-PCR. Nutritional induction of metallothionein 1, rev-erbA $\alpha$  and  $\beta$ , IGFBP-1 and 4 and repression of rPER2, and glucokinase was observed (p <0.01). Undernourished EtOH fed animals had 2-fold lower IGF1 mRNA and 10-fold lower serum IGF1 protein levels compared to undernourished controls (p = 0.0002). The present data suggest that undernutrition may potentiate the fetal toxicity of EtOH in part by disrupting IGF1 synthesis and bioavailability and zinc availability to the fetus. Supported in part by R01 AA12819 (MJR).

## DEVELOPMENTAL AND REPRODUCTIVE TOXICITY OF DI-ISOHEPTYL PHTHALATE IN RODENTS.

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Di-isoheptyl phthalate (DIHP) is a branched, 7-carbon phthalate ester. Since some phthalates of similar structure have been shown to produce developmental and/or reproductive effects in rodents, an assessment of the potential for DIHP to produce developmental and reproductive toxicity was conducted. In a developmental toxicity study, female rats were given DIHP by oral gavage on gestational days 6-20. There were significant reductions in uterine weight, increased resorptions, and reduced fetal weights in the high dose group (750 mg/kg). Fetal examination revealed malformations and variations of both the skeletal system and the viscera including ectopic testes. The intermediate dose, 300 mg/kg/day, was the no effect level. In a two-generation reproductive toxicity study, DIHP was administered in the diet at 1000, 4500, and 8000 ppm. There were significant effects on indicators of male reproductive development in the high dose group. In the first (F1) generation, anogenital distance was reduced, time to balanopreputial separation was increased, there was a significant increase in thoracic nipples and testicular abnormalities; weights of testes and accessory reproductive organs were significantly reduced. Testicular sperm counts and daily sperm productions were significantly reduced. Fertility was reduced in the high dose group. In the second (F2) generation, anogenital distance was significantly reduced, and there was evidence of reduced weight gain during lactation. The overall no effect level (NOEL) in the reproductive toxicity study was in the range of 64 - 168 mg/kg/day (gestation - lactation periods). By comparison, estimated exposures in the general human population are in the range of < 1 ug/kg/day.

ASSESSING THE ROLE OF CYTOSOLIC PHOSPHOLIPASE A (cPLA<sub>2</sub>) INHIBITION IN PHTHALATE ESTER TOXICITY.

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Fetal exposure of rats to certain dialkylphthalate plasticizers, including dibutylphthalate and diethylhexylphthalate, cause testicular malformations. These effects are associated with inhibition of fetal testosterone (T) synthesis during gestation by the monoalkylester metabolites (MBP and MEHP). Molecular and cellular targets for phthalates in the testes are unknown, causing significant uncertainty in phthalate

risk assessments. Steroidogenesis in the adult requires both LH signaling and arachidonic acid (AA) release by cPLA<sub>2</sub>. Our research includes dose response modeling, molecular modeling of cPLA<sub>2</sub>, and *in vitro* steroidogenesis and enzyme inhibition studies. In our hypothesis, MEHP binds to cPLA<sub>2</sub>, inhibiting T production and reducing AA release from membrane phospholipids. Using *in vitro* and *in vivo* dose-response models, the concentration of MEHP required to cause 50% inhibition (IC<sub>50</sub>) in high affinity cPLA<sub>2</sub> activity in human platelets was 10  $\mu$ M. The IC<sub>50</sub> for MEHP inhibition of fetal T production (estimated with a kinetic model in the female rat) was similar (4  $\mu$ M). We also studied the effect of MEHP and the cPLA<sub>2</sub> inhibitor chloroquine (CQ) on LH-stimulated progesterone (PG) synthesis in MA-10 cells. Assays were run at a maximum stimulating LH concentration (100 ng/mL LH) with inhibitor concentrations of 1, 3, 6, 10, 30, 60 and 100  $\mu$ M. Both CQ and MEHP inhibit LH-stimulated PG production. The lowest concentration of MEHP causing inhibition was 10  $\mu$ M. This value is similar to the IC<sub>50</sub> for inhibition of cPLA<sub>2</sub> and is consistent with cPLA<sub>2</sub> inhibition having a role in phthalate toxicity. Our molecular modeling of the Ca<sup>2+</sup>-binding portion of cPLA<sub>2</sub> indicates that CQ and MEHP could compete for enzyme binding with the phosphatidyl choline head group of the phospholipid substrate. Specificity of binding to the Ca<sup>2+</sup> activated portion of cPLA<sub>2</sub> may account for the structural specificity of phthalate toxicity. Studies assessing inhibition of cPLA<sub>2</sub> from various cell types will be important for confirming cPLA<sub>2</sub> as a target of these phthalates.

## MEDIUM CHAIN CHLORINATED PARAFFIN(MCCP)-INDUCED HAEMORRHAGIC LESIONS IN NEONATAL SPRAGUE-DAWLEY RATS. A ROLE FOR ALTERED VITAMIN K DISPOSITION?

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Previously MCCPs administered to female rats prior to pregnancy and during gestation have elicited increased pup mortality associated with internal haemorrhages. This study was designed to provide data to elucidate the mechanism of haemorrhaging. F0 rats were allocated to one control and one treated group. The treated group rats received 6250ppm MCCP (Cereclor S52, C14-17) in the diet. The F0 animals were treated for 4 weeks prior to pairing, then throughout the mating, gestation and lactation periods. On Days 1 and 4 of lactation pups were sampled and blood samples obtained. Dam milk samples were obtained on Days 1 and 4 of lactation. At study termination (day 12 of lactation) further milk and pup samples were obtained, together with maternal plasma samples. Mating performance and the duration of gestation were similar in treated and control groups. Slight differences in litter size at birth, and pup mortality from birth to Day 4 were too small to be attributed to treatment, but pup mortality after Day 4 increased substantially in the MCCP-treated group such that few pups survived until the study was terminated. Many treated group pups had severe bruising, with visible subcutaneous fluid. During lactation, treated-dam plasma levels of Vitamin K were 10% of control plasma values. This was reflected in the milk where concentrations of vitamin K in treated-dams varied from 0%-50% of control values. Although dam clotting times were unaffected, Factor VII activity was depressed (50% of control). In the pups, there were no changes in plasma Factor VII activity at day 1. However, from day 4 onwards the activity was decreased to 20% of control pup activity. A similar decrease in Factor X activity was seen. Thus, temporal decreases in plasma clotting factor activities in the pups from the MCCP-treated rats correlated with increased pup bruising and mortality. These data suggest that modulation of Vitamin K disposition may be pivotal in the neonatal toxicity induced by this MCCP.

## EFFECTS OF EXPOSURE TO 1-BROMOPROPANE IN GESTATION AND LACTATION PERIOD ON DAMS AND OFFSPRING IN RATS.

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1-Bromopropane (1-BP), an alternative for ozone-depleting solvents, is known to exhibit neuro-reproductive toxicities in adult rats and humans. The present study investigated the effects of exposure to 1-BP in the gestation and lactation period on dams and offspring in rats. Experiment 1: Fifty Wistar-Imamichi adult female rats were divided into 5 groups of ten each. After confirmation of pregnancy, the 4 groups were exposed to 1-BP throughout gestation and lactation period at 0, 100, 400 and 800 ppm, respectively, for 8 h/d with a 2.5-h rest between two 4-h exposures. After delivery, the number of offspring was reduced to 4 males and 4 females. The rats were put back in their cages during an intermission between exposures to nurse their offspring after delivery. The remaining one group was not moved to chambers throughout the experiment. Experiment 2: Forty female rats were divided into 4 groups of ten each. One group was exposed daily to 1-BP at 800 ppm

throughout the gestation and lactation period, and the other 3 groups were given only fresh air in chambers. After delivery, the offspring of the exposed-group and the non-exposed group were exchanged. Offspring (Group I) of the non-exposed dams were nursed by the exposed dams, and those (Group II) of the exposed dams were nursed by the non-exposed dams. The offspring of the remaining two non-exposed groups were then exchanged (Group III and IV). In experiment 1, the survival rate up to weaning decreased dose-dependently. In experiment 2, the survival rate and body weight of offspring in Group I and II were significantly lower than in Group III and IV. The comparison of Group I and II did not show any difference in survival rate, but Group I showed significantly lower body weight gain than Group II. The results suggested that the survival rate might be affected by both exposures in the gestation and lactation period, but that the body weight gain might be affected mainly by exposure in lactation.

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L-THREO 3, 4-DIHYDROXYPHENYL SERINE (DOPS) TREATMENT DURING PERINATAL DEVELOPMENT DOES NOT ALTER THE IMPACT OF DIETARY COPPER DEFICIENCY IN MICE.

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Sponsor: K. Wallace.

Alteration in catecholamine metabolism results in pathophysiological consequences. Blockage of norepinephrine (NE) synthesis in mice by deletion of the cuproenzyme dopamine beta monoxygenase (DBM) results in embryonic death. Dietary copper (Cu) deficient mice have lower NE but higher dopamine (DA) levels and poor pup survival. Might there be a connection? DOPS can be converted to NE without DBM and has been shown to rescue DBM knockout mice. Thus, perinatal Cu deficiency was induced in Swiss albino mice to investigate the effect of DOPS (a gift of Sumitomo Pharmaceuticals, Osaka, Japan) on pup survival and catecholamine levels in a 2 x 2 factorial design. Pregnant mice were placed on one of four treatments 14 days after mating. Pups were killed at postnatal day 14 (P14). Treatments were Cu adequate (Cu+) and Cu deficient (Cu-) diets with or without DOPS (1mg/mL) in the drinking water. Mortality in P14 Cu- pups was 11/24 (46%) and not significantly improved by DOPS, 14/36 (39%). Compared to Cu+ pups, Cu- mice were smaller, anemic, and had a 92% reduction in liver Cu. DOPS treatment did not alter body weight, hemoglobin, or liver Cu content in either Cu+ or Cu- mice. Catecholamine levels measured in heart and medulla/pons by LCEC showed decreased NE levels and increased DA levels in Cu- mice compared to Cu+ mice. DOPS treatment did not alter this pattern. A repeat study with ascorbic acid (2 mg/mL) added to drinking water showed increased pup mortality in Cu- mice and no effect of DOPS. Cu status markers and catecholamine levels followed the same pattern as the first experiment. DOPS was detected in heart tissue of pups in the first experiment (range 0.04-0.32 nmol/g) well below the levels of heart NE even in Cu- pups. Although DOPS was present in the pup tissue and to a greater extent in dam heart tissue, survival and catecholamine levels were not altered by the 1 mg/mL dose of DOPS; the same dose that rescued DBM knockout mice. Supported by NIH HD 39708 and 2R25-GM53421-03A1

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EFFECTS OF PERFLUOROOCTANOIC ACID EXPOSURE DURING PREGNANCY IN THE MOUSE.

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Perfluorooctanoic acid (PFOA), a member of the perfluoroalkyl acids that have wide commercial applications, has recently been detected in humans and wildlife. The current study characterizes the developmental toxicity of PFOA in the mouse. CD-1 mice were mated overnight and the day on which copulatory plugs were found was designated as GD 1. Animals were given 1, 3, 5, 10, 20, or 40 mg/kg PFOA by oral gavage daily from GD2 to GD 18; controls received an equivalent volume (10 mL/kg) of water. PFOA treatment produced early, full-litter resorptions in a dose-dependent manner, leading to 100% loss in the 40 mg/kg group. Significant deficits in maternal weight gains were detected in dams of the 20 mg/kg group that carried pregnancy to term. On GD 18, some dams were sacrificed for maternal and fetal examinations while the rest were allowed to give birth for monitoring of postnatal survival, growth, and development of the pups. PFOA induced liver enlargement in the dams at all doses, but did not alter the number of implantations. In the 20 mg/kg group, the percent of live fetuses was reduced to 74% (compared to 97% in controls) the day before birth, and fetal weight ( $0.86 \pm 0.11$  g) was significantly lower than that of controls ( $1.05 \pm 0.02$  g). However, no discernable anatomical malformations were noted in any of the treatment groups. The incidence of live births was significantly lowered by PFOA in the 10 and 20 mg/kg groups (70% compared to 93% in the control group), and postnatal survival of the neonates was severely compromised in these treatment groups. Dose-dependent deficits of weight gains were detected in all PFOA-treated pups. Significant delays in eye-opening (by about 2 days) were noted in the 10 and 20 mg/kg groups, and

pubertal indices were significantly delayed (by about 3 days in males and females) in the 20 mg/kg group. These data indicate maternal and developmental toxicities of PFOA in the mouse measured as early pregnancy loss, compromised postnatal survival, and delays in postnatal growth and maturation. This abstract does not necessarily reflect USEPA policy.

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DEVELOPMENTAL TOXICITY OF 1-BUTANOL GIVEN TO RATS IN DRINKING WATER THROUGHOUT PREGNANCY.

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1-Butanol (CAS No. 71-36-3, n-butanol; n-butyl alcohol), a flammable colorless liquid with a rancid sweet odor, is widely used as an organic solvent and intermediate in the manufacture of other organic chemicals. Exposure of the general population is mainly through its natural occurrence in food and beverages and its use as a flavoring agent. The objective of this study was to evaluate the developmental toxicity of 1-butanol in rats. Pregnant rats were given drinking water containing 1-butanol at 0.2, 1.0 or 5.0% (316, 1454 or 5654 mg/kg/day) on days 0-20 of pregnancy. A significant decrease in maternal body weight gain accompanied by reduced food and water consumption was found at 5.0%. No significant increase in the incidence of pre- and postimplantation embryonic loss was observed in any groups treated with 1-butanol. Body weights of male and female fetuses were significantly lowered at 5.0%. Although a significant increase in the incidence of fetuses with skeletal variations and decreased degree of ossification was found at 5.0%, no increase in the incidence of fetuses with external, skeletal and internal abnormalities was detected in any groups treated with 1-butanol. The data demonstrate that 1-butanol is developmental toxic only at maternal toxic doses. No evidence for teratogenicity of 1-butanol was noted in rats. Based on the significant decreases in maternal body weight gain and fetal weight, it is concluded that the no observed adverse effect levels (NOAELs) of 1-butanol for both dams and fetuses are 1.0% (1454 mg/kg/day) in rats.

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ASSESSMENT OF ISOBUTYL HEPTYL KETONE (IBHK) BY ORAL GAVAGE IN AN OECD 422 RAT REPRODUCTION/DEVELOPMENTAL SCREENING STUDY WITH SYSTEMIC AND NEUROLOGICAL ENDPOINTS.

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Twelve CD rats/sex/group were given IBHK by daily oral gavage at 0, 100, 300, or 1000 mg/kg body weight/day (mkd). Females were dosed from 2-wk prior to breeding through postpartum day (PD) 4, then necropsied on PD 5. Males were dosed for 2-wk prior to breeding through necropsy (D39). Toxicity was assessed by daily observations, functional tests including sensory evaluation, rectal temperature, grip performance and motor activity, and terminal necropsy, organ weights, and clinical and microscopic pathology. Reproductive indices, litter size, pup survival, sex, body weight, and gross external changes were assessed. Increased salivation occurred at all doses, but was transient and in close proximity of dosing. Males had decreased hemoglobin and urinary pH at  $\geq 300$  mkd, decreased ketones at 1000 mkd, increased BUN at  $\geq 300$  mkd, and increased cholesterol, total protein, and prothrombin time at 1000 mkd; females had increased cholesterol and decreased alkaline phosphatase at 1000 mkd. Clinical pathology effects were not toxicologically significant because they were slight and near historical controls. Liver, kidney and thyroid weights were increased, with corresponding histological alterations in the liver, kidneys and thyroid of males and the liver of females. The most significant kidney lesions were degenerative, affected males  $\geq 100$  mkd, and varied in severity from very slight to moderate. Male kidney effects were accompanied by hyaline droplet formation. Hepatocellular hypertrophy occurred in the liver of males at  $\geq 100$  mkd and females at  $\geq 300$  mkd. Hypertrophy of follicular epithelial cells occurred in the thyroid of males at  $\geq 100$  mkd. There were no adverse effects on neurological function. Reproductive effects included treatment-related decreases in mean pup weights in dams given 1000 mkd. A no-observed effect level (NOEL) for general toxicity was not determined, while the NOEL for reproductive effects was 300 mkd and that for neurological effects was 1000 mkd.

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A REVIEW OF THE ROLE OF GENOTOXICITY IN THE DEVELOPMENTAL/REPRODUCTIVE TOXICITY OF AGROCHEMICALS.

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Regulatory agencies in the USA and elsewhere require toxicity tests for agrochemicals including lifetime rodent studies to measure carcinogenicity, short-term tests to evaluate genotoxic potential, as well as developmental/reproductive toxicity studies

in laboratory animals. Compounds previously identified as causing genetic toxicity and/or cancer (as evaluated by short-term assays or lifetime rodent studies, respectively) from databases such as IARC, USEPA, CDPR and the published literature were examined for their effects in developmental/reproductive toxicity studies. Genotoxicity was assessed using the comet, Ames, chromosomal aberrations, micronucleus and UDS (unscheduled DNA synthesis) assays. Two classes of pesticides currently receiving intense regulatory scrutiny, the organophosphate insecticides and the triazine herbicides, have been included. The genotoxicity of fungicides that cause reproductive toxic effects were also examined. This investigation explores the possible role of genotoxicity in causing cancer and developmental/reproductive toxicity. Tumor sites are presented and correlations drawn, where possible, between genotoxic activity and reported oncogenicity. Similarly, correlations between genotoxicity and teratogenicity and effects on reproduction were examined. Epigenetic mechanisms are described to explain cancer and/or reproductive toxicity in the absence of genetic toxicity. For example, pesticides thought to cause cancer and/or reproductive toxicity through cytotoxicity/ mitogenic stimulation, genotoxic impurities, hormonal perturbations, enzyme induction, rodent-specific metabolites, peroxisome proliferation and hypermethylation, are provided.

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#### DETERMINATION OF DART REFERENCE DOSES FOR WORKPLACE CHEMICAL EXPOSURES.

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The protection of the worker from the potential adverse effects of chemicals in the workplace is of importance to both the worker and the worker's employer. As part of a workplace program aimed at protecting workers from reproductive and developmental effects, we have been conducting screening level Developmental and Reproductive Toxicology (DART) assessments, using an approach which focuses on an individual employee's potential exposures. The DART process consists of four parts: exposure assessment, DART hazard identification, risk characterization, and risk management. The exposure assessment includes identifying chemicals to which the worker may be exposed, estimating the duration and frequency of the exposure, and a measure of the level of chemical in the workplace environment. Hazard identification involves a tiered literature search; the first source of information used is usually the American Conference of Governmental Industrial Hygienists (ACGIH) Threshold Limit Value (TLV), but if the TLV limits do not consider DART effects, other data sources such as TOXNET, Material Safety Data Sheets (MSDSs), the Environmental Protection Agency's IRIS database, and the European IUCLID database are considered. A Medline search is also performed to assure that the most recent toxicological information is included in the assessment. After a review of toxicological data, the chemical's DART potential is then classified as either Known, Suspect, Not Suspect, or Insufficient Data. For Known or Suspect chemicals, a DART Reference Dose (DRD) is calculated using either the occupational exposure limit (OEL) or animal data, whichever is more relevant. Using the individual worker's exposure data and the DRD, a hazard quotient is determined. The hazard quotient is then used by company personnel to make informed risk management decisions concerning the need for workplace exposure controls. In the first nine months of 2004, we have performed DART assessments for 15 employees and evaluated 190 chemicals, including solvents, pesticides, and pharmaceuticals.

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#### DEVELOPMENT OF QSAR MODELS TO PREDICT DEVELOPMENTAL AND REPRODUCTIVE TOXICITY OF CHEMICALS.

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Often, there is a lack of experimental toxicity data which can be used for risk assessment for a majority of the chemicals found at contaminated sites. Generating new data would require experts in toxicology to conduct and evaluate the new studies and ethical and social issues involved with animal testing plus the cost and time for these assays would further delay remedial efforts at these sites. Predictive toxicological approaches such as Quantitative Structure-Activity Relationships (QSARs) provide a means to estimate non-carcinogenic toxicity of chemicals. Currently, a few QSAR models have been developed to predict the developmental toxicity (DT) of chemicals, while none have been developed to predict their reproductive toxicity (RT). Two commercial QSAR models (TOPKAT and Multi-Case) for DT, with LOAELs as the endpoint variables and various physicochemical properties of chemicals (descriptors) as the independent variable, have qualitative models to predict the DTs of chemicals. To meet objective, toxicity data on DT and RT for rats (74 chemicals), mice (68 chemicals) and rabbits (16 chemicals) for a total of 158 chemicals have been collected from the literature. The molecular structures were optimized to their global minimum using the CONFLEX module in CAChe (Computer Aided Chemistry) software. The descriptor generator programs

McLennan-Z, CAChe and AMPAC/CODESSA were used to generate the descriptors for the QSAR model. Initially, a single QSAR model for predicting RT and DT will be developed using Human Equivalent Dose (HED), (based on body weight scaling factors) as the dependent variable, and descriptors from the three descriptor generator programs mentioned above as independent variables. If this fails, three separate QSAR models will be developed for each end point for each species.



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#### INTRODUCTION TO SYMPOSIUM: ALTERNATIVE RNA SPLICING: A MECHANISM FOR ENHANCING DIVERSITY OF GENE EXPRESSION.

C. Omiecinski. *Ctr Molec Toxicology, Penn State University, University Park, PA.*

Alternative RNA splicing is an emerging field of molecular science that has significant impact on the toxicological considerations of gene expression and protein function. The diversity of alternatively spliced transcripts has far-reaching significance in terms of understanding interindividual differences in response to xenobiotics, mechanisms of toxicity at the molecular level, tissue-specific toxicity, and the mechanisms for regulating responses to environmental and chemical challenge. This Symposium will provide an overview of basic mechanisms and toxicological significance of RNA alternative splicing, with a focus on alternatively spliced xenobiotic nuclear receptors. Importantly, the session will also address bioinformatics-related issues pertaining to the identification of splice variants, including the design of microarray and genomics platforms that facilitate variant transcript detection.



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#### DISCOVERY AND TISSUE-SPECIFIC MONITORING OF ALTERNATIVE PRE-MNRA SPLICING EVENTS USING INK-JET MICROARRAYS.

J. M. Johnson, J. Castle, P. Garrett-Engele, Z. Kan, L. Lim, C. Armour, C. Raymond and E. Schadt. *Informatics, Rosetta Inpharmatics, Merck & Co., Inc., Seattle, WA.* Sponsor: C. Marcus.

To understand the biological roles, mechanism, and regulation of alternative splicing, systematic methods to monitor the splicing of exons into mature transcripts across different tissues, treatment conditions, and stages of development are needed. Microarrays offer a sensitive and parallel means to monitor transcript structure at high resolution and detect tissue-specific alternative splicing events. We have developed and optimized microarray design, sample amplification, and data analysis methods that allow tissue-specific detection of alternative splicing events using ink-jet microarrays. These tools form a robust and scalable platform we have used for discovery and analysis of alternative splicing at exons and exon-exon junctions in a systematic survey of human genes in over 50 tissues. We have used the results of these experiments to discover novel alternatively spliced isoforms of drug target genes, map the tissue distributions of known isoforms, detect changes in splicing caused by modulation of drug targets, and study the regulatory control of alternative splicing.



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#### FUNCTIONALLY DISTINCT ISOFORMS OF THE FARNESOID X RECEPTOR (FXR).

P. A. Edwards, Y. Zhang and F. Y. Lee. *Biological Chemistry, UCLA, Los Angeles, CA.* Sponsor: C. Marcus.

The farnesoid X receptor (FXR) is a member of the nuclear receptor superfamily that function as ligand-activated transcription factors. FXR binds to FXR response elements together with RXR and is activated by bile acids. The single human and murine FXR genes produce multiple mRNAs as a result of the use of two distinct promoters and alternative RNA splicing. The distinct mRNAs encode four FXR isoforms that are differentially expressed in the liver, intestine, kidney and adrenals and differentially activate specific target genes. We show that the relative activation of target genes is in part dependent upon the presence or absence of four amino acids (MYTG) that lie immediately adjacent to the DNA binding domain of FXR. In order to better understand the function of the different isoforms we incubated hepatocytes, or injected mice, with adenovirus expressing specific FXR isoforms. The results reveal critical roles for FXR isoforms in regulating metabolic pathways.



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#### HUMAN PXR: GENERATION OF DIVERSITY THROUGH ALTERNATIVE SPLICING AND POLYMORPHISM.

E. G. Schuetz. *Pharmaceutical Sciences, St. Jude Children's Research Hospital, Memphis, TN.* Sponsor: C. Marcus.

The pregnane X receptor/steroid and xenobiotic receptor (PXR/SXR, NR1I2) is a nuclear hormone receptor activated by the binding of many endo- and xenobiotics. PXR activates transcription of many drug-detoxification genes, leading to altered

drug clearance and to drug-drug interactions and is highly expressed in the liver and intestine. To understand whether alternative splicing contributes to individual variability and organ specific expression of PXR we analyzed PXR transcripts in 36 types of human tissue. PXR was expressed in many more human tissues than had been previously determined, including bone marrow and select regions of the brain. In each of these tissues, alternative splicing of various PXR exons resulted in transcripts that encoded distinct PXR isoforms. The most abundant alternative mRNAs lacked nucleotides in the ligand binding domain and altered PXR function. We also determined if sequence variation or alternative splicing were associated with allele specific variation in PXR expression using an allelic imbalance assay and those results will be presented. The presence of PXR in many types of human tissues and the activation of PXR by tissue-specific ligands suggest additional biological roles for this receptor.

 **290** FUNCTIONALLY DISTINCT ALTERNATIVE SPLICE VARIANTS OF THE HUMAN XENOBIOTIC RECEPTOR, CAR.

C. Omiecinski, S. Auerbach and M. Stoner. *Ctr Molec Toxicology, Penn State University, University Park, PA.*

The constitutive androstane receptor, CAR, serves as a xenobiotic sensor, especially in hepatic tissues of mammalian species, functioning to transcriptionally up-regulate a variety of biotransformation pathways, including CYP2B6 and UGT1A1. Several alternatively spliced CAR isoforms have been identified recently by our laboratory that we now show are functionally distinct. For example, CAR3 is a prominent CAR splice variant containing a 5 amino acid insertion in its ligand binding domain. Unlike the constitutively active reference form of the receptor, CAR3 displays little or no transactivation activity in the absence of ligand. However, in the presence of the specific CAR ligand, 6-(4-chlorophenyl) imidazo[2, 1-b][1, 3]thiazole-5-carbaldehyde O-(3, 4-dichlorobenzyl)oxime (CITCO), CAR3 is converted into a potent transactivator, and functions to recruit transcriptional co-activators such as SRC-1. In addition to alternatively spliced forms of the receptor, additional CAR diversity is generated through the use of internal translational start codons present in the CAR mRNA. In particular, the use of a methionine start site positioned at amino acid 76 in the receptor generates an N-terminal truncated CAR variant with unique cellular localization potential and modified capacity to activate several nuclear response elements. The diversity of structurally and functionally distinct nuclear receptors enables the expansion of the receptor repertoire, together with an enhanced xenobiotic sensing capability in mammalian systems. Supported by NIH GM66411 and GM32281.

 **291** STEROID RECEPTOR COACTIVATORS PROMOTE COORDINATE TRANSCRIPTION AND ALTERNATIVE SPLICING.

B. W. O'Malley. *Molecular & Cellular Biology, Baylor College of Medicine, Houston, TX* Sponsor: C. Marcus.

Steroid receptor coactivators, such as the SRC-1 family of molecules, are known to amplify nuclear receptor induction of gene expression, acting primarily at the initiation and re-initiation steps of transcription. Many of the currently published coactivators contain enzyme catalytic sites which are involved in chromatin remodeling and in protein modification reactions which alter the integrity of the receptor-coactivator complex at the promoter. We have searched for other applications of cell biology which might be represented by the diverse and rapidly expanding list of coactivators. We have found some novel and interesting functions. We have found that additional downstream subreactions of transcription appear to be carried out by the coactivators. For example, alternative RNA splicing appears to be regulated selectively by steroid receptors and by specific coactivators. Such findings are consistent with the current opinion that transcription and subsequent alternative splicing reactions to produce mature mRNAs are 'coupled' - and that coactivators participate in the coupling. We have tested specific coactivators and have shown them to mediate either exon inclusion or exclusion depending upon the nuclear receptor, the gene and the cell type. Using amino acid mutagenesis of the coactivator CAPERbeta, we demonstrated that specific domains in the molecule mediate the distinguishable effects on transcription initiation and RNA splicing, proving that these molecules are dual regulators of both transcription and RNA splicing. We conclude that nuclear receptor coactivators may provide the mechanism by which hormones can not only determine the quantity of gene product, but also the type of gene product. Considering that ~70% of eukaryotic genes are alternatively spliced, this mechanistic information may have important implications to many physiologic, pharmacologic, and toxicologic processes.

 **292** OVERVIEW OF DIETARY ACRYLAMIDE.

P. M. Bolger. *Center for Food Safety and Applied Nutrition, US Food and Drug Administration, College Park, MD.*

Initial investigations by Swedish researchers of fried and oven-baked foods indicated that acrylamide formation is associated with high temperature cooking processes for certain carbohydrate-rich foods, such as potatoes and cereals. Since then similar findings have been reported by researchers in other countries. The discovery of acrylamide in food is a concern because acrylamide is a potential carcinogen and genotoxin, and a known human neurotoxicant. It does not appear to be present in uncooked food and is present in low or undetectable levels in foods cooked at lower temperatures, such as by boiling. One plausible mechanism responsible for acrylamide formation in carbohydrate-rich foods cooked at high temperatures is the Maillard reaction between asparagine and certain sugars. However, not enough is known about acrylamide formation to identify safe, effective, and practical modifications to food processing techniques that will clearly prevent or reduce formation. Identifying major mechanisms of formation is an important step in identifying ways to reduce or prevent acrylamide formation during cooking. There are significant uncertainties about the impact of dietary acrylamide exposure on public health, since foods reported to contain acrylamide have been consumed for many years. While acrylamide causes cancer in laboratory animals at high doses, it is not clear whether a similar response would occur at the much lower levels found in food. Several epidemiological environmental studies of workplace and dietary exposures have failed to show an increased cancer risk with acrylamide exposure. It is also conceivable that subtle effects can occur on the developing nervous system at acrylamide doses lower than those that have been studied in animals and humans. To better assess the risk of acrylamide information is needed on dietary exposure, bioavailability from food, biomarkers of exposure, and the potential to cause cancer and neurotoxic or neurodevelopmental effects when consumed in food.

 **293** TOXICOKINETICS OF ACRYLAMIDE AND GLYCIDAMIDE IN B6C3F1 MICE AND FISCHER 344 RATS.

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Acrylamide (AA) is a widely studied industrial chemical that is neurotoxic, mutagenic to somatic and germ cells, and carcinogenic in rodents. The recent discovery of AA at ppm levels in a wide variety of commonly consumed foods has energized research efforts worldwide to define toxic mechanisms, particularly toxicokinetics and bioavailability. This study compares the toxicokinetics of AA and its epoxide metabolite, glycidamide (GA), in serum and tissues of male and female B6C3F1 mice and Fischer 344 rats following acute dosing by intravenous, gavage, and dietary routes at 0.1 mg/kg AA or intravenous and gavage dosing with an equimolar amount of GA. AA was rapidly absorbed from oral dosing, was widely distributed to tissues, was efficiently converted to GA, and increased levels of GA-DNA adducts above background were observed in liver after complete elimination from serum. GA dosing also resulted in rapid absorption, wide distribution to tissues, and produced liver GA-DNA adduct levels that were higher than those from AA. While oral administration was found to attenuate AA bioavailability from the diet and from aqueous gavage, a first-pass effect or other kinetic change resulted in higher relative metabolism to GA in both mice and rats when compared to the intravenous route. A similar effect on relative GA exposure was also evident as the administered dose of AA was reduced, which suggests that as dosing rate decreases, the conversion of AA to GA is more efficient. These findings are critical to assessment of genotoxicity of AA at low doses in the food supply, which appears to depend on total exposure to GA.

 **294** REFERENCE VALUES IN THE ACRYLAMIDE IRIS ASSESSMENT DEVELOPED BY THE USEPA.

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The USEPA (USEPA) has developed an electronic database known as the Integrated Risk Information System (IRIS) that contains information on human health effects that may result from exposure to various chemicals in the environment. IRIS provides a portal to health hazard identification and dose-response assessment information. Quantitative dose-response characterization is used in conjunction with exposure data to estimate the risk to public health from exposure to a given chemical and to support risk management decisions. The on-going IRIS assessment for acrylamide contains descriptive hazard identification and quantitative information in the following categories: 1) oral reference doses and inhalation reference concentrations (RfDs and RfCs, respectively) for chronic noncarcinogenic health effects; and 2) oral and inhalation unit risks for carcinogenic effects. The noncancer reference values represent an estimate of a daily exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious noncancer effects during a lifetime. The previous IRIS assess-

ment for acrylamide (from 1988) provided quantitative estimates of carcinogenic effects from oral or inhalation exposure to acrylamide (drinking water unit risk and inhalation unit risk, respectively), and an RfD for neurological effects, but no RFC. The current assessment proposes revision to the reference values based upon more recent data from noncancer studies and an additional two-year carcinogenicity study. An RFC is also derived based upon a route-to-route extrapolation from the oral data. A number of on-going research projects are addressing data needs on the toxicokinetics and health effects of acrylamide to further improve the scientific basis of the reference values, and to reduce uncertainty. [This abstract does not necessarily reflect EPA policy.]

 **295** DIETARY ACRYLAMIDE AND RISK OF HUMAN CANCER: THE ROLE OF EPIDEMIOLOGY.

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In April 2002, the Swedish National Food Administration reported the finding of elevated levels of acrylamide in commonly consumed baked and fried foods. Despite the classification of acrylamide as a probable human carcinogen, there remains a paucity of epidemiological studies examining whether dietary acrylamide could increase cancer risk. In this talk, we summarize the existing data from three case-controls studies that have examined the association between dietary acrylamide and risk of cancers of the large bowel, kidney, and bladder. We highlight results from the first two prospective studies to assess the association between dietary acrylamide and cancer risk. In the first, we assess the association between exposure to acrylamide in diet and breast cancer risk among 43,404 Swedish women aged 30-49 in the Swedish Women's Lifestyle and Health Cohort. This contributed 435,000 person-years, from which 578 breast cancer cases occurred. In the second study, we reexamine whether acrylamide in foods is related to colorectal cancer risk in the Swedish Mammography Cohort, a prospective study of 61,467 Swedish women who were followed on average for 10 years. In this cohort, 396 cases of colon cancer and 193 cases of rectal cancer occurred. Mean intake of acrylamide in the two cohorts was 26 and 25 micrograms per day, respectively. Finally, we illustrate the strengths and limitations of epidemiological studies to examine dietary acrylamide in the context of human cancer.

 **296** AN INTERNATIONAL SAFETY/RISK ASSESSMENT OF ACRYLAMIDE.

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Acrylamide was the subject of an evaluation in February 2005 of an international committee of scientists the Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA). JECFA serves as a scientific advisory body to FAO, WHO, their member states and the Codex Alimentarius Commission regarding the safety of contaminants in food moving in international trade. The conclusions of JECFA deliberations provided to the Codex influence world trade and thus have a significant economic impact on trading countries. JECFA monographs are also valuable sources of information to the international public health and scientific communities. For acrylamide, JECFA evaluated data available to date on neurotoxicity, germ cell toxicity, genotoxicity, and epidemiology, as well as studies on metabolism, biomarkers and pharmacokinetics of acrylamide in humans and rodents. The conclusions of the deliberations of the JECFA will be presented particularly regarding levels of exposure to acrylamide in human foods which may reasonably be expected to be safe as well as possible public health risks associated with these exposure levels.

 **297** ENVIRONMENTAL FACTORS AFFECTING BREAST CANCER SUSCEPTIBILITY.

S. Fenton. *USEPA, Research Triangle Park, NC.*

Breast cancer is still the most common malignancy afflicting women in the Western world. Although substantial progress has been made in elucidating some of the genetic contributors to breast cancer (i.e., the highly penetrant susceptibility genes, BRCA1 and 2), it has been estimated that only 6-12% of all breast cancer cases are due to heritable factors. Few other specific factors have been identified that contribute significantly to an individual's lifetime risk of breast cancer. It has become apparent that elements affecting cancer susceptibility (genetic or environmental components) cannot be considered separately. Environmental factors (e.g., industrial compounds, pharmaceuticals, diet, occupational hazards) have been identified in both epidemiological and rodent studies that alter breast development and tumor formation. These constituents may act as either a mutagen or as a developmental compound able to alter susceptibility to carcinogens. In addition to the contributions of environmental factors, a large percentage of cancer cases are due to sporadic mutations that may occur as a result of spontaneous genetic events, and

the interactions between gene and environmental factors. A relatively recent focus in the breast cancer field is on the interaction between genes and environment as the causal mechanism in the disease. Primary candidates for gene-environment interaction studies have been genes that encode enzymes involved in the metabolism of established cancer risk factors and those involving oxidative stress response. There are common varying forms of these genes (polymorphisms) that may directly result in impacting the risk of cancer by altering normal metabolism, circulating hormone levels, ability to respond correctly to normal stressors, or response to environmental factors. We will present data from both epidemiological and rodent studies demonstrating the importance that environmental factors play in breast cancer susceptibility. These studies will elucidate the importance of evaluating gene-environment interactions and the various environmental factors, such as diet and endocrine disrupting chemicals, on breast cancer risk assessment.

 **298** GENE-ENVIRONMENT INTERACTIONS IN THE ETIOLOGY OF BREAST CANCER.

C. Ambrosone. *Roswell Park Cancer Institute, Buffalo, NY.* Sponsor: S. Fenton.

The observation that breast cancer rates rise in women who migrate from low risk to high risk countries (e.g., from Japan to the US), and temporal and geographic patterns within the US, indicate that environmental factors are likely associated with breast cancer etiology. A role of environmental carcinogens in breast cancer is also supported by numerous animal studies. However, there has been little support in the epidemiologic literature for increased breast cancer risk with exposure to environmental factors, other than some limited data on diet. We, and others, have suggested that clear associations are masked by evaluation of heterogeneous populations, and that risk with environmental exposures may only be evident among susceptible subsets of the population, based on genetic variability in metabolic and other enzymes. The evidence for gene/environment interactions in breast cancer etiology, with examples from diet and oxidative-stress related genes and, particularly, exposure to PCBs and associated genetic variability in cytochrome P450 1A1 shall be the focus of this presentation.

 **299** INFLUENCE OF ENDOCRINE DISRUPTING COMPOUNDS (EDCS) ON MAMMARY GLAND DEVELOPMENT AND TUMOR SUSCEPTIBILITY.

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Breast cancer risk in women is known to be influenced by genetics and prolonged exposure to estrogen. However, when known risk characteristics are considered, more than 50 percent of breast cancer cases remain unexplained. There is a growing body of evidence indicating that exposures to toxic chemicals and hormone-mimicking compounds may contribute to the development of breast cancer. Although epigenetic in nature, xenobiotics that act in an estrogenic or anti-androgenic manner may hasten development of the gland and increase the incidence of mammary tumors if they significantly alter serum estradiol levels, or if they in some way alter receptor response to endogenous estradiol levels. Nonylphenol is an example of an EDC that we have shown to hasten mammary gland (MG) development following acute *in utero* exposure. Delayed development of the MG can also be caused by *in utero* exposure to EDCs, resulting in imprinting effects in the offspring. This type of delayed glandular development could lead to increased tumor formation via an enlargement of the window of sensitivity to carcinogens. For example, a toxicant may delay MG development so that undifferentiated or dividing cells may be present for longer periods of time, thus rendering the tissue more vulnerable to a subsequent genotoxic insult. The herbicide atrazine and the polycyclic aromatic hydrocarbon, dioxin, are examples of compounds that delay MG development in rodents. These delays have been detected as early as postnatal day 4 and persist throughout sexual maturity in prenatally exposed female rats. Windows of sensitivity have been identified that lead to stunted epithelial migration and branching patterns, and extended presence of terminal end buds. These multilayered structures are sensitive to carcinogens, such as 7, 12-dimethylbenz[a]anthracene, and exposure to such agents during critical windows of susceptibility can lead to increased multiplicity or decreased latency to tumor formation. (This is an abstract of a proposed presentation and does not necessarily reflect EPA policy)

 **300** EARLY LIFE DIETARY ESTROGENIC EXPOSURES AND LATER SUSCEPTIBILITY TO MAMMARY TUMORIGENESIS.

L. A. Hilakivi-Clarke, B. Yu and M. Martin. *Oncology, Georgetown University, Washington, DC.* Sponsor: S. Fenton.

Some breast cancers may originate *in utero* as a result of an exposure to a high fetal estrogenic environment that alters normal mammary gland development and gene expression patterns. We have found that the expression of estrogen receptor (ER) -  $\alpha$  is increased in the epithelial and stromal compartments of the developing rat

mammary gland by *in utero* estradiol exposure, and this is not compensated by an up-regulation of genes that repair DNA damage, such as p53 or Brca1. We also have studied whether early life dietary exposures that modify estrogenic environment affect later breast cancer risk. For example, *in utero* dietary exposure to flaxseed that contains plant estrogens (lignans), n-Ω polyunsaturated fatty acids (PUFAs) and fiber, all of which have been previously linked to reduced breast cancer risk, surprisingly increased later carcinogen-induced mammary tumorigenesis and ER-α expression in the rat mammary glands. To identify the component in flax that may be responsible for the increased mammary tumorigenesis, we fed pregnant rats with n-Ω PUFAs or fiber. These dietary exposures reduced mammary tumorigenesis, and *in utero* exposure to fiber obtained from a whole wheat diet additionally induced a persistent up-regulation of tumor suppressors Brca1 and p53. Since the protein component of flaxseed accumulates the heavy metal cadmium to levels exceeding the maximum guidelines set by the World Health Organization, this metal might be the culprit. Cadmium and several other heavy metals bind and activate ER-α, and at human exposure levels, increase experimental mammary tumorigenesis. *In utero* exposure to heavy metals via maternal diet increases breast cancer risk in an animal model. These findings suggest that when assessing the potential effects of *in utero* dietary exposures on later susceptibility to develop breast cancer, the possibility that specific dietary components have accumulated heavy metals or contain other environmental estrogens, should be taken into account. Fetal exposure to these environmental contaminants may play a role in modifying breast cancer risk in adulthood.

### 301 DNA DAMAGE/REPAIR IN HUMAN BREAST CANCER RISK.

**J. J. Hu.** *Cancer Biology, Wake Forest U. School of Medicine, Winston-Salem, NC.*

Breast cancer accounts for nearly one-third of all cancer cases diagnosed in American women. It is estimated that about 5% of breast cancer cases are related to rare but highly penetrant genes, such as BRCA1 and BRCA2. However, low-penetrant cancer susceptibility genes may contribute to a large proportion of breast cancer cases because many of them are very common. Mammalian cells are constantly exposed to a wide variety of genotoxic agents from both endogenous and exogenous sources. Ionizing radiation, an established etiologic factor for breast cancer, and other suspected risk factors, such as chemical carcinogens, alcohol, estrogen, and diet, produce reactive oxygen species, oxidized bases, bulky DNA adducts, and DNA strand breaks. Mammalian cells have evolved distinct pathways to repair different types of DNA damage and maintain genomic integrity. Inherited reduction of DNA repair capacity may lead to deletions, amplifications, and/or mutations of critical genes that contribute to breast carcinogenesis. Previous studies have shown that deficient DNA repair and elevated DNA damage contribute to human breast cancer risk. In addition, recent data suggest that DNA-repair single-nucleotide polymorphisms (SNPs) may contribute to deficient repair function and elevated breast cancer risk. Although the functional consequences of these variant alleles may result in small changes in catalytic activity and thus a small increase in cancer risk on an individual basis, the presence of these variant alleles at polymorphic frequencies in the human population may contribute to high proportion of breast cancer cases. Furthermore, multiple DNA-repair pathways are required to maintain genome integrity, and many genes are involved in different repair pathways. For example, we provide evidence that variants of XRCC1, XRCC3, and ERCC4/XPF genes in three different repair pathways, particularly in combination, contribute to breast cancer susceptibility. In conclusion, genotypes and phenotypes in different repair pathways must be evaluated simultaneously in order to fully assess breast cancer susceptibility.

### 302 ROLE OF OXIDANT STRESS IN THE ACTIVATION OF GROWTH FACTOR SIGNALING PATHWAYS IN HUMAN BREAST EPITHELIAL CELLS BY ENVIRONMENTAL POLYCYCLIC AROMATIC HYDROCARBONS (PAHS).

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<sup>1</sup>*College of Pharmacy, University of New Mexico, Albuquerque, NM,* <sup>2</sup>*Center for Molecular Toxicology, Penn State University, University Park, PA* and <sup>3</sup>*Worldwide Safety Sciences, Pfizer Global Research and Development, St. Louis, MO.*

There is great interest in determining mechanisms by which environmental agents contribute to the increasing incidence of human breast cancer. Benzo(a)pyrene (BaP) is an important environmental carcinogen that exerts both genotoxic and non-genotoxic actions. It is a complete carcinogen that is present in emissions from the burning of fossil fuels, woodsmoke, tobacco smoke, and in the diet where it is associated with charcoal-broiled foods. Recent work has shown that BaP exerts novel actions on the epidermal growth factor receptor (EGFR) and insulin-like growth factor (IGF) signaling pathways. Studies were performed in the human mammary epithelial cell line MCF-10A, but results have also been validated in pri-

mary cultures of normal human mammary epithelial cells (HMEC). BaP and certain AhR ligands (TCDD) protect MCF-10A cells from growth factor withdrawal-induced apoptosis. Certain metabolites of BaP, most notably BaP-quinones (BPQs) stimulate cell proliferation through an oxidant stress and redox-cycling mechanism. Stimulation of cell proliferation and inhibition of cell apoptosis are both important non-genotoxic mechanisms of tumor promotion and progression. This presentation will review the background on signaling pathways activated by PAHs and will provide new data on the toxicogenomic analysis of genes altered by BPQ treatment in MCF-10A treated cells. A conceptual basis for differential susceptibility of women to PAH-induced breast cancer will be presented based upon knowledge of polymorphisms present in PAH-activating and PAH-deactivation genes. Individual susceptibility to gene-environment interactions may be based upon these toxicogenetic differences. Supported in part by RO1 ES07925 and P30 ES012072

### 303 THE MULTI-SITE AMBIENT PARTICLE STUDY (MAPS): AN INTEGRATED APPROACH TO STUDYING HEALTH EFFECTS OF PM COMPONENTS.

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<sup>2</sup>*Environmental Medicine, New York University, Tuxedo, NY.*

The World Health Organization estimates that particulate air pollution (PM) is responsible for more than 500, 000 deaths worldwide each year. A large number of epidemiology studies have associated PM mass with increased mortality, and the EPA currently regulates PM on the basis of mass in different size ranges. However, recent studies suggest that PM derived from different sources may differ in toxicity and that specific PM components may serve as markers for different sources, suggesting an alternative, more efficient way of regulating PM. The overall objective of MAPS was to collect particles from several different geographical regions, characterize their physical and chemical properties, and make them available to investigators for *in vitro* and animal instillation health studies that can relate health effects with PM components and ultimately sources. Airborne particles in the ultrafine, fine, and coarse size ranges were collected in eight different locations in the US and Europe. The sites were selected to take advantage of regional differences in PM sources and components. Weekly samples were collected for a period of a month in each location, using a 3 stage particle impactor (developed at Harvard University) which is capable of collecting several mg of material during a weekly sampling interval. The particles were then assayed for a number of chemical components and made available to investigators in several different laboratories. This symposium will describe some of the studies which have characterized health effects associated with PM and PM components from each of the different geographical locations. Relating adverse health effects to specific PM size modes and specific PM chemical components is the first step towards relating these effects to PM derived from specific sources. This will ultimately allow the EPA to more effectively implement PM standards, thereby reducing not only the health impacts now associated with PM, but also their substantial impacts on quality of life and the national economy.

### 304 SAMPLE CHARACTERIZATION AND SOURCE APPORTIONMENT OF AMBIENT PARTICULATE MATTER: COMBINING ATMOSPHERIC SCIENCE AND LUNG TOXICOLOGY.

**J. M. Veranth<sup>1</sup>, G. S. Yost<sup>1</sup>, J. C. Chow<sup>2</sup> and J. G. Watson<sup>2</sup>.** <sup>1</sup>*Pharmacology and Toxicology, University of Utah, Salt Lake City, UT* and <sup>2</sup>*Atmospheric Sciences, Desert Research Institute, Reno, NV.*

**Rationale and Scope:** Ambient particulate matter (PM) chemical composition varies with time and location, and soil dust is a significant component of PM in arid regions. The USEPA and the IMPROVE network routinely measure PM composition. In principle, the PM collected at a receptor site is a linear combination of the contributions from the sources. Source apportionment by chemical mass balance is an established technique for regional airshed management. An issue is whether the observed health effects of PM are due to characteristics shared by all particles (e.g., mass, surface area, adsorbed gas-phase species) or are due to specific chemical species (e.g., metals, PAH, acids, peroxides). **Methods:** A database of dusts from over 300 soils representing various geographical locations and land uses was analyzed. *In vitro* studies are correlating cytotoxicity and cytokine release responses of lung epithelial cells (BEAS-2B) with the speciation data on soil-derived dusts. **Results:** The database analysis provided statistics on mean, S.D., and outliers for each reported chemical species. The cell culture studies indicated that some sites produced dust that induced IL-6 release 10-fold over control, while other sites produced dust that was highly cytotoxic, but that did not induce cytokine release, and other sites produced dust that appeared benign. Endotoxin did not appear to be the source of the proinflammatory signaling responses. **Conclusion:** Soil-derived particle from different sources induce different responses in a widely-used *in vitro* toxicology model. Correlating *in vitro* cell responses with PM composition data is ex-

pected to yield new insights into the relationship between pollution sources and proinflammatory signaling in the lung. This work was supported by NIEHS K25 ES011281, SERDP CP1190, and SCERP EH-03-03.

## 305 EFFECTS OF AMBIENT PM ON OXIDATIVE STRESS AND SIGNALING PATHWAYS.

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Recent studies suggest that PM derived from different sources may differ in toxicity. The goal of this study is to characterize the *in vitro* effects of ambient PM and PM components from eight different locations in the US and Europe focusing on oxidative stress and related signaling pathways. Airborne particles in the ultrafine, fine, and coarse size ranges were collected in these locations for a period of a month in each location using a Harvard 3-stage particle impactor. A respiratory epithelial cell line (BEAS-2B) stably transfected with NF-κB-luciferase reporter plasmid was used in this experiment. BEAS-2B NF-κB cells were cultured in DMEM medium supplemented with 10% FBS, 100u/ml Penicillin, 100μg/ml streptomycin, 2mM L-glutamine in a 96 well plate at a density of  $9 \times 10^4$ /well and cultured up to 95% confluence. These cells were exposed to 0, 100, 300, and 500 μg/ml PM for 24 hrs. Immediately after the exposure, cells were rinsed and lysed with Luciferase Cell Cytotoxicity Reagent and the lysate analyzed for NF-κB reporter activities using a luminometer. The associations between NF-κB activity of BEAS-2B cells treated with particles and the major particle compositions were examined. Specifically, the response variable in log scale is regressed against the proportions of elemental concentrations using a mixture model. Stepwise procedures in S-PLUS were applied to find a parsimonious subset of explanatory variables to include in least square multiple regressions. The residuals from the final linear models were used to diagnose the regression model assumptions. The variations of the response variable (NF-κB activity) can be explained by the mixture of elements and amount of other compositions (mass minus the sum of all elements measured) with the adjustment of other covariates (locations and particle size fraction).

## 306 EFFECTS ON CYTOKINE PRODUCTION BY MACROPHAGES, ENDOTHELIUM AND EPITHELIAL CELLS.

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Using primary cultures and indicator cell lines that we have created, we have examined the effects of real world and laboratory generated particles of varying size and composition on the ability to induce gene transcription via oxidant mechanism. Mouse and human macrophages, endothelial and epithelial cells and cell lines including cell lines that have been stably transfected with an IL-8 promoter luciferase construct, were cultured in the presence of varying concentrations of coarse, fine and ultrafine size particles collected by high volume samplers at multiple sites in North America to establish a relationship between changes in gene expression and oxidant stress and size and source of ambient particles. The concentrations range from 0.5 to 19 μg/cm<sup>2</sup>. Response to these materials are assessed by measuring production of cytokines and mediators (IL-8, TNF, IL-6 MIP-2) that reflect the range of cytokine responses by these cells. Alternatively, activity of the materials was assessed by their ability to activate promoter activity of a transfected reporter. From this data we can determine the relative activity of these samples as they relate to size and composition and potential source contributions. Additionally this indicated the relative sensitivity of the different cell types to different particles. Data of this type may be helpful in comparing the role of various components of ambient PM in the induction of disease in a susceptible host. Supported by EPA Star PM Center R-827354 and P30 ES-01247.

## 307 HEALTH EFFECTS OF PARTICLES FROM TRAFFIC-RELATED AMBIENT AIR POLLUTION ON RESPIRATORY ALLERGY AND INFLAMMATION: A EUROPEAN MULTISITE STUDY.

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Airborne particulate matter represents a complex mixture of organic and inorganic substances varying in size and composition. Particulate matter has been demonstrated to exacerbate asthma in all age groups. In addition, increases are likely in

lower respiratory symptoms and reduced lung function in children, and chronic obstructive pulmonary disease and reduced lung function in adults. Further, studies have documented an increased risk of developing lung cancer by long-term exposure to particulate matter. This paper will summarize the results of two multi site studies in Europe: Respiratory Allergy and Inflammation Due to ambient Particles (www.RAIAP.org) and Health Effects of Particles from Motor Engine Exhaust and Ambient Air Pollution (www.HEPMEAP.org). Toxicological studies show that these effects are brought about through factors such as lung inflammation, heart rate variability, changes in blood viscosity and oxygen deprivation. (1) PM2.5-10 and PM0.1-2.5 samples collected throughout Europe vary considerably in their composition and their bio-reactivity. This suggests that the PM2.5 and PM10 mass parameters are relatively crude in relationship to biological events and expected not to have strong correlations with health outcomes. (2) Though PM activities vary at a given site with time, clear regional differences in activity profiles can be identified. These differences partly relate to traffic patterns. (3) Ambient particles, at least the fine fractions, stimulate both an allergic and a non-allergic immune response. (4) Coarse and fine PM samples appear to have similar toxic activities *in vitro* and *in vivo* on a per mass unit basis, though the mechanisms and sites of action differ. The findings of the studies show clear toxicity of coarse PM, which underlines the need to consider coarse as well as fine PM in risk assessments and standard setting.

## 308 EFFECT OF SIZE FRACTIONATED AMBIENT PM SAMPLES ON INDUCTION OF PULMONARY ALLERGY IN MICE.

M. Gilmour. *NHEERL, USEPA, Research Triangle Park, NC.*

There is increasing evidence that exposure to certain air pollutants including ozone and diesel exhaust can enhance allergic sensitization to allergens. Previous work from our laboratory has shown that exposure to residual oil fly ash or its associated transition metals can enhance production of IgE antibody and T-helper 2 lymphocyte activity, resulting in increased allergic lung disease. This effect was associated with increased production of TNF-alpha in the lungs, and could be blocked with antibodies against this cytokine. We have also demonstrated that PM extracted from filters from two German cities with high and low incidence of allergic illnesses has differential effects on exacerbation of allergic biomarkers. Higher allergic responses in mice treated with the PM sample from the city with high incidence of allergies were associated with increased abundance of metal in that PM sample. Additional studies comparing the effect of two different diesel samples on the development of allergic lung disease have shown that an automotive diesel that is rich in hydrocarbons has stronger pro-adjuvant effects than a more carbonaceous diesel sample derived from a forklift truck. This presentation will describe studies investigating the relative potency of size fractionated ambient samples from cities in the U.S. and Germany to enhance induction of allergic immune responses to ovalbumin antigen in mice. Endpoints will include antigen specific and total IgE antibody in serum and lung fluid, pulmonary immune cytokines and patho-physiological markers of allergic lung disease. This abstract does not reflect EPA policy.

## 309 BROMINATED FLAME RETARDANTS: NEW FINDINGS.

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The brominated flame retardants are new potential public health hazards. They resemble PCBs and are found worldwide. They can be found in carpets, styrofoam, and in household and office electrical equipment. Brominated flame retardants (BFRs), especially the common polybrominated diphenyl ethers (PBDEs) have recently been reported in humans at markedly increasing levels in blood and milk. The highest levels in humans and food worldwide have been reported in US samples. Studies of milk report 10-100 times higher levels in US than in European women. US food levels are highest in fish, then meat, and finally dairy. All human and food samples measured to date are contaminated with many of the 13 commonly reported PBDE congeners, although the congener pattern can vary in different specimens. Blood and milk levels in the USA are currently similar. PBDEs cannot be measured in 1973 blood, and are increasing markedly in humans in the USA (at the same time dioxins and PCBs are decreasing). No human health studies of these compounds have been published but cell culture and laboratory animal studies suggest certain adverse health effects, similar to those reported for PCBs which the PBDEs structurally resemble. New findings will be presented for human tissue levels and food levels in the USA and worldwide in the general population and in specially exposed workers. The uncertainty regarding toxicity of various congeners will be reviewed and compared to the dioxin toxic equivalency factor concept. No such toxicity comparison factors are available for the PBDEs at the present time

making it difficult to decide which congeners should be measured. There is currently no consensus on which to be measured; this topic will be reviewed and recommendations made. Temporal trends in human PBDE levels will be presented and compared with temporal trend for dioxins, dibenzofurans and PCBs. New toxicological findings with respect to pharmacokinetics, neurological, reproductive and developmental, endocrine, and cancer endpoints will be presented. Finally, human risk assessment will be considered.

### 310 PBDES IN US HUMANS, FOOD AND ENVIRONMENTAL SAMPLES.

A. J. Schecter<sup>1</sup>, O. Paepke<sup>2</sup>, J. Ryan<sup>3</sup>, L. Birnbaum<sup>4</sup>, D. Staskal<sup>5</sup> and K. Tung<sup>1</sup>.  
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Polybrominated diphenyl ether (PBDE) flame retardants were measured in US human milk and blood, food and environmental samples. All 59 milk samples were positive for multiple congeners measured. Milk was collected beginning in 2001 from various locations in the US. PBDEs varied from 6.2 to 419 ppb lipid. In blood, we found levels from 4 to 366 in our first 39 individual analyses. In this series men had lower levels than women, although not statistically significant. Two samples of pooled blood from 100 people each had levels of 78 and 80 ppb lipid in 2003, similar to that observed in pooled serum from 100 people the same year, 62 ppb lipid. These levels are a magnitude higher than previously observed in some earlier European studies. In contrast, the concentration in a pooled archived serum sample from 100 people in 1973 was only 0.8 ppb lipid. This increasing trend is opposite to that observed for dioxins and PCBs. 44 foods of animal origin from Texas supermarkets all tested positive for multiple congeners, including the fully brominated BDE 209 congener, with highest levels in fish (especially salmon), followed by meat and then dairy. US levels are higher than published European and Japanese studies. Daily intake of PBDEs was estimated to be 163, 200 pg/kg body weight daily in US nursing infants during the first year of life and 11, 000 pg/kg daily in Germany, approximately 3, 826 pg/kg body weight for ages 2-5, and approximately 1, 300 pg/kg BW daily for ages > 19 years. Unlike dioxins and PCBs where almost all intake is from the route of consumption of animal food, it has been speculated that respiratory and dermal intake may contribute a greater portion of intake for PBDEs. Therefore, we also measured PBDEs in swipes from computer and computer monitor casings and in carpet vacuum sweepings. All samples were positive. Comparing our findings with other recent studies, we conclude that the highest levels of PBDEs in human milk, blood and in food worldwide exist in the USA. (This abstract does not reflect USEPA policy.)

### 311 TOXICOKINETICS OF BDE 47 IN MICE.

D. Staskal<sup>1</sup>, J. J. Diliberto<sup>2</sup>, M. J. DeVito<sup>2</sup> and L. S. Birnbaum<sup>2</sup>. <sup>1</sup>*UNC Curriculum in Toxicology, Research Triangle Park, NC and* <sup>2</sup>*ETD, NHEERL, ORD, USEPA, Research Triangle Park, NC.*

Polybrominated diphenylether (PBDE) congener patterns found in environmental samples and human tissue are not consistent with congener patterns found in the commercial PBDE mixtures; it is therefore essential to understand the toxicokinetic properties associated with the individual PBDE congeners. Currently, knowledge of the absorption, distribution, metabolism, and excretion is restricted to a small number of congeners which have been examined in limited *in vivo* and *in vitro* models. In general, the toxicokinetic parameters of PBDEs are dependent on the degree of bromination. With the exception of the fully-brominated congener, the PBDEs appear to be well absorbed, slowly metabolized, and distribute to lipophilic tissues. For 2, 2', 4, 4'-tetrabromodiphenyl ether (BDE 47), the major congener found in human and wildlife samples, differences in excretion rates and routes have been reported between rats and mice. In this study, the distribution and excretion of a single, oral dose (1 mg/kg) of BDE 47 was examined in adult, female C57BL/6 mice for 21 days following administration. BDE 47 was found in all tissues at all time points and distribution was dictated by lipophilicity. Elimination of BDE 47 appears to be biphasic. Highly perfused tissues have  $\alpha$  and  $\beta$   $t^{1/2}$  values of approximately 1 and 10 days, respectively. Elimination from lipophilic tissues was roughly five times slower. Although the terminal half life is long, the majority of the dose (>80%) is eliminated during the  $\alpha$ -phase. The rapid excretion of unmetabolized BDE 47 and other congeners in urine and feces suggests the presence of an active transport mechanism which may play a major role in understanding the species differences in elimination; initial results from renal transport studies support this hypothesis. Characterization of these toxicokinetic parameters will provide data needed for the development of a PBPK model which can be used in human health risk assessment. (This abstract does not reflect EPA policy. This work was partially funded by EPA NHEERL-DESE CT826513).

### 312 DEVELOPMENTAL NEUROTOXICITY OF PBDES IN MICE AND RATS.

H. Viberg, A. Fredriksson and P. Eriksson. *Environmental Toxicology, Uppsala University, Uppsala, Sweden.*

Our environment contains a vast number of contaminant including the brominated flame retardants, polybrominated diphenyl ethers (PBDEs). The PBDEs are widely found in the environment and is increasing in human milk. This means that an individual can be exposed to PBDEs during its whole lifetime, including the lactation period. We have examined the neurotoxic effects of exposure to PBDEs during a defined critical period of rapid brain development in neonatal mice and rats. The neonatal period is characterized in many mammalian species by rapid development of the immature brain. It has been shown that numerous toxicants can induce permanent disorders in brain function when administered to the neonatal mouse during the brain growth spurt (BGS). In mice and rats this period is postnatal, spanning the first 3-4 weeks of life and in the human, the BGS begins during the third trimester of pregnancy and continues throughout the first two years of life. Our studies have identified a defined critical period during the BGS in mice when the brain is vulnerable to insults of low doses of PBDEs. It has also been shown that it is the presence of PBDEs or their metabolites in the brain during this defined critical period that is crucial to evoke neurotoxic effects. Exposure to PBDEs during the short critical period of brain development leads to permanent altered spontaneous behaviour, deficits in learning and memory and disturbances in the cholinergic system, in the adult. These effects are dose-response related and tend to get worse with age. The potency of PBDEs to induce neurotoxic effects does not appear to be gender, strain or species specific, because the neurotoxic effects are induced in both male and female mice of different strains and in rats. In conclusion, the observed developmental neurotoxic effects of PBDEs are similar to the effects seen after neonatal exposure to certain PCBs and the presence of both these compounds in the environment and in human milk suggests that further attention should be focused on the neurotoxicology of PBDEs.

### 313 PBDE LEVELS AMONG US WOMEN, DAILY INTAKE AND RISK OF HARM TO THE DEVELOPING BRAIN AND REPRODUCTIVE ORGANS.

T. A. McDonald. *Office of Environmental Health Hazard Assessment, California Environmental Protection Agency, Oakland, CA.* Sponsor: L. Zeise.

The polybrominated diphenylethers (PBDEs) are a class of flame retardants widely used in electronics, furniture and textiles. PBDEs slowly migrate from these products and are now ubiquitous, measured in indoor and outdoor air, home and office dust, streams and lakes, and terrestrial and marine biota. Like PCBs and dioxins, many PBDEs are persistent and bioaccumulative, and are long lived in the body. The more bioaccumulative commercial mixtures of PBDEs have been recently banned in California, Maine and Hawaii and the European Union. Tissue levels among residents of North America are approximately 10 to 40 times higher than those of individuals in Europe or Japan, and recent data suggest that PBDE body burdens continue to rise over time in North American wildlife and humans. Five PBDEs namely, PBDE-47, 99, 100, 153, and 154, comprise more than 90 % of the PBDEs measured in US residents. The primary health concerns of PBDEs appear to be developmental effects, including harm to the developing brain and reproductive organs. PBDE levels in serum, adipose tissue, or breastmilk have been reported for individuals in six groups of women in the US. The distribution of lipid-normalized PBDE concentrations among women residing in the US was determined to be lognormal with a mean value of 87.6 ng/g and a 95th percentile estimate of 304 ng/g. Using the US body burden data and congener-specific estimates of half-lives in humans, daily intake of the sum of the five most prevalent PBDEs in people (PBDE-47, 99, 100, 153, 154) was estimated to be 4.8 ng/kg-d (median), 11 ng/kg-d (mean), and 38 ng/kg-d (95th percentile). Finally, the potential health risks posed by the PBDEs are addressed by comparing tissue concentrations in humans to estimated and measured tissue concentrations in rodents associated with developmental neurotoxicity and reproductive effects. Assuming effects in rodents are predictive of human health, the comparison suggests that the current margin of exposure is small for a significant portion of the population.

### 314 DEVELOPMENTAL TOXICOLOGY EVALUATIONS: ISSUES WITH INCLUDING NEUROTOXICOLOGY AND IMMUNOTOXICOLOGY ASSESSMENTS.

G. S. Ladics<sup>1</sup> and L. Burns-Naas<sup>2</sup>. <sup>1</sup>*DuPont Co., Newark, DE and* <sup>2</sup>*Pfizer, Inc., San Diego, CA.*

Evaluation of offspring following maternal exposures during gestation and lactation (i.e. reproductive/developmental toxicology [RDT]) has historically been a routine part of the safety assessment process. Recently, increased attention has focused on the effects of agricultural and industrial chemicals, as well as pharmaceuticals, on

the developing nervous and immune systems of the fetus and newborn. This new focus on developmental neurotoxicology (DNT) and developmental immunotoxicology (DIT) is based on the premise that the developing nervous and immune systems may be qualitatively and/or quantitatively more susceptible to chemical perturbation compared to the adult and studies conducted currently may be insufficient to protect the young. DNT studies have become common for agricultural chemicals, following the preparation of test guidelines from the USEPA (OPPTS 870.6300, 1998) and OECD (TG 426, draft). With increased DNT testing and the prospect of new DIT test guidelines, there has been considerable interest in both DNT and DIT, with many scientific workshops, roundtables, symposia, as well as sponsored research devoted to the subjects. The intent of this session is to highlight and discuss issues that are common to RDT, DNT, and DIT, including the consequences of high dose selection and maternal toxicity; the adequacy of pup exposure during lactation; whether a different dosing paradigm should be applied to RDT vs. DNT or DIT studies; whether DIT and DNT endpoints can be incorporated into a single (RDT) study for hazard identification purposes (e.g., for screening purposes, what endpoints have proven their value and should be retained). This session will provide a forum to discuss how assessment of RDT, DIT, and DNT could be integrated for hazard identification purposes and to reduce animal usage.

### 315 INTERVIEWING SKILLS FOR GRADUATE STUDENTS AND POST-DOCS.

R. J. Mitkus<sup>1</sup> and M. Pomeroy-Black<sup>2</sup>. <sup>1</sup>*Epidemiology and Preventive Medicine, University of Maryland, Baltimore, MD* and <sup>2</sup>*Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, VA*.

The ability to interview well is a critical career skill, and keeping this skill up-to-date is even more important in a volatile social and economic environment. Moreover, interviewing skills that are considered important in one sector of the job market may be quite different from those in another. Therefore, it is no surprise that there are substantial differences in the expectations of employers looking to fill positions in these two areas of post-graduate employment. The purpose of this session is to expand the personal skills sets of graduate students and post-docs with regard to interviewing for post-doctoral research positions at an academic institution, faculty positions at a community college or undergraduate institution, positions in the federal government, as well as positions in the private sector. This goal will be accomplished through formal presentations on interviewing skills, followed by question-and-answer sessions. An informed presentation on interview questions that are legally permissible across all hiring sectors is an essential part of this session. Because acquiring and processing knowledge of current interviewing skills and practices is a prerequisite for putting these skills into practice, this workshop is a must for all toxicologists who are making the transition into the workforce.

### 316 INTERVIEWING FOR AN ACADEMIC POST-DOCTORAL POSITION.

M. Ehrlich. *Biomedical Sciences and Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, VA*.

The purpose of post-doctoral training is to provide the new toxicologist with experience as a semi-independent investigator, to ease the transition to an independent investigator. Responsibilities are considerably greater than those of a graduate student, and may include exploring new frontiers for his/her mentor, managing a project (including laboratory personnel working on the project), and/or contributing to proposals that increase laboratory funding. Post-doctoral training is best done in a new environment, so the interviewer will ask why the applicant chose to do post-doctoral training, why this particular position is of interest, what the applicant can bring to the laboratory, the expectations of the applicant while employed as a post-doctoral research associate, and plans that go beyond the post-doctoral appointment. In addition to answering these questions to the best of the applicant's ability, it is important that he/she be honest about data available. To come to the interview with knowledge of current and past work of the laboratory contributes to creating a favorable impression.

### 317 INTERVIEWING SKILLS FOR A TEACHING POSITION AT AN UNDERGRADUATE INSTITUTION.

R. Resau. *Biology, McDaniel College, Baltimore, MD*. Sponsor: R. Mitkus.

There are numerous and varied colleges and universities where teaching is the primary responsibility of the faculty member and research is secondary. Some of these institutions do not expect any research effort at all. Others require research involving undergraduates, and still others expect occasional publications from the faculty member. These differences are very substantial, and require that the candidates for any given position know which alternatives they prefer, and investigate the specific characteristics of the institution offering the position. This presentation explores how to use such information to best advantage in the interview.

### 318 INTERVIEWING SKILLS FOR A POSITION IN THE FEDERAL GOVERNMENT: AN INTERVIEWER'S PERSPECTIVE.

R. J. Mitkus and A. C. Mosby. *Office of Communications and Government Relations, USEPA Region III, Philadelphia, PA*. Sponsor: R. Mitkus.

Research by the Office of Personnel Management and other organizational experts, including Dr. Paul Green, ("More than a Gut Feeling"), has determined that past performance is a valid predictor of future performance. Traditional interviewing techniques have focused on areas not directly related to the knowledge, skills and abilities required for a particular position. A newer, behavioral approach, which uses a "structured interview" process, emphasizes gaining specific examples of what a person has done in his or her education and career in order to predict future performance. This part of the workshop will discuss how the interviewer plans for and conducts the structured interview. In turn, it will allow the potential candidate to understand this process and how to prepare for a structured interview.

### 319 INTERVIEWING SKILLS FOR A POSITION IN THE PRIVATE SECTOR.

J. C. Lamb. *The Weinberg Group, Washington, DC*.

The best employers are looking for enthusiastic, informed and intelligent candidates with the ambition to grow their career. How you approach the interview can profoundly affect the impression you make. You should know to be on time, dress appropriately and most importantly, be prepared. The basic principles for a successful job search and interview in private industry are not very different from academia or government but you may have access to information on the web for private firms that can help you tremendously. There is no excuse for showing up at a job interview without knowing significant facts about the company you going to meet. The interview is a two-way communication, so be sure that you are ready to participate, not just listen. You should have a list of questions appropriate for every company you interview. Ask the personnel office what to expect at each step of the hiring process: Who will you meet? How many interviews should you expect? How long will the process take? When will they make a hiring decision? Who would your supervisor be? What is the position description? Do they have written down goals for the employee? What knowledge or skills are expected from the successful candidate? How long have others stayed in this position? Where did they go? Such questions are useful in the interview because it demonstrates your ambition to succeed. If ambition scares them, look elsewhere! Also, find out about educational assistance opportunities. There are questions you might get answered from the web that could affect your enthusiasm: How large is the firm, or the office? How long have they been in business? Are they profitable? Do their products or services interest you? Can you tell whether they have scientific problems or issues that might intrigue you, or match your training particularly well? Ask the interviewers how they see your role and your advancement potential. Check the web, go to the library, ask your friends about the firm. Do not get discouraged by an uncomfortable interview. You will learn at each one, and if it was uncomfortable at the interview, it could have been worse on the job! Good luck

### 320 WHAT INTERVIEWERS CAN AND CANNOT ASK BY LAW.

A. Mosby. *OPM, USEPA Region III, Philadelphia, PA*. Sponsor: R. Mitkus.

It is essential to ensure the development of fair and effective questions that do not violate law, when planning a logical, structured interview. This part of the workshop will raise the participant's awareness of those questions that cannot legally be asked during a structured interview. It will also address the appropriateness of other types of questions that may be asked by an interviewer during the interviewing process.

### 321 TEACHING UNDERGRADUATE TOXICOLOGY IN THE 21<sup>ST</sup> CENTURY.

T. W. Simmons<sup>1</sup> and J. B. Tarloff<sup>2</sup>. <sup>1</sup>*Department of Biology, Indiana University of Pennsylvania, Indiana, PA* and <sup>2</sup>*Department of Pharmaceutical Sciences, University of the Sciences in Philadelphia, Philadelphia, PA*.

Undergraduate toxicology course offerings and teaching methods for delivering these courses have dramatically changed over the last twenty years. Historically, toxicology was a discipline that was more or less restricted to graduate and postdoctoral training. This emphasis was reflected by a symposium on Some Special Toxicology Education and Training Programs that was sponsored by the National Institute of Environmental Health Sciences in 1980. The idea of undergraduate toxicology offerings was debated and discussed, and four Bachelor of Science programs were highlighted at this workshop. In 1992 a follow-up forum on Undergraduate Toxicology Coursework was convened at a Society of Toxicology annual meeting. The objective of this Forum was to reach a consensus on the content of a one-year course in toxicology to be offered at the undergraduate level by

colleges and universities. Interestingly, Dr. Donald Reed stated in his opening remarks that by the 21st century there could be tens of thousands of undergraduate students taking toxicology. Although the number of undergraduate students enrolled today in toxicology courses may not number in the tens of thousands, it is clear that a significant number of students are taking courses in major, minor and track toxicology programs. In addition, many more students are enrolled in courses offered by non-toxicology/pharmacology degree programs. Along with this increase in toxicology course offerings; topics, methods and tools for teaching toxicology have also grown. New technologies including software programs and internet resources, current events including bio-terrorism, television programming such as Crime Scene Investigation, and new teaching strategies incorporating scientific inquiry, constructivism, and reflective practice are transforming the toxicology classroom. The goal of this workshop is to provide a forum to improve teaching and learning in the toxicological sciences, by sharing experiences related to these trends.

### 322 OVERVIEW OF UNDERGRADUATE COURSE OFFERINGS: TOXICOLOGY TEACHING PROJECT.

**T. W. Simmons.** *Department of Biology, Indiana University of Pennsylvania, Indiana, PA.*

The Toxicology Teaching Project sponsored by Indiana University of Pennsylvania and the Allegheny-Erie Society of Toxicology promotes and supports undergraduate teaching of toxicology. The Project identifies colleges and universities that offer undergraduate majors, minors, tracks, and courses in toxicology; and offers an online course survey for professors to identify characteristics of these course offerings. Presently, there are at least 8 universities offering undergraduate degrees, 3 offering minors, and 1 offering a track in toxicology. Based on the responses to a survey from directors of 7 of the 8 undergraduate degree programs, the programs range in age from 19 to 30 years (X = 23); total enrollment in these programs is 260 (X = 41); and enrollment is increasing at 3 programs and remaining steady at 4 others, but 1 of these programs will be discontinued in 5 years. Approximately 36% of students graduating from these programs pursue post-undergraduate education including toxicology-related fields, and 54% obtain employment in a toxicology or pharmacology-related field. In addition, a larger number of undergraduate students are being introduced to the field in non-toxicology/pharmacology degree programs. There are at least 59 schools with undergraduate degree programs in environmental health, industrial hygiene, safety science, occupational health, environmental science, biology, entomology & plant pathology, microbiology, biochemistry, chemistry, nursing, pharmacy, food science or engineering; that offer 97 courses in toxicology. In addition, 2 community colleges have been identified with associate degree programs in environmental science or chemistry; that offer 2 courses in toxicology.

### 323 LABORATORY EXPERIENCES IN THE CLASSROOM.

**J. B. Tarloff.** *Department of Pharmaceutical Sciences, University of the Sciences in Philadelphia, Philadelphia, PA.*

Laboratory experiences are critical for students hoping to enter industry. We have put together two laboratory courses that emphasize problem solving, report writing, and practical exercises to help students become familiar with multiple techniques. The first semester we concentrate on handling of small animals, dosing, and measurement of outcomes. We also introduce students to the concept of tissue fractionation and enzyme assays. In the second semester, students are introduced to various separation techniques (electrophoresis, high performance liquid chromatography, Western blotting, thin layer chromatography) and cell culture. Feedback from investigators who have hired our students has guided revision of these courses through the last ten years and we continue to evolve.

### 324 CURRENT EVENTS IN THE CLASSROOM.

**S. M. Ford.** *College of Pharmacy & Allied Health, Saint Johns University, Jamaica, NY.*

This presentation will describe innovative strategies to engage undergraduate toxicology students in real-life issues with respect to the social, economic, political, and scientific values that impinge on their resolution. Specific courses and curricular design will be discussed with reference to implementation and student experience.

### 325 LOBO PHARMACOKINETICS COMPUTER SOFTWARE: INTERACTIVE PHARMACOKINETIC MODELING.

**C. Marcus<sup>1</sup>, D. Godwin<sup>1</sup>, E. Danoff<sup>1</sup> and R. Galinsky<sup>2</sup>.** <sup>1</sup>*College of Pharmacy, University New Mexico, Albuquerque, NM* and <sup>2</sup>*College of Pharmacy and Pharmacal Sciences, Purdue University, W. Lafayette, IN.*

This presentation will focus on the utilization of LOBO PK, a real-time interactive pharmacokinetic modeling software package developed to teach the principles of pharmacokinetics to undergraduate and professional students in the biomedical sci-

ences. The presentation will demonstrate how this software package can be utilized in a didactic classroom milieu to illustrate basic pharmacokinetic principles, competition kinetics, and many of the underlying molecular and physiological mechanisms by which drug/drug interactions can occur and how pharmacogenomic variations in drug metabolizing enzymes can contribute to large inter-individual responses to therapeutic drugs. The presentation will also demonstrate how this software package can be utilized in a student-centered problem based learning mode in an instructional laboratory milieu as well.

### 326 DISTANCE EDUCATION: ONLINE TEACHING OF TOXICOLOGY.

**K. L. Willett and A. Bouldin.** *Department of Pharmacology, University of Mississippi, University, MS.*

The presentation will describe the development and implementation of a semester-long online, undergraduate Introduction to Toxicology course. This course has been offered at the University of Mississippi for two semesters. An expanded course evaluation was developed to assess student attitudes and perceptions of the online version of the course, along with perceived comparisons between online and traditional environments. Results of these assessments and comparison to an assessment given to students in the same course but taught in the traditional classroom setting will be presented. Finally, potential challenges associated with the online teaching delivery method along with recommendations to others considering this teaching format will be discussed.

### 327 ENVIRONMENTAL TERRORISM: DEVELOPMENT OF EVACUATION, RE-ENTRY AND RE-USE GUIDELINES FOR CHEMICAL, BIOLOGICAL AND RADIOLOGICAL AGENTS.

**M. A. Maddaloni.** *USEPA, New York, NY.* Sponsor: **A. Jarabek.**

The collapse of the World Trade Center (WTC) presented a host of challenges to the governmental agencies charged with making decisions on evacuation, re-entry and long-term re-habilitation for the impacted public in Lower Manhattan. Occupational standards, ranging from immediately dangerous to life and health (IDLH) concentrations to long-term permissible exposure limits (PELs) existed for many of the building components and combustion by-products released from the WTC disaster. However, these standards are generally not well-suited for application to the general public (i.e. residents and office workers). Conventional environmental standards and guidelines have focused on establishing long-term exposure limits for chemical contaminants in the ambient environment. That leaves a lot of poorly charted territory for assessing environmental terrorism incidents that may involve biological or radiological agents within indoor settings, as well as in the ambient environment. The anthrax bioterrorism event of October, 2001 made that abundantly clear. The environmental response to the WTC disaster involved numerous governmental agencies along with the inevitable overlapping of jurisdictions and responsibilities. A future environmental terrorism event will likely follow suit. Accordingly, this workshop will engage the key governmental agencies involved in protecting the public health from environmental terrorism. The workshop will focus on comparing and contrasting available methods for deriving short term (i.e. evacuation), subchronic (i.e. re-entry) and long-term (i.e. re-habitation) exposure criteria a for chemical, biological and radiological agents. Of particular interest will be the role of background levels, analytical detection limits, social and economic disruption, and other miscellaneous factors in the setting of exposure limits. Risk levels for the different exposure scenarios and agent classes will be evaluated for comparability. Data gaps and methodological shortcomings will be identified for representative exposure scenarios within each class of agents.

### 328 AN EPA PERSPECTIVE ON CHEMICAL CONTAMINATION CLEAN-UP.

**S. Sterling, I. P. Baumel and C. Sonich-Mullin.** *ORD/NHSRC, USEPA, Washington, DC.* Sponsor: **M. Maddaloni.**

The U. S. Environmental Protection Agency's (EPA) National Homeland Security Research Center (NHSRC) is responsible for supporting EPA field staff in the event of a terrorist incident by assisting in the development of human health risk assessments. Based on experience at the World Trade Center, USEPA field staff have identified several needs in the area of risk assessment. In response to these needs, NHSRC is compiling risk-based information for acute, subchronic and chronic exposures that would be applicable to such sites. In the case of acute exposure, NHSRC is relying heavily upon existing information such as the Acute Exposure Guideline Levels (AEGLs) to provide useful data. For subchronic exposures of one

month to two years, NHSRC has initiated an effort to develop and apply a methodology for deriving subchronic health benchmarks from existing data. For long-term exposures, NHSRC is working with other EPA offices to strengthen the methodology for developing preliminary remediation goals. This presentation will provide an update on NHSRC's efforts to make available health benchmarks for exposure to chemicals resulting from terrorist activities.

### 329 A MILITARY APPROACH TO ASSESSING HEALTH RISK.

V. Hauschild and B. Thran. *Directorate of Health Risk Management, US Army Ctr for Health Promotion and Preventive Medicine, Aberdeen Proving Ground, Edgewood Area, MD.* Sponsor: M. Maddaloni.

Quantitative risk assessment approaches, particularly those used to assess chemical exposures, have historically resulted in the development and application of specific exposure limits or standards to protect occupational, public, or environmental settings. Despite the calculated values that represent risk in such applications, there is substantial variation in the endpoint selection and the degree of uncertainty associated with the established exposure limits. In application, use of exposure limits results in a loss of appreciation for the dose response continuum and the range of risk associated with varying exposure and human susceptibility. For certain regulatory purposes, this approach may be rationalized. In situations where risk-based goals cannot be estimated due to lack of available scientific data, or when the goals are unachievable (due to inadequate detection capabilities or mitigation/clean-up technologies), or when risk acceptance is necessary to achieve a critical goal, other approaches must be applied. The military makes use of a standardized qualitative risk characterization approach for such situations. While it is necessary to have scientific underpinnings for describing risk, this qualitative risk characterization approach can minimize the impact of variability and uncertainty. The military has successfully used this approach to facilitate rapid, systematic risk prioritization and risk communication. This presentation will discuss efforts to apply the military's qualitative risk characterization approach to two types of health hazards: chemical warfare agents and biological hazards. An example will illustrate how quantitative dose response data (that includes consideration of exposure and population variability) can be portrayed with qualitative risk ranges. The presentation will include a discussion of how a qualitative approach may be applied in response to an incident involving a weapon of mass destruction.

### 330 BIOTERRORIST THREATS TO THE US FOOD SUPPLY SYSTEM: A RISK ASSESSMENT PERSPECTIVE.

B. Hope. *Air Quality Division, Oregon Department of Environmental Quality, Portland, OR.* Sponsor: A. Jarabeck.

Since 2001, awareness of, and concerns about, terrorist use of biological agents have increased dramatically at state and federal levels and in the popular press. Vulnerability of valuable US agricultural assets to bioterrorism has received less attention. The food supply system is a key component of US agriculture. In a bioterrorism context, concerns focus on use of the system to deliver an intentionally introduced foodborne bioagent to human consumers or use of a bioagent to contaminate and disrupt system operations or to undermine confidence in the safety of the food supply. However, achieving effective agriculture security programs requires replacing such broad, and potentially intractable, generalizations with specific threat scenarios that can be evaluated, anticipated, and managed. Structured risk assessment methods can be used to provide this specificity. This point is illustrated by using fault tree analysis to undertake prospective risk assessments for two scenarios wherein human consumers are exposed to a bioagent intentionally introduced into the US food supply. This analysis suggests that the food supply system may be a less than optimal bioagent delivery mechanism, particularly if the objective is a mass casualty attack, as a number of events must occur simultaneously with reasonable probability if a bioagent is likely to be deployed effectively. In addition, an environmentally stable bioagent would be required if only the food system were used to deliver it to human consumers, but early detection of an intentional release common bioagent may be difficult absent specific intelligence or unusual epidemiological circumstances.

### 331 A PRIMER ON RADIATION ISSUES RELATED TO NUCLEAR TERRORISM.

S. L. Simon. *Radiation Epidemiology Branch, National Cancer Institutes, Bethesda, MD.* Sponsor: M. Maddaloni.

Strategies to advise authorities and the public on how to minimize individual and public harm as a result of terrorist actions that involve radiation and/or nuclear materials requires an understanding of the fundamentals of radioactivity, radionu-

clides, radiation dose, and how long-term health risks change incrementally with changes in exposure received. The similarities between exposure to radiation and exposure to chemicals or biological agents are limited primarily to exposure pathways. The types and qualities of radioactive materials, the radiations they emit, how radiation interacts with living systems and the resulting effects differ substantially from other agents. The purpose of this presentation is to provide a basic understanding of the concepts of radiation, radiation exposure, and health risk, in the context of intentionally harmful actions involving radioactive materials. A limited discussion will also be presented to explain some of the possible exposure scenarios that have been considered.

### 332 DEVELOPMENT OF OPERATIONAL GUIDELINES FOR CONSEQUENCE MANAGEMENT OF RADILOGICAL DISPERSAL DEVICE INCIDENTS.

S. Domotor<sup>1</sup>, A. Wallo<sup>1</sup>, C. YU<sup>2</sup>, D. LePoire<sup>2</sup> and S. Kamboj<sup>2</sup>. <sup>1</sup>*US DOE, Washington, DC* and <sup>2</sup>*Argonne National Laboratory, Washington, DC.* Sponsor: A. Jarabeck.

The Department of Homeland Security (DHS) is leading the development of a risk management framework for responding to and mitigating potential effects caused by terrorist use of a Radiological Dispersal Device (RDD). This initiative is being coordinated through the interagency Consequence Management Subgroup (CMS). The CMS is led by DHS and comprised of subject-matter experts in radiological and nuclear preparedness and response from a number of agencies, to include the Environmental Protection Agency (EPA), Nuclear Regulatory Commission (NRC), Department of Defense (DoD), Department of Energy (DOE), and others. The framework addresses early, intermediate, and late phases (e.g., recovery and cleanup) of response, and provides Protective Action Guides (PAGs) to aid decision makers on when to take protective actions (e.g., sheltering, evacuation, relocation) to reduce exposure to workers and members of the public from radiation or other hazards. An optimization process is provided for addressing the long-term cleanup and recovery issues from an RDD. Protective Action Guides (PAGs) are the projected doses to a reference individual, from an accidental or deliberate release of radioactive material, at which a specific protective action to reduce or avoid that dose is recommended. Operational Guidelines are derived levels of radiation or concentrations of radionuclides that can be measured by radiation detection and monitoring equipment, and then related or compared to the PAGs to quickly determine if protective actions and associated contaminant mitigation need to be implemented. For example, there may be an urgent need to make decisions on whether or not to evacuate or relocate the public, and to restore critical infrastructure services such as access to roads, medical facilities, and power, water and sewer facilities in the hours and days following the incident. Although some values already exist (e.g., derived intervention levels for food), there are many more Operational Guidelines that need to be developed.

### 333 HIGH THROUGHPUT SCREENING APPROACHES IN GENETIC TOXICOLOGY.

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Recent progress in combinatorial chemistry, molecular biology, genomics, and automation has enabled identifying a relatively large number of compounds capable of reacting with intended pharmacological targets. However, 40% of drug candidates ultimately fail during clinical development due to safety related issues. The overall attrition of drug candidates due to genetic toxicology issues alone includes about 12% of drug candidates. This causes delays in the introduction of vital drugs to patients and significant economic losses. Therefore, the development of relevant mechanism-based high throughput screening technologies to assess genetic toxicity at the early stages of drug discovery with relatively limited amount of chemical is extremely important. Genetic toxicology provides the necessary information for assessment of the genotoxic risk associated with the use of drugs. Since the beginning of genotoxicity testing in the early 1970s, many different test systems have been developed and used. Since no single test is capable of detecting all genotoxic agents, the current standard *in vitro* genotoxicity testing consists of evaluating mutagenicity (bacterial reverse mutation assay) and chromosome damage (lymphocyte aberration assay or mouse lymphoma assay). Unfortunately, these standard *in vitro* assays are not amenable to high throughput testing and their application in early phases of drug discovery is not feasible. Therefore, the research and assay development efforts have been directed to developing alternative approaches, technologies and/or endpoints. The speakers in this session present emerging state-of-the-art technologies under development and/or currently used in the pharmaceutical industry. The topics covered in this symposium comprise high throughput versions of the Salmonella assay, assays for DNA deletions, chromosome aberrations, the comet assay and a gene expression reporter assay. The presentations will cover exciting developments that may spark further interest in the automation of genotoxicity assays.

**334****BIOLUMINESCENT SALMONELLA REVERSE MUTATION ASSAY: A HIGH THROUGHPUT APPROACH FOR DETECTION OF MUTAGENICITY.**

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We have developed a novel bioluminescent high throughput Salmonella reverse mutation assay applicable to the screening of large numbers of compounds including combinatorial libraries. The bioluminescent Salmonella assay utilizes genetically engineered standard Salmonella tester strains TA98 and TA100 expressing the luxCDABE operon from *Xenorhabdus luminescens*. In principle, the assay employs bioluminescence as a sensor of metabolic activities in living cells. The assay provides highly concordant data with the outcome of the standard Salmonella reverse mutation assay. Since the results of the standard Salmonella assay are required by various regulatory agencies for approval of new drugs, the bioluminescent Salmonella assay can be effectively used for prioritization of compounds in drug discovery. Because of its high throughput attributes, the assay permits effective, fast and economical screening of a large series of structural analogs enabling the investigation of structure activity relationships. In addition, the application of bioluminescent sensors for detection of metabolically active cells might have a broad application in the design of other high throughput clonogenic assays in various cell systems.

**335****BIOLUMINESCENT YEAST DEL ASSAY TO DETECT CARCINOGENS AND CLASTOGENS.**

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Genomic rearrangements including DNA deletions are involved in carcinogenesis. DEL assays in yeast, human cells and *in vivo* in mice that quantify DNA deletion events detect a wide variety of carcinogens, many of which are negative in other short-term genotoxicity tests. For instance, of 69 tested chemicals of known carcinogenic activity, the yeast DEL assay correctly identified 86%. In contrast, the widely used Salmonella reverse mutation assay correctly classified only 33%. Furthermore, the yeast DEL assay was also capable of correctly differentiating between carcinogen / non-carcinogen structural analogs. To assess the utility of the yeast DEL assay as a screening tool for detection of genotoxicity we have evaluated the correlation of the DEL assay with commonly used tests for mutagenicity and clastogenicity (Salmonella reverse mutation assay and the *in vitro* micronucleus assay). Our study indicates that the DEL assay is capable of accurately detecting both clastogenic and mutagenic compounds. Although the original yeast DEL assay is robust and relatively simple, the original assay format limits its utility as a screening tool. Therefore, we have developed a bioluminescent version of the DEL assay suitable for automation. The assay employs bioluminescence as a sensor of metabolic activities in living cells for visualizing cells that underwent DEL recombination events. Several chemicals have been shown to be detectable in this new bioluminescent DEL assay format, which is currently being further developed into an HTS format.

**336****APPLICATION OF A HIGH THROUGHPUT COMET ASSAY IN DRUG DISCOVERY.**

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The alkaline comet assay is a microgel electrophoresis technique which is becoming established as an *in vitro* and *in vivo* test for industrial genotoxicity screening. This assay is an indicator test that detects primary DNA lesions such as direct and DNA repair-induced strand breaks and alkaline-labile sites. DNA damage is expressed as increased DNA migration measured in individual cells in an agarose gel mounted on microscope slides. Although the genetic endpoint of the comet assay is not the induction of mutations, *in vitro* data demonstrates similar sensitivity and specificity for detection of genotoxicity as compared to clastogenicity tests such as the micronucleus test or chromosomal aberration assay. Comparative data of *in vitro* studies with various cell types commonly used for industrial genotoxicity testing will be presented. Furthermore, data demonstrating that comet assay is less prone to false-positive results related to excessive cytotoxicity - a known confounding factor for *in vitro* clastogenicity tests - will be presented. For the conduct of an *in vitro* comet assay, only very small cell sample sizes are required. Adaptations of the protocol will be discussed that utilize small scale suspension cultures of multi-well culture dishes. This approach allows the conduct of genotoxicity screening at early stages of drug development, at which usually only very limited quantities of drug candidates are available. Establishing automated image analysis aided by a robot- feeded microscope can significantly enhance the throughput of comet assay samples. New approaches will be presented which can speed up the test performance by directly

using multiwell plates for microscopic analysis replacing the time-consuming preparation of microscope slides. The comet assay is also being established for detection of *in vivo* genotoxicity in pilot toxicity studies with rodents aiming at early profiling of new drug candidates. Approaches will be discussed how the comet assay can be applied to investigate *in vivo* genotoxicity in a variety of rodent organs.

**337****A FLOW CYTOMETRIC PROCESS FOR DETECTING ANEUGENIC AGENTS *IN VITRO*.**

P. A. Muehlbauer and M. J. Schuler. *Genetic Toxicology, Pfizer Global R & D, Groton, CT.*

A major barrier in moving forward with a rapid screening platform for detecting chemical agents that induce numerical chromosomal aberrations (aneuploidy) *in vitro*, is that most test systems rely on microscopic assessment of the aneuploid effects. Typically, microscopic analysis is technically arduous and is oftentimes restricted to scoring a few test concentrations for a discrete sub-population of aneuploid cells (e.g. hyperdiploid and/or polyploid). Thus incomplete data sets are typically produced and the inter-relationships between the different aneuploid populations cannot be easily discerned. The potential to automate a screening platform for detecting aneuploid agents has been expedited with the advent of molecular mitosis specific markers and the commercial availability of antibodies to them. Using these mitosis specific antibodies in combination with 2-color flow cytometry provides a unique opportunity to discriminate the mitotic (M) population of actively dividing cells on the basis of DNA content. The reliability of this process to isolate and measure mitotic populations in chemically treated human lymphocyte cultures has been previously shown in our laboratory. Estimates of the mitotic sub-populations (i.e. hypodiploid, normal, hyperdiploid and polyploid) are realized by place region gates over the mitotic population based on DNA content and applying Becton Dickinson Cell Quest software region statistics to calculate the percent gated. The flow cytometry-based process is an automated process that detects and characterizes aneuploid agents thus it offers a tremendous timesaving advantage for investigators over methodologies that rely on a microscopic assessment. Being able to observe all the aneuploid populations concurrently provides a rare opportunity for an investigator to discern the inter-relationships between the aneuploid populations and thus gain a tremendous insight and confidence with regards to the chemical hazard characterization.

**338****HIGH-THROUGHPUT SCREENING USING STRESS-RESPONSIVE REPORTER GENE ASSAYS.**

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In recent years, the maturation of microarray technology and high-density expression data analysis tools has lead to the increasing use of whole genome expression analysis to generate highly informative predictions as to the mechanisms of compound pharmacology and toxicology. While microarrays can provide information on the expression levels of thousands of genes at a time, current platforms are not suited to rapid screening of the dozens to hundreds of compounds typical of an early stage discovery program and there remains a need to translate the predictive signature information obtained from microarray and other gene expression analyses into useful high-throughput screens that can be employed against compounds earlier in the discovery process. With this aim in mind the Discovery Technology Group at OSI Pharmaceuticals generated a panel of stably-transfected mammalian cell lines expressing luciferase reporter genes under the control of various promoters and genetic elements responsive to a broad set of cellular stresses (genotoxic, oxidative, metabolic, endocrine, etc.). Assay conditions were optimized for each cell line and methods developed for performing the assays on fully automated robotic high-throughput screening (HTS) platforms. Pilot experiments generated over 18, 000 data points per 48 h run when assaying a small library of model toxins and controls over 6 dose points in triplicate against up to 13 different cell lines in parallel. We were able to identify expression profiles characteristic of genotoxins and other toxin classes and to identify expression profiles elicited by early discovery compounds that distinguished mechanism of action from potential toxicity. Stress gene expression profiles for compounds of interest to drug discovery programs may be used to flag undesirable characteristics early in the discovery process and will assist discovery program managers in deciding which compounds or structural classes are the best candidates for progression.

**339****ELECTROCARDIOGRAPHY SAFETY EVALUATION STUDIES: NEW TECHNIQUES AND APPROACHES.**

L. B. Kinter<sup>1</sup> and D. J. Murphy<sup>2</sup>. <sup>1</sup>*Safety Assessment, AstraZeneca, Wilmington, DE* and <sup>2</sup>*Drug Safety Assessment, GlaxoSmithKline, King of Prussia, PA.*

Examination of cardiac function using clinical electrocardiography (ECG) techniques can detect potential cardiotoxic and arrhythmogenic effects of new chemical entities in animals and humans. However, application of traditional technologies in

animals not trained to accommodate collection of these endpoints can result in spurious data artifacts, and arguably contributed to failure to recognize proarrhythmic potential of some drugs subsequently associated with clinical QT interval prolongation and ventricular tachyarrhythmia. Recent advances in telemetry technologies permit collection of high fidelity ECGs from naive animals in safety pharmacology or general toxicology studies. Topics presented will include 1) ECG theory and its application in preclinical safety evaluation and human risk assessment; 2) technical considerations for implanted and non-implanted ECG systems and selection of surface, intracardiac and intravenous lead placements; 3) collection of quality ECG endpoints within safety pharmacology and general toxicity study designs; and 4) analysis and interpretation of telemetry-collected ECG data. This Roundtable Session is suggested for scientists considering and currently using telemetry systems for preclinical assessments of cardiac toxicity and arrhythmogenic potential.

#### 340 CARDIAC ELECTROPHYSIOLOGY FROM THE SINGLE CELL TO THE INTACT HEART.

R. Hamlin. *Veterinary Cardiology, Ohio State University, Columbus, OH.* Sponsor: L. Kinter.

Theory of contemporary veterinary electrocardiography (ECG) and its applications to assessments of new chemical entities for arrhythmogenic potential will be briefly reviewed. Considerations for selection species, lead placements, and analysis and interpretation of ECG data for human risk assessment will be discussed.

#### 341 TECHNIQUES AND ELECTROGRAPHIC LEAD PLACEMENTS FOR IMPLANTED ECG MONITORS.

R. Coatney. *Animal Modeling & Imaging, GlaxoSmithKline, King of Prussia, PA.* Sponsor: L. Kinter.

ECG telemetry systems come in two basic configurations, systems that are entirely implanted below the body surface using survival surgical techniques, and those that have one or more sets of components carried above the body surface, usually in a jacket or other protective covering. Technical considerations for successful surgical placement of implanted ECG telemetry components and systems will be presented, including species selections, lead and transmitter placements, and welfare considerations.

#### 342 INTRACARDIAC AND INTRAVASCULAR LEAD UTILIZATION: METHODS FOR IMPROVED DATA COLLECTION.

C. Hessler. *Safety Pharmacology, Battelle Memorial Laboratories, Columbus, OH.* Sponsor: L. Kinter.

Catheter technologies permit formulation of vascular ECG electrodes which when placed in cardiac blood vessels permit regional ECG analyses using telemetry systems. Technical considerations for application of surface and intracardiac ECG electrode placements, and construction, placement and telemeterization of intracardiac lead systems will be discussed.

#### 343 OBTAINING QUALITY ECG ENDPOINTS IN SAFETY PHARMACOLOGY AND GENERAL TOXICOLOGY STUDIES. ARE RESTRAINT AND SURGICAL INTERVENTION NECESSARY?

M. Zawada and L. B. Kinter. *Safety Assessment, AstraZeneca, Wilmington, DE.*

Traditional ECG technologies are of limited utility in preclinical safety pharmacology and toxicology studies because the restraint necessary to maintain proper lead placements usually limits the duration of ECG collection to a few minutes, and stress artifacts and high heart rates further limit the utility and interpretation of the data collected under these conditions. Advent of telemeterized systems now permits collection of high quality ECG data of nearly unlimited duration, and has greatly increased the utility of ECGs to detect acute cardiotoxicity and arrhythmogenic potential in safety pharmacology studies. However, while general toxicology studies are an excellent platform for telemetric monitor for cardiotoxic and arrhythmogenic biomarkers with subchronic and chronic exposures, prestudy surgical implantation of telemetry systems is usually inconsistent with the primary objectives and endpoints and non-implanted technologies are preferred. Considerations for and examples of applications of fully-implanted and non-implanted telemetry ECG systems in safety pharmacology and general toxicology study designs will be discussed.

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#### BIOLOGICALLY-BASED MATHEMATICAL MODEL FOR GENE EXPRESSION DUE TO JP-8 EXPOSURE TO SKIN.

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Skin irritation is not completely understood at the molecular level. Biologically-based (toxicodynamic) models of the irritant process can be very useful for prediction of the severity and extent of the problem, as well as tools for the development of therapeutic and prophylactic interventions. The purpose of this study is to develop a biologically-based mathematical model based on results of changes in gene transcripts measured by Affymetrix Gene Array (U34A chip). After one-hour exposures to JP-8 jet fuel, we measured changes in mRNA one, four and eight hours after initiation of the exposure in rats. Among early responses, several genes related to the proinflammatory cytokine, Interleukin 1 (IL-1 $\alpha$ ), signaling pathway were increased. We developed a mathematical model for a part of the inflammatory pathway involved in these gene changes. The model captures the series of biochemical events initiated from IL-1 $\alpha$  binding to IL-1 receptor (type I) on the cell surface that activates the transcriptional factor NF-kappa B and leads to production of a responsive protein, IL-6 and an inhibitor of NF-kappa B. Using a deterministic approach, the model describes the system with a set of ordinary differential equations assembled from reaction rate laws and divides it into three compartments: the nucleus, cytosol and extracellular space. Temporal changes in protein concentrations are described by forward and backward reaction rate terms for binding, degradation, activation, translocation and synthesis. The model prediction shows qualitative agreement with gene array results. We conclude that this model is a useful tool for the future investigation and understanding of the irritant process occurring in the skin. (Supported by Air Force Office of Scientific Research and a Wright State University Major Collaboration Grant)

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#### MODELLING THE RELATIONSHIP BETWEEN NICKEL AND CHROMIUM EXPOSURE AND CONTACT DERMATITIS.

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Metal induced contact dermatitis is an important occupational health problem and both chromium and nickel are known to cause both irritant and allergic responses. In an *in vitro* study conducted by the Health and Safety Laboratory, human keratinocytes were dosed with increasing concentrations of nickel and chromium ( $0.01\text{--}10^5 \mu\text{M}$   $\text{K}_2\text{Cr}_2\text{O}_7$  and  $0.01\text{--}10^4 \mu\text{M}$   $\text{NiCl}_2$  for 24hrs) to determine their effect on cell respiratory activity, viability, metal association and production of inflammatory mediators (in particular, IL-1 $\alpha$  and IL-8). Keratinocytes are the principal skin cells in the epidermis and their response to nickel and chromium was found to differ markedly. The observed amounts of metal associated with the cell, the decrease in cell viability and the release of IL-1 $\alpha$  by the keratinocytes were all much greater for chromium than for nickel at the same concentration, suggesting the former is much more cytotoxic. In order to predict the effects of nickel and chromium *in vivo*, a mathematical model describing the innate and acquired immune responses to such a toxic insult has been formulated. Mechanistically based mathematical modelling is a powerful predictive tool that effectively tests the validity of toxicological assumptions as well as helping to guide experimental work. Our model incorporates the diffusion and reaction of extracellular metal ions within the epidermis, the association of the antigen with the cells, the resulting damage to the viable keratinocytes, the release of cytokines and the influx of immune and T-cells, and provides predictions of the spatial and time evolution of these components within the epidermis. Using experimentally determined parameter values, the model was validated against the measured outputs from the *in vitro* study described above and initial results were found to agree well with the observed behaviour. Once validated, the mechanistic basis of the model allows us to extrapolate from *in vitro* to *in vivo* in order to gain insight into the relationship between metal dose and the immunological response.

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#### COMPUTATIONAL MODELS FOR THE ACQUISITION OF NEOCORTICAL NEURONS IN THE DEVELOPING HUMAN, MONKEY, AND MOUSE: CROSS SPECIES COMPARISON OF TOXICODYNAMICS.

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For interspecies extrapolation of toxicity data for risk assessment purposes, it is particularly pertinent to acknowledge the importance of the neocortex as the defining feature of human evolution. Important morphological and behavioral attributes associated with the enlarged human neocortex are absent in the rodent models used

in most toxicology studies. This necessitates quantitative research into the development and evolution of the neocortex, in order to gain the knowledge necessary for protecting our most unique and arguably most valuable feature as humans. Here we develop computational models of human neocortical development while comparing it to our previously developed rhesus monkey and rodent models. We predict the vast increase in size of the neocortex in humans by changing two key parameters, namely the cell cycle length and the length of the neurogenesis period. We compare our results to independent stereological studies estimating neocortical neuron number in adult and developing human. We have also evaluated the contribution of cell death to neocortical development in these three species. When compared to mouse, we predict cell death may play a larger role in shaping the primate neocortex. Furthermore, when applying research performed in the rat on ethanol-induced inhibition of the cell cycle during neocortical neurogenesis, our models suggest developing humans may be more sensitive to long-term neocortical neuronal deficits based on the prolonged neurogenesis period (84 days compared with 6 days in the rodent or 60 days in the rhesus monkey) during fetal development.

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#### MAGNETIC RESONANCE IMAGING AND COMPUTATIONAL FLUID DYNAMICS SIMULATIONS OF RABBIT NASAL AIRFLOWS FOR HYBRID CFD/PBPK MODELING OF METHYL IODIDE.

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Methyl iodide (MeI), an intermediate in the manufacture of some insecticides and pharmaceuticals, is under review for US registration as a non-ozone depleting alternative to methyl bromide in the pre-plant soil fumigation market. Both acute and subchronic inhalation exposures to MeI in rats have resulted in lesions of the nasal olfactory epithelium. Fetal effects have been observed in pregnant rabbits exposed to MeI vapors and the uptake of MeI by nasal tissues may impact systemic dosimetry. Computational fluid dynamics (CFD) models for nasal tissue dosimetry have been developed for rats, monkeys and humans but not rabbits. Thus, the purpose of these studies was to develop CFD models of the rabbit nasal airways that can be used in modeling MeI or other materials requiring a quantitative understanding of rabbit nasal airway dosimetry. 3D computational meshes were derived from magnetic resonance images of 3 adult female NZW rabbits for use in CFD simulations. The percentages of total airflows over the nasal respiratory and olfactory epithelium of female rabbits were calculated from these simulations under steady-state inhalation conditions. These airflow calculations are critical parameters that will be used in hybrid CFD/PBPK models that describe the nasal as well as systemic dosimetry of MeI in rabbits. Although there was considerable inter-animal variability in the fine structures of the nasal turbinates and airflows in the anterior portions of the nose (maxillary turbinate region), there was remarkable consistency between rabbits in the percentage of total inspired airflows that reached the olfactory epithelium lining the ethmoid turbinate region (~20%). These latter results were consistent with previous published estimates for the male F344 rat. (Supported by Arvesta Corporation).

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#### IN SILICO PHARMACOGENETIC ANALYSIS OF R-WARFARIN METABOLISM.

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To better understand the way in which commonly prescribed drugs are metabolized and to define their mechanism of action, we are applying a recently developed haplotype-based computational method that was shown to correctly identify the genetic basis for differences in biologically important traits among inbred mouse strains (Liao *et al.*, *Science*, 2004, in press). Warfarin is a commonly used anticoagulant with a narrow therapeutic index that has a large variation in individual dosing. Its metabolism was analyzed by administering <sup>14</sup>C-labeled R-warfarin to males of 13 inbred mouse strains. Plasma samples were collected at different time points following treatment for liquid scintillation counting and HPLC-radiometric characterization of the amount of drug and metabolites in each inbred strain. Interestingly, these inbred strains had a unique profile for warfarin and 9 identified metabolites. The pattern of variation in the amount of glucuronidated 7-hydroxylated metabolite of warfarin was computationally correlated with genetic variation found within cytochrome P450 2c29 (*Cyp2c29*). The involvement of this cytochrome in warfarin metabolism was confirmed by experiments showing that liver microsomes could biotransform warfarin to its 7-OH metabolite *in vitro*; and this biotransformation could be inhibited by a *Cyp2c* isoform specific substrate (tolbu-

tamide). In addition, expressed recombinant *Cyp2c29* also mediated the biotransformation of warfarin to its 7-OH metabolite. These results demonstrate that factors responsible for inter-individual pharmacokinetic differences for clinically important drugs can be identified by computational genetic analysis in mice. (Supported by NIH RO1 GM068885-01).

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#### AN INTEGRATED QSAR-PBPK MODEL FOR SIMULATING PHARMACOKINETICS OF CHEMICALS IN MIXTURES.

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Chemicals in mixtures may compete for metabolism, thereby altering their tissue dose in the organism and possibly the toxicity outcome. To predict the change in tissue dose of chemicals during mixed exposures, physiologically-based pharmacokinetic (PBPK) models are of particular use. These models integrate information on physiology, tissue and blood solubility characteristics as well as metabolism and inhibition constants to provide simulations of pharmacokinetics of chemicals in mixtures. The animal and human physiological characteristics are generally known, but the solubility and metabolic parameters for all mixture components need to be estimated and this process can be expensive and time-consuming. The present study integrated quantitative structure-activity relationships (QSARs) within PBPK models to simulate the pharmacokinetics of chemicals in mixtures. The approach involved the development of QSARs for maximal velocity of metabolism and Michaelis-Menten constant of several organic chemicals based on the group contribution approach of Gao. The Michaelis-Menten constant for each chemical was then set equal to the inhibition constant for modeling competition for enzyme binding sites. Previously published QSARs for partition coefficients were used to calculate solubility parameters for all chemicals. The adequacy of this integrated QSAR-PBPK model was evaluated by comparing the predictions with experimental data on the altered blood kinetics of dichloromethane, toluene, ethylbenzene, o-xylene, p-xylene, m-xylene, trichloroethylene and styrene in rats exposed for 4 hr to 50 ppm each of these chemicals in mixtures. The QSAR-PBPK modeling methodology provided predictions of altered pharmacokinetics of chemicals in mixtures that were within a factor of 0.8 to 1.5 of the experimental data. This study for the first time has succeeded in predicting the change in pharmacokinetics of chemicals in mixtures, on the basis of animal physiology and QSARs for chemical-specific parameters including the metabolic inhibition constants.

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#### ISSUES IN THE USE OF PBPK MODELING IN THE DEVELOPMENT OF CANCER SLOPE FACTORS FOR PERCHLOROETHYLENE.

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The current California Environmental Protection Agency Public Health Goal (PHG) for perchloroethylene (PCE) was derived using an upper-bound estimate of the fractional metabolism of PCE in humans. This upper-bound estimate was obtained from an evaluation of the variability in human metabolism of PCE that was conducted using a physiologically based pharmacokinetic (PBPK) model in a Markov Chain Monte Carlo (MCMC) analysis. However, the data used in the MCMC analysis were limited to post-exposure concentrations of PCE in blood and exhaled air from a single inhalation study: 4-hr exposures at 72 or 144 ppm. The use of post-exposure concentrations of parent chemical for estimating metabolism in the human is problematic because PBPK model predictions of these exposures provide only an indirect indication of the amount metabolized. Recent data on the blood concentrations and urinary excretion of the major PCE metabolite, trichloroacetic acid (TCA), following exposure of human subjects to lower concentrations of PCE (10 to 30 ppm) than in previous studies, made it possible to compare the high- to low-dose extrapolation capability of several published models of PCE. We found that a model based on the work of Gearhart *et al.* (1993), which is the only model developed using data on TCA kinetics, gave the closest predictions of the TCA blood concentrations and urinary excretion observed in the low-concentration exposures. Other models overestimated metabolite excretion in this study by 5- to 15-fold. A modification of the model of Gearhart *et al.* (1993) to include metabolism of PCE to TCA in the kidney markedly improved agreement with the experimental time-course data, without altering predictions of liver metabolism. MCMC analysis of the variability in metabolism of PCE was conducted using this model together with data from three different human studies. The resulting upper 95% confidence limits of the fraction of PCE metabolized by the inhalation and oral routes were 3.4 and 7.9%, respectively, compared to 58 and 79% used in the derivation of the PHG.

S. M. Hays. *Summit Toxicology, Lyons, CO.* Sponsor: D. Pyatt.

Lead poisoning continues to be a public health threat for individuals in special situations and communities of people exposed to elevated levels of lead, often in their drinking water. Lead is regulated based on a safe blood concentration. Predicting the blood lead level associated with a set of exposure assumptions requires the use of a toxicokinetic model. While several lead toxicokinetic models exist, only the lead physiologically based pharmacokinetic (PBPK) model can predict blood lead levels in both children and adults, for both short (including intermittent) and long term exposures, and accommodate special circumstances like rapid rates of bone loss. Two recent public health concerns that have been assessed using the lead PBPK model include; 1) children exposed to elevated levels of lead in their school's drinking water, and 2) assessing the likely blood lead levels in astronauts who spend extended periods of time in microgravity whereby they may lose substantial amounts of bone mass. The current EPA action level for lead in school drinking water supplies is 20 ppb. Using the PBPK model, it is predicted that this level of lead in the drinking water causes an extremely small increase in blood lead levels. Likewise, substantially higher levels of lead in school water supplies have only minimal impact on blood lead levels in children. A linear relationship between school drinking water concentration and incremental increase in blood lead has been developed using the PBPK model and is provided for communicating this important issue to children and parents facing elevated levels of lead in their schools. Astronauts may lose substantial percentages of their bone mass while in microgravity. The lead PBPK model was used to simulate the potential impact that variable rates of bone loss have on blood lead levels. The results indicate that there exists the potential for significant elevations in blood lead levels with these high rates of bone loss, even after only short periods in space. Using the lead PBPK model to simulate these two scenarios has provided some valuable insight on the potential public health threats posed by each.

G. Johanson, K. Stamyr and P. Nord. *Work Environment Toxicology, Karolinska Institute, Stockholm, Sweden.*

It has been estimated that fire smoke causes 5-10, 000 fatalities per year in the US alone. Carbon monoxide is presumably the major single agent, however, the contribution of hydrogen cyanide (HCN) might be severely underestimated. HCN is released in large amounts during combustion of nitrogen containing materials, such as polyurethane foam in furniture. Fast diagnosis and treatment of intoxication may significantly reduce the number of fatalities. Aim The aim of this study was to investigate if it is possible to detect HCN poisoning by analyzing exhaled air. Modeling A PBTK-TD model for HCN was used to predict expected breath HCN levels after life-threatening exposures. The model was validated against available animal (detoxification rates, time to lethality vs dose, half times in blood and plasma etc) and human (blood and plasma cyanide levels in surviving and deceased fire victims) data. Experimental The time-course of cyanide appearance in exhaled air was measured on-line with an electrochemical detector in 10 volunteers during and after 1 min x 10 ppm HCN exposure. The study was approved by the Regional Ethical Review Board and performed according to the Helsinki Declaration. The experiment revealed an average half time of 16 (range 10-24) s in breath. These low dose experiments suggest that, even after massive peak exposures, the contribution from washout from the airways will be negligible and below the instrument (0.2 ppm) as well as the true HCN background (0.01-0.03 ppm) in breath within a few minutes. In contrast, the model suggests that acutely toxic exposures will result in elevated breath levels for several minutes. For example, a 30 min x 131 ppm (lethal within 30-60 min) exposure results in a breath level of 0.8 ppm 5 min after exposure and 0.5 ppm 30 after min. Conclusion It appears feasible to detect systemic cyanide poisoning via breath analysis (supported by the Swedish Council for Working Life and Social Research (FAS) and the Swedish National Board of Health and Welfare).

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The hallmark of Aryl Hydrocarbon receptor (AhR) activation is induction of Cyp1a1 and related phase I and II metabolism genes. Because AhR can be activated by dioxin-like compounds that are linked to teratogenesis, carcinogenesis, and im-

mune and endocrine modulation, induction of AhR-related genes such as Cyp1a1 may be of significant concern for preclinical drug candidates. To study mechanisms of AhR activation, we used a large chemogenomic database (DrugMatrix) containing expression profiles from short-term, repeat dose rat studies with 600 reference drugs and toxicants. Of the 539 compounds profiled in liver, heart and kidney, 219 significantly induced Cyp1a1 in at least one tissue, many of which are approved drugs (184). Interestingly, 49 of these Cyp1a1 inducers did not induce the expression of other AhR responsive genes. This suggests possible alternative mechanisms for Cyp1a1 induction and/or AhR activation distinct from receptor binding. To evaluate whether Cyp1a1 is a suitable biomarker for AhR activation, an *in vitro* gel-shift assay utilizing rat hepatic cytosol was used to screen 150 compounds for their ability to stimulate AhR transformation and DNA binding and 24 positive compounds were identified. The results indicated 83 compounds that induce Cyp1a1 *in vivo* failed to activate the AhR *in vitro*. The *in vitro* results also identified many novel AhR ligands structurally distinct from known ligands. Using *in vivo* gene expression profiles, a support vector machine algorithm signature was derived that can distinguish between Cyp1a1 inducers that bind to and activate the AhR from those compounds that do not. These results also contribute to an improved understanding of alternative pathways of Cyp1a1 induction and its relevance to the toxic and biological effects of activators of the AhR signaling pathway.

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Endometrial cancer is one of the major gynecological malignancies in women. RL95-2 endometrial cancer cells which are estrogen- and Ah-receptor positive were used for the study. After an initial screening, quercetin, amentoflavone, kaempferol, and myricetin were identified for further study. RL95-2 cells were induced with TCDD (10 nM/24 hrs) or BaP (1  $\mu$ M/48hrs). To investigate the CYP1 inhibition pattern in intact induced cells, the four selected flavonoids (0.5 – 5  $\mu$ M) were added 30 min prior to the EROD assays. Quercetin, kaempferol and myricetin inhibited EROD activity in a dose-dependent manner (TCDD/BaP-induced EROD activity: IC50= 2.49/2.25, 3.72/3.99, 2.93/2.16  $\mu$ M respectively); while amentoflavone was inactive. To determine the relative antagonism of CYP1A1 and CYP1B1 RNA expression, quantitative real time reverse transcription PCR was done. Quercetin inhibited TCDD-induced CYP1A1 mRNA levels while kaempferol (5  $\mu$ M) inhibited TCDD-induced CYP1B1 mRNA levels significantly. In western blots on cell microsomes, quercetin (5  $\mu$ M) also significantly inhibited TCDD-induced CYP1A1 protein levels, however it did not inhibit BaP-induced CYP1A1 protein. In contrast, CYP1B1 protein levels were not significantly different among various treatments. However, basal levels of CYP1B1 protein were ~12 pmol/mg as compared to ~1 pmol/mg for CYP1A1 protein. CYP1A1 message, enzyme activity and protein levels were inhibited by quercetin, and CYP1B1 message was inhibited by kaempferol in the RL95-2 endometrial cells. Because the CYP1 family has been implicated in detoxifying chemotherapeutic drugs and activating xenobiotics to genotoxic intermediates, these results suggest that quercetin and kaempferol may be effective in cancer chemoprevention. (Supported by American Association of Colleges of Pharmacy and Mississippi Functional Genomics Network, NIH/NCRR).

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Although an increasing proportion of the American population is obese, there is only limited information on whether obesity or weight loss influence drug metabolism in humans. Cytochrome P450 (CYP) isoform expression is very variable in human liver samples that are used in toxicity testing and research. This variation is due to factors such as genetic polymorphism, environmental and pharmaceutical exposure to inducers and pathological and post mortem changes. It complicates the study of endogenous regulators of human CYP. Gastric bypass surgery is used in chronically obese patients as an aid to weight-loss as it both reduces food intake and absorption. Typically, once patients have lost weight a second surgery is performed to remove excess skin and muscle from the abdominal cavity. We obtained liver biopsy samples (with informed consent) from patients at both surgeries so that we could compare the effects of weight loss directly in the same patients. We investigated CYP isoform expression in these samples using isoform selective activities and western blotting. Microsomal preparations were assayed for a range of monooxygenases including acetanilide 4-hydroxylase (CYP1A2), chlorzoxazone 6-hydroxylase (CYP2E1) testosterone 6 $\beta$ -hydroxylase (CYP3A) and lauric acid 11-hydroxylase

(CYP4A) activities. Of these, CYP3A-selective testosterone hydroxylase activity was consistently increased in samples from the second surgery as compared to corresponding samples from the first (obese) surgery  $215 \pm 40\%$ ,  $P < 0.05$ ). Small increases in acetanilide and lauric acid hydroxylases were also observed ( $+52 \pm 28\%$ ,  $+35 \pm 12\%$  respectively), whereas chlorzoxazone hydroxylase activity was slightly decreased ( $-18 \pm 11\%$ ). This suggests that obesity does influence the expression of CYP3A and possibly other CYP isoforms in human liver.

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#### BIPHENYL PROPARGYL ETHERS AS INHIBITORS OF CYP 1A1, CYP 1A2, AND CYP 2B1.

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Cytochrome P450 enzymes are a superfamily of hemoproteins involved in the metabolism of endogenous and exogenous compounds including many drugs and environmental chemicals. Certain aromatic acetylenes have been shown to be inhibitors of these enzymes. Size of the ring system, the location of the acetylenic moiety, and the distance of the triple bond from the ring influence the specificity, efficiency and mechanism of inhibition. Previously we found that 4-(1-propynyl)-biphenyl is a potent inhibitor of CYP 1A1 and 1A2, but 4-(1-butynyl)-biphenyl is only a moderate inhibitor. In an attempt to identify potent inhibitors of CYP 1A and 2B enzymes, we synthesized a series of mono- and di-substituted biphenyl propargyl ethers and mono-substituted biphenyl methyl propargyl ethers, including 2-biphenyl propargyl ether(2BPhPE), 4-biphenyl propargyl ether(4-BPhPE), 2, 2'-biphenyl dipropargyl ether(2, 2BPhDPE), 4, 4'-biphenyl dipropargyl ether(4, 4BPhDPE), 2-biphenyl methyl propargyl ether(2BPhMPE), and 4-biphenyl methyl propargyl ether(4BPhMPE). The presence of an oxygen atom on the substituent changes the polarity and orientation of the triple bond. The compounds were synthesized by coupling the appropriate biphenyl alcohol with propargyl bromide in the presence of sodium hydride. These compounds have been tested as inhibitors of CYP 1A1, 1A2, and 2B1 with methoxyresorufin (1A2), ethoxyresorufin (1A1), or benzyloxyresorfin (2B1) as the substrate. The preliminary results indicate that 2BPhPE is a potent inhibitor of 1A2 and 2B1 but only a moderate inhibitor of 1A1. 4-BPhPE and 2BPhMPE are potent inhibitors of 1A2 but only moderate inhibitors of 1A1 and 2B1. 4, 4BPhDPE is a moderate inhibitor of 1A1 and 1A2 but a poor inhibitor of 2B1. 2, 2BPhDPE is a moderate inhibitor of all the CYP enzymes tested. 4BPhMPE acts as a potent inhibitor of all the CYP forms tested, but may be a tight binding alternate substrate for 1A1. Experiments continue to determine the mechanism of inhibition as well as the inhibitory parameters.

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#### USE OF A HUMAN HEPATOCYTE-DERIVED CELL LINE TO SIMULTANEOUSLY ASSESS CYP3A4 INDUCTION AND TIME-DEPENDENT INHIBITION.

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Cytochrome P4503A4 (CYP3A4) is the major drug-metabolizing enzyme in human liver and can be subject to both induction and inhibition. Changes in CYP3A4 expression and activity are frequently associated with drug-drug interactions. We report the use of a human hepatocyte-derived cell line to simultaneously assess CYP3A4 induction and time-dependent inhibition. Fa2N-4 cells were plated and cultured for two days, then exposed to varying concentrations of test compound in cell culture medium. After 48 hours, cells were washed, then incubated with medium containing a CYP3A4 probe substrate (midazolam) in order to assess catalytic activity. Cells were then harvested, RNA isolated, and CYP3A4 mRNA measured. Replicate sets of plated cells were treated in parallel, and viability of cells assessed using a mitochondrial dehydrogenase activity assay. Treatments that affected cell viability were not used in subsequent mRNA and activity determinations. The prototypical inducers rifampicin (30  $\mu$ M) and dexamethasone (50  $\mu$ M) produced 15- and 2-fold increases in CYP3A4 mRNA in these cells after 48 hours of treatment, and 9- and 2-fold increases in enzymatic activity, respectively. In contrast, compounds that were both inducers and time-dependent inhibitors of CYP3A4, produced increases in CYP3A4 mRNA, but decreased CYP3A4 catalytic activity in these cells. For example, ethinyl estradiol (10  $\mu$ M) and ritonavir (1  $\mu$ M) each produced approximately 4-fold increases in CYP3A4 mRNA, but reduced midazolam 1-hydroxylase activity relative to control by 75% and 50%, respectively. Since compounds were washed from the cells prior to incubation with probe substrate, the decrease in CYP3A4 catalytic activity was not likely a result of competitive inhibition, but was likely due to time-dependent inhibition. These results indicate that this assay can be used for early identification of CYP3A4 inducers as well as time-dependent inhibitors of CYP3A4 in drug discovery.

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#### HUMAN NAPHTHALENE METABOLISM.

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The polycyclic aromatic hydrocarbon naphthalene is an environmental pollutant, a component of jet fuel and since 2000 reclassified as a potential human carcinogen. Few studies of *in vitro* human metabolism of naphthalene are available, and these studies are mostly focused on lung metabolism. Therefore, the current studies were performed to characterize the metabolism of naphthalene mediated by human cytochromes P450 (CYP) using pooled human liver microsomes (pHLM) and CYP isoforms. The major metabolites from naphthalene mediated by pHLM were trans-1, 2-dihydro-1, 2-naphthalenediol (dihydrodiol), 1-naphthol (1-ol), and 2-naphthol (2-ol). Their kinetic parameters showed Km values of 23, 40, and 115  $\mu$ M, and Vmax values of 2860, 268, and 22 pmol/mg protein/min., respectively. Through human CYP isoform screening of naphthalene metabolism, CYP1A2 was identified as the most efficient isoform among those tested for producing dihydrodiol and 1-ol while CYP3A4 was the most effective for 2-ol production. Only CYP1A2 generated 1, 4-naphthoquinone from naphthalene. Metabolism studies of primary metabolites of naphthalene were also performed in order to identify secondary metabolites. While 2-ol was readily metabolized by pHLM to produce 2, 6- and 1, 7-dihydroxynaphthalene, dihydrodiol and 1-ol were inefficient substrates for pHLM. To further explore the metabolism of dihydrodiol and 1-ol, a series of human CYP isoforms was applied. 1, 4-naphthoquinone and four unknown metabolites from 1-ol were observed, and 1A2 and 2D6<sup>1</sup> were identified as the most active isoforms for the production of 1, 4-naphthoquinone. Dihydrodiol was metabolized by CYP isoforms to three unidentified metabolites with CYP2A6 having the greatest ability toward this substrate among those tested. The metabolism of dihydrodiol by CYP isoforms was lower than that of 1-ol. These studies identify both primary and secondary metabolites of naphthalene mediated by pHLM and CYP isoforms. The dihydrodiol appears to be a relatively stable biomarker of human exposure to naphthalene.

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#### ROLE OF PULMONARY CYTOCHROME P450 3A1 IN 1-NITRONAPHTHALENE BIOACTIVATION AND INJURY IN ADULT AND POSTNATAL RATS.

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Cytochrome P450 (CYP) monooxygenases play an important role in the bioactivation of hazardous air pollutants such as 1-nitronaphthalene (1NN). Despite similar rates of 1NN metabolism in postnatal and adult rat lungs, postnatal rats are more susceptible to 1NN pulmonary injury than adults. The exact isozyme responsible for 1NN metabolism is unknown. The purpose of this study was to determine if CYP3A1, a major constituent of human liver and lung, is involved in the bioactivation of 1NN and if the amount of CYP3A1 explains the difference in susceptibility between postnatal and adult rats. We evaluated airway and liver microsomes for CYP3A1 activity using the substrate Midazolam. Seven-day old rats have lower rates of baseline Midazolam metabolism in lung and liver compared to adults. Ketoconazole, a CYP3A inhibitor, decreased total Midazolam metabolism in lung and liver microsomes from 7-day old and adult rats by at least 50%, *in vitro*. *In vivo*, ketoconazole inhibited total Midazolam metabolism by 51% in adult rat lungs, 26% in adult liver, 35% in 7-day old lungs, and 55% in 7-day old liver. To determine if ketoconazole prevents 1NN pulmonary injury, rats received an IP injection of carrier or ketoconazole prior to administration of 50mg/kg 1NN. Lungs were evaluated by high resolution light microscopy 24-hrs later. Extensive areas of vacuolated and exfoliated cells and bare basement membrane were detected in the bronchi of adult rats treated with carrier+1NN, whereas only a few vacuolated cells were detected in the bronchi of adult rats treated with ketoconazole+1NN. Ketoconazole did not significantly alter 1NN pulmonary injury in 7-day old rats as observed in adults. We conclude that pulmonary CYP3A1 is involved in the bioactivation of 1NN. We hypothesize the different injury patterns observed between adult and 7-day old rats could potentially be due to inhibition of first pass 1NN metabolism in 7-day rat liver allowing more 1NN to reach the lung. NIH ES06700, USEPA (R827442010) ES 004311 NIH Training Grant HL007013

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#### MODIFICATION OF PULMONARY CYP2B1 AND INDUCED CYP1A1 ACTIVITIES BY INTRAVENOUS INJECTION OF IRON DEXTRAN.

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Cytochrome P450s (CYPs) are membrane-bound heme-containing proteins (hemoproteins) that catalyze oxidation of xenobiotics producing less lipophilic substances thereby facilitating their excretion. The heme moiety of this protein is iron-

protoporphyrin IX, which plays the major role in the catalytic activity of the enzyme. We hypothesized that the bioavailability of iron modifies the activity of inducible (CYP1A1) and constitutional (CYP2B1) CYP isozymes in rat lungs. Therefore, male Sprague-Dawley rats were injected i.v. with 50 or 200 mg/kg BW iron as iron dextran. Saline was injected as a control. Eleven days later, rats were injected intraperitoneally with the classic CYP1A1 inducer, beta-naphthoflavone (BNF; 50 mg/kg BW), to induce CYP1A1. Three days after BNF injection, rats were sacrificed and the lungs were perfused by phosphate buffer saline (PBS) through the pulmonary artery to remove blood. Immediately after washing, the microsomes of the right lung lobes were freshly prepared to measure the CYP1A1-dependent enzymatic activity [ethoxyresorufin-O-deethylase (EROD)] and the CYP2B1-dependent enzymatic activity [pentoxyresorufin-O-deethylase (PROD)]. Before separation of microsomes by differential centrifugation, an equal portion of lung homogenate was freeze dried for measurement of lung iron. The results showed that pulmonary iron concentration was increased in rats receiving 50 mg/kg (310 + 31.45 µg/gm) and 200 mg/kg (796 + 37.8 µg/gm) iron compared to control (180 + 13.33 µg/gm). The activities of PROD and induced EROD were significantly decreased by injection of 200 mg/kg iron. These findings suggest that increasing rat lung iron content modifies xenobiotic metabolism in lung by decreasing the activity of CYP2B1 and inducible CYP1A1.

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#### ROLE OF CYP2E1 IN THE OXIDATION OF ACRYLAMIDE (AA) TO GLYCIDAMIDE (GA) AND FORMATION OF DNA AND HEMOGLOBIN ADDUCTS.

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AA is a known animal carcinogen, neurotoxin, and reproductive toxin. AA is formed in baked and fried carbohydrate-rich foods such as french fries. Our earlier studies showed that CYP2E1 is the primary enzyme responsible for AA oxidation to GA. Using CYP2E1-/- (KO) mice, subsequent studies in this laboratory showed that AA metabolism to GA is required for the induction of male germ cell mutagenicity. To assess the role of CYP2E1 in the epoxidation of AA and the formation of GA-DNA adducts as well as AA and GA hemoglobin (HGB) adducts, AA was administered to KO or wild-type (WT) mice at 50 mg/kg IP. Six hours later, mice were euthanized and blood and tissues were collected. Using LC-ES/MS/MS, AA, and GA, as well as DNA and HGB adducts were measured. While the plasma levels of AA and GA were 115.0±14.0 and 1.7±0.31 µM in KO mice, respectively, they were 0.84±0.80 and 33.0±6.3 µM in the plasma of AA-treated WT mice, respectively. Dosing of AA to WT mice caused a large increase in N7-GA-Gua and N3-GA-Ade adducts in the liver, lung, and testes. Further, while traces of N7-GA-Gua adducts were measured in all three tissues from KO mice treated with AA, the levels were significantly lower than in WT mice (52- to 66-fold). Significant elevation of both AAVal and GAVal adducts was seen in WT mice treated with AA. In AA-treated KO mice, the levels of AAVal were roughly twice as high as those in WT mice. The amount of GAVal in AA-treated KO mice was low but was significantly higher than in vehicle-treated KO mice, and were  $\geq$  33-fold lower than that found in AA-treated WT mice. In conclusion, these results demonstrated that CYP2E1 is the primary enzyme responsible for the epoxidation of AA to GA, which leads to DNA adduct formation. Oxidative pathways other than CYP2E1 can lead to formation of GA and GA-DNA adducts, but the contribution of these pathways is negligible by comparison.

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#### GENE EXPRESSION ALTERATIONS IN IMMUNE SYSTEM PATHWAYS FOLLOWING EXPOSURE TO IMMUNOSUPPRESSIVE CHEMICALS.

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Exposure to xenobiotics can affect a number of adverse immunological outcomes, including infectious disease, hypersensitivity, and autoimmunity. It has been proposed that related disorders may share causative genetic alterations. Diethylstilbestrol (DES), Dexamethasone (DEX), Cyclophosphamide (CPS), and 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD) are immunosuppressive chemicals that can induce similar pathophysiological endpoints, however the mechanism of toxicity is different for each compound. The primary goals of these studies are to correlate changes in gene expression with alterations in functional immune endpoints, study the mechanisms of action, and evaluate the commonalities among the test compounds. Female B6C3F1 mice were treated with one of the four chemicals daily for five days. The dosages, routes and vehicles selected had been previously optimized for each compound. Splenocyte RNA was analyzed using Illumina Sentrix™ arrays and AnEx software. A dose-dependent trend was evident in differ-

ential gene expression for each of the chemicals as compared to controls. DES and TCDD induced the greatest and least number of changes in gene expression, respectively. Many of the differentially expressed genes are known to play a role in apoptosis, host defense, and cell growth, differentiation, and adhesion. The majority of gene alterations were unique to a single compound, however a number of genes were similarly altered across compounds. Alterations common to three different chemicals include upregulation of IL-18, lymphotxin B receptor, and colony stimulating factor receptor, and downregulation of RANTES and histocompatibility antigens. These findings are consistent with observed alterations in immune function. Genomic analysis revealed several gene expression changes that may be commonly associated with xenobiotic-induced immune system perturbations. However, distinct gene profiles were also found in association with chemicals that target similar immune parameters.

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#### THE ROLE OF CYCLIN DEPENDENT KINASE INHIBITOR P21 IN TCDD-INDUCED THYMIC ATROPHY.

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Thymic atrophy is a hallmark of exposure to dioxins, of which 2, 3, 7, 8-tetrachloro dibenzo-p-dioxin (TCDD) is the most potent congener. TCDD-induced thymic atrophy may be regulated by multiple pathways that are triggered following activation of the aryl hydrocarbon receptor (AhR). In the current study, we tested the hypothesis that TCDD exposure results in p21-mediated cell-cycle arrest in the thymus, and that TCDD-induced thymic atrophy results in part from a blockade of thymocyte proliferation. To test our hypothesis, p21 KO mice and age-matched wild-type mice were exposed to 10 g/kg TCDD for 48-72 hrs. We found that TCDD induced G0/G1 cell cycle arrest in thymocytes of wild-type but not in p21 KO mice. In addition, p21 KO mice were resistant to TCDD induced thymic atrophy. We also noted that lack of p21 rendered mice resistant to TCDD-induced thymocyte apoptosis. Finally, we analyzed the effect of TCDD on CD4-/CD8- (DN) thymocyte subsets, defined by expression of CD44 and CD25, which is comprised of immature progenitors that proliferate to become CD4+/CD8+ (DP) thymocytes. TCDD exposure resulted in a blockade of thymocyte maturation at the highly proliferative DN2 stage (CD44+/CD25+), as indicated by the significant increase in the proportion of DN2 thymocytes, and concomitant decrease in DN3 (CD44-/CD25+) thymocytes. In addition, the increase in DN2 thymocytes was accompanied by G0/G1 cell cycle arrest of thymocytes in the DN compartment. However, thymocytes from TCDD-exposed p21 KO mice showed no alteration in the distribution of thymocytes within the DN compartment. Taken together, these results suggest that TCDD exposure results in p21 mediated cell cycle arrest of thymocytes and that the resulting blockade of thymocyte proliferation contributes, at least in part, to apoptosis and thymic atrophy.

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#### TCDD SUPPRESSES ANTIGEN-SPECIFIC INTERACTIONS BETWEEN OTII CD4 T CELLS AND OVA-LOADED DENDRITIC CELLS.

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Exposure to the environmental contaminant TCDD (dioxin) causes suppressive effects on the immune system. The mechanism(s) of action at the cellular and molecular levels underlying the potent suppression of T cell-mediated immune responses is not understood. We hypothesize that TCDD exposure impedes activation of CD4 T cells due to a lack of persistent interactions with dendritic cells (DC). To test this hypothesis, C57Bl/6 recipient mice were gavaged with vehicle or TCDD (15 µg/kg), injected i.v. with OT-II ovalbumin (OVA)-specific naïve T cells, and immunized with OVA-loaded DC. The fate of both the DC and antigen-specific T helper cells in the draining lymph nodes (LN) was then evaluated on multiple days after immunization. On days 3, 4 and 6, TCDD-treated mice had significantly decreased numbers of donor DC, and on day 6 a greater frequency of these cells were PI-positive suggesting their loss was due to increased cell death. Following re-stimulation with OVA, LN cells from TCDD-treated mice produced significantly lower amounts of IL-12. Furthermore, the ability of the OVA-loaded DC to induce clonal expansion of the OT-II CD4 T cells in TCDD-treated mice was significantly suppressed on days 4 and 6 post-immunization when compared to the vehicle-treated controls. OVA-specific T cells in mice treated with dioxin were also less activated based on lower expression of CD11a and CD44, and suppressed production of IL-2 and IFN- $\gamma$  following *ex vivo* re-stimulation. Additional studies utilizing OVA-loaded DC from AhR wildtype (AhR /) and knockout (AhR -/-) mice demonstrated that the TCDD-induced loss of DC was mediated exclusively via an AhR-dependent process. However, suppression of the OVA-specific CD4 T cell

clonal expansion in TCDD-treated mice was only partially restored following the immunization of adoptively transferred mice with OVA-loaded AhR-/- DC. These results suggest that both the DC and activated T lymphocytes are directly affected following activation of the AhR- $\gamma$ ii

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SUPERANTIGEN-PRIMED T CELLS EXPOSED TO 2, 3, 7, 8-TETRACHLORODIBENZO-P-DIOXIN (TCDD) UNDERGO APOPTOSIS DURING THE FIRST ENCOUNTER AND EXHIBIT ANERGY UPON RESTIMULATION WITH THE ANTIGEN.

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In the current study, we investigated the effect of TCDD on the ability of staphylococcal enterotoxin A (SEA)-primed T cells to divide by dual-labeling the cells with 5, 6-carboxyfluorescein diacetate succinimidyl ester (CFSE) and antibodies against the specific T cell receptors. To this end, C57BL/6 wild-type mice were injected ip with TCDD (10 microg/kg body weight) followed by hind footpad injections of SEA (10 microg/footpad). The draining popliteal lymph nodes (PLN) were harvested 1-4 days post treatment, labeled with CFSE and cultured for 1-4 days without further stimulation or in the presence of the recall antigen. TCDD-exposed SEA-reactive Vbeta3+ and Vbeta11+ T cells showed decreased cell divisions upon *in vitro* culture in the absence of any stimulation, which also correlated with increased levels of apoptosis. Moreover, the recall cell-division response was also defective in SEA-reactive T cells isolated from TCDD-exposed mice. However, during the recall response, cells from TCDD-exposed mice did not exhibit a defect in apoptosis, thereby suggesting that the defective recall response may result from a state of anergy rather than increased apoptosis. Using AhR knock out (KO) mice, we found that AhR was involved in the regulation of defective cell division and apoptosis induced by TCDD. Together, these data demonstrate while TCDD-induced apoptosis may account for the decreased primary T cell proliferative response, the reduced cell division seen during subsequent exposure to the recall antigen may result from a state of anergy. The current study also demonstrates that combined use of superantigen and CFSE may offer a simple and useful tool to monitor the ability of immunotoxins to alter the proliferative responsiveness of antigen-specific T cells (This work was supported in part by NIH grants R01AI053703, R01ES09098, R01 AI058300, R01DA016545 and R01HL058641).

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B CELL MATURATION AND TCDD-INDUCED MODULATION OF THE 3'α ENHANCER.

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2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), a known disruptor of B cell differentiation, induces binding of the aryl hydrocarbon receptor (AhR) nuclear complex to dioxin responsive elements (DRE). Effects on B cell differentiation appear to be dependent on the AhR and the 3'α enhancer has been identified as a molecular target of TCDD. Transcriptional regulation of the immunoglobulin heavy chain gene involves several regulatory elements including the 3'α enhancer, which is composed of four distinct regulatory domains. DNA binding sites for several transcription factors, including B cell-specific activator protein, NF- $\kappa$ B and Oct have been identified within the 3'α enhancer domains and are believed to be important in regulating 3'α enhancer activity. Previous studies have focused on mature B cells and have demonstrated TCDD-induced inhibition of 3'α enhancer activity which may be, at least in part, mediated by AhR-DRE binding within the 3'α enhancer. However, one domain within the 3'α enhancer, independent of the other domains, appears to be active in pre-B cells potentially implicating a role in B cell maturation. Therefore the objective of the present studies was to determine if TCDD could modulate 3'α enhancer activity within B cell lines representing various B cell maturation states. Preliminary studies with transiently transfected luciferase reporter constructs demonstrate a TCDD-induced inhibition of LPS-activated 3'α enhancer activity within the immature B cell line, WEHI-231. These results suggest that TCDD may target B cell maturation. (Supported by the School of Medicine, WSU)

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SYNTHETIC AND ENDOGENOUS PPARγ AGONISTS, GW7845, 15-DEOXY- $\Delta$ <sup>12,14</sup>-PROSTAGLANDIN J<sub>2</sub>, AND MONO-(2-ETHYLHEXYL) PHTHALATE, ACTIVATE COMPLEX CASPASE CASCADES IN PRO/PRE-B CELLS.

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Humans receive significant exposure to multiple PPARγ agonists, including anti-diabetic drugs (thiazolidinediones) and environmental contaminants (phthalates). Synthetic PPARγ agonists, including GW7845 and mono-(2-ethylhexyl) phthalate

(MEHP), as well as the endogenous agonist, 15-deoxy- $\Delta$ <sup>12,14</sup>-prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>), induce lymphocyte apoptosis, suggesting that PPARγ agonists are immunotoxins. Here, we compared the caspase cascade activated by each of these PPARγ agonists in the non-transformed pro/pre-B cell line, BU-11. A similar suite of caspases was activated by each of the PPARγ agonists; however the time required to initiate caspase activation and induce apoptosis differed. Significant apoptosis was induced within 1, 8, and 5 hr by optimal GW7845 (40  $\mu$ M), MEHP (150  $\mu$ M), and 15d-PGJ<sub>2</sub> (10  $\mu$ M) doses, respectively. Treatment with any of the PPARγ agonists resulted in the release of cytochrome c from the mitochondria, a potentially apical event in PPARγ agonist-induced apoptosis. Cytochrome c release was accompanied by mitochondrial membrane depolarization following treatment with GW7845, but not with MEHP or 15d-PGJ<sub>2</sub>. Caspases-3 and -9 appeared to be activated concurrently with cytochrome c release. In addition, treatment with any of the PPARγ agonists resulted in significant caspase-8 activation. Furthermore, formation of truncated Bid suggested a role for caspase-2 and/or caspase-8 in apoptosis. Using peptide inhibitors, we show that multiple initiator caspases may be required to fully activate the PPARγ agonist-induced apoptosis program. These data demonstrate that complex, but similar, apoptotic programs may be initiated by structurally diverse PPARγ agonists.

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CB2 CANNABINOID RECEPTOR AGONIST, JWH015 TRIGGERS APOPTOSIS IN IMMUNE CELLS: POTENTIAL ROLE FOR CB2 SELECTIVE LIGANDS AS IMMUNOSUPPRESSIVE AGENTS.

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Cells of the immune system uniquely express a cannabinoid receptor designated CB2, whose precise function remains unclear. Delta-9-tetrahydrocannabinol (THC), a major psychoactive component found in marijuana and other cannabinoids that interact with both CB1 and CB2 receptors are known to induce apoptosis in immune cells and cause immunosuppression. Such studies suggested the possible use of cannabinoids as anti-inflammatory/immunosuppressive agents. However, cannabinoids that bind CB1 receptors are also psychoactive thereby limiting their clinical use. In the current study, we therefore investigated whether the synthetic CB2-selective agonist JWH015, devoid of psychoactivity, would mediate apoptosis and cause immunomodulation *in vivo*. We found that JWH015 inhibited the proliferative response of splenocytes to T and B cell mitogens *in vitro* which correlated with induction of apoptosis. In addition, JWH015 triggered apoptosis in thymocytes *in vitro*. Mechanisms of apoptosis revealed that JWH015 caused release of cytochrome c and Smac, and cleavage of caspase-8, caspase-9, caspase-3 and Bid, as well as caused loss of mitochondrial membrane potential ( $\Delta$ V<sub>m</sub>). Use of caspase inhibitors showed that JWH015 induced apoptosis through cross-talk between the extrinsic (death receptor) and the intrinsic (mitochondrial) pathways of apoptosis. Finally, administration of JWH015 into C57BL/6 mice caused thymic atrophy and apoptosis as well as decreased peripheral T cell response to mitogens. Together, this study suggests for the first time that CB2 selective agonists can induce apoptosis in immune cells and cause immunosuppression. Thus, such cannabinoids may serve as novel anti-inflammatory/immunosuppressive agents and would be ideal candidates for clinical use due to lack of psychotropic effects.

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THE ENDOGENOUS CANNABINOID, 2-ARACHIDONYL-GLYCEROL, SUPPRESSES NF-AT NUCLEAR TRANSLOCATION AND INTERFERON- $\gamma$  PRODUCTION.

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2-Arachidonoyl-glycerol (2-AG), an endogenous ligand for both CB1 and CB2 cannabinoid receptors, has previously been demonstrated to modulate immune functions including suppression of interleukin-2 (IL-2) expression and nuclear factor of activated T cells (NF-AT) DNA binding activity. As NF-AT is a critical transcriptional regulator of both IL-2 and interferon- $\gamma$  (IFN- $\gamma$ ), the objective of the present studies was to investigate the effect of 2-AG on IFN- $\gamma$  expression and associated upstream signaling events. IFN- $\gamma$  expression was assessed using an enzyme-linked immunosorbent assay (ELISA) and reverse transcriptase polymerase chain reaction (PCR). Translocation of NF-AT was measured by Western analysis and calcium determinations were made with a dual excitation spectrometer. Pretreatment of splenocytes with 2-AG markedly suppressed phorbol ester plus calcium ionophore induced IFN- $\gamma$  secretion in a cannabinoid receptor-independent manner. In addition, 2-AG suppressed IFN- $\gamma$  steady state mRNA expression in a con-

centration- and time-dependent manner. Time of addition studies revealed that 2-AG treatment up to 12 hours post cellular activation still resulted in suppression of IFN- $\gamma$ , which was consistent with a time course conducted with cyclosporin A (CsA), an inhibitor of NF-AT activity. Coincidentally, 2-AG perturbed the nuclear translocation of both NF-AT1 and NF-AT2 proteins, and blocked thapsigargin-induced elevation in intracellular calcium, suggesting that altered calcium regulation might partly explain the suppression of NF-AT nuclear translocation. Taken together, these data demonstrate that 2-AG suppresses IFN- $\gamma$  expression in murine splenocytes in a cannabinoid receptor-independent manner, and that the mechanism might involve suppression of intracellular calcium signaling and perturbation of NF-AT nuclear translocation. (Supported by NIH grant DA12740).

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#### CONCEPTUAL PRINCIPLES FOR UTILIZING OMIC TECHNOLOGIES IN MECHANISTIC RISK ASSESSMENT.

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Omics technologies promise tremendous advancements for toxicology and risk assessment, including identification of more sensitive biomarkers of exposure and effect, of pre-toxicological changes, and of molecular mechanistic steps in toxicologic pathways. Omics technologies may thus help identify fundamentally similar modes of toxic action based on similar DNA, RNA, protein, or metabolic profiles and help categorize chemicals accordingly. Although some of these promises are attainable, it is important to appreciate that omics technologies alone are unlikely to elucidate the complete mechanism or mode of toxic action. To explore how best to utilize omics technologies in mechanistic research, we first considered the classic definitions of mode and mechanism of action and evaluated the types of data required to satisfy those definitions using a classic hepatotoxicity model. Our evaluation suggests several principles. Polyomic approaches will be necessary to define the sequence of molecular steps in a mechanism of toxicity. Because it is critical to understand the causal links between individual mechanistic steps and to integrate this understanding at several levels of biological organization, polyomic approaches will augment, but not replace, classical mechanistic research. Bioinformatic technology will be essential for interpreting and integrating polyomic data according to fundamental tenets of pharmacology and toxicology, such as dose-response, and in turn, to make interpretations from the data that are relevant for risk and safety assessment. It is critical to develop approaches that differentiate between pre-toxicological, compensatory, and protective responses to chemicals, and that can identify the dose-dependent transitions in mechanisms that most impact risk estimation.

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#### USE OF GENOMICS DATA TO PREDICT MODE OF ACTION IN CHEMICAL HAZARD CHARACTERIZATION.

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The process of characterization of chemical hazard and human carcinogenic potential is evolving from their traditional dependence on tumor endpoints to the inclusion of data that describe the mode of action (MOA) at the cellular and molecular level. Information from gene expression profiling has the potential to greatly improve this process by predicting key mechanistic pathways involved in the chemical mode of toxicity and the human relevancy of the effects observed in animals by parallel inter-species comparisons. Hypothetical MOA(s) can then be generated for a more targeted testing protocol of the chemical of concern. Dimethylarsinic acid (DMA) will be used as an example to illustrate how gene microarray data can more rapidly provide mechanistic information comparable to that developed from years of research using more traditional toxicological endpoints. Exposure to DMA has been shown to result in rat urinary bladder tumors. Studies specifically designed to evaluate the mode of action for these bladder tumors suggest that DMA-induced cancer is associated with increased cell proliferation, generation of reactive oxygen species and cytotoxicity. Gene expression analysis of the target cell population for bladder cancer after *in vivo* exposure characterized the molecular changes involved in DMA-induced toxicity and demonstrated that oxidative stress response, cell proliferation and apoptosis are all involved in the toxicity response. The present example shows how genomics can be used as a cost- and time-effective tool to inform MOA and to generate hypothesis-driven and targeted chemical testing procedures for improved human health risk assessments. (This abstract does not reflect EPA policy).

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#### ASSIGNING OCCUPATIONAL HAZARD CATEGORIES (OHCs) FOR MATERIALS USED AND SYNTHESIZED IN THE PHARMACEUTICAL INDUSTRY.

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A chemical hazard categorization scheme used by a broad range of industries has been published by the UK HSE as part of the COSHH program. This scheme allows ranking of chemical hazards and targeted implementation of health-protective controls. There is no counterpart used widely across US industry, although NIOSH and other groups have coordinated recent discussions aimed at popularizing this approach. Within GlaxoSmithKline, the Occupational Hazard Category (OHC) program is recognized as a critical tool for ranking potential health hazards of chemicals. Solid materials are classified according to hazard into appropriate categories from OHC-1 (least hazardous substances) to OHC-4 (most hazardous). Notations accompanying the OHC designation include Carcinogen, Skin and Respiratory Sensitiser, Corrosive, Reproductive Hazard, etc. The OHC and notations of special hazard are linked to the GSK Exposure Control Matrix (ECM) which specifies hierarchical engineering controls as the primary means for control and containment of solid materials in order to avoid occupational exposure. Assignment of an OHC is derived from a matrix based upon the hazard classification of materials (EU R-phrase, toxicological characterization & pharmacological effect/potency). The assignment of the GSK OHC to raw materials, isolated intermediates, novel materials and pharmaceutical products supports global product development and manufacture. Externally established OELs can be converted to an OHC to aid in selection of workplace controls. Business circumstances and timing may dictate that an OHC can be established based on a limited data set. In this case, professional judgment and comparison to similar materials is used to guide the selection of an OHC. In summary, the methodology for OHC assignment currently in use by GSK is based on unifying several different approaches adopted by governmental and business groups and has been adopted for global application to the GSK product development process.

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#### USING STRUCTURE-ACTIVITY RELATIONSHIPS FOR CHEMICAL HAZARD SCREENING UNDER THE SUSTAINABLE FUTURES PROGRAM.

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Sustainable Futures is a voluntary EPA program with the goal of encouraging application of pollution prevention principles and the development of inherently low hazard new chemicals submitted as premanufacture notices (PMNs) under Section 5 of the Toxic Substances Control Act (TSCA). As part of the program, training and support are provided to companies in the evaluation of new chemicals, with limited available information, using EPA's hazard and risk characterization models and protocols. Physical/chemical properties, persistence, bioaccumulation, aquatic toxicity, and human health hazard from noncancer and cancer effects are identified. For this screening assessment, a broad search is conducted for relevant data on the chemical of interest. In most cases, data on the chemical are very limited, and so toxicology data on structural analogues and degradation products, as well as QSAR predictions, are used. Toxicological judgement is used to identify preferred analogs, focussing on key reactive structural groups that are likely to determine toxicity. Decision criteria for identifying potential ecological or health concerns and for selecting analogs have been developed and refined in the implementation of this program. Using these data, effect levels are identified, and combined with estimates of general public, occupational and aquatic exposure to develop risk assessments for these various scenarios and targets. This risk screening approach allows for the rapid collection and review of datasets and identification of potential toxicological and ecological concerns early in product development, and illustrates the utility of structure activity relationships for human health and risk assessment.

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#### SCIENTIFIC CRITERIA TO TEST INGREDIENTS ADDED TO CIGARETTES.

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The Life Sciences Research Office (LSRO), a nonprofit research organization, evaluates the potential of ingredients to change the relative risk of adverse human health effects from smoking cigarettes. LSRO convened a multidisciplinary, ad-hoc advisory panel and received public comments at two open meetings in 2002 and 2003. LSRO also evaluated more than 3,800 papers and 80 books, as part of the Added Ingredients Review (AIR). LSRO's recently released initial report concluded that testing of ingredients is feasible and worthwhile. LSRO's AIR Panel held six

meetings about the second report, which LSRO is in process of publishing. In summary, this report describes the following criteria for testing. Data submitters could compare smoke from identically manufactured cigarettes containing different amounts of the ingredient. Data submitters could aim to show that neither the added ingredient (nor its pyrolysis products): (a) transfers into cigarette smoke, (b) changes the physics, chemistry, or biological activity of the smoke, or (c) alters smokers' exposures to smoke. If any of these criteria are not met, a data submitter could decrease the ingredient and retest, or provide additional data, showing that the ingredient does not change the relative risk of adverse health effects of cigarette smoking. As an alternative, epidemiological studies, comparing identically manufactured cigarettes, with and without the ingredient, could provide definitive evidence of the effects of an ingredient on human health. While predictive, such studies are generally infeasible, because of their long durations and relative insensitivities. Thus, if performed at all, epidemiological studies probably would become part of post-marketing surveillance.

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#### AN INTEGRATED DECISION TOOL FOR EVALUATING CHEMICAL SAFETY IN LABORATORY RESEARCH STUDIES INVOLVING ANIMALS.

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Toxicology and pharmacology studies using whole animal exposures remain a cornerstone of chemical and drug safety evaluation. These studies involve potential health consequences for researchers, laboratory personnel, and animal handlers. Therefore, approaches for assessing potential health concerns associated with study protocols are needed. To meet the requirements of Institutional Animal Care and Use Committees (IACUC) and other groups, we have developed an integrated approach for risk assessment of activities associated with conducting animal research experiments. This approach integrates tools to support the traditional elements of risk assessment. First, an evergreen database structure has been developed that summarizes key hazards of toxicants or drugs used in each newly evaluated protocol. This database synthesizes and provides a critical evaluation of toxicological data for each agent. Second, a decision-logic for evaluating the potential exposure has been developed based on physical and chemical characteristics, routes of administration and use scenarios, and toxicokinetic data. Third, a qualitative approach for aligning potential hazard and exposure to corresponding control strategies has been developed. This approach has been applied to over 300 laboratory use protocols, and offers a tool for increasing the consistency in defining hazards, improving the documentation for certification, and clarifying the underlying rationale for workplace control recommendations.

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#### REAL WORLD TOXICOLOGY: A FRAMEWORK FOR EVALUATING TORT CLAIMS IN THE COURTROOM.

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The United States is the most litigious society in the world, and toxic tort cases are increasing in Western Europe and developing nations as well. The toxicologist plays a critical role in evaluating evidence, and providing an opinion for a judge or jury to evaluate. Most tort claims are based on an initial perception that an exposure or environmental contamination has occurred, and liable parties are held responsible. A recent court decision, Daubert, allows the judge to act as a gatekeeper regarding the expert's opinion and supporting evidence. We present a systematic approach to evaluate any legal case involving toxicology with 5 questions. Specific examples are provided where this process has been successfully used in tort claims. This approach also provides a framework to communicate principles of toxicology in plain language easily understood by individuals without professional training or education in the field.

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#### A FRAMEWORK/APPROACH FOR INCORPORATING PBPK MODELING INTO CUMULATIVE RISK ASSESSMENT OF CHEMICAL MIXTURES.

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Improving methods for cumulative risk assessment (CRA) of chemical mixtures has been a major goal and challenge. Toward this end, a framework/approach for incorporating physiologically-based pharmacokinetic (PBPK) modeling into CRA has

been developed, complementing existing CRA guidance, and refining its ten-step process to address PK issues. Principally, this allows the reevaluation of exposure and dose-response assessment to be transitioned from external to internal measures of exposure. PBPK modeling, as applied to chemical mixtures, can address both pharmacokinetic as well as human enzyme kinetic interactions during CRA. Doing so requires specific data. Phase I (Initial Analysis) is comprised of 5 steps culminating with the grouping of chemicals by toxic mechanism, identifying chemicals which may pose a significant amount of the mixture risk and a decision to embark on a comprehensive analysis of the mixture. PBPK analysis occurs in Phase II (Comprehensive CRA), which is comprised of 5 steps initiating with a dose-response analysis, and concluding with characterization of risk. The development of this framework/approach, not the actual CRA, utilized two model sets of chemical mixtures: Mixture 1 consists of 6 OP pesticides (methyl-parathion, parathion, chlorpyrifos, fenthion, diazinon, and fenitrothion) which share the same mode of action (acetylcholinesterase inhibition); Mixture 2 consists of 4 volatile organic chemicals (trichloroethylene, tetrachloroethylene, 1, 1, 1-trichloroethane, and chloroform) which have different modes of action and a variety of toxic endpoints. We present the advantages of integrating PBPK modeling and credible human tissue studies into CRA, as well as the methodologies involved in generating interactive PBPK models and quality human tissue studies. (Supported by USEPA Contract 3C-R102-NTEX)

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#### DEVELOPMENT OF A CHILDREN'S HEALTH RISK ASSESSMENT FRAMEWORK USING A LIFE-STAGE APPROACH.

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The draft Framework for Children's Health Risk Assessment (CHRA) has been developed to provide a comprehensive resource for CHRA. This document is not a guideline, but rather describes the overall structure and the components considered important for CHRA. The document emphasizes the need to consider potential exposures to environmental agents during preconception and all stages of development and focuses on the relevant adverse health outcomes that may occur as a result of exposures. The framework addresses the question of why an improved CHRA will strengthen the overall risk assessment process. The value added to this approach will include: 1) a more complete evaluation of the potential vulnerability at different life-stages, 2) evaluation of potential for toxicity after exposure during all developmental life-stages, 3) integration of adverse health effects and exposure information across life-stages and 4) a focus on the underlying biological events and critical developmental periods for incorporating mode of action considerations. This framework expands upon the ILSI (2001) framework that included problem formulation, analysis, and risk characterization. The CHRA framework expands on the life-stage-specific nature of the analysis with scoping and screening level questions for hazard characterization, dose response and exposure assessment. The risk characterization step recognizes the need to consider life-stage specific risks and explicitly describes the uncertainties and variability in the data base. It is important to note that lack of data at a specific life-stage using this approach will not automatically lead to increased uncertainty in the risk assessment (*The views of the authors of this abstract do not represent Agency policy or endorsement*).

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#### CASE STUDIES: DEVELOPING A DECISION-MAKING FRAMEWORK FOR OCCUPATIONAL HEALTH AND SAFETY CLEARANCE OF NEW CHEMICALS.

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Chemical manufacturers are expected to assess fully the potential occupational risks or hazards which could arise from the processing and/or manufacturing of new chemicals. However, in the absence of any standard or comprehensive methodology there has been room for error and/or inconsistency. Although a variety of hazard categorization and ranking schemes have been developed to remedy this gap, published approaches for expanding these into more robust occupational risk assessments are more limited. In order to remedy this gap, we have developed a comprehensive occupational hazard identification and risk assessment framework. The framework includes a decision flow chart that guides the user through specific criteria for completing the four basic steps of human health risk assessment: hazard identification, dose-response assessment, exposure assessment, and risk characterization. The procedure was designed specifically to allow for the maximum use of

the available data and current risk assessment methods. Criteria for developing toxicity and exposure potential ratings were developed drawing from a variety of published methods, with a scientific rationale presented for the selection of the recommended approaches. A set of risk characterization tools that integrate information from the toxicity and exposure assessments, as well as their interpretation was also developed. Full implementation of the procedure provided: (1) improvement in the quality of in-house risk assessment processes, (2) consistency and confidence in risk assessment output (e.g., hazard and risk ratings), and (3) strong support for safety and health decision-making for the clearance of new chemicals for use at facility operations.

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A RETROSPECTIVE REVIEW OF STUDIES UTILIZING GAVAGE DOSING OF PRE-WEANING RATS DEMONSTRATES NO ADVERSE CONSEQUENCES OF DOSING PROCEDURES.

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In some studies that are conducted to assess potential hazard, dose response, and age-related sensitivity during development, it may be necessary to administer test substance directly to pre-weaning animals in order to ensure adequacy of exposure during critical early life stages. The USEPA Office of Pesticide Programs has received a number of studies that have utilized such direct dosing procedures in rats. These include 10 guideline developmental neurotoxicity (DNT) studies, performed in 5 laboratories, and over two dozen range-finding and cholinesterase inhibition studies, performed in 7 laboratories, under GLP regulations. All of the DNT studies included the following procedures: Wistar or Sprague-Dawley rats were used; pups were born in the performing laboratory; pups were dosed once daily; vehicles included water or corn oil; and dosing volume was 5 or 10 ml/kg bw. Direct doses to pups were administered between postnatal days (PND) 7 and 21, with durations of 11-15 days. An evaluation of vehicle control data across the guideline DNT studies demonstrates no adverse impact of direct gavage dosing to pre-weaning rats. DNT control group sizes ranged from 24-38 litters, resulting in 200-300 post-culling control pups (2400 pups from 294 litters). Following initiation of dosing to pups of any age, the number of mortalities per control group ranged from 1-9 (mean=3.9) during the dosing period; only seven of these deaths appeared to be due to dosing error. Except for these seven pups, there were no adverse findings attributable to gavage dosing: clinical and functional observation data, scheduled necropsy results, and mean pup body weight gain data appear normal. In conclusion, when performed by trained technical staff, direct gavage dosing of pre-weaning rat pups appears to be a valid, non-traumatic methodology for use in toxicology studies. (This abstract does not necessarily reflect the views or policy of the USEPA.)

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RISK ASSESSMENT IN NEPHROTOXICOLOGY – SENSITIVITY OF RENAL TESTS.

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The kidney is a critical target organ for many chemicals. Toxicology studies often include the evaluation of a variety of renal toxicity endpoints, including organ (kidney) weight change, histopathology as well as clinical chemistry findings. Kidney weight change is often observed at doses below those that have clear implications for functional renal deficits. As a result, the appropriate use of this endpoint for risk assessment is an area of considerable debate. To help resolve this issue existing animal toxicity findings and risk assessment interpretations were evaluated for a set of kidney toxicants. We compiled risk value data from current risk assessments for nephrotoxicants to determine the frequency at which various combinations of effects were identified as sufficiently adverse to serve as the basis for assigning critical effect levels. We also evaluated the sensitivity of kidney weight change as a marker of nephrotoxicity as compared to more traditional measures. Our results show that a significant portion of risk values are based on kidney weight change alone, suggesting that risk assessment scientists have interpreted kidney weight as an adverse effect. In our analysis of the sensitivity of kidney weight as a predictor of nephrotoxicity, we found that kidney weight change had a calculated sensitivity of 80% in predicting histopathological change or renal function impairment as measured by clinical chemistry changes. No clear relationship between specific mechanisms of action and likelihood for identifying kidney weight as an early effect marker was identified. Based on this analysis, we conclude that increased kidney weight is sufficiently predictive of more severe renal damage to be used as the basis for selecting adverse effect levels for risk assessment.

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RELEVANCE OF RODENT FORESTOMACH TUMORS IN CANCER RISK ASSESSMENT.

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Studies have shown that most chemicals, which are known to be human carcinogens, also cause cancer in experimental animal models, but the converse is not as well established in that known animal carcinogens are not equally predictive of human carcinogenicity. A particularly controversial area in the use of animal data for human risk assessment is rodent forestomach tumors as a predictive tool for human carcinogenicity. Humans do not have a forestomach, but have histologically similar organs, including the oral cavity, pharynx, esophagus and glandular stomach. If tumors of the rodent forestomach are derived by a genotoxic mechanism, it is more likely that the agent is a human carcinogen as well. However, proliferative lesions in the rodent forestomach, including hyperplasia and compound-related tumors, may result from a combination of effects related to toxicokinetics, epithelial irritation, and/or dosing regimen and are less likely to be relevant for evaluating human carcinogenic potential. Hyperplasia is recognized as a preneoplastic lesion leading to forestomach papillomas and squamous cell carcinoma, but establishing the mechanism of action is critical to determining relevance to humans. We review the comparative anatomy and physiology of the rodent forestomach with human tissues and organs, forestomach tumor biology, regulatory practices and risk assessment guidance regarding the use of forestomach tumors for cancer risk assessment, examining specific chemicals, including benzo(a)pyrene, 1, 3-butadiene, epichlorohydrin, ethyl acrylate, hexavalent chromium, and mercuric chloride. A weight of evidence approach that combines criteria related to human exposure patterns, rodent tumor biology, genotoxicity, kinetics and comparative/mechanistic toxicology is proposed to evaluate human carcinogenic potential. Forestomach tumors resulting from chronic irritation associated with high-concentration drinking water and gavage dosing should not be used as the basis for determining carcinogenicity in humans or quantitative cancer potency estimates.

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APPLICATION OF QSTRS TO SELECT A SURROGATE CHEMICAL FOR A CHEMICAL LACKING ORAL TOXICITY DATA.

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Chemicals of concern (CoCs) at hazardous waste sites are cleaned up based on their potential toxicity to humans and the ecosystem. Often, there is a lack of experimental toxicity data for developing oral reference doses (RfDs) for a CoC in the literature. This research describes a method for identifying a surrogate chemical for any given CoC using Quantitative Structure Toxicity Relationships (QSTRs). A commercial QSTR model, TOPKAT, was used to establish structural and descriptor similarity between the CoC and the compounds in the QSTR model database using the Oral Rat Chronic LOAEL model. All database chemicals within a similarity distance (Euclidean distance between descriptor values of database chemical and CoC) of  $\leq 0.200$  from the CoC are considered as potential surrogates. If the CoC fails to satisfy model considerations for the LOAEL model, no surrogate is suggested. Potential surrogates that have toxicity data on Integrated Risk Information System (IRIS) or Health Effects Assessment Summary Tables (HEAST) become candidate surrogates. If more than one candidate surrogate is identified, the chemical with the most conservative RfD is suggested as the surrogate. The procedure was applied to determine an appropriate surrogate for dichlorobenzophenone (DCBP), a metabolite of chlorobenzilate, dichlorodiphenyltrichloroethane and dicofol. 47 potential surrogates were identified that were within the similarity distance of  $\leq 0.200$ , of which only five chemicals had an RfD on IRIS or HEAST. Among the 5 potential surrogates, chlorobenzilate with an RfD of 2E-2 mg/kg-day was chosen as a surrogate for DCBP as it had the most conservative toxicity value. This compared well with surrogate selection using available metabolic information for DCBP and its metabolites or parent compounds in the literature and the provisional toxicity value of 3E-2 mg/kg-day that was developed using a sub-chronic study. In addition, 90% of 359 IRIS chemicals tested with this procedure had a surrogate chemical whose RfD was more conservative than the parent RfD on IRIS.

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A HISTORICAL PERSPECTIVE ON LONG-TERM ANIMAL BIOASSAYS.

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In addition to being an academic discipline, the history of toxicology is relevant to forensic matters. For example, the history of toxicology can be a component in cases where past decisions regarding toxicological testing or data interpretation are evaluated in the context of the then-existing state of scientific knowledge. To illustrate how the state of scientific knowledge changed over time, we conducted an

analysis of chronic cancer bioassays from the 1940s to the present. Our analysis demonstrated that the cancer bioassay evolved through several stages - assays were initially used to recreate known human diseases in animal models; subsequently they were used to evaluate the safety of chemicals in foods, drugs and cosmetics; later they were used to evaluate the safety of environmental and occupational chemicals; and more recently they have been used to obtain data to calculate acceptable exposure levels. For example, in 1969 Innes et al. reported carcinogenicity data for 120 chemicals tested for the National Institutes of Health. All of the chemicals were either pesticides or chemicals with existing evidence of carcinogenicity in humans. It was not until the mid- to late-1970s that toxicologists began using data from animal tests to quantitatively estimate human cancer risks in the absence of human epidemiological data (e.g., Mantel et al. 1975, Schmahl et al. 1977). Thus, the purpose of testing changed only with an increasing level of confidence in the predictive ability of such tests for evaluating exposures outside the observable range of effects. Using lists of chronic bioassays published by the US Public Health Service since the 1950s, we have evaluated the critical events in the evolution of chronic bioassays in toxicology. By describing the "state of the art" in toxicology over time, we can place contemporaneous knowledge in an appropriate context. We also demonstrate how this analysis may be illuminating in interpreting contemporary progress made in developing and implementing methods to test for endocrine active chemicals.

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THE T25 METHOD FOR CANCER HAZARD CHARACTERISATION. COMPARISON WITH HAZARD CHARACTERISATION BASED ON EPIDEMIOLOGY.

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Recently we have described a simple method for quantitative risk assessment of non-threshold carcinogens based on the dose descriptor T25. In the present study quantitative hazard estimates calculated with the T25 method have been compared with results obtained using quantitative methods based on epidemiological studies. "Known" and "Likely/Probably" human carcinogens were identified from the USEPA database IRIS. In cases where the hazard characterisation was performed on the basis of epidemiological studies, the IARC monographs were used to identify animal studies by oral or inhalation exposure suitable for hazard characterisation by the T25 method. Six agents were identified: benzene, benzidine, 1, 3-butadiene, cadmium, nickel subsulfide and vinyl chloride for which USEPA had made hazard estimations based on data from epidemiological studies. Animal data suitable for hazard characterisation were also available. For comparing hazard characterisations based on epidemiological and animal data, it was pragmatically decided to do this by comparing the chronic doses expressed as those representing a lifetime cancer hazard of  $10^{-5}$  (both data sets were derived by linear extrapolation). In all cases the difference between the chronic doses determined from animal studies by the T25 method differed from those determined from epidemiological studies by a factor of less than three. Although a limited number of carcinogens were studied, the results demonstrate a very good agreement between the hazard characterisation based on epidemiological investigations and animal experiments spanning a range of more than  $10^4$ .

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DOSE-RELATED ISSUES IN THE DESIGN AND INTERPRETATION OF CHRONIC TOXICITY AND CARCINOGENICITY STUDIES IN RODENTS.

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Building on a 1997 ILSI Risk Science Institute (RSI) monograph, Principles for Dose Selection in Chronic Rodent Bioassays, a 24-member multi-sectorial international working group convened by RSI has developed guidance on a number of dose-related issues in the design and interpretation of chronic toxicity and carcinogenicity studies in rodents. Among the topics covered by the working group are the role of specific study objectives in the selection of dose levels, characterization of the design decision process, key factors in study design and their implications for interpretation and assessment of studies, and perspectives on new technologies and the future of the rodent bioassay. The working group noted that chronic rodent studies should be seen as scientific experiments, designed with well-articulated objectives and conducted to address specific questions. Six possible objectives and several combined objectives are identified and discussed. The working group described a generic 7-step process for dose selection, beginning with consideration of the scientific, regulatory/risk management, and practical contexts. Optimal dose placement may differ, depending on whether the primary purpose of the bioassay is screening, dose-response characterization, NOAEL/BMD determination, or safety assessment. Factors affecting study design are discussed, ranging from kinetics to nutritional and hormonal effects to tissue pathology. Case studies illustrate the applica-

tion (or omission) of these concepts in practice. Finally, the implications of potential paradigm shifts in biology, toxicology and risk assessment for study design are considered. (Supported by EPA Cooperative Agreement R-83049601, Health Canada Contract No. H4045-03-EXSD015/4500063129, and the ILSI Risk Science Institute)

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STATISTICAL PROPERTIES OF CARCINOGEN THRESHOLD ESTIMATES USING LOG-LINEAR REGRESSION.

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The objective of this study is to examine the statistical properties of a method that has been proposed for estimation of dose-response thresholds for carcinogens. Simulation studies have been conducted to examine the statistical properties of a method proposed by Dr. W. J. Waddell, based on log-linear regression. The simulations were based on published data sets that have been analyzed by this method. For this presentation we focus on the simulation results for an NTP study of the food additive 2, 4-hexadienal. The simulation study was done by fitting 1-stage, logistic, piece-wise linear ("hockey-stick"), and log linear regression models to the data. These models all fit the data adequately, based on a chi-square goodness of fit test using a p-value  $\geq 0.1$  as the acceptance criterion. Monte Carlo methods were used to generate sets of 2000 stochastically-varying simulated data sets. Dose-response thresholds estimated using log-linear regression were compared with the known properties of the underlying dose-response relationship, and the performance of the log-linear regression method was characterized. The primary result was that simulations based on no-threshold models (1-stage and logistic) resulted in an above-zero threshold estimate in every case, using log-linear regression. Simulations based on threshold models (log-linear regression and piece-wise linear regression) returned similar threshold estimates. However, the estimates using piece-wise linear regression exhibited considerably better precision than estimates using log-linear regression. These results suggest that the log-linear regression procedure is unreliable for determining the presence of a dose-response threshold, and sub-optimal for determining the location of the threshold in cases where the threshold is assumed to exist.

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AN EMPIRICAL APPROACH TO DOSE-DURATION-RESPONSE MODELING AND TIME-SCALING.

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An approach to deriving a dose-duration-response curve for dichotomous data is described with objectives of: (1) taking exposure duration, as well as exposure level, into account; (2) bringing to bear as much data as possible, of suitable quality, in a unified analysis; (3) testing co-factors that may affect response (e.g., sex, species, lab); (4) determining a time-scaling relationship (effect of a unit change in exposure level relative to a unit change in exposure duration). The idea behind the empirical approach is to extend a dose-response model that fits the data at individual exposure durations to a single model for data over all exposure durations. This is accomplished by using the duration-specific parameter estimates to determine a parametric function to explicitly incorporate duration into the dose metric, creating a dose-duration metric. The data over all exposure durations are then fit simultaneously using the dose-duration metric with addition of any co-factors to be tested. The approach is demonstrated using several studies on mortality of rats exposed to hydrogen sulfide (H2S) for durations between 10m and 6h. The resultant dose-duration metric coincides with one of the choices available in EPA's program for categorical regression (CatReg). The resultant time-scaling equation differs from the classical time-scaling equation of ten Berge et al. (1986) for concentration (C) and time (t) of the form  $C^n t = k$  (for n a parameter, k positive constant), which has been applied previously in a draft EPA guideline document on H2S. The dose-duration model with the derived metric fits the data significantly better than when a dose-duration metric consistent with the ten Berge equation is substituted. Time-scaling from the derived metric produced increasingly more conservative (i.e., lower) concentration levels than the ten Berge alternative, for comparable risk, as exposure durations decreased from 1h.

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STATISTICAL METHODOLOGY FOR THE SIMULTANEOUS ANALYSIS OF MULTIPLE TYPES OF OUTCOMES IN NONLINEAR THRESHOLD MODELS.

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Multiple outcomes are often measured on each experimental unit in toxicology experiments. These multiple observations typically imply the existence of correlation between endpoints, and a statistical analysis that incorporates it may result in improved inference. When both discrete and continuous endpoints are measured,

methodology to appropriately analyze the endpoints is scarce. We propose a new methodology to simultaneously analyze binary, count, and continuous outcomes for nonlinear threshold models that incorporates the intra-subject correlation. This methodology uses quasi-likelihood estimation and a working correlation matrix, and is therefore appropriate when the mean response of each outcome is of primary interest and the correlation between endpoints is of secondary importance. The methodology is illustrated with data collected from a mixture of five organophosphorus pesticides. Five outcomes—gait abnormality, tail pinch response, motor activity, and brain and blood cholinesterase—were analyzed using nonlinear threshold models. Compared to the individual analyses of each endpoint, the simultaneous model that incorporates the correlation between all five endpoints resulted in similar parameter estimates but had increased power to detect differences due to more precise estimates. In particular, the dose-threshold parameters for three endpoints were found to be statistically different from zero in the simultaneous analysis while none was significant in the individual analyses. Supported by T32 ES07334-01A1 (NIEHS, NIH). This is an abstract of a proposed presentation and does not necessarily reflect EPA policy or the official views of the NIEHS, NIH.

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TOXICITY ESTIMATION OF LOW LEVEL SHORT-TERM EXPOSURES TO CHEMICAL MIXTURES.

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The recent national report on human exposure to environmental chemicals indicates that over 100 chemicals have reached detectable levels in the U. S. human population. Thus, concerns exist that low level exposure to environmental chemical mixtures may adversely affect human health and wildlife. Health hazards assessment from exposure to chemical mixtures are most often performed using the hazard index approach that uses potency weighted dose or response addition. The Agency for Toxic Substances and Disease Registry (ATSDR) has developed a mixtures research program that supports targeted experimental research coupled with *in vitro* and limited *in vivo* testing to detect subtle health effects and verify or resolve built-in assumptions in the assessment methods for chemical mixtures. Mixtures of polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and dioxins have been evaluated using the toxicity equivalency factor method that is based on the principle of additivity. Meta-analysis of recent experimental studies supported by ATSDR, with mixtures of chloro-organic hepatotoxicants, neurotoxicants, and metals using standard toxicological endpoints did not demonstrate deviations from expected toxicity based on additivity at the lowest dose level tested. These findings suggest the need for further research into more sensitive molecular biomarkers-based parameters in order to detect possible ongoing cellular effects.

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PURITY ANALYSES FOR TOXICOLOGY STUDIES - A CASE STUDY WITH ANTHRAQUINONE.

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Anthraquinone is used as an intermediate in the manufacture of dyes and pigments, an additive in the kraft pulping process in the paper industry, a catalyst in the isomerization of vegetable oils, an accelerator in nickel electroplating, and as a bird repellent. The National Toxicology Program conducted studies of the chemical in feed for 14 weeks and 2 years in male and female F344/N rats and B6C3F1 mice. In response to questions regarding the purity of this material raised by a supplier of the anthraquinone made by a different synthesis route, work was done to identify and quantify small impurities seen in the original analysis. The original analyses indicated an overall purity of 99.4% with a gas chromatographic (GC) analysis using flame ionization detection indicating a purity of 99.9% with one impurity of 0.1% and a high performance liquid chromatographic (HPLC) analysis using ultraviolet detection indicating a purity of 99.5% with two impurities (one of 0.2% and one of 0.3%). Beyond quantitation by peak area percent, these impurities were not further characterized during the original analyses. The current work identified the two impurities as 9-nitroanthracene and anthracene by mass spectrometry and retention time matching using both GC with flame ionization detection and HPLC with UV detection. They were found to be present at concentrations of 0.06 and 0.05 (anthracene) and 0.11 and 0.09% (9-nitroanthracene) by HPLC and GC respectively. In addition anthrone and phenanthrene were found to be present at 0.002% or less. The chemical was also checked for the presence of several other suspected impurities with structural similarities to anthraquinone. No other impurities were found.

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THE TCDD TEQ IN HUMAN BLOOD FROM DIETARY VS. ANTHROPOGENIC DIOXINS: A DIETARY STUDY.

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Numerous naturally occurring compounds in the human diet can bind to the Ah receptor (AhR). The purpose of this study was to provide data for evaluating whether dietary Ah receptor binding compounds are contributors to the non-

PCDD/F and PCB blood TCDD TEQ (2, 3, 7, 8-tetrachlorodibenzo-p-dioxin toxicity equivalent quotient). Ten human volunteers were enrolled in the study only after giving full informed consent. The study protocol was reviewed and approved by an external Institutional Review Board. Volunteers maintained a specified (vegetable free or vegetable rich) diet over the course of two 4-day periods. Two volunteers participating in the study continued the vegetable-rich diet for an additional two-day period, while also ingesting an I<sup>13</sup>C extract. Meals were to be consumed at specified times. One 100 mL blood sample was taken before commencing the controlled diets, which was used for GC/MS analysis of PCDD/Fs and PCBs. During the four-day baseline diet, two blood samples were collected. During the four-day endodioxin-rich diet, four blood samples were collected. Bioassay analyses of the whole blood samples were carried out using a reporter gene bioassay assay for Ah receptor ligands. The mean analytical TCDD TEQ values calculated for the group of participants ranged from 0.022 to 0.119 ppt. In participants eating a vegetable-free diet, the bioassay TCDD TEQs ranged from 13 to 66 ppt. For participants eating an vegetable-enriched diet, the bioassay TCDD TEQs were 18 to 133 ppt. For participants eating an vegetable-enriched diet and taking I<sup>13</sup>C supplements, the bioassay TCDD TEQs were 62 to 218 ppt. The results of this study indicate that PCDD/Fs and PCBs comprise a very small fraction of the total TCDD TEQ in human blood and that modifications to the diet influence the TCDD TEQ. Substances that could be responsible for the vast majority of the blood TCDD TEQ are naturally-occurring Ah receptor binding compounds found in the diet, endogenous Ah receptor ligands and/or other anthropogenic compounds (besides PCDD/Fs and PCBs).

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RELATIVE POTENCIES OF THREE DIOXIN CONGENERS BASED ON LIVER FOCI FORMATION ASSAY AND LIVER CONCENTRATIONS IN RAT AND HUMAN ESTIMATED BY PBPK MODELS.

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In the health risk assessment of dioxins, WHO-TEF (toxic equivalency factor) is presently used to express the relative potencies (REP) of 29 dioxin-like compounds. In WHO-TEF, the same TEFs are set for rodents and humans without consideration of interspecies differences, although the half-lives for rats (20 days) and humans (6.9 years) are different, as are the body sizes and organ weights. For Japanese people in general, 2378- tetrachlorodibenzo- p- dioxin (TCDD), 12378-pentachlorodibenzo- p- dioxin (PeCDD) and 23478- pentachlorodibenzofuran (PeCDF) are three large contributors to total toxic equivalency (TEQ), according to their TEFs and daily uptake levels. The relative contribution of 2378-TCDD, 12378-PeCDD and 23478-PeCDF were 8.4, 14.4, and 12.7 % of total TEQ. We tried to determine new REPs of these three dioxins for rats and humans based on a specific endpoint and the concentration in the target organ. In this study, we focused on the formation of hepatic foci as a marker of the tumor promotion activity of dioxins. Rats were initiated with diethylnitrosoamine (DEN) and a 70% partial hepatectomy was followed by repeated oral administration of dioxins. Foci formation in the liver was quantified by staining with anti-glutathione-S-transferase P1 (GSTP1) antibody. The observed relative foci formation activities of 2378-TCDD, 12378-PeCDD and 23478-PeCDF were 1.0, 0.9 and 0.6, respectively. For interspecies extrapolation of the toxicity of dioxins, we developed physiologically based pharmacokinetic (PBPK) models for humans. Interindividual differences among Japanese adults were also analyzed with a Monte Carlo simulation. These methods and the results obtained are useful for the quantitative risk assessment of dioxins.

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VARIABILITY AND UNCERTAINTY DISTRIBUTIONS FOR THE CARCINOGENIC POTENCY OF 2, 3, 7, 8-TCDD.

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There are ten long-term carcinogenicity bioassays using 2, 3, 7, 8-TCDD, in male Syrian Golden hamsters, male and female Osborne Mendel rats, male and female B6C3F1 and B6C3 mice, and two in female and one in male Sprague-Dawley (SD) rats. Carcinogenic potency estimates and their approximate uncertainty distributions were obtained from each experiment using likelihood methods applied to a linearized multistage dose-response curves. Maximum likelihood estimates (MLE) for the carcinogenic potency range from zero to 40, 000 kg-d/mg using the metric of applied dose, and 95th percentile upper confidence limits (UCL95) on potencies in rats and mice appear to be lognormally distributed, but the hamster is a low outlier. Applying a lognormal between-experiment variability distribution to the rat and mice data leads to a MLE for the median of 1, 100 kg-d/mg, with an uncertainty UCL95 on the UCL95 of the variability distribution (the 95/95 point) of 15, 000 kg-d/mg. Constraining the between-experiment variability to have the geometric standard deviation (a factor of 10) previously observed (Crouch, 1996) in animal-to-human extrapolations, and including the hamster data also, leads to an extrapolated median estimate of 80 kg-d/mg for potency in humans, but a 95/95

point of 30, 500 kg-d/mg. Using the same methods but the EPA approach of extrapolation using the 1/4 power of bodyweight ratios gives a median estimate of human potency of 7, 000 kg-d/mg with 95/95 point of 52, 900 kg-d/mg (rat and mouse data only). A better dose metric for extrapolation to humans may be the lifetime average concentration in the body. This metric was estimated for two experiments with measured concentrations (both in female SD rats) based on the dosimetric model of Carrier (1995). The MLE for carcinogenic potency are 0 and 0.46 per  $\mu\text{g}/\text{kg}$ , but these are not significantly different. The combined uncertainty distribution has median 0.18 and UCL95 0.41 per  $\mu\text{g}/\text{kg}$ . All uncertainty and variability distributions are available in tabulated and parametric form.

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#### COMPARING ENVIRONMENTALLY RELEVANT PCBs TO TCDD IN CYP1A2 NULL AND WILDTYPE MICE.

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The role of CYP1A2 on the interactions of TCDD (2, 3, 7, 8-tetrachlorodibenzo-p-dioxin, dioxin), dioxin-like (DL) and non-dioxin-like (NDL) polychlorinated biphenyl (PCBs) was compared in multiple responses of different laboratory-defined mixtures, based on mass ratios found in food, using *Cyp1a2* null (KO) and C57BL/6J wildtype (WT) male and female mice. The chemical groups were TCDD alone, DL Mix A, NDL Mix B, and combination Mix C and the doses ranged from 0.0, 0.001, 0.01, 0.1, 1.0, and 10.0  $\mu\text{g}$  TEQ/kg body weight for Mix A, B and C, and the same plus 100.0  $\mu\text{g}$  /kg for TCDD. Liver weight was increased only in Mix C in both male and female, WT and KO. No effects were observed in the KO mice on markers of oxidative stress. Both male WT and KO showed greater decreases in levels of total triiodothyronine (TT3), while Mix A and Mix C resulted in a 50% reduction in levels of total thyroxine (TT4) in all groups. With exposure to TCDD or DL mixes, but not to NDL PCBs, there was an increase in ascorbate levels. Based on these findings, there does not appear to be a strain difference in response to chemical exposure, indicating CYP1A2 is not required for these biochemical responses. However, there does appear to be a gender difference between the both the knockout and wildtype, reflecting a possible endogenous estrogenic component to maintenance of TT3 and ascorbic acid cycling levels. Based on these findings, it appears that CYP1A2 does play a role in the processes leading to increased liver weight, increased enzymatic induction, and disruption of thyroid homeostasis, but does not appear to play a role in responding to oxidative insult. Neither in the presence nor absence of CYP1A2 can the TEQ approach predict all effects measured when DL and NDL PCBs are present. (This abstract does not reflect EPA policy. DB supported by EPA CT902908 and NIEHS T32-ES07126)

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#### CANCER POTENCY ESTIMATION FOR ACRYLAMIDE.

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A widely-recognized carcinogen, acrylamide produces tumors at multiple sites in rats and mice. Sources of exposure to acrylamide include cigarette smoke, the diet (i.e., formation occurs during high temperature cooking of many plant-based foods), and occupational uses. A thorough evaluation of the available evidence on the carcinogenic mode of action indicated that a genotoxic mechanism was most likely operative. Thus, a low-dose linear approach to dose-response characterization was taken. In addition, the analysis explicitly took into account the difference between humans and rats in the conversion of acrylamide to its DNA-reactive epoxide glycidamide, the longer half lives of acrylamide and glycidamide in humans than rats, and interspecies differences in pharmacodynamics. Calculation of the cancer potency of acrylamide was based on data from multiple acrylamide-responding tumor sites observed in four long-term drinking water studies in male and female rats. For each experiment, a probabilistic Monte Carlo procedure was used to derive a multisite cancer potency distribution, which combined dose-response relationships for each acrylamide treatment-related cancer site. The multisite potency distributions were used to obtain a geometric mean of potencies from the four studies, using Monte Carlo techniques. The upper 95 percent confidence bound of the geometric mean distribution was taken as the cancer potency for acrylamide. This potency estimate for acrylamide is lower than the value currently used by California EPA and USEPA. Also discussed are sources of interindividual variation in sensitivity to acrylamide-induced cancer due to variability in activation and detoxification enzymes, age, and co-exposures to other carcinogens or promoters. Finally, the cancer potency estimate based on the rat bioassays was compared with a cancer potency estimate based on pancreatic tumors observed among acrylamide-exposed workers, as a measure of the quantitative consistency and plausibility of the rat-derived estimate.

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#### ACRYLAMIDE TOXICITY - IS A REAPPRAISAL NECESSARY?

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Acrylamide has long been considered an exclusively man-made chemical. In biomonitoring studies however, even unexposed persons displayed a background level of DNA adducts specific for acrylamide exposure. In 2000 acrylamide formation in foodstuff has been detected, which may explain this level in biomonitoring studies. Risk assessment for acrylamide exposure has been based both on animal experiments and epidemiological studies; besides neurotoxicity the carcinogenic potency in animals has been led to the classification of acrylamide as a probable human carcinogen. Based on food exposure and comparison of likely acrylamide uptake in low and high food exposure groups the carcinogenic risk of acrylamide to humans should be reevaluated. Studies in industrial plants do not unanimously indicate an increase in human tumor incidence. Animal experience did not show a specific organ or organ group preference in high exposure groups, as opposed to specific acting carcinogens. Specifically, at 2  $\text{mg}/\text{kg}$  bw, i.e. close to a toxic concentration female Fischer rats developed tumors in the mammary gland, uterus, thyroidea and CNS, at nearly equivalent rates. Studies addressing the mutagenicity resulted in divergent phenomena - not mutagen but clastogen. Although these studies usually are interpreted as evidence for a specific carcinogenic potential also at low doses the following results favor a toxic mechanism with carcinogenicity likely due to cell toxicity: diverse tumor organs; weight loss as toxic phenomenon at the effect level; relevant incidence of spontaneous tumors in control animals. Whereas for a purely technical chemical prudence should prevail a different view on relevance may be necessary for compounds present in the natural environment. We will reassess epidemiological data with regard to human toxicity of acrylamide and background acrylamide exposure from foodstuff.

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#### DOSE-RESPONSE MODELING OF IN VIVO GENOTOXICITY DATA: ITS RELEVANCE TO RISK ASSESSMENT ILLUSTRATED BY AN APPLICATION TO ACRYLAMIDE.

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Genetic toxicity results are typically summarized as being either positive or negative, with no further consideration of the dose-response patterns that can be estimated from such studies. This analysis explores the use of two possible modeling approaches that extend the utility of such data: Poisson regression of counts of genetic effects per cell and dynamic modeling of the time-course of micronucleus production and loss as a function of exposure. Benchmark doses and predictions of response rates for predetermined doses of interest can be used to assess the relevance and role of the genetic toxicity results in a risk assessment. With respect to the acrylamide case study, the results suggest that the doses at which genetic damage is predicted to occur do not appear to be consistent or congruent with the major tumor endpoints (in the thyroid) observed in two long-term bioassays in rats; at doses equivalent to the highest bioassay dose level (where there was a 16% incidence of thyroid tumors) the model-predicted changes in chromosome aberration or micronucleus rates were less than 5%. Benchmark doses derived from the genetic toxicity data base do not appear to be the critical ones for acrylamide risk assessment. The benchmark doses derived from multigeneration reproductive studies were between 3 and 14  $\text{mg}/\text{kg}$  whereas those derived using the genotoxicity data ranged from 14 to 847  $\text{mg}/\text{kg}$ . This analysis suggests that issues associated with dose metric selection, extrapolation from one type of study to another and from one species to another, and mathematical modeling approaches need careful consideration.

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#### ISSUES RELATING TO THE DERIVATION OF ORAL TOXICITY VALUES FOR CHLORDECONE.

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Chlordecone (Kepone®) insecticide production in the United States ended in 1975 due to severe intoxication from industrial exposure at a Hopewell, VA facility. The primary noncancer health effects of oral exposure in humans and animals include liver and kidney effects, neurotoxicity, and male reproductive toxicity. Other reproductive effects (i.e., persistent vaginal estrus, impaired reproductive success) and developmental effects were observed in laboratory animals at higher doses. Some of these effects may be related to the endocrine disrupting properties of chlordecone. Renal toxicity (i.e., proteinuria and glomerulosclerosis) occurred at the lowest

chronic dose in animal studies. Noncancer toxicity values (e.g. RfDs or ADIs) could be derived using benchmark dose modeling of the renal data and application of appropriate uncertainty factors. No data on the carcinogenicity of chlordcone in humans are available. A single chronic dietary study provides evidence for carcinogenicity following high-dose exposure in rats and mice but lacks appropriate dose-response data. Therefore the study is considered to be limited in its usefulness for low-dose extrapolation and assessment of human cancer risk. The available data suggest that chlordcone is not genotoxic, but acts as an epigenetic carcinogen and a tumor promoter. Because the mode of action for carcinogenicity has not been completely elucidated, both linear and nonlinear approaches could be applied to dose-response assessment. A time-to-tumor approach could be utilized to adjust for the significant mortality in the bioassay. A nonlinear approach would consider the available data for precancerous liver hyperplasia that has been associated with subsequent liver tumor formation. (The views expressed in this abstract are those of the authors only and do not necessarily reflect the views or policies of the USEPA.)

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#### RE-EVALUATION OF THE RISK OF LUNG CANCER FROM DIESEL EXHAUST EXPOSURE.

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In the past several years, important developments have occurred (and are ongoing) that call for a critical review of the database on the association between exposure to diesel exhaust (DE) and lung cancer. Indeed, several assessments of DE exposure and epidemiological data are ongoing, and should provide a greatly improved database for DE within the next few years. For instance, these assessments should provide more definitive data regarding the potential carcinogenic risks from historical and current DE, and some will be particularly relevant for assessing the impacts of future advancements in diesel technology. With respect to the current diesel database, changes in diesel engine technology have significantly changed the composition of DE such that the epidemiological studies evaluating the effects of diesel engines in the 1950s through the 1980s, many of which are cited in contemporary assessments of DE, will not accurately predict the carcinogenic potential of current or future DE exposures. Further, a reevaluation of the available epidemiological and toxicological databases demonstrate that they are, in fact, insufficient to support a connection between exposure to DE and lung cancer. As several noted researchers have recently concluded, the significant deficiencies in the epidemiology studies make them inadequate to link DE to increases in lung cancer. In fact, new data demonstrate that DE and gasoline exposures are inseparable in all industries except mining. Thus, studies in miners present the best data for evaluating the potential linkage between DE and lung cancer. Finally, a contemporary assessment of the available evidence demonstrates that the toxicological database does not provide an adequate basis for finding an association between DE and lung cancer, that data regarding the mutagenicity of DE extracts have limited utility for conducting an assessment of the association between exposure DE and lung cancer, and that meta-analyses are unsuitable for evaluating the potential linkage between DE exposure and lung cancer.

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#### IMPORTANCE OF PEROXISOME PROLIFERATION IN UNDERSTANDING POTENTIAL MODE(S) OF ACTION FOR TCE AND ITS APPLICATION IN RISK ASSESSMENT.

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Peroxisome proliferators include a variety of chemicals such as phthalates, chlorinated paraffins, chlorinated solvents such as trichloroethylene and perchloroethylene, certain pesticides and hypolipidemic pharmaceuticals. These chemicals can be of concern to regulatory agencies if their potential for carcinogenic action is indicated by long-term carcinogen bioassays using rodents. The purpose of this presentation is to discuss the current state of knowledge for known peroxisome proliferators, including the scientific controversies, and describe how this information could be used in assessing human health risk for trichloroethylene (TCE). Peroxisome proliferators have often been thought to elicit their effects without causing mutation of DNA or chromosome damage. They are heterogeneous in action and the receptors they activate are extremely pleiotropic. Studies have demonstrated that peroxisome proliferator-activated receptors (PPARs), particularly activation of PPAR alpha, may be an obligatory factor in peroxisome proliferation in rodent hepatocytes. Recent studies in human and rodents, however, have reported that there are also other actions of PPAR alpha activation. TCE and two of its metabolites (trichloroacetic acid and dichloroacetic acid) have been shown to induce peroxisome proliferation in rodents though each has a different toxicity profile. Understanding the responses of PPAR alpha agonism for the determination of a key event or as a possible mode of action for TCE effects is crucial for its risk assessment. (Disclaimer: The views expressed in this abstract are those of the authors and do not represent the policy of the USEPA).

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#### INCORPORATION OF TRICHLOROACETIC ACID PLASMA BINDING IN HUMAN AND MOUSE IN TRICHLOROETHYLENE RISK ASSESSMENT.

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Trichloroacetic acid (TCA) is a metabolite of the solvent trichloroethylene (TCE), which remains an important risk assessment issue because of its carcinogenic potential. PBPK-model generated TCA dose metrics were employed in the most recent draft TCE risk assessment by USEPA (2001). Subsequently, Lumpkin et al. (2003) found that the proportion of bound TCA to free TCA is substantially higher in human plasma than mouse plasma. TCA binding equations were incorporated into 2nd generation PBPK models for TCE and TCA for mice (Abbas and Fisher, 1997) and humans (Fisher et al., 1998). Cancer bioassay conditions (NTP, 1990; NCI, 1976) were simulated using the 2nd generation mouse model (with binding), and average daily liver, total blood, and free blood AUC and peak TCA concentrations were calculated. All TCA AUC values were very similar when binding was incorporated into the PBPK models using either a single, saturable binding equation or using interpolation of the binding data. This finding indicates that the low dose predictions of the TCE PBPK models are robust to alternative descriptions of TCA plasma binding. The linear multistage model was fit to PBPK generated internal dose-metrics vs. liver tumor incident rates in male B6C3F1 mice. Mouse internal dose ED10 and LED10 values were extrapolated to human external doses with the 2nd generation human model that included TCA binding in plasma. Human ED10 and LED10 estimates based on TCA AUC in liver exceeded those based on liver dose metrics calculated previously (without TCA binding in plasma) (EPA, 2001; Rhomberg, 2000) by one to two orders of magnitude. Calculations of risk (e.g. incremental risk per mg/kg/day) would, thus, decrease by 10 to 100 times (Supported by DOE Coop. Agreement DE-FC09-02CH11109).

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#### USE OF A PHYSIOLOGICALLY BASED PHARMACOKINETIC MODEL FOR CHLOROFORM TO EVALUATE BIOMONITORING DATA.

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Biomonitoring data are evidence of exposure to environmental chemicals but do not, by themselves, say anything about the risk of adverse health effects. Physiologically based pharmacokinetic (PBPK) modeling can be used to estimate exposures consistent with biomonitoring data. Exposure estimates and knowledge of exposure-adverse effect and tissue dose-adverse effect relationships do, however, provide a basis for risk estimation. In anticipation of the need to evaluate biomonitoring data for chloroform, three published data sets describing blood and exhaled breath levels in people exposed to chloroform under controlled conditions were evaluated with an existing PBPK model. Model predictions agreed well with the measured data. We then defined a plausible exposure regimen combining inhalation, oral and dermal exposures associated with residential use of water containing low levels of chloroform. The following inputs to the PBPK model were chosen to reflect realistic exposure scenarios: (1) water concentration (0 - 80 ppb), (2) water intake (927, 2016, 3588 mL/day), (3) shower/bath duration (3, 10, 15, 60 min), (4) ambient concentration in air (1.2, 2.1, 4.1 ppb), and (5) water-air mass transfer coefficient (2.5, 8.9, 21 L/min). Time-course predictions of exhaled breath and blood levels using these inputs showed that inhalation and dermal exposures, and drinking tap water could each contribute substantially to total chloroform exposure when showering was considered. PBPK modeling of variability in the input parameters, as indicated above, predicted that variability in drinking water intake would, by itself, be expected to have little effect on the overall exhaled breath and blood levels. In summary, this study indicates that (a) multiple routes of exposure to chloroform should be considered during the evaluation of biomonitoring data and (b) PBPK modeling will be useful in the analysis of biomonitoring data for chloroform and other halomethanes.

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#### MONTE CARLO ANALYSIS OF SOURCES OF VARIABILITY IN CHLOROFORM-INDUCED HEPATIC CYTOLETHALITY AND REGENERATIVE PROLIFERATION IN B6C3F1 MICE.

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Characterization of sources of interindividual variability in toxic responses supports population-based risk assessment and helps to define how responses should be extrapolated from laboratory animals to humans. Physiologically based pharmacokinetic (PBPK) modeling can be used to study how PK behavior differs between individuals in a population and among species, as in the extrapolation from

laboratory animals to people. We extended a PBPK model for chloroform to describe a plausible mechanism of hepatic cell killing and regenerative proliferation, which is a risk factor in carcinogenesis (*Toxicol. Sciences* 75, 192-200, 2003). This pharmacodynamic (PD) extension allowed simulation of labeling index (LI), which is a measure of regenerative cellular proliferation in response to cytotoxicity. In the current study we analyzed sources of variability in LI data from female B6C3F1 mice that inhaled chloroform for 7 consecutive days (*Toxicol. Sciences* 66, 201-208, 2002). A Monte Carlo implementation of the PBPK/PD model was developed in which PK parameters and the control rate of hepatic cell division were varied. This analytical approach tests the hypothesis that the measured interindividual variability in LI is due to a combination of PK variability and variability in the control rate of hepatic cell division. The distributions, means, and standard deviations (SD) for the PK parameters were obtained from literature; the distribution, mean, and SD of the control cell division rate were calculated from 10 control LI data points. Monte Carlo simulations were compared to measured data by plotting measured and predicted mean LI against measured and predicted variance of the LI. With 1,000 iterations of the Monte Carlo model, all measured values fell within the cloud of predicted data points. Extension of these studies to male B6C3F1 mice and to rats will help to define appropriate treatment of interindividual and interspecies variability in human health risk assessments for chloroform.

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DOSE-RESPONSE MODELING AND BENCHMARK CALCULATIONS FROM SPONTANEOUS BEHAVIOR DATA ON MICE NEONATALLY EXPOSED TO 2, 2', 4, 4', 5 - PENTABROMODIPHENYL ETHER.

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In this work the benchmark dose (BMD) method was introduced for spontaneous behavior data observed in 2-, 5-, and 8-month-old male and female C57Bl mice exposed orally on postnatal day 10 to different doses of 2, 2', 4, 4', 5 - pentabromodiphenyl ether (PBDE 99). Spontaneous behavior (locomotion, rearing, and total activity) was in the present work quantified in terms of a fractional response defined as the cumulative response after 20 minutes divided by the cumulative response produced over the whole 1h test period. The fractional response contains information about the time-response profile (which differs between the treatment groups) and has appropriate statistical characteristics. In the analysis male and female mice could be characterized by a common dose-response model, i.e. they responded equally to the exposure to PBDE 99. As a primary approach, the BMD was defined as the dose producing a 5 or 10 % change in the mean fractional response. According to the Hill model, considering a 10 % change the lower bound of the BMD for rearing, locomotion, and total activity was 1.2, 0.85, and 0.31 mg PBDE 99 / kg body weight, respectively. A probability based procedure for BMD modeling was also considered. Using this methodology the BMD was defined as corresponding to an excess risk of 5 or 10 % of falling below cut-off points representing adverse levels of fractional response.

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ADVANTAGES AND LIMITATIONS OF BENCHMARK DOSE MODELING AND THE NOAEL/LOAEL APPROACH IN PERFORMING DOSE-RESPONSE ASSESSMENT BASED ON SMALL STUDIES: SUBCHRONIC RFD FOR CHLOROBENZENE AS A CASE STUDY.

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Benchmark dose modeling has advantages over the traditional NOAEL/LOAEL approach for dose-response assessment when suitable data are available. However, the benchmark dose methodology is highly sensitive to certain characteristics of the data to be modeled. One such character is group size. In the benchmark dose methodology, a benchmark response (BMR) of 10% is conventionally used for quantal data. USEPA guidance notes that 10% response is at or near the limit of sensitivity in a typical cancer bioassay. Group sizes in such studies are usually 50 or more animals. Yet, the benchmark dose methodology and 10% BMR have frequently been applied in assessments based on data from subchronic studies, which may include 10 or fewer animals per group. Application of the benchmark dose methodology and 10% BMR to small studies such as these can be problematic. A case study in which a subchronic RfD is derived for chlorobenzene is used to illustrate the issues involved. The critical study for this assessment was a 13-week study in dogs (4/sex/group). The point of departure for the subchronic RfD was derived by both benchmark dose modeling with a 10% BMR and by the NOAEL/LOAEL approach, and the results contrasted. Limitations of both approaches in handling

small group sizes are discussed. This case study suggests that the NOAEL/LOAEL approach is less sensitive to small group sizes than benchmark dose analysis using the 10% BMR. This is because the NOAEL/LOAEL approach requires less resolution from its statistical analysis, and because it can incorporate a weight-of-evidence analysis based on multiple endpoints simultaneously. Risk assessors have underappreciated the difficulties associated with application of benchmark dose methodology and 10% BMR to small studies, and should consider carefully whether group sizes are sufficient to support benchmark dose modeling in their assessments.

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BENCHMARK DOSE MODELING OF DEVELOPMENTAL RESPONSES IN OFFSPRING OF RATS EXPOSED TO TRICHLOROACETIC ACID.

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Trichloroacetic acid (TCA) is formed in drinking water as a byproduct of disinfection with chlorine. Selected fetal and litter data from a developmental study in Long-Evans rats (Smith et al. 1989) were analyzed by benchmark dose (BMD) modeling as part of the EPA effort to update information on health effects of TCA for IRIS. Pregnant rats were exposed to 0, 330, 800, 1200, or 1800 mg/kg-day of TCA by oral gavage on gestation days (GD) 6-15 and sacrificed on GD 20. Incidence data for fetuses with visceral malformations (levocardia was the principal lesion) and continuous data for fetal body weight (BW) and fetal crown-rump length (CRL), converted into quantal form, were modeled. Conversion of BW and CRL data involved 1) an assumption of normal distribution and 2) the use of the estimated distribution of the controls to define a response (BW or CRL decreased more than 1 SD below the control mean). Converted data for fetal BW and CRL were analyzed using three models, each of which included dose and litter size as explanatory variables and accounted for intralitter correlation by assuming a beta-binomial distribution for individual fetal responses. The TERALOG, TERAVAN, and TERAMOD programs (ICF Kaiser International, Ruston LA) were used to fit these models by maximum likelihood methods to the available data. Litter incidence data for levocardia were modeled using all available quantal models in the EPA BMDS program (v. 1.3.1). Overall, the most sensitive modeled responses were fetal BW and litter incidence of levocardia. The 95% lower confidence limits on the BMD values for these endpoints were 28 mg/kg-day (average from three models) and 31.3 mg/kg-day, respectively, for a benchmark response of 5% extra risk. These values are similar to the NOAEL of 32.5 mg/kg-day obtained for hepatic effects in F344 rats exposed to TCA in drinking water for two years. (Opinions expressed in this abstract are the authors and do not necessarily represent EPA policy.)

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EMPIRICAL EVALUATION OF SUFFICIENT SIMILARITY FOR A MIXTURE OF NINE HALOACETIC ACIDS (HAA'S) ON CHINESE HAMSTER OVARY CELL CHRONIC CYTOTOXICITY USING A FIXED-RATIO RAY DESIGN.

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Ray designs based on relevant fixed-ratio rays are useful for testing hypotheses of additivity for large numbers of chemicals. Relevant ratios of chemicals in industrial or environmental processes may not remain constant. While ray designs decrease experimental effort, all possible rays cannot be experimentally evaluated. Interest may be focused on statistical two-sided equivalence tests for additivity or one-sided equivalence tests for lack of synergy along an observed reference ray, and whether the same inference applies to an unobserved candidate ray. Borrowing ideas from the statistical equivalence literature, we have developed methodology that permits us to claim equivalent inference from an observed reference ray to an unobserved relevant candidate ray. Based on this methodology, equivalence margins associated with biologically meaningful deviations are chosen. A confidence region is computed for the reference ray and for the candidate ray based on the variability from the observed data. If both confidence regions are completely contained within the equivalence margins then the candidate ray may be concluded to be sufficiently similar in inference to the reference ray. The regions of equivalence are depicted graphically for interpretation. The cytotoxic effects of nine HAA's formed during disinfection of drinking water were evaluated in Chinese hamster ovary cells using single chemical concentration-response curves and a reference mixing ratio. Based on the observed reference ray, which shows lack of synergy, the graphical methods described above are used to illustrate that a mixing ratio from another disinfection process is sufficiently similar in inference as defined by selected equivalence margins. (This abstract does not reflect USEPA policy).

## EVALUATION OF PROPOSED THRESHOLD DOSES FOR CHRYSOTILE EXPOSURE AND RESPIRATORY DISEASE.

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Some researchers have suggested that a cumulative chrysotile exposure of approximately 25 f/cc\*year is a minimum threshold dose for increased risk of lung cancer and asbestos. However, to our knowledge, the scientific merits of this suggested threshold have not been tested via a comparison to occupational doses derived from individual exposure data. Brake mechanics provide a unique opportunity to conduct such an analysis, because 1) they are exposed to the chrysotile form of asbestos only, 2) there is a substantial amount of published industrial hygiene data characterizing asbestos exposures in brake servicing facilities, and 3) numerous epidemiology studies have consistently demonstrated that brake mechanics are not at an increased risk of developing asbestos-related respiratory diseases. In this analysis, we developed distributions of occupational tenure data for automobile mechanics and 8-hour TWA concentration data from long-term personal samples collected by NIOSH in brake service garages in the US (more than 80 samples were collected during the years 1976 through 1987 at 22 brake repair facilities). A Monte Carlo analysis was used to derive an upper bound (95th percentile) cumulative asbestos exposure of approximately 3 f/cc\*year for brake mechanics in the US. This upper bound is below the 25 f/cc\*year threshold and, therefore, our exposure analysis is consistent with the negative epidemiology data for lung cancer and asbestososis in auto mechanics. Although a chrysotile threshold dose has not been suggested for mesothelioma (indeed there is considerable debate in the scientific community as to whether chrysotile asbestos is a causative factor in mesothelioma), numerous studies have consistently concluded that brake mechanics are not at an increased risk of developing mesothelioma and therefore the upper bound cumulative exposure estimate of 3 f/cc\*year for brake mechanics could serve as a preliminary estimate of a minimum threshold chrysotile dose for mesothelioma.

## CHRYSOTILE ASBESTOS EXPOSURE ASSOCIATED WITH REMOVAL OF AUTOMOBILE EXHAUST SYSTEMS (CIRCA 1946-1970).

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For decades, asbestos-containing gaskets were used in almost every mechanical application. A gasket is a sealing device which frequently must be resistant to extreme temperatures and pressures. Prior to the mid-1970s, some automobile exhaust systems contained asbestos gaskets either at flanges along the exhaust pipes or at the exhaust manifolds of the engine. Additionally, a limited number of automobile mufflers briefly contained asbestos paper inside the muffler. Recent claims have suggested that there might be appreciable health risks associated with asbestos exposure during the repair of automobile exhaust systems. A simulation study was conducted to characterize personal and bystander exposures to asbestos during the removal of automobile exhaust systems (circa 1946-1970) containing asbestos gaskets. Personal, area, bystander, and area background samples were collected for about one hour during the repair work. Sixteen pre-1974 vehicles with old or original exhaust systems were studied. Air samples were analyzed by NIOSH PCM and TEM methods and bulk samples were analyzed by PLM and XRD. Results showed eighteen of thirty air samples collected on the lapel of the worker showed PCM results below the limit of detection (0.003-0.13 f/cc). TEM analysis indicated that only two lapel samples showed asbestos in which concentrations were 0.02 f/cc for chrysotile. Thirty-three of fifty-one area bystander samples were below the limit of detection (0.004-0.093 f/cc for PCM) and TEM analysis indicated that six bystander samples showed asbestos in which the highest concentration was 0.02 f/cc for chrysotile. Under a worst-case scenario where a mechanic might repeatedly conduct exhaust work over an eight-hour workday, these results indicate that exposure to asbestos from work with automobile exhaust systems containing asbestos gaskets was substantially below 0.1 f/cc, the current PEL for chrysotile asbestos.

## EXPOSURE RECONSTRUCTION OF HISTORICAL AIRBORNE BENZENE CONCENTRATIONS: CASE STUDY OF A DECK CREWMAN ON BOARD CRUDE OIL AND CHEMICAL TANKERS.

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High benzene exposures on board tankers transporting cargo containing benzene have been reported. Several factors influence the degree of benzene exposure under this occupational setting including the type of loading/unloading tasks performed

and the ventilation systems present. A reconstruction of historical airborne benzene exposures associated with deck work on board two tankers was conducted (one crude oil and one chemical). Work history of a shipmate was used as a basis for this case study. Parameters considered in the exposure model, included quantity of benzene-containing cargo, time involved with specific tasks (loading/unloading, manual gauging, transport, tank cleaning), voyage information including transit time at sea, and general conditions on board the tankers. Exposure estimates for specific operations believed to be comparable to the cargo and conditions on board these tankers were identified from the scientific literature. Monte Carlo methods were then used to characterize the plausible range of airborne benzene exposures a deck crewman might have historically experienced on board these tankers. Based on the specific work history of this case study, exposure model results showed that cumulative benzene exposures to a deck crewman on board tankers transporting crude oil and chemical cargo with less than five percent benzene were relatively low (less than one ppm/year). Tank cleaning operations provided the opportunity for the highest potential benzene exposures and appeared to drive the exposure estimates. Under the assumptions used in this case study, a worker exposed for a forty-year working lifetime would have a cumulative exposure to benzene well below levels shown to be associated with an increased risk of disease. These findings suggest that benzene exposure under the conditions modeled in this assessment do not pose a health risk to deck crewmen.

## DIESEL-RELATED BENZENE EXPOSURES DURING REFUELING OPERATIONS AT TWO GROCERY DISTRIBUTION CENTERS.

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Airborne benzene exposures were assessed during diesel refueling of tractor trailer trucks and generator fuel tanks for refrigerated trailers used for food transportation. Fuel was dispensed from a fuel truck tank by a nozzle with no vapor recovery system. Benzene samples included near-source levels before refueling, approximate 1-min breathing zone levels while refueling, integrated breathing zone levels for an industrial hygienist shadowing a refueler, background air levels, and diesel fuel concentrations. Summa canisters were analyzed by EPA-TO-15 and passive dosimeter badges by NIOSH 4500. Airborne benzene levels above the tank openings immediately after cap removal averaged 16.0 + 7.9 ppb (mean + S.E., n = 3) for tractor tanks and 4.3 + 1.1 ppb (n = 6) for generator tanks. Breathing zone levels obtained for about 1 min while the refueler was dispensing fuel averaged 38.7 + 19.7 ppb (n = 3) for tractor tanks and 11.8 + 7.1 ppb (n = 6) for generator tanks. Integrated breathing zone levels for the industrial hygienist shadowing the refueler averaged 8.8 + 5.3 ppb (n=2) while refueling tractor and generator tanks. Yard area samples including locations downwind from the entrance where diesel trucks entered the facility averaged 2.0 + 0.04 ppb (n = 7). Benzene concentration in the diesel fuel averaged 0.0034 + 0.0010 wt.‰ (n=3). These data suggest that airborne benzene exposures during diesel refueling are lower than levels associated with gasoline. Benzene levels in the yard with diesel traffic were similar to regional historical averages and suggest that diesel exhaust is not a significant source of benzene exposure to yard workers. Average workday exposure levels to refuelers of tractor trucks in open areas could be higher if diesel refueling operations involved more vehicles, but it seems unlikely that benzene exposure would exceed the OSHA Permissible Exposure Limit of 1.0 ppm (8-hour time-weighted average).

## OCCUPATIONAL PESTICIDE EXPOSURE DURING SEED CORN PRODUCTION IN MICHIGAN.

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A dermal exposure and work activity assessment was conducted to determine if the EPA's occupational risk assessment methodologies adequately protect workers from pesticide exposure in seed corn production. Seed corn is a crop that requires significant amount of hand labor, often conducted by sensitive subpopulations such as adolescents and migrant workers. A variety of practices such as rogueing (removing genetically undesirable plants) and detasseling are unique to this crop and can be considered high-risk from previously applied pesticides. Therefore, the purpose of the study was to determine: 1) dislodgable foliar residues, 2) dermal exposures, and 3) a generic transfer coefficient for risk assessments on tall/row crops for Tilt® (propiconazole) and Warrior® ( $\lambda$ -cyhalothrin). A 152-acre field used for seed corn production was sprayed with Tilt® (4 ounces/acre) and Warrior® (3 ounces/acre). Leaf samples were taken at Days 0 through 7 after application for dislodgable foliar residue pesticide analysis. The residue dissipation curves displayed typical first order decay; the R-squared was 0.84 and 0.86 for Warrior® and Tilt®, respectively. On day 2, the field was reentered by 15 volunteers wearing inner/outer whole body

dosimeters to determine the amount of leaf residue transferable to the participant. All volunteers roged for a 4-hour period after which the inner/outer dosimeters were divided into lower and upper leg, lower and upper arm, and front and back torso sections and submitted for pesticide analysis. The majority of the pesticide residues were distributed on the upper leg, lower leg, and lower arm. Residue penetration from the outer to the inner dosimeter was 5.3% and 7.2% for Warrior® and Tilt®, respectively. Day 2 foliar residues were used to calculate transfer coefficients (TC) for each section of the dosimeter. The average overall TC for Tilt® and Warrior® were 7800 and 6800, respectively. A preliminary estimate indicates that the margin of exposure for this crop and activity far exceeds EPA's target of 100 for occupational risk.

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**PESTICIDE EXPOSURE ASSESSMENT: MOISTURE ENHANCES MALATHION TRANSFER TO HARVESTER GLOVES AND CLOTHING, BUT DOES NOT INCREASE ABSORBED DOSE.**

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When strawberries are protected from pests with insecticides, fungicides, miticides, or molluscicides, field entry intervals are an important means to minimize harvester exposures. Establishment of safe entry intervals requires knowledge of dosages that produce experimental adverse effects and field measurements of harvester exposure. The influence of morning dew on harvester exposure was studied using latex glove and cotton forearm dosimeters (ug per harvester), and urine biomonitoring. Using a crossover design groups of 12 workers harvested malathion-treated in either the morning or in the afternoon during a 10-day period. Worker production during the day did not differ. The dislodgable foliar residues ranged from 0.22 to 0.014 ug/cm<sup>2</sup>. This abstract reports the first 3 d of 10 that are included in this report. Latex gloves in the morning retained 5.3 ± 1.1 mg malathion and 1.2 ± 0.66 mg malathion in the afternoon. A similar pattern of residue retention was found on the forearm dosimeters: morning residues were 1.2 ± 0.61 and 0.28 ± 0.81 during the afternoon harvest. Urine specimens normalized for daily creatinine excretion were obtained for measurement of malathion mono- and diacids. Even though the gloves and shirtsleeves represented morning: afternoon ratios of about 4 (day 3-5 post malathion application), the excreted malathion acids following morning exposures (0.95 ug/kg-3d) and afternoon exposures (1.26 ug/kg-3d) did not differ (>0.05). Based upon these observations, morning dew enhanced pesticide transfer from treated foliage, but it did not increase malathion absorption of harvester. Pesticide exposure estimates based upon external dosimeters may be excessive in the presence of moisture e.g., dew, rain, and perhaps perspiration. Supported in part by the California Strawberry Commission and DB Specialty Farms, Santa Maria, CA.

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**CHEMICAL EXPOSURE ASSESSMENT: SURFACE DEPOSITION OF ENVIRONMENTAL TOBACCO SMOKE (ETS).**

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ETS is commonly regarded as a source of bystander exposure to mainstream and sidestream smoke. Dermal exposures may be more important than inhalation to semivolatile (< 10<sup>-3</sup> mm Hg) chemicals in ETS. Nicotine was used as an exposure marker due to its concentration in ETS, stability, suitability for sensitive biological and environmental analysis, and toxicity. Nicotine is readily absorbed from inhaled air, ingestion, and skin contact. Dermal contact may be an important route of exposure for non-smokers, and this exposure may begin early in life. Carpet swatches were placed for 1, 2, and 4 weeks in 9 homes of cigarette smokers. Transferable carpet residue (TCR; µg nicotine/cm<sup>2</sup>) was sampled with a cotton cloth pressed beneath a 30-lb. roller. Nicotine was measured via GC/MS following Soxhlet extraction of carpet swatches with ethyl acetate. Nicotine concentrations in carpet swatches contained as much as 4.3 µg/cm<sup>2</sup> with a mean deposition of 1.29 µg/cm<sup>2</sup>. Carpet deposition increased about 0.3 µg/cm<sup>2</sup>/week. Mean nicotine concentrations after a four-week exposure were 1.7 µg/cm<sup>2</sup>. Potential daily dose (µg/person) is the product of TCR x TC x exposure hours. TCRs (TC; cm<sup>2</sup>/h) are used by the USEPA to evaluate and re-register pesticides for residential use. Assuming 10% nicotine transferability and the USEPA TC of 8700 cm<sup>2</sup>/hr, a potential daily dermal dose of 1, 500 µg/person-h. Assignment of dose is tentative since the transferable carpet residue is more tightly bound than organophosphate and pyrethroid pesticides that have been studied in this laboratory. Transferable surface residue to a cotton cloth utilizing a 30 lb roller was unsuccessful (LOQ 5 µg/ml). Dermal absorption could contribute significantly to ETS exposure measured using biomarkers such as cotinine and attributed to inhalation exposure (e.g. Jurado et al. 2004 and Kim et al., 2004). Many persons object to the irritancy of ETS in indoor settings, but primary exposure to tobacco smoke constituents, may result from dermal contact rather than inhalation.

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**VALIDATION OF A SURROGATE MIX TO DETERMINE CONCENTRATION OF INDIVIDUAL COMPONENTS OF JP-8 IN AEROSOL AND VAPOR SAMPLES BY GC/MS.**

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JP-8 is a kerosene-based jet fuel containing several hundred hydrocarbons and is used in a variety of military vehicles, including aircraft. The chemical complexity of JP-8 makes analysis of individual components difficult. The objective of this study was to develop an analytical method, with an accuracy and precision of 20% or better, for JP-8 aerosol and vapor samples using gas chromatography/mass spectrometry (GC/MS). Preliminary analysis of the hydrocarbon composition of aerosol and vapor samples of JP-8 collected from a nose-only mouse exposure chamber at the University of Arizona was undertaken. Thirty-four of the primary components detected in the atmosphere were used to create a surrogate mixture for GC/MS analysis. Our surrogate mixture contained n-alkanes ranging from n-octane to n-heptadecane, toluene, n-ethylbenzene, xylenes, n-substituted cyclohexanes, 3-ethyltoluene, 2-methylnonane, trimethylbenzenes, indene, methyldecanes, 1, 2, 3, 4-tetrahydronaphthalene, naphthalene, methylnaphthalenes, and dimethylnaphthalenes. Three separate runs containing a standard curve, ranging from 1.25 µg/mL to 250 µg/mL, and six replicates each of the surrogate at concentrations of 1.25 µg/mL, 4.0 µg/mL, 75 µg/mL, and 200 µg/mL were analyzed. The average precision obtained for the thirty-four components was 8% or better, while the average accuracy was 13.5% or better. The precision of the standard curves was 18% or better at all concentrations of each of the components. Based on these results, we attained our desired precision and accuracy and have thus developed a suitable method for analyzing JP-8. This method will be used in the future to determine the concentration of major components in JP-8 samples acquired from the nose-only exposure chamber at the University of Arizona. Funded by AFOSR [grant no F49620-03-1-0157].

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**PHYSICOCHEMICAL CHARACTERISATION OF COMBUSTION PARTICLES FROM RESIDENTIAL WOOD SMOKE AND VEHICLE EXHAUST.**

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Air pollution is associated with negative health effects. The particle characteristics responsible for these effects are still debated, but size, surface area and chemistry seem to influence the potency of the particles. This work is part of a VISTA\* project investigating possible relationships between physicochemical characteristics of particles and their biological effects. Since residential wood smoke and vehicle exhaust are the primary sources of particulate air pollution in Norway, combustion particles collected from a traditional Norwegian wood stove and in a road tunnel were compared. In this study, the morphology of the particles was characterised by transmission electron microscopy (TEM) and the ratio of organic to total carbon content (OC/TC) was determined by thermal-optical transmission analysis. The only combustion particles observed in the samples were carbon aggregates of different sizes, consisting of spherical primary particles with diameters between 20 and 60 nm. The mean diameters of primary particles from wood smoke was 39 ± 11 nm, whereas the diameter for vehicle exhaust from the road tunnel sample was 26 ± 7 nm. The OC/TC ratios for wood smoke and vehicle exhaust were 0.43 ± 0.04 and 0.65 ± 0.03, respectively (4 measurements). For equal particle masses, a decrease in particle diameter results in an increase in surface area. In the comparison of combustion particles from the two sources, vehicle exhaust particles were found to have a larger surface area per unit mass and a higher content of organic material, as compared to wood smoke particles. Increases in surface area and organic carbon content have been associated with increases in certain biological effects. Based on these parameters, combustion particles from vehicle exhaust may be considered more harmful than particles from residential wood smoke. \* VISTA - The Norwegian Academy for Science and Letters and Statoil. [www.vista.no](http://www.vista.no)

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**COMPARISON OF TRACER METHODS USED TO MEASURE IN-VEHICLE CONCENTRATIONS.**

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Two studies<sup>1</sup> used different tracer methods to estimate diesel particulate matter (DPM) inside school buses attributable to bus exhaust. One used continuous monitors for black carbon, PM2.5, PAH, and SF<sub>6</sub> (a tracer gas released into the school

bus exhaust pipe). Objectives included characterizing student exposures, and conditions associated with high concentrations. It provides in-bus concentrations for several buses over a range of conditions. However, instrument instability due to sample line switching and tracer release rates that didn't vary with emission rate resulted in inconsistent, and in some cases, unrealistic estimates of self pollution. Based on correlations between SF<sub>6</sub> and other species, the study reports that about one-fourth of in-bus DPM concentrations, or 2-5 µg/m<sup>3</sup>, were due to bus exhaust. Regressions were inconsistent, and the few showing statistical significance may indicate only that DPM from other sources is entering the bus at the same time as the tracer. The second study focused exclusively on self-pollution, using iridium (Ir) tracer in the fuel. The DPM:Ir emission ratio was measured on a dynamometer; in-bus integrated samples were collected for Ir and PM2.5. DPM from the bus was detected with a sensitivity of 0.001 µg/m<sup>3</sup>. The average concentration was 0.22 µg/m<sup>3</sup>, 10-25X lower than the SF<sub>6</sub> self-pollution estimate. This difference appears to arise from the SF<sub>6</sub> study's inability to distinguish the bus's emissions from those of other sources. The iridium tracer method is specific and highly sensitive. Continuous data for SF<sub>6</sub> and other species improve the ability to identify conditions affecting concentrations, but are less sensitive and non-specific. Design of future studies should insure that adequate sensitivity for all parameters is achieved, and that bus exhaust contributions can be explicitly distinguished from on-road background. <http://www.arb.ca.gov/research/schoolbus/schoolbus.htm>; [http://www.greendieseltechnology.com/tracer\\_091003.html](http://www.greendieseltechnology.com/tracer_091003.html)

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#### SIMULATION AND ASSESSMENT OF OCCUPATIONAL EXPOSURES TO ISOCYANATES AND VOCs DURING APPLICATION OF A URETHANE PRODUCT SUITE UNDER WORST-CASE CONDITIONS.

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Some reports claim health effects for workers exposed to isocyanates and VOCs while applying sprayed-on paint and urethane coatings. However, no exposure data are available for workers using troweled-on urethane putty and associated cleaners and primers in confined work spaces such as coal mines. Therefore, laboratory tests and work simulations were performed to assess the potential, worst-case occupational exposure to VOCs and isocyanates resulting from the use of a urethane product suite to repair rubber conveyor belts typically used in the coal mining industry. Tests were conducted in small chambers to identify airborne chemicals that could be measured during belt repair simulations. A method was also developed to assess potential dermal exposure to isocyanates using treated cotton sleeves as a surrogate skin surface. A series of six belt repair simulations were performed by an experienced belt repair technician in a walk-in exposure chamber. These simulations were designed to quantify potential exposure to isocyanates and VOCs while using the product suite to repair a rubber conveyor belt. A target list of 24 compounds, including 3 isocyanates, was developed from the small chamber data and the product's MSDSs. These compounds were sampled during the belt repair simulations. No airborne isocyanates were detected. The measured average airborne VOC concentrations were well below established occupational exposure limits. Low and intermittent levels of isocyanates were detected in dermal sleeve samples. These dermal exposures resulted from incidental self-application by the worker while wearing gloves having surface residues of uncured urethane putty. The results of the laboratory tests and large chamber simulations suggest that the normal and intended use of the urethane product suite is not likely to result in VOC or isocyanate exposure to workers at levels sufficient to produce adverse health effects

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#### OCCUPATIONAL EXPOSURE TO ULTRAFINE PARTICLES AND POLYCYCLIC AROMATIC HYDROCARBONS FROM CANDLE EMISSIONS.

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Recently attention has been focused on occupational exposures to ultrafine particles (UFPs). Exposure to UFPs may include candle emissions in restaurants, spas and churches. Recent environmental studies indicate that there may be an increased risk of cardiovascular disease and pulmonary distress associated with exposure to UFPs in urban environments. Environmental concentrations of UFPs in urban environments is much greater than rural environments due to soot generated from vehicles, power plants and other fuel sources. Previous claims have been made that candle soot contains polycyclic aromatic hydrocarbons (PAHs). Comparatively, using candles for a limited time period exposes a person to less UFPs than ambient outdoor air of a typical city. A single candle and groups of 5 and 10 candles were burned inside a test room. Candles were selected based on the types typically used in churches, restaurants and spas. To accurately measure submicrometer-sized particles, a condensation nucleus counter was used. PAHs were sampled and analyzed according to OSHA Method 58. These data were extrapolated to churches, restaurants and spas using modeling equations. Candle emission data shows that the exposure to UFPs from candle use is not much different than that inhaled during an outdoor 24 hour time period. PAH analysis demonstrates that airborne concentrations are below detectable limits and do not impact lifetime cancer risk. Moderate use of candles is not a risk in occupational and residential settings because of transient and infrequent exposures and low levels of air constituents. Lifetime daily average dose (LADD) of ultrafine particles (adjusted for alveolar deposition) from ambient sources such as parking lots and roadways ranged between 0.775-1.10 E9 particles, inside shops 3.46 E8 particles and inside restaurants with secondary sources and the absence of candles, 5.82 E9 particles. LADD calculations for candle soot in occupational workplaces where candles are burned ranged from 1.86-9.36 E9 particles. The increased exposure to candle soot is not a measurable public health hazard.

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#### EXPOSURE ASSESSMENT FOR PERCHLORATE IN MILK.

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Perchlorate has been measured in nearly 100% of commercial milk samples collected in Texas and California from three separate studies. It is presumed that perchlorate occurs in cow's milk because it concentrates in the leaves of alfalfa consumed by dairy cows. The alfalfa contains perchlorate because it is grown with perchlorate affected irrigation water. The measured levels of perchlorate in milk are low ranging from 1.5 to 10.6 ppb, with a mean for all three surveys of 3.6 ppb. However, analytical data validation information are not available for most of these samples. We calculated the dose of perchlorate for the US population, children ages 1 to 6 years, females of childbearing age, and pregnant or nursing females using USDA food consumption survey data, and considered all forms of liquid cow's milk and buttermilk. The fetus and neonate may be more sensitive to perchlorate than adults. In the survey, 67 percent of the US population reported consuming milk, whereas 92 percent of children, age 1 to 6 years, and 83 percent of pregnant and/or nursing women consumed milk. Using the mean concentration of perchlorate in milk reported in the three studies, the dose of milk for each population was calculated for the mean, and 50th and 90th percentiles of milk consumption rates. Body-weight adjusted doses were highest for children, ranging from 0.086 at the 50th percentile to 0.17 µg/kg-day at the 90th percentile of milk consumption rates. Doses for pregnant or nursing women were approximately 30% higher than those for all women of childbearing age (14 to 49 years), and ranged from 0.017 at the 50th percentile to 0.039 µg/kg-day at the 90th percentile of milk consumption rates. The dose to pregnant and nursing women does not exceed either the USEPA draft perchlorate reference dose (RfD) of 0.03 µg/kg-day or the RfD used by CalEPA to determine a perchlorate public health goal of 0.37 µg/kg-day. The dose to children does not exceed the CalEPA RfD, but exceeds the draft USEPA RfD by a factor of 2 (at the 50th percentile) and 6 (at the 90th percentile). These doses are lower than the dose which causes inhibition of iodide uptake in adults, a known precursor to thyroid dysfunction.

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#### CURRENT ALCOHOL USE IS ASSOCIATED WITH A REDUCED RISK OF HOT FLASHES IN PERI-MENOPAUSAL WOMEN.

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Hot flashes are one of the most common physiologic symptoms reported by women transitioning to menopause. Previous studies indicate that hot flashes are precipitated by low levels of endogenous estrogens. While many factors are associated with low estrogen levels, a few studies suggest that alcohol use may be associated with low estrogen levels in peri-menopausal women. Thus the purpose of this study was to examine the relation between current alcohol use, estrogen levels, and hot flashes in peri-menopausal women using a case-control study design. Cases were peri-menopausal women who reported ever experiencing hot flashes (n=317). Controls were peri-menopausal women who reported never experiencing hot flashes (n=222). Each participant completed a questionnaire and provided a blood sample that was used to measure estradiol and estrone levels by enzyme-linked immunosorbent assay. The results indicate that current alcohol users had lower odds than non-users of experiencing any hot flashes (odds ratio (OR): 0.63, 95% confidence interval (CI): 0.42, 0.93), independent of age, race, obesity and smoking habits. Also, the hot flashes experienced by current alcohol users were not any more severe (OR: 0.7, 95% CI: 0.46, 1.08), or any more frequent (OR: 0.56, 95% CI: 0.31, 1.02) than those experienced by non-users. In addition, current alcohol users had similar levels of estradiol (geometric mean: users 95.9 pg/ml, non-users 91.8 pg/ml; p<0.3) and estrone (geometric mean: users 117.5 pg/ml, non-users 112.2 pg/ml; p<0.3) compared to non-users of alcohol. These data suggest that current alcohol use is associated with a reduced risk of hot flashes in peri-menopausal women by a mechanism that may not include changes in estrogen levels. Supported by NIH AG18400.

ENVIRONMENTAL EXPOSURE TO CADMIUM AT A LEVEL INSUFFICIENT TO INDUCE RENAL TUBULAR DYSFUNCTION DOES NOT AFFECT BONE DENSITY AMONG FEMALE JAPANESE FARMERS.

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Some recent researches suggest that environmental exposure to cadmium (Cd), even at low levels, may increase the risk of osteoporosis and that the bone demineralization is not just a secondary effect of renal dysfunction induced by high doses of Cd as previously reported. To investigate the effect of exposure to Cd at a level insufficient to induce kidney damage on bone mineral density and bone metabolism, we conducted health examinations on 1,380 female farmers from five districts in Japan who consumed rice contaminated by low-to-moderate levels of Cd. We collected peripheral blood and urine samples and medical and nutritional information, and measured forearm bone mineral density. Analysis of the data for subjects grouped by urinary Cd level and age-related menstrual status suggested that Cd accelerate the increase of urinary calcium excretion around the time of menopause and the subsequent decrease in bone density after menopause. However, multivariate analyses showed no significant contribution of Cd to bone density or urinary calcium excretion, indicating that the results mentioned above were confounded by other factors. These results indicate that environmental exposure to Cd at levels insufficient to induce renal dysfunction does not increase the risk of osteoporosis, strongly supporting the established explanation for bone injury induced by Cd as a secondary effect. This research protocol was approved by the Committee on Medical Ethics in Jichi Medical School.

HEALTH EFFECTS REPORTED IN TEXAS HOMES WITH VISIBLE MOLD AND/OR WATER DAMAGE.

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Although the presence of indoor mold has been associated with allergy symptoms, there is inadequate data quantifying the airborne mold in homes reporting health effects in the occupants. In order to determine the average mold spore levels in homes with reported health effects, a retrospective analysis was performed of indoor air quality investigations conducted in Texas homes from the years 2001 to 2003 in response to homeowner complaints of visible mold and/or water damage. As part of the investigations, homeowners were questioned about symptoms that were present in the home occupants. These data describe the reported health effects, as well as the indoor and outdoor spore concentrations in 94 investigated homes. Airborne mold spores were collected using Zefon Air-O-Cell spore traps collected for five minutes at a flow rate of 15 liters per minute. The samples were analyzed by direct microscopy to determine the genus of the molds and the concentration of spores was calculated (spores/m<sup>3</sup>). The mean indoor and outdoor airborne spore levels were 3,014 spores/m<sup>3</sup> (range 160-20, 352 spores/m<sup>3</sup>) and 9,315 spores/m<sup>3</sup> (75-156, 323 spores/m<sup>3</sup>), respectively. *Stachybotrys* was found in 21 homes (mean = 227 spores/m<sup>3</sup>). Symptoms were reported in 72% of the homes with visible mold and/or water damage. The most commonly reported symptoms were allergies (42%), upper respiratory problems (19%) and asthma (13%). There were no significant differences between the indoor airborne spore concentrations of homes with or without reported symptoms. However, the mean outdoor spore concentration was significantly higher outside homes that reported allergies (3-fold, P<0.01), respiratory problems (4-fold, P<0.01) or asthma (7-fold, P<0.05) than in homes that reported no adverse health effects. In summary, the mean outdoor spore concentration was significantly higher for homes that had occupant reported health effects than in those with no reported symptoms.

AN INVESTIGATION OF HOME DAMPNESS AND ADVERSE HEALTH EFFECTS ON A NATIVE AMERICAN RESERVATION.

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Over the past 15 years, indoor dampness and mold have been perceived as a growing public health issue. Home dampness has been implicated in a variety of health effects including asthma, upper respiratory disease, and allergic rhinitis. It is still unclear what mold contributes to the risk of respiratory disease associated with damp houses. In 2001, we conducted a cross-sectional study of Native Americans who lived in flooded rental homes on the Turtle Mountain Reservation. Households were randomly selected for the study (N=137) which included visual

inspection, environmental sampling and responses to a health questionnaire. We used non-parametric and logistic regression analyses to look for relationships among environmental factors, demographic and lifestyle factors, home dampness, presence of molds and adverse health symptoms. Nearly all (98.5%) homes had one or more factors that contributed to home dampness and 67.7% had living space relative humidities above the EPA recommended range (30%-50%). Inspectors found visible mold in the living spaces of most homes (71.4%). The amount of mold in these areas was significantly correlated with the number of dampness contributors ( $\beta=0.33$ ,  $p<0.001$ ) found. Most study participants (93.7%) reported experiencing at least one adverse health symptom, with an average of 5.2 symptoms. The average number of self-reported health symptoms increased with the number of dampness contributors in the home ( $\beta=0.24$ ,  $p<0.001$ , trend line  $R^2=0.65$ ). Dampness contributors were significantly correlated with the total number of respiratory symptoms ( $\beta=0.28$ ,  $p<0.001$ ) and rhinitis symptoms ( $\beta=0.26$ ,  $p<0.001$ ). Living in a cluttered home ( $\beta=0.30$ ,  $p<0.001$ ) or having a pet indoors ( $\beta=0.30$ ,  $p<0.001$ ) were associated with the total number of self-reported symptoms. Logistic regression modeling demonstrated that *A. versicolor* in household dust samples was the mold most often associated with adverse health symptoms. More discriminate strategies are needed to assess the individual and synergistic contributions of these factors on adverse health outcomes.

UNIVOCAL DECISION RULES FOR THE ASSESSMENT OF CHRONIC SOLVENT-INDUCED ENCEPHALOPATHY; A PROPOSAL.

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The two current 1985 international classification systems for chronic solvent-induced encephalopathy (CSE) lack univocal criteria. Our purpose was to develop univocal decision rules for patient data, based on the principles of these systems. We scrutinised our diagnostic methods, which were derived from the 1985 systems. Subjective procedures were replaced by objective ones as much as possible. Present implicit decision rules were refined and explicitly formulated for the following fields: (1) test-based neuropsychological impairment, (2) cognitive and somatic symptoms, (3) symptoms of disturbed mood and well-being, (4) the extent of solvent exposure, (5) differential diagnosis, and (6) the combination of these five fields to a final CSE diagnosis, in which three stages of deficit are discerned. By applying these rules to the data that we collected in the evaluation of 1543 alleged patients, 14% were diagnosed as CSE-only and 5% as CSE combined with another disease. We consider this proposal as a feasible example of the necessary elaboration of the 1985 classification systems. The proposed system keeps its transparency if cut-off criteria are set at a different level. We hope that it will contribute to an improved and internationally acceptable classification for CSE.

MATERNAL DDE SERUM LEVELS AND NEUROLOGICAL DEVELOPMENT IN INFANTS. PRELIMINARY FINDINGS.

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Experimental studies have shown that pp-DDE (DDE) has the potential to alter neurodevelopment. However scarce human evidence is available. From an ongoing cohort study, 87 pregnant women with no delivery complications and their infants were identified in Morelos, Mexico, whose Southern area used to be endemic for malaria. Mental and psychomotor development was evaluated in the infants at one month of age by the Neonatal Behavioral Assessment Scales (NBAS) and the Bayley Scales of Infant Development (BSID-II) and at three months of age by the BSID-II only. The BSID-II included indices of mental development and psychomotor development. Serum samples from the mothers were obtained during the first trimester of their pregnancy to determine DDE and other organochlorine pesticides by electron capture gas-liquid chromatography. The median of maternal DDE levels was 1,460.17 ng/g (range 95-11,445.). The median DDE level in serum is common in Mexican women living in similar areas and was comparable to that observed in women residing in California in 1971. Better scores for mental and psychomotor indexes at first month of age were observed in breastfed children independently of DDE maternal levels. A borderline significant reduction in the psychomotor index at three months of age ( $\beta=-2.09$ ,  $p=0.08$ ) was observed among breastfed children whose mothers had DDE levels higher than the median during

the first trimester of pregnancy, as compared to breastfed children whose mothers had DDE levels below the median. Data were adjusted by maternal IQ and home scale. Our preliminary results suggest a negative interaction between DDE exposure and breastfeeding on the motor development of infants at three months of age. Further studies in larger populations are needed to confirm the magnitude and the reversibility of this finding. (Supported by: Fogarty International Center/NIH, Grant D43 TW00640; CONACyT grants 31034-M and #41708).

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#### ASSESSING THE PUBLIC HEALTH IMPACTS OF THE 1991 GULF WAR IN SAUDI ARABIA: A QUALITATIVE RISK ASSESSMENT APPROACH.

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During the 1991 Gulf War, Iraqi military forces invaded and occupied Kuwait, deliberately contaminating the State's natural resources. The burning of oil wells, release of oil into the Arabian Gulf, and disruption of the desert surface by troop movements also impacted Kuwait's neighbor, Saudi Arabia. These activities contributed to increased levels of oil residues in seafood, and combustion products, soot, and airborne particulate matter (PM) in the atmosphere. As part of a larger program to evaluate the health impacts in Saudi Arabia from the war and its aftermath, a qualitative risk assessment was performed to identify populations experiencing adverse health effects from exposure to pollutants, stressful situations, or trauma. This assessment was also designed to determine relevant exposure pathways and contaminants of concern, and to identify war-related health effects; all for the purpose of designing a large-scale Exposure and Health Survey. Environmental monitoring data were obtained from a number of sources including air monitoring and modeling efforts and other published sources. Analyses of these data indicated that much of the eastern half of the Kingdom was impacted by air pollution, primarily airborne PM, which was a complex mixture of oil fire soot, soot-contaminated dust, and vehicle exhaust. Shoreline and seafood contamination data were obtained from previously published studies that measured trace metal and hydrocarbon concentrations in sediment and/or biota. Overall findings of the analysis established that health outcomes of concern could be broadly categorized as follows: cardiovascular, respiratory, systemic, and post-traumatic stress disorder (PTSD).

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#### GETTING THE LEAD DOSE-RESPONSE CURVE RIGHT: IMPLICATIONS FOR PUBLIC HEALTH AND POLICY.

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Population lead study researchers sample a range of lead exposure and often frame blood lead as a continuous independent variable, against which they regress the health effect. Publications sometimes use natural log transformed lead, sometimes linear lead with no formal testing of the functional form best describing the dose-response curve for lead-health effect. We use data from a pooled analysis study of blood lead on child IQ (N=1333) and a study of blood lead on elevated blood pressure in pregnant women (N=1735) to show that untransformed blood lead and natural log transformed blood lead are both highly significant predictors of the health effects. We use diagnostic tests (J-test and generalized additive models with spline fitting) to show that natural log transformed blood lead describes the drop in child IQ with increasing lead significantly better than the linear lead function (J-test, p=0.009) and that the log-linear dose-response curve is not due to residual confounding by improperly specified control variables. The same procedure for the blood lead-systolic blood pressure function shows marginally significant improvement in the dose-response function using the log-linear form (J-test, p=0.053). We compare linear-linear and log-linear dose-response functions for blood lead-IQ in a published economic benefits model showing dollar savings from the US nationwide blood lead reduction between 1976 and 1999. The log-linear dose-response function for lead-IQ showed 2.2 times the economic savings (\$319 billion) predicted by the linear-linear dose-response function (\$149 billion). A log-linear dose-response function for lead on IQ implies that 70% of the decrease in child IQ (12.4 IQ points) measured over the study lead range of 0.1-71.7 µg/dL (17.8 IQ points) occurs from 0.1-10.0 µg/dL. The 10 µg/dL CDC action limit for children does not prevent the majority of damage to child intelligence. We can duplicate the economic benefit already achieved by further reducing national blood lead to 0.2 µg/dL.

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#### MECHANISMS OF METAL-ASSOCIATED VASCULAR DISEASE.

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There is increasing evidence associating several toxic metals and cardiovascular outcomes. However, it is generally assumed that these risks involve high exposures and often renal toxicity. We have reported that small increments in blood lead in adults (from 1 µg/dL to 3 µg/dL) are associated with significant increases in risks of hypertension and premature mortality due to cardiovascular disease and stroke. To explore mechanisms by which low level lead (Pb) and cadmium (Cd) exposures may increase risks of peripheral arterial disease (PAD), we used data from two National Health and Nutrition Examination Surveys conducted from 1999-2000 and from 1999-2002. There was a dose-effect relationship of blood Pb with increased odds of PAD among US adults as levels increased from below 1 to approximately 5 µg/dL. For Cd, blood levels above 0.7 µg/L and urine levels above 0.24 µg/L (along the full dose range) were associated with increased odds of PAD. These associations were not confounded by biomarkers of lipid or renal function, arguing against a major role of these mechanisms in the observed effects of Pb or Cd. Elevations in blood Pb were also strongly correlated with elevations in serum homocysteine (HC) at levels as low as 1.6-2.2 µg/dL. Our analysis indicates that Pb may confound the observed associations between HC and vascular disease. It is possible that HC may be a biomarker of Pb-induced oxidative stress. We also examined gene-environment interactions for Pb and vascular disease, to test the role of nitric oxide-related pathways. In a study of Pb-exposed workers in Korea, the nitric oxide synthase gene G→T 894 polymorphism was not associated with blood pressure nor modified the Pb-blood pressure relation. These results demonstrate the utility of epidemiological studies in hypothesis generation for mechanistic toxicology. In addition, they support the need for research on metal-induced vascular dysfunction at levels within the range of current exposures. *Research supported by American Heart Association, CDC, NHLBI, and NIEHS.*

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#### CATEGORIZATION OF THE ASSOCIATIONS BETWEEN EXPOSURE TO THE HERBICIDES USED IN VIETNAM OR THEIR CONTAMINANTS AND HEALTH OUTCOMES.

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Between 1962 and 1971 military forces sprayed herbicides over Vietnam. In 1991, because of ongoing uncertainty about the long-term health effects on Vietnam veterans of the herbicides sprayed, Congress passed the Agent Orange Act of 1991. That legislation directed the Secretary of Veterans Affairs to request the National Academy of Sciences, a non-profit organization that provides independent, objective advice on scientific issues, to perform a comprehensive review and evaluation of scientific and medical information regarding the health effects of exposure to Agent Orange, other herbicides used in Vietnam, and the various chemical components of those herbicides, including dioxin. In assessing the evidence of an association between exposure to the herbicides and their components and a health outcome, the committees responsible for the reports review the available literature and categorize the evidence as 1) sufficient of an association; 2) limited or suggestive of an association; 3) inadequate or insufficient to determine whether an association exists; or limited or suggestive of NO association. In addition, conclusions regarding the biological plausibility of the health effects and the risk to Vietnam veterans are also made. Biennial updates are produced; the latest of those reports is *Veterans and Agent Orange: Update 2004*, which will be released in December 2004. The assessments in the reports are used by the Department of Veterans Affairs to make policy decisions regarding compensation to veterans for service-related illnesses. The methods used by the committee as well as the most recent conclusions made by the committee will be discussed.

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#### THE IMPACT OF REDUCTION OF THE OCCUPATIONAL EXPOSURE LIMIT FOR FORMALDEHYDE IN QUEBEC: A RE-EVALUATION OF HEALTH RISKS.

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As part of a study on the impact of a reduction of the occupational exposure limit (VEA) for formaldehyde (FM) in Quebec, which value is currently 2 ppm, the health risks related to occupational exposures to FM was re-evaluated. A critical analysis of the available literature data following acute, subchronic or chronic exposure was conducted. Specific criteria were used to select and classify most pertinent studies. This analysis showed that exposure to concentrations near the VEA should not induce adverse effects on the respiratory system, such as asthma. Also, with the

CIIT model, the probability of FM-induced cancers in the 143, 491 Quebec workers exposed to 1 ppm for 40 years was calculated to be less than one case. In the range of concentrations of the VEA, the critical biological effects appear to be nose, throat and eyes irritations; preventing these effects should avoid the appearance of other effects. To assess the impact of a reduction of the VEA, a dose-response relationship was thus established between exposure to FM and the occurrence of irritations as a critical effect. This was best achieved by quadratic regression rather than linear regression on raw irritation data combined from selected published human studies, and compiled by concentration ranges and by degree of severity. Results showed that at concentrations below to 0.75 ppm, there is little probability for FM-induced irritations to occur. Between 0.75 and 1 ppm, 6.3% of exposed individuals could experience mild eye irritations, 1.6% mild nose and throat irritations and none severe eye irritations. Between 1 and 2 ppm, 10.1% and 0.8% of exposed individuals could manifest mild and severe eye irritations respectively, and 4.5% mild nose and throat irritations. The application of this risk assessment to the context of the 143, 491 Quebec workers potentially exposed to FM shows that a decrease of the actual exposure limit towards 1 ppm could prevent mild irritations for 300 to 400 workers and severe eye irritations for 50 workers. This research was funded by the IRSST.

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EFFECTS OF INHALED COMBUSTION-DERIVED PARTICULATE MATTER ON INDICES OF CARDIAC, PULMONARY, AND THERMOREGULATORY FUNCTION IN SPONTANEOUSLY HYPERTENSIVE RATS.

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Epidemiological studies have shown a positive relationship between elevated levels of ambient particulate matter (PM) and rates of morbidity and mortality; these correlations are further strengthened when limited to individuals with preexisting cardiopulmonary diseases. While similar effects have been demonstrated in animals, the mechanism(s) by which these effects are mediated are unresolved. To further investigate this phenomenon, we examined the cardiac, pulmonary, and thermoregulatory effects of a combustion-derived PM (HP12), a particle with a more relevant metal profile ( $\uparrow$ Zn,  $\downarrow$ V) and bioavailability than the residual oil fly ash used in many previous studies. Spontaneously Hypertensive (SH) rats were surgically implanted with radiotelemeters capable of continuously monitoring electrocardiogram (ECG), heart rate (HR), systemic arterial blood pressure (BP), and core temperature ( $T_{co}$ ). Animals were divided into air and HP12 groups and exposed via inhalation (0 and 11 mg/m<sup>3</sup>, respectively) for 6hr/dx4d while inside whole-body plethysmograph chambers. This methodology permitted continuous, simultaneous monitoring and acquisition of cardiac and pulmonary physiological parameters in unanesthetized, unrestrained animals. Rats underwent blood collection and were sacrificed to obtain bronchoalveolar lavage (BAL) at 7 days post-exposure. We observed no changes in HR, BP, or  $T_{co}$  with exposure to inhaled HP12. However, ECG abnormalities, consisting of general rhythm disturbances and premature contractions, were observed in  $\approx$ 50% of HP12-exposed rats. There were slight increases in select blood and BAL indices, while there were only minor effects of HP12 on respiration parameters. Thus, this study demonstrated minimal deficits in cardiac function in SH rats after inhalation exposure to a low-metal content, "ambient-like", combustion-derived particle. (Abstract does not represent USEPA policy. This research was supported by EPA CT826513.)

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CARDIOPULMONARY EFFECT OF PARTICULATE MATTERS ON DIABETIC RATS: FROM CAPS TO ULTRAFINE PARTICLES.

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The association between ambient particulate matter (PM) and cardiovascular diseases has been demonstrated in epidemiological studies. Recent studies suggest that diabetic patients are at greater risk for PM-associated cardiovascular events. However, the biological mechanism remains unclear. In this study, we used concentrated ambient particles and ultrafine carbon black particles to examine the cardiopulmonary effect of particles on diabetic rats. First, streptozotocin-induced diabetic and healthy rats were intratracheally administered with PM2.5 collected from traffic busy area in a dose of 200 $\mu$ g. Cell and differential counts as well as protein and LDH activity were determined in BALF. 8-OHdG, ET-1 and NO, as well as CRP, IL-6 and TNF- $\alpha$  in peripheral blood were also determined. Our results showed that diabetic rats were associated with increased 8-OHdG, IL-6, ET-1 and decreased NO. In non-diabetic rats, PM exposure was also associated with increased 8-OHdG, IL-6, TNF- $\alpha$ , CRP and decreased NO. Interestingly, increases of

8-OHdG and ET-1 after PM exposure were more prominent in diabetic rats as compared to non-diabetic rats. General linear model further indicated that there were interactions between diabetes and PM on 8-OHdG ( $p<0.01$ ) and ET-1 ( $p=0.08$ ). Subsequently, we exposed diabetic and non-diabetic rats to 0, 125 and 500 $\mu$ g ultrafine carbon black particles (ufCB). The results revealed that ufCB caused significant in lung inflammation and plasma IL-6 in diabetic and non-diabetic rats. We also observed significant alteration in plasma ET-1 and NO in diabetic rats exposed to ultrafine carbon black while, similar finding were not observed in non-diabetics. We concluded that ambient fine particles may enhance the risk of cardiovascular diseases in diabetics through the interaction between PM and diabetes that results in excess ROS generation and endothelial dysfunction. The results also suggest that inert ultrafine particles may induce cardiovascular toxicity in diabetics. We also found that STZ-diabetic rats may be a useful model for PM toxicity study.

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TOXICOGENOMIC EXPRESSION PROFILES OF SPONTANEOUSLY HYPERTENSIVE RATS EXPOSED TO CONCENTRATED AMBIENT PARTICLES.

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Particulate air pollution has been associated with morbidity and mortality of cardiopulmonary disease. However, the mechanism remains unclear. The goal of this study is to use cDNA microarray technology to examine the global gene expression patterns of different target organs in diseased animal models exposed to concentrated ambient particles (CAPs). Six male spontaneously hypertensive rats (SHR) were exposed to CAPs in a traffic busy EPA supersite at Hsing-Chung area, Taipei. Five male SHR without CAPs exposure were prepared as controls. After 6 hr/day, 1 day/week CAPs exposure for 10 weeks, rats were sacrificed and the lung, heart and aorta tissue were collected and prepared for total mRNA extraction. cDNA microarray were performed in triplicate experiments using Rat Chip 8K. The ratio of means of each target organ was calculated for analysis. In our study, we observed differential gene expression profiles in lung, heart and aorta tissue. Higher expressions of IL-1 $\beta$ , IFN, MIP-1 $\gamma$ , ICAM-1, and MnSOD were observed in lung in SHR after CAPs exposure. Interestingly, we found several genes related to blood pressure control, systemic inflammation, coagulation and atherosclerosis were significantly expressed in aorta among cardiovascular compromised SHR. In aorta, higher expression of ANP, IFN, MIP-1, MCP-1, TGF- $\beta$ , VEGF, VCAM-1, fibrinogen-like protein, and PAI-1 were observed. These expression profiles may represent manifestations of PM-related thrombosis formation and subsequent cardiovascular outcome, especially in susceptible populations. Furthermore, these markers may be used in elucidating ambient PM toxicity.

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TIME-COURSE OF INFLAMMATION AND TISSUE DAMAGE IN THE MOUSE LUNG CAUSED BY FINE PARTICULATE MATTER FROM SIX EUROPEAN CITIES (PAMCHAR).

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In contrast to epidemiological findings, coarse particles have had after 16 to 48-h exposures a higher inflammatory potency in the animal lung than fine particles. We investigated the time-course of different inflammatory markers using fine particles (PM<sub>2.5-0.2</sub>) collected with an optimized high volume cascade impactor during selected seasons in six European cities: Duisburg (autumn), Prague (winter), Amsterdam (winter), Helsinki (spring), Barcelona (spring) and Athens (summer). Healthy C57BL/6J mice were intratracheally exposed to the PM samples (10mg/kg) using three exposure durations: 4h, 12h and 24h. At the end of the exposures, the lungs were lavaged and the bronchoalveolar lavage fluid (BALF) was assayed for indicators of inflammation and tissue damage: cell number microscopically, total protein and lactate dehydrogenase (LDH) spectrophotometrically and cytokines (TNF- $\alpha$ , IL-6 and KC) immunochemically. After 4-h exposures, the PM<sub>2.5-0.2</sub> samples increased all the cytokine concentrations with large potency differences between cities. After 12-h exposures, the BALF cytokine concentrations were no longer increased with these samples, but the total cell number and total protein concentration most often peaked at this time point. In line with earlier reports, after 24-h exposures, none of the PM<sub>2.5-0.2</sub> samples induced significant changes in BALF cytokine concentrations and the total cell numbers and total protein concentrations were only slightly higher than the basal levels. In contrast, the coarse PM<sub>10-2.5</sub> samples induced clear increases in all of these parameters at 24 h. In conclusion, exposure times longer than 12 h seem inappropriate for the assessment

of inflammatory activity and tissue damage in the healthy mouse lung after a single intratracheal instillation of urban air fine particles. Grants: EC-FP5 contract QLK4-CT-2001-00423 and The Academy of Finland and TEKES.

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#### EXPOSURE TO CONCENTRATED AMBIENT PARTICLES DOES NOT AFFECT ENDOTHELIAL VASOMOTOR FUNCTION IN PATIENTS WITH ISCHEMIC HEART DISEASE.

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There is extensive evidence of endothelial dysfunction in patients with atherosclerosis, systemic inflammation, and in cigarette smokers. We hypothesised that exposure to concentrated ambient air particles will cause systemic inflammation and have detrimental vascular effects. Twelve male patients with stable ischemic heart disease and 12 age-matched non-smoking volunteers were recruited. These individuals were exposed to concentrated ambient urban fine + ultrafine particles (CAP) in Edinburgh, Scotland or filtered air (FA) during 2 hrs of intermittent exercise. Bilateral forearm blood flow and inflammatory markers were measured 6-8 hrs after CAP or FA exposure in response to intra-brachial acetylcholine (5-20  $\mu$ g/min), sodium nitroprusside (2-8  $\mu$ g/min) and bradykinin (100-1000 pmol/min). Pulmonary inflammation following CAP and FA exposure was assessed in 8 subjects using exhaled breath condensate. Average exposure concentrations  $190 \pm 37.2$   $\mu$ g/m<sup>3</sup>. Levels of 8-isoprostone in exhaled breath increased 6 hours after exposure to CAP (CAPs  $16.9 \pm 8.5$  pg/ml vs FA  $4.9 \pm 1.2$  pg/ml,  $p < 0.05$ ). There was no difference in whole blood leukocyte count, serum C-reactive protein concentration, or resting forearm blood flow following exposure to CAP or FA. Although there was a dose dependent increase in blood flow with each vasodilator ( $p < 0.001$ ), both endothelium-dependent and independent vasodilation were unaffected by CAP or FA in either group. A 2 hour exposure to Edinburgh urban CAP, low in combustion-derived component, causes pulmonary oxidative stress but not systemic inflammation. On average a 6-fold increase over ambient air, exposure to Edinburgh urban ultrafine + fine CAP did not affect vascular function in either middle-aged healthy volunteers or patients with stable ischaemic heart disease.

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#### EXPOSURE TO CONCENTRATED AMBIENT PARTICLES IN DETROIT ALTERS HEART RATE VARIABILITY IN SPONTANEOUSLY HYPERTENSIVE RATS.

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Elevations in airborne particulate matter (PM) are linked to increased mortality and morbidity in humans with cardiopulmonary disease. Clinical studies show that PM is associated with altered heart rate variability (HRV) and suggests that loss of autonomic control may underlie cardiovascular toxicity. To assess the effect of PM on HRV in a rodent model of cardiovascular disease, we exposed spontaneously hypertensive rats (SH) and Wistar-Kyoto (WKY) rats to concentrated air particulates (CAPs) generated from ambient air in urban Detroit. Rats ( $n=4$ /group) were exposed to CAPs or filtered Air for 8h/day (7am-3pm), for 13 consecutive days inside a mobile laboratory equipped with whole body exposure chambers and a Harvard type fine concentrator that enriches fine PM (<2.5  $\mu$ m). Rats were implanted with radio transmitters and electrocardiograms were recorded by telemetry. Beat to beat intervals were used to calculate SDNN and RMSSD as determinants of HRV. Average mass concentration of CAPs was  $502 \pm 272$   $\mu$ g/m<sup>3</sup> (range:103-918). SDNN increased in CAPs/SW rats on Day 1 compared to Air/SW controls (13.5 vs. 15.6 ms; air vs. CAPs), but decreases in SDNN were observed in CAPs/SW rats during successive exposures and were consistent through Day 13 (17.5 + 1.5 vs. 14.9 + 0.5 ms). In CAPs-exposed SH rats, a trend for decreased SDNN in the early morning hours and in the early days of exposure was evident compared to baseline (0.8 - 2.7ms decrease). Conversely, decreased SDNN in the afternoon hours was not present early, but developed by Day 13 in CAPs-SH rats (2.3 ms decrease). CAPs-induced decreases in RMSDD occurred in later exposure days in SH rats. Changes in HRV were not dependent on PM mass. No consistent changes in SDNN or RMSDD were present in CAPs/WKY rats. These data suggest that CAPs-induced changes in HRV in rats with systemic hypertension are dependent on the length and frequency of exposure. Research supported by EPRI.

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#### EXPOSURE TO CONCENTRATED AMBIENT PARTICLES IN DETROIT, MICHIGAN CAUSES HEART RATE AND THERMOREGULATORY CHANGES IN SPONTANEOUSLY HYPERTENSIVE AND WISTAR-KYOTO RATS.

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Cardiovascular effects have been reported in rats exposed to concentrated ambient particles (CAPs) in different regions of the United States. The objective of this study was to evaluate the cardiovascular effects of CAPs in a region dominated by mobile source emissions. We stationed a portable particle concentrator in an area heavily impacted by diesel emissions in Southwest Detroit, MI. Spontaneously hypertensive (SH) and Wistar-Kyoto (WKY) rats were exposed to CAPs 8 hours/day for 13 consecutive days. Animals were implanted with telemeters, and heart rate (HR) and temperature (T) data were collected continuously. Four groups of animals were studied: SH-CAPs, SH-sham (filtered air), WKY-CAPs, and WKY-sham ( $n=4$  in each group). Extensive exposure monitoring was conducted, including gaseous pollutants and particulate matter (PM) components. CAPs exposure concentrations were  $103-918 \mu\text{g}/\text{m}^3$  (mean= $502 \mu\text{g}/\text{m}^3$ ). We observed a significant reduction in HR over the exposure period in CAPs-exposed SH and WKY compared with sham animals. Mean HR in CAPs and sham SH rats was  $300 \pm 13$  bpm and  $292 \pm 11$  bpm, respectively ( $p=0.001$ ). CAPs-exposed WKY and sham animals had mean HRs of  $312 \pm 8$  bpm and  $305 \pm 7$  bpm, respectively ( $p < 0.0001$ ). There was a suggested negative association between HR and CAPs concentration, with  $R^2$  values of 0.18 ( $p=0.15$ ) for SH and 0.21 ( $p=0.12$ ) for WKY. Core body T in WKY rats decreased with CAPs exposure, although this effect did not reach statistical significance ( $p=0.15$ ). As with HR, there was a suggested trend toward reduced T with increasing CAPs concentration ( $R^2=0.25$ ;  $p=0.08$ ). These results are important in light of the small sample size studied, and suggest that CAPs exposure causes cardiovascular and thermoregulatory changes in rats. Additional analyses incorporating detailed exposure characterization and source apportionment data from this study will provide valuable insight into the CAPs components associated with these changes.

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#### SUBCHRONIC EFFECTS OF CONCENTRATED AMBIENT PARTICLES (CAPS) IN SENESCENT MICE (AKR).

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The objectives of this subchronic (3 months) CAPs inhalation study in senescent mice (AKR) was to determine the effects of CAPs on the progressive changes in cardiac function and heart-rate regulation in an animal model of a susceptible human population. AKR mice ( $n=8$ /group) were exposed to northeastern regional background CAPs or filtered air for 6 hr/d, 5d/wk. from Feb. to May 2004. Six died during experiment (two from control group, four from exposure group). Ten second ECG, body temperature, and activity data were sampled from each mouse every 5 minutes using implanted DSI transmitters throughout the study. In the surviving mice, daily average of the 5-minute heart rate (HR) was decreased in both exposure (52 bpm) and control (23 bpm) groups during the experiment. Most mice exhibited very strong circadian rhythm in their daily standard deviation of normal to normal beat (SDNN, a measure of heart rate variability, HRV), which had a very strong negative correlation ( $R=-0.9$ ) with body temperature and activity counts. The differences of the 24 hr average of non-exposure weekend day SDNN between CAPs exposed and filtered air controls were significant after three weeks of CAPs exposure, and the difference continually widened until the end of the study. Differences of daily 24hr average SDNN between CAPs and control in the first six weeks are 0.01, 0.02, -0.06, -0.07, -0.3, -0.25 respectively. The results of this study suggest that CAPs exposure significantly altered cardiac autonomic function in senescent mice.

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#### CARDIAC INJURY FROM LONG-TERM EPISODIC EXPOSURE TO PARTICULATE MATTER (PM): SOLUBLE COMPONENTS OR SOLID PARTICLES?

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Long-term exposure to PM has been associated with cardiac injury in rats. The purpose of this study was to investigate if cardiac injury was due to soluble metals (i.e., zinc), insoluble PM, or pulmonary injury/inflammation. Male Wistar Kyoto rats ( $n=8$ ) were exposed intratracheally (IT), once/wk x 8 wks to either saline, Mount

St. Helens ash (MSH, no soluble metals) - 2.3 mg/kg/wk, environmental combustion particles (PM, zinc as soluble metal) - 1.15 or 2.3 mg/kg/wk, the saline-leachable fraction of PM (PM/L) - 2.3 mg/kg/wk or ZnSO<sub>4</sub> (Zn) - 33.4 µg/kg/wk (equivalent soluble zinc in PM). Another group of rats was exposed to half the concentration of all components above but for 16 wks. All exposures, except saline, caused marked lung inflammation including MSH. Lavage fluid protein, albumin, and LDH also increased in all groups relative to saline. PM high dose demonstrated the greatest effects. No changes were apparent among the hematological parameters. Four sections of each cardiac tissue were examined microscopically. Lesions were scored in a blinded manner for extension (e.g., focal or multifocal) of inflammation, fibrosis and myofiber degeneration. Minimal to no lesions were noted in controls. Mild to moderate myocardial lesions were noted in all other groups including MSH; the mean severity was highest in PM high dose group. Subepicardial and random myocardial lesions were characterized by inflammation with fibrotic degeneration. Gene expression arrays are being evaluated to understand potential differential roles of the PM components. In summary, long-term episodic IT exposure to insoluble PM or Zn causes persistent pulmonary inflammation/injury as well as varying degrees of cardiac lesions. These findings support the hypothesis that both soluble and insoluble components may cause cardiac injury. (Does not reflect USEPA policy. Supported in part by #CR829522 between EPA and UNC).

**442 HEART RATE CHANGES IN 24-MONTH OLD FISHER 344 RATS EXPOSED TO CONCENTRATED PARTICULATE MATTER (PM2.5) CLOSE TO A FREEWAY IN DIAMOND BAR, CA.**

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High incidences of mortality and morbidity from cardiovascular-related diseases exist in the United States. A portion of those incidents has been associated with air pollution episodes according to a good number of epidemiological studies, especially in the geriatric population. Epidemiological studies serve as a starting point and strongly support the need for additional research. Thus we see the need for using geriatric rats as susceptible-population experimental models. Eight 24-month old Fisher 344 rats were implanted with telemetry-based EKG monitors. The rats were used as their own controls. In week one the rats were exposed to a particle-free and gas-free atmosphere. In week two, the same rats from week one were exposed to concentrated fine particulate matter (PM2.5). Each week exposures ran for three days for four hours each day. Exposures took place in Diamond Bar, CA downwind of a major freeway in our mobile exposure vehicle and utilizing a particle concentrator. We observed an increase in heart rate in the rats when exposed to PM2.5 as compared to purified air. This increase might result from pulmonary irritation induced autonomic nervous system stimulation or proinflammatory cytokine induction of autonomic neural reflexes affecting the heart. These results suggest potentially serious effects on susceptible individuals in the geriatric population if exposed to elevated levels of inhaled particulate matter.

**443 EFFECTS OF ON-ROAD AEROSOLS IN AGED RATS.**

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Ambient particulate pollution has been associated in epidemiological studies with adverse health effects in elderly individuals with cardiopulmonary diseases. We hypothesize that inhaled ultrafine particles (UFP) and their gaseous co-pollutants contribute to these effects, especially when vehicle passengers are exposed directly to freshly-generated, concentrated highway aerosols. To test this hypothesis, we exposed old rats (20-22 mo, F-344) to freshly-generated highway aerosols (particles (<1µm)/gas phase, gas phase only, or filtered air) using an on-road tractor-trailer exposure system. Inlet pipes at the end of the trailer allowed sampling of the diluted engine exhaust plume emitted from the front of the tractor. Rats were treated with inhaled endotoxin or instilled influenza virus either before or after on-road exposures to prime respiratory tract cells. Controls were treated with saline. Exposures (6 hr; 1 or 3 days in a row) in compartmentalized whole-body chambers occurred while driving between Rochester and Utica (NY I-90). Endpoints related to lung inflammation, inflammatory cell activation, and acute phase responses were measured after exposure. The primary particle size was 13-19 nm and the number concentration was 1.6-4.3 x 10<sup>6</sup>/cm<sup>3</sup>. The expected significant changes in response due to priming agent (endotoxin, virus) were observed. Exposure atmosphere had no effect on lavage PMN percentage, cell oxidant release, or biochemical indices of cytotoxicity. However, a significant increase in total lavage cell number, increases in macrophage intercellular adhesion molecule-1 expression, a decrease in the percentage of blood PMNs, and changes in plasma fibrinogen were due to the particle/gas

phase mixture. Some of these changes were evident three days after exposure. These results show that freshly-generated exhaust aerosols have significant effects on the pulmonary and cardiovascular systems in compromised, old rats.

**444 COLLECTION, AEROSOLIZATION, AND CHARACTERIZATION OF PAVED ROAD DUST FOR AN INHALATION TOXICITY STUDY.**

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Resuspended dust, including paved road dust, contributes to both PM10 and PM2.5 in ambient air. Although studies on the toxicity of inhaled road dust are limited, recent work (Wellenius et al., 2003) has shown associations between cardiovascular response to concentrated ambient particles and the concentration of silica, an indicator of resuspended dust. In order to characterize the respiratory and cardiovascular toxicity of inhaled dust directly, a collection, processing, suspension, and characterization strategy has been developed and tested. The collection strategy evaluates a modified indoor surface sampler versus a commercial street sweeper. Samples were processed by sieving to 40 microns and resuspended by a Wright Dust feeder into a rodent inhalation chamber. Particle size, metals, carbon, inorganic ions, and fungal/bacterial content were characterized. The composition of the resuspended dust in the inhalation chamber was consistent with previous laboratory studies used to define resuspended dust composition.

**445 CHEMICAL, MICROBIOLOGICAL AND TOXICOLOGICAL CHARACTERIZATION OF FINE PARTICULATE MATTER (PM2.5) FROM AN INDOOR ENVIRONMENT IN PUERTO RICO.**

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Fine atmospheric particles (PM2.5) were sampled at an indoor working environment consisting of 3 analytical laboratories located in the same building. The objective of this study was to determine the individual composition of particulate matter and to evaluate its chemical, toxicological and microbiological characteristics. We compare our results with that of the immediate surrounding outdoor environment and with other published indoor studies. All samples were digested for metal analyses including their appropriate field blanks. Six metals were analyzed in this study (As, Cd, Fe, Ni, Pb and V) by means of Atomic Absorption. Some of the filters were expended for the determination of fungi. Fungi were grown in malt extract agar and glycerol agar culture media. Aqueous extracts from particulate matter were evaluated by a immuno-toxicological assay (Bio-Plex Cytokine Array System) in human lymphocytes. The amount of PM2.5 indoor ranged from 4.5-12 µg/m<sup>3</sup> as compared to 21.5 µg/m<sup>3</sup> outdoor. Although the levels of PM2.5 were higher outdoor the concentration of metals per mass of PM2.5 was greater indoors. The metals per mass in the indoor air ranged from 7.1- 172, 195 µg/g. All metal analyzed were significantly higher indoor when compared to outdoor with an I/O ratio as high as 10 for Ni. These indoor metal concentrations are high when compared to other studies. The main fungi species found in the indoor environment was Aspergillus versicolor. The concentrations of these fungi were relatively low as 2 CFU/m<sup>3</sup>. The Aqueous extracts significantly increased the release of IL-8, IL-17 and MCP-1 by human lymphocytes. Relative increases in IL-5 and IL-13 were also observed for these extracts. This research provides the tools for studying the effects of specific components in indoor air particulate matter on immune response.

**446 HIGH NO<sub>2</sub>/NO<sub>X</sub> RATIO IN DIESEL ENGINE EMISSION AEROSOL MAY REPRESENT A MORE IMPORTANT ACUTE TOXIC TRIGGER THAN PARTICULATE MATTER (PM) FOR LUNG TISSUE.**

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We assessed the PM and gas phase respective contributions to lung toxicity profile induced by Diesel engine emission aerosols. Organotypic cultures of rat lung tissue were exposed to continuously sampled and diluted Diesel engine emission aerosols. PM content and size distribution were measured using an AVL415 and SMPS respectively. CO, NO, NO<sub>2</sub>, and HC were monitored using a Horiba Mexa 7000 analyser. Filtration of diluted emissions through a silicon carbide monolith removed >99% PM from the emissions and did not alter the gas phase pollutant content. Engine was operated at different speeds and loads with various fuels (Sulphur and aromatics), and after treatment devices to quantitatively modulate pollutant

emissions. Endpoints for Inflammatory reaction (TNF $\alpha$ ) and oxidative stress (GSH, Catalase, GPx, SOD, 8-oxoguanine) where assessed in lung tissue and/or culture medium. Beside the wellknown inflammatory impact of PM emission evidenced at low NO<sub>2</sub>/NO<sub>x</sub> ratio (<0.1) (Bion et al. CBT 2002;18:301-14), we have identified that aerosol gas phase was responsible for oxidative stress. We could correlate oxidative injury to increasing NO<sub>2</sub>/NO<sub>x</sub> ratio in the exhaust which varied from less than 0.1 up to more than 0.55, with almost no variation of total NO<sub>x</sub>. Neither CO nor HC were assignable for these effects. For NO<sub>2</sub>/NO<sub>x</sub> ratio >0.2, emission filtration could no more modify the toxic response pattern suggesting a predominant impact of the gas phase over the PM phase. For high NO<sub>2</sub>/NO<sub>x</sub> ratios >0.4, we observed lung tissue cytotoxicity and cell necrosis. Oxidant enzyme profiles varied with increasing NO<sub>2</sub>/NO<sub>x</sub> ratio, suggesting a modulation of ROS profile according to the experimental combination. We suggest that NO<sub>2</sub>/NO<sub>x</sub> ratio could be a good marker of the pro-oxidative potential of combustion engine emission aerosols and thus be considered as a useful tracer of lung detrimental biological potential of these emissions. Supports from EC QLK4-CT62002-02357 MAAPHR program and Region Haute Normandie are greatly acknowledged

#### 447 EFFECTS OF DIESEL EXHAUST ON TLR3 SIGNALING IN RESPIRATORY EPITHELIAL CELLS.

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There are a variety of intrinsic as well as extrinsic factors, such as exposure to air pollution that can affect the pathogenesis of respiratory infections. Diesel exhaust (DE) emissions can significantly contribute to air pollution levels and exposure to DE can alter host defense and immune responses. Double stranded RNA (dsRNA), a by product of viral infections, is a ligand for toll-like receptor 3 (TLR3). In this study, we examined the effects of DE extract (DEE) on dsRNA-induced responses in respiratory epithelial cells. A549 cells were exposed to DEE for 2 hours, subsequently challenged with polyribonucleic acid:polyribocytidyl acid (poly I:C), a synthetic form of dsRNA, and 24 hours post-exposure analyzed for the expression of IL-6 and IFN-beta. We found that pre-exposure of epithelial cells to DEE significantly increased the expression of IL-6 and IFN-beta in response to poly I:C. Flow cytometric analysis showed that TLR3 is predominantly expressed in the cytoplasm of A549 cells. Exposure to DEE increased TLR3 mRNA levels in these cells. In addition, we examined the expression of the TLR3 adaptor protein Toll/IL-1 receptor domain containing adaptor inducing interferon- $\beta$  (TRIF) in response to DEE exposure. We found that exposure to DEE caused an increased expression of TRIF within 3-4 hours post-exposure. Upon activation of TLR3, TRIF associates with TNF receptor-associated factor 6 (TRAF6). TRAF6 is an intracellular signaling protein required for the TLR3-dependent activation of NF- $\kappa$ B and Interferon regulatory factor 3 (IRF3), which regulate the expression of IL-6 and IFN-beta, respectively. Overexpression of a dominant negative version of TRAF6 reversed the effects of DEE on poly I:C-induced IL-6 expression. Taken together these results indicate that exposure of respiratory epithelial cells to DE could potentially alter the response to viral infections by increasing the expression of components involved in the TLR3-dependent signaling cascades.

#### 448 NEUROTROPHIN RECEPTOR BLOCKADE ATTENUATES DIESEL EXHAUST PARTICULATE MATTER (DEP) ENHANCEMENT OF ALLERGIC RESPONSES.

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Recent investigations have linked neurotrophins including NGF, NT-3, and BDNF to allergic airways diseases. Antibody blockade of NGF attenuates airway resistance associated with allergic airway responses in mice. Mice administered an antibody against the low affinity pan-neurotrophin receptor p75, or p75 knockout mice have reduced pulmonary airway inflammation. DEP has been linked to asthma exacerbation in many cities with vehicular traffic congestion. Cell types affected by DEP, including eosinophils, macrophages, lymphocytes and the airway epithelium, also produce neurotrophins and/or express neurotrophin receptors. We tested the hypothesis that DEP-induced enhancement of the hallmark features of allergic airway disease in a murine model is dependent on p75 function. Nonallergic (NA) and ovalbumin (OVA)-allergic C57BL/6J mice were intranasally instilled with an antibody against the p75 receptor or saline alone 1 hour before OVA challenge. The mice were then exposed nose-only to the PM<sub>2.5</sub> fraction of SRM2975 DEP (0.85 mg/m<sup>3</sup>) or air alone for five hours, 1 hour later (n=9/group). Two days later, air exposed OVA-allergic mice developed increased levels of bronchoalveolar lavage fluid eosinophils, macrophages, neutrophils and IL-4, enhanced serum OVA-specific

IgE, and had a small but insignificant increase (22%) in methacholine (Mch)-induced airway obstruction (PenH; Buxco), relative to air-exposed NA mice. DEP-exposed OVA-allergic mice had a significantly greater degree of airway obstruction than all other groups (68% greater vs. air-exposed NA mice). Instillation of anti-p75 significantly reduced mean PenH values in DEP-exposed OVA-allergic mice to levels similar to NA mice. The data demonstrate that neurotrophins play a role in DEP exacerbation of allergic responses, particularly airway obstruction, and may be relevant to human DEP exposure (Supported by NCSU/USEPA CT829470. This abstract does not reflect EPA policy).

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#### YM1/2 CHITINASE PROTEINS IN MURINE NASAL EPITHELIUM AFTER A 13-WEEK INHALATION EXPOSURE TO ULTRAFINE CARBON BLACK PARTICLES.

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Mice exposed for 13 wk to airborne, ultrafine carbon black particles (Cb) develop nasal lesions including chronic rhinitis, mucous cell metaplasia in respiratory epithelium (RE), degeneration of olfactory epithelium (OE), and accumulations of an unique eosinophilic protein in both RE and OE. The purposes of the present study were to identify the Cb-induced eosinophilic protein and determine the exposure-response relationship for Cb exposure and protein expression in murine nasal epithelium. Female mice were exposed to 0, 1, 7, or 50 mg/m<sup>3</sup> Cb for 6 h/day, 5 days/wk for 13 wk. Mice were sacrificed 1 day, 13 wk, or 11 mo post-exposure (PE). Cb aerosols had a primary particle size of 17 nm with aerodynamic diameters ranging from 1.2-1.6 microns. Nasal airways were processed for light microscopy and immunohistochemically stained for Ym1/2 chitinase proteins (Ym). Morphometry was used to determine the volume density of Ym in RE and OE. No or scant amounts of Ym were present in RE or OE of mice exposed to 0 or 1 mg/m<sup>3</sup> Cb. Mice exposed to 7 mg/m<sup>3</sup> Cb and sacrificed 1 day and 13 wk PE had mean values of 3.4 and 5.3 nl Ym/mm<sup>2</sup> basal lamina at 1 day and 13 wk PE, respectively. Mice exposed to 50 mg/m<sup>3</sup> had three fold more Ym in RE than 7 mg/m<sup>3</sup> Cb-exposed mice at similar times PE. Only 50 mg/m<sup>3</sup>-Cb-exposed mice had marked amounts of Ym in sustentacular cells of OE at 1 day PE, and an 18-fold-Ym increase in OE, compared to air controls, at 13 wk PE. No Cb-induced increases of Ym in RE or OE were found at 11 mo PE. These results indicate that inhaled, low toxicity, ultrafine particles may cause marked and persistent increases of Ym expression in murine nasal epithelium. The role of mammalian chitinase proteins in particle-induced airway epithelial injury and repair in mice or humans is yet to be determined. (Research sponsored by the International Carbon Black Association).

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#### PARTICLE SURFACE AREA AND PARTICLE NUMBER, BUT NOT PARTICLE MASS, PREDICT THE ADJUVANT EFFECT OF PARTICLES ON ALLERGIC RESPONSES IN MICE.

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Airborne particles have been shown to enhance allergic responses in humans and mice. Traditionally, particle exposure has been monitored as mass concentration of PM<sub>10</sub> or PM<sub>2.5</sub>. Large particles strongly influence the mass concentration, thus particle mass is a poor measure for characterizing the amount of the small, possibly more biologically potent particles. The aim of this study was to examine whether fine particles exert a stronger adjuvant effect on allergic responses than coarse particles, and if so, whether particle surface area or particle numbers predict the increased IgE responses better than particle mass. We used polystyrene particles (PSP) ranging in diameter from 0.0588 to 11.14  $\mu$ m, carbon black (CB) and diesel exhaust particles (DEP), to study the adjuvant effect of particles on the immune responses to the allergen ovalbumin (OVA) after subcutaneous injection into the footpad of BALB/c mice. At a given mass per mouse, the small particles (0.0588 and 0.202  $\mu$ m PSP, CB and DEP) increased the OVA-specific IgE serum levels to a higher degree than the larger particles (1.053, 4.64 and 11.14  $\mu$ m PSP). Furthermore, during the primary response to OVA in the draining popliteal lymph node, the particles' synergistic effect increased with decreasing particle size, measured as cell numbers, expression of surface markers (CD19, MHC class II, CD86 and CD23) and *ex vivo* production of IL-4 and IL-10. Linear regression analyses indicated that the IgE response was predicted by the total particle surface area ( $R^2=0.64$ ), number of particles ( $R^2=0.62$ ) and particle diameter ( $R^2=0.58$ ), but not by particle mass ( $R^2=0.06$ ). This conclusion was confirmed by linear regression analyses for the cellular parameters. To conclude, ultrafine and fine particles had

stronger adjuvant effects on allergic responses than larger particles at equal administered mass. Accordingly, the total particle surface area and particle number, but not the currently used particle mass, predicted the adjuvant effect of particles on allergic responses.

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#### CHEMICAL CHARACTERISTICS AND TOXICITY OF POLAR ORGANIC EXTRACT OF DIESEL EXHAUST PARTICLES.

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Previous studies show that diesel exhaust particles (DEP) induce inflammatory responses. Chemical characteristics such as oxidative capacity may be related to the responses. The present study was designed to elucidate the relationship between chemical characteristics of organic extract of DEP and toxicity. DEP were collected with quartz filter. The organic compounds of DEP were extracted using dichloromethane. The organic extracts (OE-DEP) were fractionated into n-hexane soluble fraction (H-SOL) and n-hexane insoluble fraction (H-INSOL). GC-Mass, IR, and NMR spectra of OE-DEP, H-SOL and H-INSOL were measured. Oxidative capacity was measured using dithiothreitol (DTT) consumption. Induction of heme oxygenase-1, as a marker of oxidative stress, in alveolar epithelial cells was measured by ELISA. Cytotoxicity was assessed by LDH release. Induction of inflammatory response was evaluated the number of neutrophils infiltrated into peritoneal cavity of BALB/c mice 24 h after administration of the extracts. IR spectra show that H-INSOL not H-SOL has functional groups related to oxygenation such as hydroxyl, ketone, aldehyde, carboxyl groups. Relative intensity of these peaks were H-INSOL>OE-DEP>H-SOL. DTT assay also showed that relative oxidative capacity was H-INSOL>OE-DEP>H-SOL. Induction of heme oxygenase-1, by H-INSOL is also stronger than those of OE-DEP or H-SOL. H-INSOL injured alveolar epithelial cells concentration dependently. The relative strength of the cytotoxicity was H-INSOL>OE-DEP>H-SOL. The analysis of the number of the infiltrated neutrophils in the intraperitoneal cavity also showed that H-INSOL caused inflammation dose dependently. The induction of the inflammatory responses by H-INSOL was stronger than OE-DEP or H-SOL. These results suggest that polar organic extract, H-INSOL, contains oxygenated compounds. Some of them have oxidative capacity. They may be responsible for the induction of oxidative stress and inflammatory responses.

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#### ORGANIC EXTRACT OF DIESEL EXHAUST PARTICLES STIMULATES EXPRESSION OF IA AND COSTIMULATORY MOLECULES ASSOCIATED WITH ANTIGEN PRESENTATION IN RAT PERIPHERAL BLOOD MONOCYTES BUT NOT ALVEOLAR MACROPHAGES.

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Diesel exhaust particles (DEP) consist of carbonaceous cores that adsorb many organic compounds. DEP and organic compounds in them were shown to induce oxidative and inflammatory effects. Previous reports have been shown that DEP induce allergic asthma. Our present study is designed to clarify whether DEP may affect 1) the expression of Ia and B7 molecules in alveolar macrophages (AM) as a mature cell or peripheral blood monocytes (PBM) as an immature cell, 2) the antigen-presenting (AP) activity of PBM. The organic extract of DEP (OE-DEP) was prepared using dichloromethane. AM and PBM were prepared from normal male Wistar rats (8-10 weeks). T cells for use in the experiments of the AP activity were prepared from lymph nodes of ovalbumin-sensitized rats. AM and PBM were exposed to whole DEP, OE-DEP or residual particles from DEP (washed DEP) for 24 hrs. The expression of cell surface molecules associated with antigen presentation was measured by flow cytometry. AP activity was assessed by ovalbumin-specific T cell proliferation. Whole DEP and OE-DEP significantly increased the expression of Ia and B7 molecules in PBM but not in AM concentration dependently. The effect of washed DEP was not observed in this study. Low concentration of organic extract also increased AP activity of PBM. These results suggest that DEP may enhance AP activity in immature APC and the effect contributing to the organic compounds rather than the residual carbonaceous particles of DEP. Furthermore, our recent study shows that OE-DEP had ability to induce oxidative stress (induction of heme oxygenase-1) in pulmonary cells equivalent to whole DEP, but the activity of washed DEP was low. Thus, the oxidative stress may be a key component causing the exacerbation of allergic reaction by DEP.

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#### WOOD SMOKE PARTICLES INCREASE ALLERGIC RESPONSES IN MICE, BUT LESS THAN DIESEL EXHAUST PARTICLES (DEP).

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There is ample evidence that ambient air particles increase the severity of asthma and allergic diseases. In this project financed by VISTA\* we have used well-characterized combustion particles to study the relationships between particle properties and their capacity to increase allergic responses. Diesel exhaust particles (DEP) and chemically and physically characterized particles from wood smoke and road traffic were used to study the adjuvant effect of combustion particles on the immune responses to the allergen ovalbumin (OVA). OVA, particles with and without OVA and vehicle alone (HBSS), were injected into one hind footpad of BALB/cA mice. Particles together with OVA significantly increased the level of OVA-specific IgE in serum, as compared to the groups given OVA or particles alone, although wood smoke particles with OVA induced significantly lower IgE levels compared to the other particles. However, wood smoke particles with OVA induced higher levels of OVA-specific IgG2a than DEP and road traffic particles. The primary responses to particles with or without OVA, were studied using cells in the draining popliteal lymph node. As compared to OVA and particles alone, all particles with OVA increased cell numbers, cell proliferation, *ex vivo* secretion of IL-4 and IL-10 after Con A stimulation and expression of several cell surface molecules (CD19, MHCII, CD86 and CD23). Overall, DEP with OVA seemed to be most potent in inducing primary cellular responses, and wood smoke particles induced somewhat higher responses than road traffic particles. In conclusion, all combustion particles tested had the capacity to enhance allergic sensitization, although to different degrees. DEP was the most potent allergy adjuvant, while the potency of wood smoke and road traffic particles depended on the parameters measured. The antibody experiments indicated that while DEP was a clear Th2 adjuvant, wood smoke and road traffic particles appeared to give a mixed Th1/Th2 associated response. \* The Norwegian Academy for Science and Letters and Statoil

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#### COMPARISON OF PULMONARY TOXICITY BETWEEN HEALTHY SPRAGUE-DAWLEY RATS AND SPONTANEOUSLY HYPERTENSIVE RATS EXPOSED TO DIESEL EXHAUST PARTICLES.

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Epidemiologic studies have demonstrated an association between ambient particulate matter and cardiopulmonary morbidity and mortality. Diesel exhaust particles (DEP), a major contributor to ambient particulate matter, have also been reported to be associated with lung cancer and cardiopulmonary diseases. It has been proposed that the reactive oxygen species (ROS) may contribute to the cardiopulmonary toxicity of particulate matter including diesel particles. Spontaneously hypertensive (SH) rats, a model with antioxidant deficiency, have been used in many studies investigating PM toxicity. Here, we compare the pulmonary and systemic inflammation and oxidative DNA damage of DEP between healthy Sprague-Dawley (SD) rats and SH rats. Both SD rats and SH rats were administered with different doses of DEP (SRM 2975, NIST) (0, 125, 500 µg per animal) via intratracheal instillation. After 24 hours, cell and differential counts as well as total protein were determined in bronchoalveolar lavage fluid. Plasma 8-OHdG, IL-6 and TNF- $\alpha$  were also determined in peripheral blood. After DEP exposure, significant lung inflammation and injury with dose-response relationship were observed in both SH rats and SD rats. However, there is no difference on these parameters between SD rats and SH rats. We conclude that diesel exhaust particles may cause lung inflammation and injury, which may not be mediated through ROS production. Further studies are needed to clarify above findings.

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#### APOE MOUSE MODEL OF ATHEROSCLEROSIS CONFER SUSCEPTIBILITY TO EXTRAPULMONARY EFFECTS OF DIESEL EXHAUST.

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Particulate matter air pollution (PM) has been associated with increased incidence of cardiovascular mortality. Analysis of specific cause of death suggests that coronary artery disease (CAD) represents a major susceptibility trait. To test the hypothesis that CAD confers vulnerability to adverse PM health effects, we used a mouse model of hyperlipidemia and atherosclerosis, the ApoE knockout mouse fed a high fat diet for 16 weeks. During the 15th week of the high fat diet, ApoE mice and their genetic control (C57BL/6J) were implanted with radiotelemetry device to permit the acquisition of electrocardiographic (ECG) signals. After 7 days of recovery, mice (n=5 per group) were exposed to filtered air or diesel exhaust (0.5 or

5.0 mg/m<sup>3</sup>) for 6 h/d  $\times$  3 d. Raw ECG traces were recorded digitally to enable post hoc analysis. Mice were euthanized 18 hours following cessation of the final exposure. Lipid staining of the aortas confirmed the increased severity of atherosomatous disease in ApoE mice compared to C57BL/6J mice. Bronchoalveolar lavage analysis indicated significant pulmonary inflammation only in mice (both strains) exposed to the highest diesel concentration. Significant ECG findings, primarily decreases in heart rate ( $\Delta$ max = 70 bpm below filtered air-exposed mice), were present in ApoE mice but not C57BL/6J mice exposed to the high concentration of diesel. Measures of systemic oxidative stress (oxidized low-density lipoproteins) were also elevated (>5-fold) in the high concentration-exposed ApoE mice. These findings support the use of the ApoE mouse to investigate mechanisms of PM-associated adverse cardiovascular health effects. (This study was funded by an EPA STAR grant #R830839-010. This abstract does not necessarily reflect EPA policy.)

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#### RELATIVE EFFICACY OF COMBUSTION EMISSION PARTICULATE MATTER TO ADSORB THE NEUTROPHIL-ATTRACTING CHEMOKINE IL-8.

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Previous data indicated that when diesel exhaust (DE) particulate matter (PM) was incubated with a solution of IL-8, the ELISA-detectable activity in the supernatant of the suspension was depleted. Furthermore, the particles then gained the ability to activate neutrophils, suggesting that the IL-8 became adsorbed to the surface of the particles and remained in a bioactive form (Seagrave et al., 2004). These results suggested that inhaled PM could locally concentrate the neutrophil chemokine, potentially causing a strong localized inflammatory responses. We have extended these studies to examine the mechanisms of adsorption, determine whether other forms of PM had similar IL-8-adsorptive capacities, and quantify the neutrophil activating potential of the DE-PM-associated IL-8. Radiolabeled IL-8 associates with the particles, confirming that the loss of ELISA-detectable IL-8 is not loss of immunoreactive epitopes. Binding to DE-PM does not seem to be through either ionic or hydrophobic mechanisms, but is blocked weakly by larger proteins and somewhat more strongly by smaller proteins, suggesting intercalation into micropores. Dissociation of the chemokine is very slow in protein-free buffer, but is increased in the presence of competing proteins, suggesting rapid re-binding. We examined a wide range of particles including DE-PM from 5 sources, 2 types of gasoline engine emissions PM, 3 types of ambient PM, woodsmoke PM, and 3 forms of carbon black. Results showed a wide range of adsorptive capacity, even among the various types of DE-PM. DE-PM-bound IL-8 appeared to be slightly less effective in activating neutrophils than equivalent amounts of soluble IL-8. These data support the hypothesis that IL-8 adsorption to inhaled PM could contribute to localized inflammatory responses in regions of high PM deposition. This work was supported by the Health Effects Institute and the National Institutes of Health.

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#### DIESEL EXHAUST PARTICLES SUPPRESS LPS-STIMULATED PRODUCTION OF PGE2 IN HUMAN ALVEOLAR MACROPHAGES: ROLE OF P38 MAPK AND ERK PATHWAYS.

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Studies have reported associations between exposure to ambient particulate matter (PM) and adverse health effects, including respiratory and cardiovascular effects. Diesel exhaust particles (DEP) compose a significant fraction of PM in some areas. Alveolar macrophages (AM), the key players in lung host defense mechanisms against bacteria, function by releasing soluble mediators such as cytokines and prostanglandins (PG). This study was designed to investigate the effect of DEP on human AM responsiveness to LPS by monitoring the release of PGE2 and also to study the signal transduction pathways involved in the expression of cyclooxygenase 2 (COX2). AM were exposed *in vitro* to Standard Reference Material (SRM) 2975, SRM 1650, SRM 1975 (dichloromethane extract of SRM 2975) (SRMs from NIST) or carbon black (CB) for 24hr, and subsequently incubated with LPS (1ng/ml) for 24hr. DEPs, but not CB, suppressed the release of PGE2 by LPS-stimulated AM. This suppression is significant after 4h of LPS stimulation. mRNA expression of COX2 was not affected by all DEP treatment at 2h of LPS treatment, but there was a decreased COX2 mRNA expression (>50%) after 4h of LPS treatment, suggesting a feedback inhibition. However, COX2 protein levels were equivalent in vehicle and DEPs exposed AM. Further studies to investigate the effect of DEPs on p38 MAPK and ERK signaling pathways showed that DEPs alone (24 hr) did not inhibit the phosphorylation of p38 or p42. However, DEP exposure in-

duced a decrease in phosphorylated p38 and p42 (vs vehicle) with LPS stimulation. The data indicate DEPs suppress the ability of AM to produce PGE2 in response to LPS, despite having no effect on the COX-2 protein expression. The data suggest that organic extract was capable of inducing these responses without the particle core. Alteration of macrophage signaling pathways may play a role in some PM-induced health effects. [This abstract may not represent official EPA policy. Support: EPA-UNC CEMALB CR829522]

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#### SULFORAPHANE, A POTENT PHASE 2 INDUCER, INHIBITS THE ADJUVANT EFFECT OF AEROSOLIZED DIESEL EXHAUST PARTICLES IN A MURINE MODEL FOR OVALBUMIN SENSITIZATION.

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Human epidemiological studies suggest a correlation between exposure to ambient particulate matter and adverse health effects while animal studies indicate diesel exhaust particle (DEP) exposure can enhance asthma-like symptoms. One mechanism whereby DEP is thought to exacerbate endpoints associated with asthma is by oxidative stress. Our previous studies show that antioxidants can mitigate increases in antigen specific IgG1 and IgE levels caused by DEP. Here we show that D, L-sulforaphane (broccoli extract), a potent phase 2 inducer, was able to mitigate antigen specific increases in IgG1 and IgE caused by DEP. 24 balb/c mice were treated with 4.5 umol/mouse/day D, L-sulforaphane and 24 were treated with olive oil (vehicle) by gavage for 7 days prior to a 10-day inhalation treatment, the D, L-sulforaphane treatment continued through the 10-day inhalation treatment. The mice were placed into 4 different inhalation exposure groups (6 mice/group), saline (20 minute/day), ovalbumin (OVA, 20 minutes/day), DEP (60 minutes/day) and DEP+OVA and treated by inhalation for 10 days. Sera samples were taken 4 days after the last inhalation treatment. Mice that did not receive D, L-sulforaphane treatment presented the typical pattern of OVA-specific IgG1 induction, i.e., DEP+OVA treated mice showed high levels of OVA-IgG1 (307 ng/ml) while saline, OVA only and DEP only treated mice all had OVA-IgG1 levels below 100 ng/ml. However, mice treated with D, L-sulforaphane prior to DEP+OVA treatment showed a decrease in OVA-IgG1 levels (20ng/ml). This same pattern held true for OVA-IgE levels, i.e., OVA+DEP treated mice presented high OVA-IgE levels compared to mice treated with saline, OVA only or DEP only and mice pre-treated with D, L-sulforaphane prior to DEP+OVA treated had significantly reduced OVA-IgE levels. This data suggests that induction of phase 2 enzymes can be protective against the adjuvant effects of DEP.

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#### THE INCREASE IN TOTAL IGE IN SERUM OF FEMALE MICE AFTER INTRANASAL EXPOSURE OF CARBON BLACK PARTICLES WITH POLLEN.

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Carbon black particles (CB) have been widely used for commercial products. The risk of allergic diseases induced by inhalation of carbon black particles is a growing concern. We evaluated allergic effects of CB in an intranasal exposure model in BALB/c mice. CB (Mitsubishi Kasei) and purified pollen were suspended in PBS. Female BALB/c mice, 6-8 weeks of age, were intranasally instilled by pipetting a total volume of 10  $\mu$ l of the suspensions for three consecutive days. The amount of CB administered for each group was 0, 33.5, 100 and 300  $\mu$ g, and that of pollen for the groups was 5  $\mu$ g. For additional groups, one group was administered 300  $\mu$ g of CB only, and the other group was administered PBS only. Ten days after administration, each mouse was intranasally instilled suspended 5  $\mu$ g of pollen in PBS. At 15 days after the first administration, blood was collected from a tail of each mouse. Serum total IgE and pollen-specific IgE were determined by ELISA. The mean value of total IgE of the group administered 100, or 300  $\mu$ g of CB with pollen was significantly higher than that of the group administered pollen only. The means of total IgE of the high-dose groups were also significantly higher than that of the group administered PBS. The mean value of total IgE of the group administered CB only was slightly higher than that of the group administered pollen or PBS only. The mean values of pollen-specific IgE of the groups administered CB and pollen were higher, however, it did not reach the significant level. The administration of CB with pollen by instillation increased total IgE at the levels of 100  $\mu$ g and over. Since the mean values of pollen-specific IgE was not different significantly among the groups, CB may have a direct effect of increasing IgE. High dose exposure of CB may be related to allergic diseases.

DIESEL AND HARDWOOD SMOKE EMISSIONS DIFFERENTIALLY AFFECT CLEARANCE AND INFLAMMATION OF INTRATRACHEALLY INSTILLED PSEUDOMONAS AERUGINOSA.

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Diesel exhaust (DE) and wood smoke are contributors to indoor, outdoor, and occupational air pollution. Of the many cardio-respiratory health outcomes associated with episodic and long-term exposures to air pollution, respiratory infection is a major contributor to morbidity and mortality. As part of a larger health assessment of multiple anthropogenic source emissions, mice were exposed to DE and hardwood smoke (HWS) and instilled with an inoculum of a common respiratory pathogen, *Pseudomonas aeruginosa*. Bacterial clearance and pulmonary inflammation were assessed. DE was generated from a 2000 model Cummins 5.9L, turbo diesel engine operated on national average certification fuel on a modified version of the EPA heavy-duty test cycle. HWS was generated by burning consistent weights and sizes of oak in an uncertified stove operated on a 3-phase cycle: kindling, high-burn, and low-burn. DE and HWS were diluted to particulate matter (PM) mass concentrations of 30, 100, 300, and 1000  $\mu\text{g}/\text{m}^3$  respectively. Young C57Bl/6 mice were exposed to DE and HWS for 6h/d, 7d/ wk, for 1wk and 6mo, instilled with bacteria, and sacrificed after 18h in clean air. Colony counts of serial diluted lung homogenate and histopathology were assessed. HWS had no overall effect on bacterial clearance or inflammatory histopathology at either time point. Consistent with our previous observations in a model of respiratory syncytial virus infection, DE decreased bacterial clearance and exacerbated inflammatory histopathology. These results indicate that resistance to respiratory bacterial infection is differentially affected by PM-containing pollutant mixtures from different sources. This abstract is funded by the National Environmental Respiratory Center (NERC) with support of multiple government and industry sponsors including the USEPA. This work is not intended to represent the view of any NERC sponsor.

SAFETY EVALUATION OF AEROSOLIZED CYCLOSPORINE IN RATS AND DOGS.

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Lung transplantation treated by current systemic regimens has the highest rate of rejection associated with any current transplant procedure. Local immunosuppressive therapy with aerosolized Cyclosporine Inhalation Solution (CyIS) has the potential to improve survival after lung transplants by delivering high concentrations of cyclosporine to the site of rejection while reducing systemic drug exposure and side effects. The objective of these studies was to evaluate the potential toxicity of CyIS when given by nose-only inhalation to rats and dogs for 28 consecutive days followed by a recovery period. Sprague-Dawley rats received total inhaled doses of 0 (air control), 0 (vehicle, propylene glycol), 7.4, 24.3 and 53.9 mg cyclosporine/kg/day. Daily administration of CyIS did not result in any observable accumulation in blood or lung tissue. Treatment-related effects were observed in all dose groups including mortality (high dose only), clinical observations, body weight, food consumption, and clinical and anatomic pathological parameters. Systemic effects were consistent with those known for cyclosporine. There were no respiratory or systemic effects of high doses of the propylene glycol vehicle relative to air control. A NOAEL of 24.3 mg cyclosporine/kg was established for local effects of CyIS in the respiratory tract. In a separate study, beagle dogs were exposed to 0, 4.4, 7.7 and 9.7 mg cyclosporine/kg/day. Minimal, focal laryngeal ulceration was observed in the mid dose males on Day 29 and the high dose males after recovery. There were no adverse effects in clinical observations, body weights, food consumption, electrocardiograms, ophthalmic examinations, respiratory function, or clinical and anatomic pathology. The NOEL is 4.4 mg cyclosporine/kg in the dogs. Toxicokinetics from the rat study showed that the exposure of cyclosporine was ~18 times higher in the lung tissue compared to the blood. These studies support the use of CyIS for prophylaxis of lung rejection in patients receiving allogeneic lung transplants.

TOXICITY OF A SELECTIVE INHIBITOR OF LCK IS DUE TO INHIBITION OF LCK AND IS NOT CHEMOTYPE-RELATED.

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Lck, a Src family tyrosine kinase involved in the activation of T cells, is a potential target to prevent acute rejection of transplanted organs. We have developed potent inhibitors of Lck that block T cell activation and prevent acute rejection in the rat

heterotopic heart transplantation model. However, a src-family selective Lck inhibitor showed extensive toxicity when dosed at 10 X efficacious AUC in 14-day rodent toxicity studies. Histologic changes caused by this compound included mixed inflammatory infiltrate and focal necrosis in the heart, lymph nodes, eye and harderian gland, tubular necrosis in the kidney, sperm granuloma in the epididymis, and thymic and splenic atrophy. This Lck inhibitor also caused significant loss of body weight, lymphopenia, neutrophilia and decreases in plasma proteins and serum albumin. We needed to determine whether the toxicity of these inhibitors was due to inhibition of the target Lck or was linked to the chemotype. Structure-activity analysis showed the critical nature of a methoxy group of this compound for activity against Lck; hence we synthesized a structurally close desmethoxy analog of this LCK inhibitor. As expected, the desmethoxy analog was 15-fold less potent against Lck compared to the parent compound but exhibited relative similar potency against other Src family members tested and analogous PK characteristics. Doses of both compounds were administered that gave similar exposure in a 14-day rat toxicity study to compare the toxicology of the two compounds. The desmethoxy analog did not show any of the same toxicity as parent compound. It is concluded that the toxicity of this Lck inhibitor is a result of inhibition of Lck and is not linked to the chemotype of these compounds. Thus, the inhibition of Lck as a therapeutic modality appears to be limited by mechanism-based toxicity.

THE IMMUNOHISTOCHEMICAL EXPRESSION OF UROPLAKIN III IN RAT BLADDER TISSUE.

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This poster presents the results of an investigation into the immunohistochemical staining pattern of the differentiation marker, uroplakin III, and its correlation with the proliferation marker Ki67 in urothelial lesions. Several current drug development strategies involve the assessment of PPAR (peroxisome proliferator-activated receptor) agonists as potential therapeutics for the treatment of Type II (matute-onset) diabetes and dyslipidaemias. Histopathology in preclinical rodent toxicity studies on this class of compounds has revealed multi-organ pathology, including an increased incidence of pre-neoplastic and neoplastic bladder lesions. Ninety percent of the apical surface of mammalian urothelium is covered by rigid looking proteinaceous plaques, known as asymmetric unit membranes (ASMs). As the principal differentiation product of urothelium they are believed to play a role in urothelial structure and function. ASMs contain four major sub-units of uroplakins, of which uroplakin III possesses domains accessible for use in immunolocalisation studies. Retrospective samples of bladder were obtained from in-house rat carcinogenicity studies to include normal tissue, and bladder showing hyperplastic, benign and malignant lesions as observed by H&E histopathology. Immunohistochemical staining patterns using an antibody against uroplakin III in these tissues were assessed. Uroplakin III expression was also correlated with expression of Ki67, a general cell proliferation antigen.

BORTEZOMIB (VELCADE®) DOES NOT INDUCE THE FORMATION OF PROTEINASE K-RESISTANT PRION PROTEIN IN MURINE AND HUMAN NEURONAL CELL LINES *IN VITRO*.

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Prion diseases are associated with the conversion of cellular prion protein (PrP<sup>C</sup>) to a protease-resistant isoform (PrP<sup>Sc-like</sup>). A PrP<sup>Sc-like</sup> form accumulated when PrP transfected cells were subjected to complete proteasome inhibition, suggesting a potential theoretical link between proteasome inhibition and the pathogenesis of prion disease. However, these findings were not duplicated in nontransfected, but normal wild-type PrP-expressing neuronal cell lines and primary neurons. This discrepancy has been attributed to the potential of proteasome inhibitors to selectively induce transcription from expression constructs carrying a heterologous viral promoter. We determined if pharmacologically relevant concentrations of bortezomib resulted in the accumulation of proteinase K resistant forms of PrP in nontransfected mouse (N2A, GT-1) and human (NT-2) neuronal cells. Cells were exposed to bortezomib and other unrelated proteasome inhibitors (lactacystin, epoxomicin, and MG 132) for 16 hours and were examined by Western. Verification of functional proteasome inhibition was shown by accumulation of c-jun after exposure of each cell line to the proteasome inhibitors. All cell lines had abundant normal PrP<sup>C</sup> expression and no indication of an increase in the amount of endogenous PrP<sup>C</sup> or the conversion of it into a PrP<sup>Sc-like</sup> form upon treatment with any of the proteasome inhibitors. In

conclusion, this study and recent literature indicates that *in vitro* studies with non-transfected neuronal cell lines expressing wild-type PrP exposed to bortezomib do not show increased levels of PrP, nor the formation of proteinase-resistant forms. Previous preclinical studies with bortezomib indicated that it does not appreciably cross the blood-brain barrier, and in toxicity studies, there has been no evidence of prion-related disease pathology. Thus, the experimental data indicates that there is negligible risk associated with proteasome inhibition and prion-related disease.

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#### PHARMACOLOGY AND TOLERABILITY OF INS50589, A REVERSIBLE P2Y<sub>12</sub> RECEPTOR ANTAGONIST WITH ANTI-PLATELET AGGREGATION ACTIVITY, ADMINISTERED BY CONTINUOUS INTRAVENOUS INFUSION IN RATS AND DOGS.

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The objective of this study was to evaluate the pharmacology and tolerability of INS50589 when administered by continuous intravenous infusion to Sprague-Dawley rats and Beagle dogs. Animals were infused for up to 24 hours with INS50589 at concentrations from 0.3 to 30 mg/kg/h in rats and 0.03 to 1.5 mg/kg/h in dogs. Blood was collected for assessment of ADP-stimulated *ex vivo* platelet aggregation and determination of drug plasma levels. Platelet counts were determined by standard hematology. Tolerability to treatment was assessed by clinical observations and serum chemistry. The results indicate that infusion of INS50589 produced a rapid, dose-dependent inhibition of *ex vivo* platelet aggregation in rats and dogs. No treatment-related changes in platelet count were observed in either species. In rats, infusion of INS50589 at doses from 3 to 20 mg/kg/h resulted in 70-100% inhibition of platelet aggregation at all time points. Half-maximal inhibition of platelet aggregation was observed at plasma levels of INS50589 of approximately 185 nM. No adverse clinical signs were observed during or after infusion of rats with INS50589 at doses up to 10 mg/kg/h for 24 h. In dogs, maximal inhibition of platelet aggregation was observed at doses between 0.3 and 1.0 mg/kg/h. The inhibitory effect reached steady state within 30 minutes, was maintained for the duration of infusion at all concentrations tested, and reversed rapidly after the end of infusion. Half-maximal inhibition of platelet aggregation was attained at plasma levels of INS50589 of approximately 63 nM. The plasma half-life of INS50589 was 7 minutes in dogs. INS50589 was well tolerated in dogs when infused at a dose of 1.5 mg/kg/h for 24 hours. In conclusion, intravenous infusion of INS50589 induced rapid, reversible inhibition of platelet aggregation, and was well tolerated at doses up to 1.5 mg/kg/h in dogs and 10 mg/kg/h in rats.

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#### RANGE-FINDING TOXICITY STUDIES WITH SQ109, AN ANTI-TUBERCULAR AGENT, IN RATS AND DOGS.

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Pre-clinical range-finding studies were conducted with SQ109 (N-adamantan-2-yl-N'-[3, 7-dimethyl-octa-2, 6-dienyl]-ethane-1, 2-diamine dihydrochloride) to determine its relative toxicity. Doses of 0, 7.2, 36, and 54 mg/m<sup>2</sup>/d i.v., and 0, 144, 360 and 900 mg/m<sup>2</sup>/d p.o. (gavage) were given to male Fischer 344 rats (3/group) for up to 14 days. Lethality occurred with doses  $\geq$ 360 mg/m<sup>2</sup>/d p.o. (Days 3-9). Microscopic evaluation of tissues from these rats showed hypertrophy and glycogen depletion of liver, depletion or necrosis of lymphoid tissue and bone marrow, necrosis of GI tract and hyperplasia of urinary bladder. Hyperplasia of urinary bladder was also observed for 1/2 rats surviving to Day 15. Dose-dependent lymphopenia ( $\geq$ 360 mg/m<sup>2</sup>/d p.o.) and liver toxicity (900 mg/m<sup>2</sup>/d p.o.) could be identified early with clinical pathology analyses. No toxicity was seen with 144 mg/m<sup>2</sup>/d p.o. or  $\leq$ 54 mg/m<sup>2</sup>/d i.v. Male and female Beagle dogs (1/sex/group) received SQ109 doses of 0, 100, 200, 240 (female) and 400 mg/m<sup>2</sup>/d i.v., and 0, 100, 400 and 1200 mg/m<sup>2</sup>/d p.o. (gavage) for up to 28 days. Dogs receiving  $\geq$ 400 mg/m<sup>2</sup>/d p.o. were also given ondansetron to prevent emesis. Both dogs in the 400 mg/m<sup>2</sup>/d i.v. group were euthanized moribund on Day 2. The male dog had moderate inflammation of the lung and minimal to mild degeneration of liver, kidney and heart, hemorrhage in gall bladder, and necrosis or depletion of lymphoid tissues upon microscopic evaluation. Hepatobiliary and kidney toxicity were identified with clinical pathology analyses prior to euthanasia. Toxicologically relevant changes in body weights and clinical pathology were not observed with  $\leq$ 240 mg/m<sup>2</sup>/d i.v. or  $\leq$ 1200 mg/m<sup>2</sup>/d p.o. SQ109 was present in plasma at all oral doses administered, and plasma concentrations increased with repeated dosing. The MTD for SQ109 is  $\geq$ 54 (i.v.) and  $\sim$ 144 (p.o.) mg/m<sup>2</sup>/d for rats, and NOAEL is 240 (i.v.) and  $\sim$ 1200 (p.o.) mg/m<sup>2</sup>/d for dogs (N01-CM-87103 and N01-CM-07110).

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#### SAFETY ASSESSMENT OF A NOVEL P38 INHIBITOR IN CYNOMOLGUS MONKEYS FOLLOWING DAILY ORAL DOSING.

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Inhibitors of the p38 MAP kinase enzyme are thought to be a viable agents for the treatment of inflammatory diseases. A small molecule inhibitor of p38 MAP kinase (e.g. AMG 548) has been evaluated in a repeated dose toxicology study in cynomolgus monkeys. Monkeys (4/sex/group) were dosed orally for at least 28 consecutive days with doses of 0 (control article was 1% Tween® 80 and 2% hydroxypropyl methylcellulose), 3, 25, and 75 mg/kg/day. Recovery animals (2/sex) were included for the control and high dose levels and were evaluated following a 4-week recovery period. Animals were evaluated for clinical observations, body weight, food consumption, clinical pathology, electrocardiography, ophthalmology, pharmacodynamic effects, toxicokinetics, and histopathology. Several animals had clinical observations of watery, liquid stool throughout the study that appeared to be related to treatment. In extreme cases the liquid stool resulted in dehydration, lack of food consumption, and morbidity. Minor, reversible effects on clinical pathology were noted and the gastrointestinal tract was the target organ. There were no test-article related effects noted on electrocardiographic measurements, ophthalmic examinations, body temperatures, or body weights. AMG 548 had a pharmacodynamic effect to inhibit lipopolysaccharide (LPS)-induced production of tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin-1 beta (IL-1 $\beta$ ). No accumulation or consistent sex difference in exposure was observed; exposure on day 26 increased greater than dose-proportionally across the dose range. Based upon clinical observations, clinical pathology, and histopathology, a no observed adverse effect level (NOAEL) was considered to be 3 mg/kg/day (male) and 25 mg/kg/day (female) following 28 days of repeated oral dosing in cynomolgus monkeys.

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#### CYTOFLAVINE REDUCES HYPOXIA CAUSED BY SEVERE ALCOHOLIC INTOXICATION AND MILD HEAD TRAUMA.

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**Background:** Severe alcoholic intoxication is accompanied by hypoxia, which may be worsened even by mild head trauma. Cytoflavine (C) is a novel antioxidant; each 10ml vial of it contains succinate (1g), N-methylglucamine (1.65g), riboflavin (0.02g), nicotinamide (0.1g) and inosine (0.2g). It can reverse hypoxic damage via improvement of mitochondrial function. The aim of our study has been to assess C influence on parameters of oxygen transfer and consumption. **Methods:** 21 patients (pts) with blood ethanol level more than 0.25g/dl sustaining head trauma were included into single blind study of C. All of them were comatose and mechanically ventilated. 12 pts received C (0.15ml/kg b.i.d. IV). Arterial and venous blood and expired air were assessed for O<sub>2</sub> and CO<sub>2</sub> content. According to Fick's principle the parameters of ventilation, circulation, oxygen transfer and consumption were calculated. The data are presented as means $\pm$ SEM. **Results:** The admitted pts had dead space to tidal volume ratio increased up to 0.54 $\pm$ 0.04 (compared with normal values 0.32 $\pm$ 0.04), and increased right to left shunt to 13% $\pm$ 2% of cardiac output. Oxygen consumption and extraction parameters were non-significantly decreased. By the 3rd day the oxygen consumption and extraction restored in C group. In the control group the oxygen consumption progressively decreased to 104 $\pm$ 12ml/min\*m<sup>2</sup> (normal values 149 $\pm$ 16). Oxygen extraction has also been reduced to 19 $\pm$ 2ml/l in the control group (normal values 37.8 $\pm$ 0.4). There was a trend to reduction of right to left shunt in C group, but it remained disturbed in the control group. A trend to decreased duration of coma and incidence of complications was found in pts received C. **Conclusion:** Cytoflavine restores oxygen transfer and utilization in severe alcoholic intoxication complicated with mild head injury. The mechanism includes succinate-dependent improvement of mitochondrial respiration described earlier.

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#### TEMPO ATTENUATION OF DICLOFENAC ADDUCTION AND ENTEROPATHY.

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**Background:** Therapy with diclofenac and other non-steroidal anti-inflammatory drugs (NSAIDs) is occasionally complicated by small intestinal permeability and other indicators of enteropathy. One strategy reported to protect against NSAID enteropathy is pretreatment with Tempo (2, 2, 6, 6-tetramethyl-1-piperidinyloxy),

a nitroxide radical scavenger (Davies and Jamali, 1997). Objective: We investigated the unknown mechanism responsible for the protective effect of Tempo pretreatment using our established rat model of diclofenac enteropathy where addition of enterocytes by the reactive acyl glucuronide metabolites of diclofenac appears to be a causal factor in small intestinal ulceration. Experimental Design: Sprague-Dawley rats were pretreated at 1 hr with Tempo (100 mg/kg) or vehicle and then treated with diclofenac (50 mg/kg). Extent of enteropathy was measured by counting ulcers and assessing intestinal leakage by measuring serum protein at 3, 12 or 24 hours. Patterns of diclofenac addition to proteins in liver and intestinal tissues were assessed by western blot analysis and immunohistochemistry. Serum concentrations of diclofenac were determined by HPLC. Results: Compared to rats treated with diclofenac alone, which typically had ~90 intestinal ulcers by 12 hrs, Tempo pretreated rats had < 5 ulcers at 12 hrs and showed sustained protection against intestinal leakage at 24 hrs. At 3 hr, Tempo pretreated rats had 65% lower serum concentration of diclofenac and markedly less drug-protein addition in liver and intestine. Conclusions: Possible effects on diclofenac uptake, distribution or bioactivation could explain why the observed protection against diclofenac enteropathy in Tempo pretreated rats was associated with lower systemic drug levels and less tissue addition. (Supported by NIH DK 56494, DK56338, and NIEHS ES06676)

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ENDOSCOPY STUDY OF THE GASTROINTESTINAL TOLERANCE OF STRONTIUM RANELATE IN CYNOMOLGUS MONKEYS.

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The objective of this study was to evaluate the digestive tolerance of strontium ranelate, a new antiosteoporotic drug, and to compare it to that of another strontium salt, strontium chloride. Strontium ranelate, strontium chloride or placebo were administered orally in capsules to three groups of two male and two female cynomolgus monkeys once daily for 7 days at a dose-level of 2g/day, which is the recommended dose in therapeutic use. Endoscopic examination of the esophagus, stomach and the first part of the duodenum was performed in fasted animals approximately 3 hours after the first (Day 1) and last dosing (Day 7). Where lesions were found on Day 7, further examinations were performed on Days 8 and 14 to assess recovery. Treatment with placebo and strontium ranelate did not result in any visible damage in the gastric mucosa, esophagus or first part of the duodenum. In contrast, acute superficial gastric damage was noted in all animals receiving strontium chloride, consisting of hemorrhagic and erosive lesions (ulceration in one male and marked congestive arthritis in one female). These effects were reversible after cessation of treatment. Microscopic examination of biopsy samples from the site of these gastric lesions revealed moderate granulocyte infiltration, indicating a local irritative origin of the lesions. It is concluded that oral administration of strontium ranelate is well tolerated by the gastric mucosa of cynomolgus monkeys while strontium chloride induces reversible superficial gastric lesions. This good gastrosophageal tolerance has been confirmed during the clinical trials of strontium ranelate.

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TC 1734-112: PRECLINICAL SAFETY ASSESSMENT OF A SELECTIVE  $\alpha 4\beta 2$  NICOTINIC ACETYLCHOLINE RECEPTOR AGONIST.

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TC-1734-112 is an agonist of the central  $\alpha 4\beta 2$  nicotinic acetylcholine receptor with a high binding affinity to membrane preparations from rat brain. The compound is currently under development in our laboratories in Phase 2 clinical trials for the treatment of AAMI indication. To support safety, TC-1734-112 has been tested in preclinical animal models including acute, subacute 2- and 4- week studies, subchronic 90 day studies, chronic 6-month, Segment 2 reproductive, mutagenicity, and safety pharmacology studies. TC-1734-112 was well tolerated in all repeat dose studies in the rat and dog and changes were observed at high treatment doses and blood levels: 1) bradycardia in the dog at 10 and 30 mg/kg; 2) testicular and accessory sex organ pathologies in the rat at 150 and 200 mg/kg; mild centrilobular hypertrophy with alkaline phosphatase elevations in the rat at 50, 150, and 200 mg/kg; and alveolar histiocytosis in the rat at 150 mg/kg. Dosages of 3 mg/kg in the dog and 5 mg/kg in the rat were considered the NOEL. There were no ophthalmoscopic or electrocardiogram alterations. TC-1734-112 was not mutagenic or teratogenic. For the toxicokinetics, the absorption of TC-1734 was rapid with a Tmax at about one hour. The Cmax and the AUC were generally greater for females than males and there was a general trend for values to increase as the study proceeded. Accordingly, if the maximum clinical dose is projected at about 1 mg/kg, a sufficient margin of safety exists to support long term clinical trials.

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OPIOIDS: OCCUPATIONAL CONTACT DERMATITIS AND THE LOCAL LYMPH NODE ASSAY (LLNA).

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Allergy to some opioid phenanthrene derivatives, including morphine and codeine, is recognized as a hazard in health care and pharmaceutical workers handling these materials. Retrospective assessment of human cases of occupational allergy by patch test has been used to categorize a limited number of opioids as dermal allergens. However, very little information is available on the use of laboratory assays to prospectively assess the allergenic potential of specific opioids. Recent trends in opioid chemistry suggest that this gap should be addressed in evaluating the potential impact of less well characterized opioids on occupational health. For example, *Papaver somniferum* (opium poppy) strains have been selectively developed to express high levels of the phenanthrene derivatives thebaine and oripavine as a means to provide raw materials for synthesis of newer medically important opioids. In addition, chemical syntheses yield stable, isolated substances such as 14-hydroxynormorphinone (NHM) from bioactive, native opioids. NHM is non-analgesic and relatively safe to transport and process, but can be readily chemically converted to medically useful opioid antagonists. As an initial step in quantifying the allergenic potential and potency of materials such as NHM, thebaine, and oripavine, we have assessed these materials in the LLNA. In addition, since opioids can trigger histamine release, these materials have been assessed for potential to irritate eye and skin in the GSK tiered worker safety test battery. The LLNA predicts NHM as a sensitizer with concentration-dependent increases >3-fold in stimulation index observed at applied concentrations of 10% and 25% and a <3-fold SI at 5%. NHM was non-irritant to skin but was a mild eye irritant. NHM has also been identified recently as a human dermal allergen. Very similar LLNA and dermal/ocular irritation findings for oripavine have also been obtained. In summary, the LLNA used in conjunction with assays for direct irritant potential provides useful data for prospectively assessing occupational hazards of opioids.

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USE OF NALTREXONE TO PREVENT ADVERSE EFFECTS OF SUFENTANIL IN BEAGLE DOGS.

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Sufentanil citrate is a potent analgesic used in the maintenance of balanced general anesthesia. It is also used in a proprietary drug formulation for an implantable drug delivery device, which is expected to be valuable in the treatment of chronic pain. Performance testing of such devices requires implantation in different animal species including non-rodents. The beagle dog is widely used in non-clinical safety studies. The lethal dose for IV administration in male beagle dog is approximately 10  $\mu$ g/kg. In order to evaluate the final human configuration of the device, which carries a high concentration of Sufentanil, purpose-bred adult male beagle dogs were each subcutaneously implanted with five CHRONOGESIC® Sufentanil osmotic pumps (DURECT Corp, Cupertino, CA) and carefully observed for more than one month. Animals were treated with naltrexone to minimize adverse effects of Sufentanil. Each osmotic pump delivered Sufentanil at 320  $\mu$ g/day. Animals (body weights 11.2 and 13.3 kg) received 1600  $\mu$ g/day total; daily dosage levels were 142 and 120  $\mu$ g/kg. Starting one day prior to implantation, animals were given tablets of naltrexone HCl (50 mg, b.i.d. at 6 a.m. and 3 p.m., orally). No clinical signs of Sufentanil overdose were observed. Animals showed only minor decreases in body weight (mean 0.4 kg) during the first week after implantation, followed by steady gains of 0.1, 0.4, and 0.2 kg during the following three weeks. Food consumption (mean 398 g/day before treatment) was also slightly decreased during the first week (255 g/day) and then showed a steady increase of 314, 323, 346 g/day during the following weeks. Thus, the used naltrexone treatment regimen was effective in prevention of the adverse effects of otherwise lethal doses of Sufentanil in beagle dogs.

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FLORIDA RED TIDES: MOLECULAR THERAPEUTICS FROM A SEA OF RED.

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Florida red tides produce potent neurotoxic chemicals known as brevetoxins. Brevetoxins also induce severe pulmonary effects, including bronchoconstriction and inhibition of tracheal mucus velocity when inhaled. Recently, an antagonist known as brevenal has been isolated from both field samples and laboratory cultures. Brevenal is a trans-syn polyether material possessing 5 rings in a 67677

motif. A number of active derivatives of brevenal are found naturally. Brevenal displaces brevetoxin binding on voltage-gated sodium channel site 5, antagonizes the acute animal toxicity of brevetoxins, and reduces or eliminates both the inhibition of tracheal mucus velocity and the bronchoconstrictor effects of brevetoxin exposure by inhalation. The antagonistic effects elicited by brevenal occur at picomolar to nanomolar concentrations, which makes them equipotent with the brevetoxin molecules whose effects they antagonize. Brevenal, when applied at picomolar concentrations alone in a sheep pulmonary system, increases mucociliary transport as evidenced by roentgenographic techniques. The effects of brevenal on TMV occur at approximately 1 million-fold lower concentration than for amiloride with nearly equal duration of effect. The discovery of brevenal is a step forward in the discovery of therapeutic agents to counteract seafood poisonings due to brevetoxins and pulmonary effects encountered during Florida red tides. More important, the brevenal structure may provide insight into development of therapeutics for mucociliary diseases including cystic fibrosis. Sponsored by P01 ES10594 funded by NIEHS, NIH, DHHS.

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EFFICACY OF PIROXICAM IN ALLEVIATING TETRACYCLINE-INDUCED MUSCLE DAMAGE.

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Oxytetracycline long-acting (TLA) is used in the treatment of canine ehrlichiosis. Intramuscularly (IM) administered TLA produces a severe pain and muscle damage. This study evaluates the efficacy and safety of piroxicam in alleviating adverse effects of an IM therapeutic dose of TLA in six healthy dogs. The dogs were administered TLA alone and, after a two-week interval, were injected with TLA and piroxicam concurrently. All the dogs were physically examined after each injection. Blood samples were collected 24h pre- and at 0, 0.5, 1, 3, 6, 9, 12, 24, 48, and 96h post-treatment for serum creatinine kinase (CK) activity. Swelling was noticed at the injection site of TLA in all six (100%) dogs and all were reluctant to move due to muscle stiffness, and lost appetite. Five dogs (83%) felt pain at the injection site. Four were unable to use the injected legs temporarily. Injecting TLA and piroxicam concurrently suppressed all the signs except for muscular stiffness, which was observed in two (33%) of the dogs for 12h. Transient increases in CK activities were observed after both treatments. Mean muscle damage was  $14.95 \pm 8.78$  g after IM TLA. Muscle damage was not significantly elevated ( $27.57 \pm 17.2$  g;  $P \geq 0.05$ ) after injecting TLA and piroxicam concurrently. These data show that piroxicam suppressed the side effects of an IM dose TLA but did not suppress muscle damage.

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THE EFFECTS OF CONTINUOUS INTRAVENOUS INFUSION OF SALINE ON BODY WEIGHT GAIN, FOOD INTAKE AND CLINICAL PATHOLOGY IN THE GÖTTINGEN MINI PIG.

M. Mus and C. Copeman. IPN, CTBR Bio-Research Inc., Senneville, QC, Canada. Sponsor: M. Vezina.

The Göttingen minipig has frequently been used as a non-rodent model for pre-clinical intravenous infusion safety studies at CTBR. Data from non-catheterized animals (oral and dermal routes) ranging from 4 to 8 months of age were evaluated to determine any effects of the presence of a femoral catheter and continuous intravenous infusion on the body weight gain, food consumption and clinical pathology parameters. The animals were surgically prepared with an indwelling medical grade catheter, placed in the vena cava via the femoral vein. The catheter was then brought subcutaneously to the exteriorization point on the animal's back. The catheter was connected to a clinical grade infusion pump via a tether and jacket system. The pigs were infused with 0.9% Sodium Chloride for Injection U.S.P. continuously for 26 weeks at a rate of 4 mL/hour. Body gains, food consumption and clinical pathology were unaffected when compared with historical control values for non-catheterized animals. It is concluded that in our laboratory the presence of an indwelling catheter and continuous intravenous infusion of saline in pigs had no adverse effects on the animals when compared to non-catheterized pigs.

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BACKGROUND INCIDENCE OF EXPERIMENTAL DESIGN-RELATED EVENTS ON INTRAVENOUS INFUSION STUDIES.

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Intravenous (IV) infusion has become a common route of administration, in particular with the expansion of the biotechnology industry. Conducting IV infusion in preclinical laboratory species can present procedure-related difficulties that require close control to avoid impacting the outcome of a study. Background inci-

dence of such events plays an important role in the overall interpretation of a study. Compilation of the historical incidence of experimental procedure-related deaths, catheter repairs and rate of infection at the infusion site in rat, dog and primate IV infusion studies of up to 6 months duration conducted over the past 15 years was extracted from an average of 30 rat, 15 dog and 8 primate studies/year. Procedure-related deaths at the time of surgical cannulation or during post-operative maintenance has been maintained at or below 1 to 2 % for rats and <1 % for dogs and primates. During the conduct of the studies, experimental-related deaths associated with infusion (or inability to progress with the infusion to study termination), in saline infused animals is  $\leq 1\%$ . Repair of catheters is anticipated in infusion studies, however the frequency for saline-infused animals remains low, at <1 % for all species. The use of appropriate sterile procedures during the surgical cannulation and additional precautions used during the conduct of the study to minimize bacterial infiltration have permitted maintenance of a <1% infection rate at the site of infusion. These success rates are also feasible with other relatively innocuous vehicles (eg. 5% Dextrose in Water, USP, phosphate buffered saline). The rates/volume and duration of infusion will greatly influence the success of the infusion. Infusates with extremes of pH or osmolality or that are irritant for other reasons will also negatively influence the success of the infusion. Based on this background data, it is considered that the low incidence of infusion-related adverse events does not interfere with the outcome of short or long-term infusion studies in our laboratory.

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VALIDATION OF A NEWLY INVENTED PORT CATHETER SYSTEM FOR CONTINUOUS INFUSION STUDIES.

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For continuous infusion studies in restrained or unrestrained rodent and non-rodent species, the choice of the appropriate port model is pivotal. Available vascular access ports (VAP) are being used depending on the selected species and the method of application. We developed a new modular multi-functional titanium port of unique shape, reduced size and adaptability to different applications. For initial validation, two male and two female cynomolgus monkeys (*Macaca fascicularis*, 3.8 - 6.4 kg and 4 - 8 years of age) were subcutaneously implanted with the new Covance VAP (patent pending). Due to the rocket-like shape and particularly due to smaller size, the incrustation process and post surgery recovery were accelerated in comparison with other ports, commonly available on the market. The small holes around the modular device facilitated the subcutaneous fixation. The catheter of choice for nonhuman primates was connected to the port and the animals will be administered intravenously for 13 weeks with 0.9% NaCl at 0.25 mL/kg, using the Pegasus Infusion Pump, stored in a back pack, and connected either by Gripper or SFN-port needles. Clinical signs, neurological evaluations, determination of body temperature, food consumption, 4-weekly clinical pathology, x-ray imaging using a contrast medium and a full macro- and microscopic evaluation were recorded. The sc port and the silicone membrane could easily be palpated and required less frequent needle exchanges than conventional ports. Clinical signs observed were a reddish and slightly swollen area at the port, isolated episodes of low food consumption and occasionally irregular feces. These observations were considered as incidental background findings. Body weight development was normal. One animal was terminated on study day 55. X-ray diagnostic in week 8 confirmed the proper VAP location and the free-flow into the vena cava caudalis. In conclusion, these preliminary results demonstrate that the new multi-functional VAP system appears well tolerated and should be suitable for various applications due to its modular design.

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CONTINUOUS SUBCUTANEOUS INFUSION IN RODENTS AND NON RODENTS.

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Intravenous infusion pre-clinical safety studies have been routinely performed at CTBR for close to 20 years on a wide-range of pharmaceutical and biotechnology products and in several species of laboratory animals. The subcutaneous route is often used for drug administration in humans. Continuous subcutaneous infusion has been developed to provide an alternative to intravenous infusion and subcutaneous injection and can assess the potential biological effects of compounds having a short half-life. Subcutaneous infusion was selected as the route of delivery for various drug types intended for the treatment of rheumatoid arthritis, auto-immune and inflammatory diseases, anti-obesity, and type II diabetes. Continuous subcutaneous infusion in rodents as well as non-rodents was successfully performed for up to 3 months. This method allows for higher infusion volumes to be administered, with an expected accuracy of delivery within  $\pm 10\%$  of the nominal dose. Infusion

rates as high as 1.0 mL/kg/h for rodents and 0.208 mL/kg/h for non-rodents have been employed using saline as the preferred vehicle. These rates correspond to approximate daily dose volumes of up to 0.6 mL for a 23 g mouse, 7.2 mL for a 300 g rat, 15 mL for a 3 kg monkey and 50 mL for a 10 kg dog. Advantages of such a system include low risk of infection, low stress to the animals, and unhindered animal activity. Since the pump and infusate remain external to the cage, the potential for the animal to interfere with the infusion is eliminated and handling of the animals during daily dosing procedures is minimized. Furthermore, the set-up does not interfere with daily study-related activities such as clinical observations, body weights, food consumption, blood sampling, etc. Overall, our method of infusion has been demonstrated to be both well-tolerated and without biologically significant effects on clinical pathology or pathology, which were generally transient in nature.

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#### A METHOD FOR LONG-TERM CONTINUOUS SUBCUTANEOUS INFUSION IN RATS AND DOGS.

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Continuous subcutaneous infusion is occasionally employed as a route of administration in humans. In order to mimic this route for nonclinical development of such drug candidates our laboratory developed a surgery and dosing technique in rats and dogs. The procedure involved an incision in the mid-scapular region, followed by insertion of the catheter in a caudal-lateral direction (approximately 1 inch for rats and 3 inches for dogs) by means of a trocar. In the case of dogs the surgery employed only a local anesthetic, while in rats general isoflurane anesthesia was used. The external portion of the catheter was connected to a cage-mounted infusion pump by means of a jacket and tether and infusion of control or test article was initiated at rates of up to 0.13 mL/kg/hour. Depending on the irritant nature of the test material, the catheters were replaced at intervals of 1 to 2 weeks, and the newly inserted catheters were directed away from the previous catheter path. Rats and dogs were maintained on continuous infusion for 6 months, during which time the following toxicological parameters were assessed: clinical condition, body weight, food intake, clinical pathology, ophthalmology, ECGs (dogs only) and toxicokinetic sampling. At the end of the treatment period animals were subject to terminal examinations, including organ weights and histopathology. Evaluation of data from controls (6 dogs/sex and 24 rats/sex) infused by this method for 6 months revealed minor, transient infusion site findings, but no effect on general physical condition, body weight gain or food intake, no ophthalmological changes, no ECG changes, and no changes in clinical pathology parameters relative to normal values. Histopathological findings of mild to moderate fibrosis and inflammation were seen in the tissue in the area of the catheter, but were considered to reflect a normal reaction to the presence of a foreign material. In conclusion, this technique was considered to be suitable for use with drugs to be administered by long term subcutaneous infusion.

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#### SUCCESSFULL PREVALIDATION OF AN *IN VITRO* RECONSTITUTED HUMAN CORNEA MODEL TO ASSESS THE EYE IRRITATING POTENTIAL OF CHEMICALS.

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This multi-centre study aimed at evaluating the reliability (reproducibility) and relevance (predictive power) of a new commercially available human corneal epithelial (HCE) model (SkinEthic Laboratories, Nice, France) to assess acute ocular irritation. A prevalidation approach (protocol optimisation, transfer and performance) was followed and at each of the 4 participating laboratories, 20 reference chemicals, covering the whole range of irritancy, were tested. The compounds were applied topically to the HCE cultures and the level of cytotoxicity (tissue viability and histological analysis) was determined. Once a standardized protocol was established, a high level of reproducibility between the laboratories was observed. In order to assess the capability of the HCE model to discriminate between irritants (I) and non-irritants (NI), a classification prediction model (PM) was defined based on a viability cut-off value of 60%. The obtained *in vitro* classifications were compared with different *in vivo* classifications (e.g. Globally Harmonized System) which were calculated from individual rabbit data described in the ECETOC data bank. Although an overall concordance of 80% was obtained (sensitivity = 100% and specificity = 56%), the predictive power of the HCE model substantially increased when other sources of *in vivo* and *in vitro* data were taken into account.

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#### *IN VITRO* SKIN IRRITATION : STRONG REPRODUCIBILITY FOR 50 CHEMICALS TESTED ON THE SKINETHIC RECONSTITUTED HUMAN EPIDERMAL MODEL.

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Evaluation of the irritancy potential to human skin of any chemical or formulation used in the chemical, pharmaceutical, and cosmetics industries is a necessity. Most of the available data come from the rabbit Draize test, but its reproducibility is questionable. Toxicologists develop tests on various skin models but the emergence of new tools such as reconstituted human skin tissues offers the most promising future to alternative methods. We have tested fifty chemicals (20 compounds chosen by ECVAM and 30 products previously tested in a human *in vivo* patch test) with two *in vitro* skin irritation test protocols. One is a direct topical application test and the other an *in vitro* patch test. Both protocols were performed using multiple endpoint analysis including cell viability (MTT reduction), histology, and IL-1 $\beta$  release. A prediction model is proposed to classify the chemicals as irritant or non irritant, and the results are compared to available rabbit and human data. The strong reproducibility observed by endpoint, and by compound, combined with the enhanced convenience and the reduced costs obtained by the use of reconstituted human epidermis prove all the usefulness and the potential of this tool.

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#### DETERMINATION OF THE DOSE-RESPONSE RELATIONSHIP FOR SURFACTANTS USING THE BCOP.

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The Bovine Corneal Opacity And Permeability Assay (BCOP) is a very sensitive assay in which it determines a safe allowable use for different types of surfactants (i.e. anionic, cationic). With this assay, we are able to determine an acceptable amount of surfactants to balance into the formula of our bubble bath products to avoid irritant potential. This method enables us to guide in the development of final product formulas when formulating with surfactants. There were multiple series of bubble bath formulas with surfactants at different concentrations tested in 1% and 10% dilutions in sterile, deionizer water. A set of four corneas was incubated in the presence of the test article at 32 ± 1°C for 60 minutes. A set of three corneas was incubated in the presence of the positive control at 32 ± 1°C for 10 minutes. After the 10 and 60-minutes exposure times, the control or test article treatments were removed. The epithelial side of the corneas was washed at least three times with the Complete MEM and an opacity measurement was performed. The corneas exposed to the positive control (10-minutes exposure) were returned to the incubator for approximately 2 hours. The corneas exposed to the negative control and test articles (60-minute exposure) were returned to the incubator for 1 hour. After the designated post-exposure incubation times, a final measure of opacity was obtained. The optical density at 490 nm (OD<sub>490</sub>) was determined using a Molecular Devices Vmax kinetic microplate reader. A 360  $\mu$ L sample of each 1:5 dilution was transferred to the specified well on the 96-well plate. The plate was read again and the final reading was saved. After results were calculated and graphed, a range was then determined from non-irritating to mildly irritating effects of surfactant levels. Whereby from this relationship a safe use level for the surfactants can be recommended in experimental formulations.

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#### EVALUATION OF A SOLUBILITY PROTOCOL FOR *IN VITRO* CYTOTOXICITY TESTING.

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A standardized solubility protocol was tested as part of a NICEATM and ECVAM multi-laboratory validation study to evaluate the usefulness of two *in vitro* basal cytotoxicity assays for estimating rodent and human acute systemic toxicity. The intent of the protocol was to guide the selection of solvent for 72 coded chemicals and to evaluate the interlaboratory variation in solvent selection. Chemicals were tested in each of four labs for solubility in culture medium, dimethyl sulfoxide (DMSO), and ethanol (ETOH) in a hierarchical solubility protocol that favored the order listed. Solubility in DMSO and ETOH was tested at concentrations 100X greater than medium to adjust the solubility test concentrations of all solvents to equivalent concentrations to be used in the cytotoxicity assays. Chemicals were tested at

successively lower concentrations that differed by fixed intervals. The criterion for solubility was a clear solution with no evidence of precipitate after vortexing, sonating, and mild heating, as necessary. All four labs agreed on the solvent for 52 (72%) chemicals, and on both solvent and maximum concentration for 42 (58%) chemicals. For 7 of the remaining 20 chemicals, three of four labs agreed on the solvent and maximum concentration. For the remaining 13 chemicals, at least two labs agreed on the solvent, but not necessarily the highest concentration. The final solvents selected were media for 37 chemicals, DMSO for 35 chemicals, and ETOH for 0 chemicals. These data show that the protocol provided good interlaboratory reproducibility for most chemicals, but that differences in solvent selection may occur that could affect the maximum tested concentration and study outcome. Supported by: N01-ES-35504, N01-ES-75408; EPA IAG DW-75-93893601-0; European Commission 19416-2002-04 F2ED ISP GB

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#### INTEGRATED DISCRETE MULTIPLE ORGAN CULTURE: A NOVEL *IN VITRO* EXPERIMENTAL SYSTEM FOR TOXICITY STUDIES.

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Multiple organ interactions may play an important role in the effects of a toxicant *in vivo*. A toxicant may be biotransformed in the liver, and the metabolites may be toxic to a distant organ such as the central nervous system. To model the *in vivo* multiple organ interactions, a novel cell culture system, the Integrated Discrete Multiple Organ Culture (IdMOC; patent pending) system, has been developed in our laboratory. The system applies a wells-in-a-well concept (several "inner wells" within a single "containing well"), where cells from multiple organs are cultured in the inner wells, with each cell type cultured in a separate well. After culturing, the multiple cell types are "integrated" via an overlying medium that covers all the inner wells in the containing well. The system thereby models an organism with multiple organs, with the organs situated physically separated from each other, but connected via the circulatory system. The advantages of the IdMOC includes the following: 1. Simultaneous treatment of multiple cell type can be achieved via the addition of the toxicant into the overlying medium, allowing the assessment of differential cytotoxicity under virtually identical experimental conditions. 2. The presence of cells from multiple organs allows the assessment of total metabolites and effects as *in vivo*. 3. The effects of treatment on individual cell types can be evaluated with relevant (e.g. pharmacology or toxicity) endpoints. The application of IdMOC is demonstrated with tamoxifen, a known anticancer agent for the treatment of breast cancer, via the co-culturing of normal human cells (hepatocytes, astrocytes, aortic endothelial cells, renal proximal tubule cells, small airway epithelial cells) and a cancer cell (MCF-7 breast adenocarcinoma cells). Tamoxifen, while toxic to all cell types, was most toxic towards the MCF-7 cells. IdMOC with human cells models human *in vivo* and represents a useful experimental system for drug development.

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#### EFFECTS OF NANOSPHERES ON THE ADHESION AND CELL VIABILITY OF ASTROCYTES.

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In recent years, there has been an increasing interest in the potential of using magnetic nanoparticles as carriers for drug delivery by pharmaceutical companies. However, data concerning the effects of these nanospheres on the nervous system is still limited. This study tested the hypothesis that magnetic carriers, nanospheres, (1) can inhibit adherence of astrocytes to culture plates and (2) cause cytotoxicity or termination of growth. Astrocytes were obtained from the cerebral cortices of newborn Sprague-Dawley rats. Using light microscopy, changes in plating pattern were determined by visual assessment. Cell counting 4 days after plating revealed a significant decrease in the number of viable cells in treated groups ( $p < 0.0001$ ). To determine the cytotoxic effects of nanospheres, astrocytes were allowed to adhere to culture plates prior to treatment. Membrane integrity and mitochondrial function were measured using colorimetric analysis of lactate dehydrogenase (LDH) and 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), respectively. Treatment with nanospheres did not significantly alter astrocytic LDH release ( $p = 0.27$ ) in controls ( $100\% \pm 1.56$ ) versus treatment ( $97.18\% \pm 2.03$ ). However, a significant increase in MTT conversion ( $p = 0.01$ ) between control ( $100\% \pm 3.65$ ) and treated groups ( $112.8\% \pm 3.23$ ) was observed. The latter could be interpreted as possible mitochondrial uncoupling. These data suggest that nanospheres impede the attachment of astrocytes to substratum. However, once astrocytes are attached to a substratum and grow to confluence, nanospheres may cause mitochondrial stress, but not direct cell death. Supported by a grant from NanoSonic to MA

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#### IN VITRO MODEL OF PENETRATION AND ACTIVE DECONTAMINATION OF A CORROSIVE.

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The aim of this study is to show by *in vitro* experiments the penetration of a corrosive such as sodium hydroxide (NaOH) and to evaluate the interest of a rinsing with an active solution. *In Vitro* studies were performed: 1) Penetration of NaOH through the tissues was simulated through a semi-permeable membrane depending on the concentration. A volume of NaOH is put at the surface of the membrane and the evolution of the pH is measured in a compartment containing sodium chloride (420 mosmoles/Kg). 2) A model of semi-permeable membrane was used to simulate a complete rinsing of 2N NaOH exposure. Comparison was made between a water rinsing and an active rinsing solution such as Diphtherine. Two times of exposure was tested, 20 seconds and 1 minute. The penetration of sodium hydroxide depends on its osmotic pressure. For 0.1N and 0.2N NaOH concentrations, the penetration was delayed and a final pH value after 900s was respectively about 9 and 10. When the NaOH concentration is 1N, the penetration is faster due to a higher osmotic pressure than cornea ; the final pH value after penetration is 11.5 at 900s. For 2N and 5N concentrations, the penetration is really higher than for 1 N concentration and a final pH value is respectively 12 at 900s and 12.3 at about 600s. The complete rinsing showed better efficacy with Diphtherine rinsing versus water rinsing at all times of exposure. For a 20 seconds time of contact and after 3 minutes of rinsing, the external pH value was respectively about 9 for Diphtherine and about 12.8 for water. After 45 minutes, the internal pH value is about 9 with Diphtherine rinsing and 11.5 with water rinsing. For one minute of contact, the curves of pH are similar with a delayed decrease of pH. This *in vitro* model has shown to be depending on the concentration of the corrosive and the balance of osmotic pressure. The simulation of a complete rinsing showed better efficacy with an active rinsing solution versus water rinsing after NaOH exposure.

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#### TESTING CASCADE TO PREDICT THE POTENTIAL OF DRUG DISCOVERY COMPOUNDS TO INDUCE MITOCHONDRIAL DYSFUNCTION.

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Mitochondrial toxicity has been suggested as a pharmacotoxicological effect with a variety of therapeutics. For example, adverse effects associated with certain antiviral agents, lipid-lowering drugs, and certain cancer chemotherapeutics are attributed to mitochondrial dysfunction. In the case of antiviral therapy with AZT, mitochondrial toxicity is confirmed by monitoring the extent of mitochondrial DNA depletion. Some fibrates, used to treat hyperlipidemia, have been shown to inhibit complex I (NADH-cytochrome c reductase) of the electron transport chain *in vitro*. Doxorubicin causes mitochondrial dysfunction by selectively abstracting electrons from complex I through a redox cycling mechanism. Although progress has been made in identifying the mechanisms of mitochondrial toxicity, the ideal situation is to avoid drugs with the potential of causing mitochondrial toxicity. Some chemical compound classes can be predicted to target mitochondria such as redox cycling compounds or weak carboxylic acids. With other compound classes, the potential may not be as obvious. We are interested in developing a systematic testing cascade that would allow us to predict if any of our new chemical entities have the possibility to cause mitochondrial dysfunction and to address possible mechanisms. We used well-documented model compounds such as rotenone, doxorubicin, metformin, valproic acid and several fibrates in our studies. We tested each compound in the following assays: 1. The commercially available MitoScan, which identifies inhibitors of the electron transport chain; 2. potential of compounds to inhibit complex I-IV activities, 3. effect of compounds on respiration using glutamate/malate and succinate/rotenone as substrates; 4. ability of compounds to induce the mitochondrial permeability transition and 5. the effect of compounds on mitochondrial membrane potential. We present our findings and discuss advantages and limitations of each testing regimen. We provide recommendations for what we believe could be a useful testing cascade for new drug candidates that are suspected to have the potential to cause mitochondrial dysfunction.

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#### PREDICTION OF TOXICITY PARAMETERS OF AMINO- AND NITROBENZENE DERIVATIVES.

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It is necessary to have information about the approximate toxicity of chemical substance on a stage of toxicological study design. Such information can be received by means of Quantitative Structure-Activity Relationships (QSAR) analysis. One hundred and fifty equations for prediction of a number of toxicity parameters were received on the basis of several physicochemical descriptors ( $\log P$ , Hammett parameter  $\delta$ , and molecular refractivity) for a class of amino- and nitrobenzenes. The

following quantum mechanical molecular characteristics were the most valuable for toxicity prediction: energy of the highest occupied molecular orbital (HOMO), energy of the lowest unoccupied molecular orbital (LUMO), total energy, energy of activation, maximal index of free valence (Nmax), the maximum charge of a carbon atom not bound to a functional group/substitute (Qmax), the difference between a sum of charges of a carbon atom in a benzene ring and its derivative ( $\Delta Q$ ). Toxicity parameters included acute oral and inhalation toxicity, cumulative properties, thresholds of acute, chronic and embryotoxic activity. The received multiple regression models had a high level of reliability ( $P<0.05$ ,  $R>0.7$ ). The validation of the equations has shown coincidence between actual and calculated values of acute oral and inhalation toxicity, cumulative properties, thresholds of acute activity for more than half of amino- and nitrobenzenes which were not used for a training set in obtaining these equations. Absence of sufficient experimental data limited validation of the models for prediction thresholds of chronic and embryotoxic activity. All physicochemical and quantum mechanical molecular descriptors can be calculated based on the structural formula of a new chemical. Thus, the suggested models allow predicting several toxicity properties of amino- and nitrobenzene compounds during the design of new chemical entities even prior to their laboratory synthesis.

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#### THE USE OF LASER SCANNING CYTOMETRY (LSC) AS A POTENTIAL TOOL FOR QUANTITATIVE ANALYSIS OF CYPs IN LIVER TISSUE SECTIONS.

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We investigated if LSC can be used for quantitation of CYP induction in liver sections from animals treated with CYP inducers. Rats were treated with phenobarbital (PB) at 10 and 100 mg/kg or dexamethasone (DEX) at 5 and 50 mg/kg administered by gavage for 4 days. Livers were collected and microsomal fractions and formalin-fixed paraffin-embedded sections were prepared. Sections were processed for immunohistochemistry, stained with CYP3A or CYP2B antibody, visualized with fluorescent detection and analyzed by LSC. Microsomal CYP3A and CYP2B were analyzed by Western blot (WB) using the same antibodies as for LSC detection. Microsomal samples were assayed for pentoxysresorufin O-deethylase (PROD) and ethylmorphine N-demethylase (EMD) activity, indicators of CYP2B and CYP3A, respectively. Treatment with PB or Dex resulted in a dose-dependent increase in CYP2B (PB treated) and CYP3A proteins expression (Dex or PB treated), increases in liver weight and biochemical activities. Expression of CYP2B increased by 2.3 to 4.5-fold in PB treated groups compare to control with 2-fold increase observed between the low and high dose groups as was detected by WB analysis. PROD increased 4.7 to 9.5-fold in PB treated groups compared to controls with a 2-fold increase observed between the two groups. Two fold increase has been observed between treated groups by LSC. Induction of CYP3A by PB ranged from 1.5 to 4-fold by WB. A 2.6 and 2.9-fold increase was found between a low and high dose groups as measured by WB and LSC, respectively. EMD increased 1.9 to 2.8-fold in PB treated groups with a 1.5-fold increase observed between the two dose groups. CYP3A protein increased up to 2-fold by DEX compare with untreated animals with corresponding changes detected by LSC. EMD was also increased 1.7 to 3.4-fold in DEX treated groups compared to controls with a 2-fold increase observed between the two groups. These data indicate that LSC procedure can be used for analysis of CYP levels.

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#### METABONOMICS COMES INTO THE COLD: COMPARISON OF SENSITIVITY AND REPRODUCIBILITY OF CRYOGENIC PROBES VERSUS INCREASED FIELD STRENGTH.

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Metabonomics is gaining acceptance as a tool for distinguishing metabolic perturbations resulting from drug administration [1]. The aim of this work was to compare the metabonomic results from 1H NMR spectroscopic data acquired at 700 MHz on a conventional probe with that at 600 MHz from a cryogenic probe. Male rats were administered a single dose of hydrazine, a hepatotoxicant with a well-defined metabonomic profile [2] at 10, 40, or 75 mg/kg, and urine was collected over the next 72 h. Two sets of samples were prepared by robotics and analyzed at the 2 field strengths. The resulting NMR spectral data were analyzed using multivariate statistics. In-life and clinical pathology findings reflected previous literature data [2]. Irrespective of field strength, metabonomic analysis of urine spectral data demonstrated substantial modulations in urinary metabolites that were entirely consistent with published observations [2, 3]. Treatment-related changes consisted

of decreases in citrate, 2-oxoglutarate, creatinine, hippurate, and succinate with an increase in 2-amino adipate, beta-alanine, creatine, taurine, and argininosuccinate. Direct analysis of the signal-to-noise ratio of the fumarate resonance showed that greater sensitivity was observed using a cryogenic probe at 600 MHz (260-390:1) compared to the data obtained on a conventional probe at 700 MHz (88-186:1). The overall interpretation of the metabolic profile of hydrazine toxicity was the same regardless of the field strength. Enhanced dispersion at the higher frequency provides value for the identification of novel metabolic markers, but for initial marker discovery, the better sensitivity of the cryogenic probe technology provides a rapid cost-effective improvement for metabonomic analyses. <sup>1</sup>Lindon et al, *Toxicol. Appl. Pharmacology*, 187:137, 2003; <sup>2</sup>Nicholls et al, *Chem. Res. Toxicol.*, 14:975, 2001; <sup>3</sup>Keun et al, *Chem. Res. Toxicol.*, 15:1380, 2002

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#### EFFECTS OF REPEAT INTRAMUSCULAR DOSING OF KETAMINE FOR 14-DAYS IN CYNOMOLGUS MONKEYS.

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Ketamine is a dissociative anesthetic and analgesic agent that is widely used as an adjunct to biomedical procedures performed in drug safety studies in nonhuman primates because of its capacity to rapidly produce a profound and transient state of sedation without significant loss of normal reflexes. Little was known, however, of the effects of sedative levels of ketamine on parameters commonly assessed in safety assessment studies in these species. In this study the effects of ketamine administration in the sedative range doses were assessed on body weight, food consumption, hematology, coagulation, serum chemistry, urinalysis, and organ weights in cynomolgus monkeys (*Macaca fascicularis*). Ketamine was administered by intramuscular injection at dose levels of 0, 5, or 20 mg/kg once daily for 14 consecutive days to cynomolgus monkeys (3 animals/sex/dose level). The resulting data indicates that ketamine administration had negligible effects on food consumption, body weight, serum chemistry, hematology, coagulation, ionized calcium and organ weights in this species when compared to pre-dosing baseline and concurrent control data. In conclusion, daily intramuscular administration of sedating levels of ketamine appears to have no impact on drug safety assessment parameters in a non-human primate model; and because the routine use of ketamine minimizes risk to personnel associated with collection of data from unrestrained animals, as well as effectively minimizes any influence restraint may contribute to the study parameters assessed, provides a very valuable adjunct to the conduct of nonhuman primate safety assessment studies.

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#### A CANINE MODEL FOR SAFETY EVALUATION OF PERCUTANEOUS INTRAHEPATIC INJECTION OF 10% ROSE BENGAL.

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Haepatocellular carcinoma (HCC) accounts for 90% of liver tumors and over 1 million deaths annually. Detection of the disease has been improved by advances in medical imaging techniques. The most common method of treatment is ablative therapy conducted by ultrasound guided microwave coagulation or small molecule injection. Surgical treatment may be limited or complicated by liver disease or anesthetic risk. Percutaneous administration has been reported to have similar patient survival times and response rates as invasive surgical catheter placement for colorectal liver metastases. Rose Bengal is a stable, small molecule with potential cytotoxic effects against HCC that is excreted unmetabolized via the bile, with secondary excretion by the kidneys. For safety evaluation, the canine model was selected based on considerations of anatomy, physiology and previous toxicity data. One male and one female adult dog were premedicated with ketamine/xylazine then anesthetized with isoflurane. The xypoid area was surgically prepared and a single 20 mg/kg dose of 10% Rose Bengal (w/v) in sterile saline was given by percutaneous intrahepatic injection. Hematology, coagulation and clinical chemistry parameters were measured at pre-dose and four days post-dose prior to sacrifice, and liver was collected for histopathology. Results included clinical observations of purple discolored emesis, urine and feces. On day four, there were biologically relevant increases in alanine aminotransferase and alkaline phosphatase. Histologically, the treated liver lobe of each animal was characterized by a band of subcapsular degeneration and necrosis of hepatocytes that contained small numbers of neutrophils and macrophages and subjacent sinusoidal congestion. It was concluded that the canine percutaneous intrahepatic model was appropriate to evaluate the clinical indication of this test article.

## CHARACTERIZATION OF NOVEL BIOMARKERS OF PERCHLORATE EXPOSURE IN ZEBRAFISH.

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Perchlorate inhibits iodide uptake by thyroid follicles and lowers thyroid hormone production. Although several effects of perchlorate on the thyroid system have been reported, the utility of these pathologies as markers of environmental perchlorate exposures has not been adequately assessed. This study examined time-course and concentration-dependent effects of perchlorate on thyroid follicle hypertrophy, colloid depletion, and angiogenesis; alterations in whole-body thyroxine (T4) levels; and somatic growth and condition factor of subadult and adult zebrafish. Changes in the intensity of the colloidal T4 ring previously observed in zebrafish were also examined immunohistochemically. Three-month-old zebrafish were exposed to ammonium perchlorate at measured perchlorate concentrations of 0, 11, 90, 1131 and 11480 ppb for 12 weeks, and allowed to recover in clean water for 12 weeks. At 2 weeks of exposure, the lowest observed effective concentrations (LOECs) of perchlorate that induced angiogenesis and depressed the intensity of colloidal T4 ring were 90 and 1131 ppb, respectively; other parameters were not affected (whole-body T4 was not determined at this time). At 12 weeks of exposure, LOECs for colloid depletion, hypertrophy, angiogenesis and colloidal T4 ring were 11480, 1131, 90 and 11 ppb, respectively. All changes were reversible, but residual effects on angiogenesis and colloidal T4 ring intensity were still present after 12 weeks of recovery (LOEC, 11480 ppb). Whole-body T4 concentration, body growth (length and weight), and condition factor were not affected by perchlorate. The sensitivity and longevity of changes in colloidal T4 ring intensity and angiogenesis suggest their usefulness as novel markers of perchlorate exposure. The 12-week LOEC for colloidal T4 ring is the lowest reported of any perchlorate biomarker in aquatic vertebrates.

## CROSS-TISSUE BIOMARKERS THAT PREDICT AND CHARACTERIZE THE ACUTE PHASE RESPONSE.

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We have developed a large library of microarray biomarker sets (Drug Signatures™) for important toxicities, pathologies, and pharmacological mechanisms of action to optimize compound selection during drug development. The signatures were derived from a comprehensive chemogenomic reference database (DrugMatrix™) that ties gene expression values from compound-treated rats in short term studies to pharmacological, toxicological, and pathological effects of the administered compounds. The database contains data from 600 compounds using over 15, 000 microarray gene expression arrays coupled to the clinical chemistry, hematology, and histopathology of the treated rats. We have generated drug signatures using a specialized Support Vector Machine (SVM) algorithm, and until now have specifically trained them on gene expression values derived from one particular tissue. For example, we currently have approximately 180 liver-specific signatures that can predict various endpoints such as ALT elevation or bile duct hyperplasia from microarray data. In the present experiment, we investigated whether it is possible to create signatures from microarray data from many tissues simultaneously. The ultimate goal is to identify biomarkers that can be used broadly in many different tissues as well accessible tissues such as whole blood. Since all tissues that are profiled for microarrays contain residual blood, and/or contain resident cells derived from blood cell lineage, pan-tissue signatures are likely to contain blood-borne markers. As a starting point, we made pan-tissue signatures that detect a compound-induced acute phase response within hours of compound administration — earlier than hematological perturbations, such as fibrinogen levels or WBC counts. The signature uses genes such as calgranulin B or lipocalin 2 known to be expressed in WBCs in response to inflammatory signals. Cross-tissue signatures will further expand our ability to form conclusions about mechanisms of toxicity and pharmacology from microarray experiments in under-represented tissues, and possibly allow selection of biomarkers to use in human whole blood.

## THE CHANGE IN FINGER OVAL AREA IN A NON-HUMAN PRIMATE COLLAGEN-INDUCED ARTHRITIS MODEL AND ITS CORRELATION WITH BIOCHEMICAL PARAMETERS.

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We prepared a collagen-induced arthritis (CIA) model by sensitizing with an emulsion of type II bovine collagen to cynomolgus monkeys, and have assessed its efficacy by monitoring the change of swelling of the joint which is an important site. In this study, we assessed the correlation between swelling of the finger oval area and biochemical parameters including bone metabolic parameters. Male cynomol-

gus monkeys were used and the 1st sensitization was conducted following 1-week acclimation. The 2nd sensitization was conducted 3 weeks after the 1st sensitization. During this period, score of swelling, body weight, urinalysis, hematology and serum biochemistry were examined sequentially. Finger oval area was measured on the interphalangeal (IP) joints of 16 fingers except for the thumbs of all limbs, and score of swelling was observed on 64 major movable joints in the distal areas including knee and elbow joints of all limbs. When compared with the mean IP area, 29 of 40 animals showed 105% or greater after the sensitization period. However, 38 of 40 animals showed swelling at one site or more in the score of swelling. The correlation between IP area and score of swelling was high ( $R=0.800$ ), and those with serum ALP and urine NTx were high. The coefficient of correlation with ESR and CRP which were well known as the inflammatory markers were  $R=0.313$  and  $0.262$ , respectively. The clinical significance of bone metabolic markers in rheumatoid arthritis has not been unclear, because there are insufficient evidences. However, IP oval area showed high correlations with serum ALP and urine NTx, and this suggests that bone metabolism is the type of high metabolism rotation. Additionally, it is expected that score of swelling, serum ALP and urine NTx which showed high correlation with IP oval area would be available parameters for evaluation of drug efficacy.

## EFFECTS OF BRIEF ENVIRONMENTAL TOBACCO SMOKE (ETS) AND PROPIONIC ACID EXPOSURES.

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Little is known concerning relationships among severity and duration of brief contaminant exposures and magnitude of impacts over time. To generate descriptive data needed for eventual mechanistic understanding, we exposed 20 healthy never-smokers to 8 conditions, each presented in a 100-min session conducted in a 10m<sup>3</sup> environmental chamber (ETS: 0, 15, 100 and 800  $\mu\text{g}/\text{m}^3$  RSP; PA: 0, 1, 10 and 15 ppm). Perceptual, breathing and blood pressure responses were recorded at different times. Odor, nasal irritation and eye irritation ratings indicated that: i) the ranges of the PA and ETS stimuli are approximately equal in perceived intensity; ii) 15  $\mu\text{g}/\text{m}^3$  ETS-RSP is peri-threshold after ~50 minutes of exposure; and iii) odor is the most sensitive endpoint and shows the clearest evidence of adaptation. Continuous breathing measurements showed that: i) overall, breathing responses appear at least as sensitive as perceptual endpoints; ii) PA causes a decline in breathing frequency due to increased inhalation duration but, because of a marked increase in inhalation volume, an increase in minute ventilation; and iii) with ETS, the slowed breathing (also due to increased inhalation duration) and a drop in inhalation volume accounted for a concentrated-related drop in minute ventilation. Blood pressure was altered by only the highest ETS concentration, which caused a decrease in systolic and an increase in diastolic pressure. Although it is difficult to measure a variety of responses from the same individuals repeatedly under different well-controlled conditions, this approach may be a critical step in generating the comprehensive quantitative data needed for understanding exposure situations representative of actual environments. As part of an effort to probe neural mediation of responses, anosmic (lacking olfactory input to brain) individuals will be tested next in this paradigm. Supported in part by Philip Morris USA Inc.

## RESPONSES OF MRP2-DEFICIENT TR RAT TO REPEATED DICLOFENAC EXPOSURE.

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Background: Mrp2 exporter-deficient TR rats have previously been found resistant to the enteropathy produced by a single dose of diclofenac, a widely prescribed NSAID. This resistance is attributed to their limited capacity for biliary delivery of reactive acylglucuronide metabolites of this NSAID to the intestine. However, this resistance might be overcome with repeated exposure to diclofenac if other mechanisms — not involving acylglucuronide exposure — contribute to the enteropathy of chronic NSAID treatment. Objective: We tested the hypothesis that potential changes in the response of TR rats to repeated diclofenac exposure could involve a shift in ulcer location or a disassociation of ulceration from metabolite adduction of enterocytes. Experimental Design: Male TR rats and Wistar rats were treated with diclofenac po for 5 days at a low pharmacological dose of 10 mg/kg or at a high dose of 50 mg/kg. Enteropathy was defined by measuring declines in serum albumin and assessing ulcer number and location. Adduction was assessed by immunohistochemistry. Results: TR rats were relatively resistant to the low pharmacological dose (no decline in serum albumin and <5 ulcers) which caused substantial enteropathy in Wistar rat (averaging a 23% decline in serum albumin and 67 ulcers

that were located predominately distally). However, the high dose did cause enteropathy in TR rats as indicated by an average 22% decline in serum albumin and ~20 ulcers that were predominately proximal located and associated with very sparse immunohistochemically detectable adduction, particularly at the brush border of the enterocytes. Conclusions: Thus, Mrp2-exporter deficient rats responded to repeated low diclofenac doses with a resistance to enteropathy but developed enteropathy after repeated high doses that was shifted proximally in location and accompanied by only sparse adduction of enterocytes. (Supported by NIH DK 56494, DK56338, and NIEHS ES06676).

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EVALUATION OF THE EFFECTS OF INTRANASAL ADMINISTRATION OF IL-4 TO MICE AND INHIBITION OF THESE EFFECTS BY A MURINE IL-4 RECEPTOR ANTIBODY.

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Inhibition of IL-4 receptor is being evaluated for the treatment of asthma. The ability of a murine IL-4 receptor antibody (muIL-4R Ab) to inhibit effects of IL-4 intranasal administration and to affect serum IgE levels were evaluated. A pilot study was conducted to determine the optimal dose of IL-4 to use as a challenge. Five groups (4 female BALB/c mice/group) received a single intranasal administration of saline or IL-4 at 30, 50, 100, or 300 ng/animal. All animals were sacrificed on Day 2 and lungs were harvested for ClCa3 mRNA analysis (ClCa3 is a calcium activated chloride channel associated with excessive mucus production in asthma). Intranasal IL-4 administration produced a dose-dependent increase in ClCa3 lung expression levels. A safety study was conducted to determine toxicity, toxicokinetics (TK), and pharmacodynamics (PD) of a muIL-4R Ab, when administered weekly for 4 weeks by intravenous (IV) or subcutaneous (SC) injection to BALB/c mice, and to evaluate recovery following a 4-week treatment free period. Five groups of mice (15/sex/group for toxicity, 15-36/sex/group for TK, and 5/sex/group each for IL-4 challenge and IgE analysis) were dosed at levels of 0, 4, 20, or 100 mg/kg IV or 100 mg/kg SC. Mortality, clinical signs, body weights, clinical pathology (hematology, serum chemistry and urinalysis), organ weights, gross and microscopic pathology, TK, antibody analysis, and PD effects were evaluated. There were no test article-related effects on clinical signs, body weights, clinical pathology, organ weights, gross pathology, or histopathology. The administration of muIL-4R Ab by either IV or SC did not elicit an antibody response. Administration of muIL-4R Ab over a 4-week period significantly reduced intranasal IL-4 induced ClCa3 mRNA expression in the lung and reduced total serum IgE levels. Based on the results of the study, weekly IV or SC treatment with muIL-4R Ab for 4 weeks in BALB/c mice was well tolerated and pharmacodynamic activity was observed.

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CYSTEINYLMERKATIN 1 AND 10 PROTEIN ADDUCTS OF BENZENE OXIDE AND NAPHTHALENE-1, 2-OXIDE FOR QUANTIFICATION OF DERMAL EXPOSURE TO BENZENE AND NAPHTHALENE.

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Epoxide metabolites of naphthalene and benzene are generated by cytochrome P450s in the liver and the skin where they can form protein adducts. Likely nucleophilic protein targets for adduct formation in the skin are the sulphydryl groups of cysteine residues in the head region of keratin-1 (K1) and keratin-10 (K10) proteins. We have developed polyclonal antibodies to adducts of epoxide metabolites of naphthalene and benzene and keratin proteins. S-phenyl-, S-(1-naphthyl)-, and S-(2-naphthyl)-cysteines were synthesized and incorporated by FMOC chemistry into oligopeptide sequences of K1 or K10. Antisera were collected from rabbits immunized with each of these synthesized adducts and purified by immunoaffinity chromatography with S-aryl modified K1 and K10 oligomers linked to an amino-containing resin. Antibodies purified in this manner were cross-reactive with K1 or K10 proteins, thus, they were further purified through a column containing either the K1 or K10 oligomer linked to an amino-containing resin to remove non-epitope specific antibodies. The resulting antibodies showed high specificity for a specific adduct and low cross reactivity with other adducts. Immunoaffinity purified antibodies generated, with high specificity and low cross reactivity, will be used to develop an ELISA to measure these adducts extracted from dermal tape-strip samples collected from persons occupationally exposed to naphthalene and benzene. This will allow us to determine if these adducts are formed in the skin and to quantify dermal exposure. Supported by NIEHS P42-ES05948.

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GLYCOPROTEOMIC ANALYSIS OF RAT PLASMA FOLLOWING PROTEIN PHOSPHATASE INHIBITION BY MICROCYSTIN-LR.

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Microcystin-LR (MCLR), a peptide toxin produced by freshwater blue-green algae, is a potent inhibitor of hepatic protein phosphatases 1 and 2A, and induces massive liver injury *in vivo*. Using targeted glycoproteomics profiling, a method has been developed to isolate and quantify changes in plasma mannosylated proteins relevant to MCLR-induced hepatotoxicity. In this study, male Sprague-Dawley rats were treated with MCLR (500 ug/kg, ip) or saline (control) and necropsied at 6h. Mannosylated glycopeptides were isolated and quantified in plasma samples (control and MCLR-treated) using lectin affinity chromatography and a global isotope coded labeling agent (GIST). Briefly, this procedure involves the following: 1) plasma proteins from each sample were tryptically digested into peptides, 2) the resultant tryptic peptides from the plasma samples were GIST-labeled with acetoxysuccinimid (control sample) and deuterated-acetoxysuccinimid (treated sample), then mixed together, 3) mannosylated, labeled peptides were lectin affinity selected using concanavalin A-agarose, 4) the isolated mannosylated peptides were separated using HPLC with UV detection, 5) changes in these peptides, from control and treated plasma, were quantified using MALDI-TOF mass spectrometry. MALDI-TOF mass spectrometry of GIST-labeled peptides identified approximately 60 mannosylated peptides, of which roughly half changed greater than 2-fold, when comparing the control and treated samples. Approximately 20 mannosylated peptides were down-regulated or unchanged when compared to the control sample, while about 5 mannosylated peptides were up-regulated in the MCLR-treated sample. These findings indicate that changes in mannosylated plasma proteins occur with MCLR-induced hepatotoxicity. Further refinement of this technique and its use in comparison of control to treated plasma will identify glycoproteins altered by toxicity and disease for use in mechanistic studies and diagnostics.

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FORMATION OF UVA LIGHT INDUCED OXYPEUCEDANIN DNA ADDUCTS AND DETECTION USING HPLC <sup>32</sup>P-POSTLABELING.

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Coumarins and furocoumarins occur in the leaves and fruits of many plants species. Several of the furocoumarins, including psoralen, 5-methoxysoralen (5MOP), and 8-methoxysoralen (8MOP), have well documented phototoxicological properties. Although most research has focused on 8MOP due to its high phototoxic potential and use in some therapy modalities (e.g. PUVA), several other naturally occurring furocoumarins are phototoxic. Oxypeucedanin is a furocoumarin that occurs at significant concentrations in lime oil. We sought to determine if oxypeucedanin formed DNA adducts following irradiation in the presence of DNA, and whether the DNA adduct formed from oxypeucedanin could be distinguished from those formed from other furocoumarins. Oxypeucedanin was isolated from commercially available lime oil using published methods. Oxypeucedanin and other furocoumarins were dissolved in ethanol, and mixed overnight with 1 mg/mL DNA. The solutions were irradiated with a fluorescent-lamp UVA source at doses of 0 to 50 J/cm<sup>2</sup>. The DNA was isolated and DNA adducts <sup>32</sup>P-postlabeled using a standard protocol, and separated using HPLC or TLC. Oxypeucedanin formed a single DNA adduct in a dose-dependent manner when illuminated with UVA light. This adduct was chromatographically resolved from the photo-induced DNA adducts of coumarin, psoralen, 5MOP, and 8MOP using TLC or HPLC. The HPLC and TLC <sup>32</sup>P-postlabeling procedure has been used to determine the contribution of the various naturally occurring furocoumarins to DNA adduct formation when lemon and lime oil were incubated with DNA and exposed to UVA. This method will allow us to distinguish the contribution of each furocoumarin to DNA adduct formation from naturally occurring furocoumarin complex mixtures.

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ACTIVATION OF HUMAN AH RECEPTOR SIGNALING BY POLYCYCLIC AROMATIC HYDROCARBONS AND EXTRACTS OF SOILS FROM THE NEW ORLEANS AREA.

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Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants that come from natural and human sources. Exposure to PAHs at sufficient levels causes numerous effects that include immunotoxicity, reproductive and birth defects, and

cancers. These toxic effects are mediated by the Ah (aryl hydrocarbon) receptor. Thus, monitoring the environment for pollutants that activate this signaling pathway is of toxicological importance. Sixteen environmentally relevant PAHs were tested individually and as mixtures in a yeast-based Ah receptor signaling assay. We classified these PAHs into four groups (inactive, weakly active, moderately active, and strongly active) based on their activation of human Ah receptor signaling. Indeno(1, 2, 3-cd)pyrene, chrysene, benz(a)anthracene, benzo(a)pyrene, benzo(j)fluoranthene, and benzo(k)fluoranthene were strongly active PAHs in our assay. PAH mixtures showed composition-dependent additive and synergistic effects in the Ah receptor assay. Receptor independent effects may explain the synergism of some PAH mixtures. Environmental samples from the New Orleans area were analyzed for PAH composition and quantity by gas chromatography/mass spectroscopy and then tested in this bioassay. Levels of total PAHs, active PAHs, and benzo(a)pyrene were correlated with the dilutions of the environmental samples needed to give EC25 signaling levels in the Ah receptor assay. Some environmental samples gave anomalous results in the Ah receptor assay. Samples with unexpectedly high signaling could contain additional Ah receptor ligands (PCBs, dioxins, etc.) that we did not measure. The Ah receptor signaling assay is a simple, rapid, and inexpensive tool for preliminary screening of samples that contain PAHs and other chemicals with affinity for the Ah receptor.

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### INDUCTION OF CYCLOOXYGENASE-2 IN THE LUNGS OF RATS EXPOSED TO TOBACCO SMOKE.

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Cigarette smoking is associated with lung inflammation, epithelial damage, and remodeling of the airways leading to chronic obstructive pulmonary disease (COPD). Chronic airway inflammation is likely to play a key role in the development and progression of tobacco smoke (TS)-induced pulmonary disease. However, the mechanism by which smoking contributes to COPD is unknown. Inflammatory mediators, including products of cyclooxygenase-2 (COX-2), play a critical role in the initiation and progression of inflammation. We wished to evaluate expression of COX-2 protein in a model of TS-induced inflammation and disease. Spontaneously hypertensive (SH) rats were exposed to TS at approximately 30, 60, or 80 mg TSP/m<sup>3</sup> for 6 hours/day, 3 days/week, for 7 or 14 weeks. COX-2 protein expression in lung tissue was determined using Western blots and found to be significantly increased at the high dose of smoke following 7 and 14 weeks as well as at the medium dose after 14 weeks. Immunohistochemistry demonstrated COX-2 positive cells in the cuboidal epithelium of the conducting airways. Numbers of COX-2 positive cells followed a dose-dependent pattern, especially in central and mid-level airways of the lungs. An increase in exposure length from 7 to 14 weeks resulted in fewer COX-2 positive cells in the central airway of rats exposed to the highest TS level, due in part to replacement of cuboidal epithelium by stratified squamous epithelium. Lung lavage demonstrated a significant increase in macrophages and neutrophils at both 7 and 14 weeks at the highest concentration of smoke. We conclude repeated exposure to TS is associated with increased expression of COX-2, persistent airway inflammation, and epithelial metaplasia. COX-2 expression in epithelial cells of the airways may play an important role in the inflammatory response leading to remodeling of the epithelium and progression of tobacco smoke-induced airway disease.

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### DNA AND HEMOGLOBIN ADDUCTS FROM ORAL ADMINISTRATION OF ACRYLAMIDE TO MALE FISCHER 344 RATS.

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Acrylamide (AM) is carcinogenic in rats, producing tumors in the thyroid, tunica vaginalis, mammary tissue on administration in the drinking water. Glycidamide (GA), the epoxide metabolite of acrylamide reacts with DNA to form a number of adducts, the major adduct being N-7-(2-carbamoyl-2-hydroxyethyl)guanine (N7-GA-Gua). AM and GA both react with hemoglobin to form adducts at the N-terminal valine residue. The objectives of this study were to quantitate the major DNA adduct derived from the reaction of glycidamide in tissues from rats administered AM, and to quantitate the AM- and GA-derived hemoglobin adducts. Male Fischer rats were administered either vehicle alone or 1, 2, 3-<sup>13</sup>C<sub>3</sub> acrylamide by gavage at a dose of 50 mg/kg. At 24 hours following administration, DNA was isolated from liver, lung, spleen, testis, brain, white blood cells, and thyroid. Natural abundance and <sup>13</sup>C<sub>3</sub>-N7-GA-Gua were analyzed by LC-MS/MS. The unlabeled adduct was below the limit of quantitation of the method. Similar levels of <sup>13</sup>C<sub>3</sub>-N7-GA-Gua were observed in most tissues: 13.5 ± 1.7 (white blood cells), 17.3 ± 0.7 (liver), 18.0 ± 2.4 (kidney), 17.0 ± 1.5 (brain), 15.5 ± 1.7 (spleen), 14.7 ± 2.3 (lung), 14.0 (thyroid) and 13.5 ± 1.7 (white blood cells) pmol/mg DNA. N7-GA-

Gua in testis was significantly lower than the other tissues investigated (10.1 ± 0.8 pmol/mg DNA). Hemoglobin adducts derived from AM (N-(2-carbamoyl-ethyl)valine, AAVal) and GA (N-(2-carbamoyl-2-hydroxyethyl)valine, GAVal) were measured by the modified Edman method, with quantitative analysis by LC-MS/MS. In the vehicle-treated rats, unlabeled AAVal and GAVal were 0.05 pmol/mg, and were similar in the <sup>13</sup>C<sub>3</sub>-AM-treated rats. <sup>13</sup>C<sub>3</sub> AAVal and <sup>13</sup>C<sub>3</sub> GAVal were 17.3 ± 0.5 and 7.7 ± 1.0 pmol/mg globin, respectively, in the <sup>13</sup>C<sub>3</sub>-AM- treated rats. These data indicate that GA is distributed widely in the body, with the exception of testis, in which the levels of <sup>13</sup>C<sub>3</sub>-N7-GA-Gua were lower than the other tissues.

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### CHARACTERIZATION OF MAINSTREAM CIGARETTE SMOKE-INDUCED CHRONIC INFLAMMATION IN ICR AND C57BL/6 MICE.

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To understand the pathogenesis of smoke-induced emphysema, male C57BL/6 (C57) and ICR mice were exposed nose-only to mainstream cigarette smoke (MS) for 6 months at 75, 250 and 600 µg total particulate matter (TPM)/L from 2R4F standard reference cigarettes. Blood and bronchoalveolar lavage (BAL) fluid samples were collected 24 hours post-exposure at selected times (6, 13, 22, 28 weeks and 13 week recovery period). Blood was analyzed for biomarkers of exposure (carboxyhemoglobin, nicotine, and cotinine). BAL fluid was analyzed for biomarkers of effect: oxidative stress (GSH, GSSG, and 8-isoprostanate), tissue injury (elastase and collagenase), inflammation (cells, LDH, NAG, and proteins), and apoptosis. Overall, minimal strain differences were observed. Biomarkers of exposure increased in a dose-dependent manner. Changes in biomarkers of effect were primarily observed at 600 µg TPM/L. There were no significant changes in most oxidative stress and tissue injury biomarkers; however 8-isoprostanate increased following 13 week recovery period. BAL macrophage and neutrophil counts were rapidly (6 weeks) and consistently (13, 22, 28 weeks) elevated while lymphocyte counts gradually increased over time. Apoptotic activity declined with repeated exposures. Following 28 weeks of exposure, minimal emphysema of the alveolar ducts was observed in ICR (20%) and C57 (10%) mice. Additionally, inflammatory infiltrates were observed around alveolar ducts and adjacent vasculature. Morphometric analysis of the lung was inconclusive (i.e. Lm increase < 20%). In summary, changes in macrophages, neutrophils, and lymphocytes reported in smokers with emphysema were comparable to MS-induced changes observed in this mouse model. Therefore, ICR and C57 mice chronically exposed to MS may be utilized to investigate the role of inflammation in the pathogenesis of emphysema.

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### NANBIOTECHNOLOGY: AUTOMATED REAL-TIME MEASURES OF TOXICANT INFLUENCE ON CELL SPEED IN CHEMOTACTIC ENVIRONMENTS.

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Cellular movement is a critical aspect of many physiological processes, including inflammation, immune responsiveness, and metastasis. This movement can be directed by soluble signals called chemokines and is potentially sensitive to toxicant influence both by changes to the molecular machinery of cell movement and by changes to the external signals that influence this movement. We have developed an automated real-time assessment of cell movement, and have used this ECIS/taxis system to evaluate the influence of various metal toxins and oxidative conditions on chemotactic cell movement. This system employs a target electrode as the detector. When cells cross this electrode as they respond to a chemotactic gradient, they impart an increased resistance to current that is passed through the electrode. The time of cell arrival at the target electrode can be precisely defined by the characteristic change in resistance that results, and the magnitude of the cell response is proportional to the total increase in resistance that occurs. Employing this technology, we have defined the influence of various extracellular matrix proteins on the chemotactic response of Jurkat T cells to SDF-1a. Collagen, fibronectin and laminin each significantly diminish the speed of cell movement in response to SDF-1a under certain conditions. In addition, we have found that metallothionein (MT) has chemotactic activity, suggesting that cells exposed to toxins will alter cell trafficking by releasing metallothionein to the extracellular environment. Finally, we have used ECIS/taxis to define the capacity of neutrophils to move in an oxidative environment, and the different effects oxidant has on cells with different MT levels. Toxicant-mediated changes that increase or decrease cell movement will have important implications for the progression of inflammatory disease, for the development of protective immune responses, and for the establishment of secondary tumor foci. NIEHS ES07408, NIBIB EB00208.

MODULATION OF EXTRACELLULAR MATRIX MARKS  
EARLY DEGENERATION OF KIDNEY PAPILLAE  
INTERSTITIAL CELLS (IC) OF RATS DOSED WITH  
INDOMETHACIN (IND).

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Regionspecific renal damage such as renal papillary necrosis (RPN) is difficult to monitor and predict. Using both gene expression profiling (GEP) of papillae total RNA and 2-D gel electrophoresis of urine (protein id by Maldi-ToF), we sought to identify early markers for RPN. Rats were dosed with 0, 8.4, 14 or 16.8 umol/kg/day IND sc for 3 or 7 days. Urine was collected on days 7-8. On days 4 or 8, animals were sacrificed and the papilla removed from one kidney for RNA isolation; the 2nd kidney was used for histologic evaluation. Early, time- and dose-dependent interstitial matrix increases and IC degeneration were noted for IND-dosed animals. No significant changes in a battery of urine chemistry parameters, including urinary electrolytes, NAG and creatinine, were observed. By contrast, the pathologic changes were accompanied on days 4 or 8 by altered expression of a number of genes involved in extracellular matrix remodeling, including 12/15 lipoxygenase, syndecan, MMP inhibitor, haptoglobin, T-kininogen, latexin, MMPs, plasminogen activator, proteoglycoprotein, alpha-1 protease inhibitor and others. Uromodulin, clusterin and contrapsin-like protease inhibitor mRNA expression were also altered. Expression changes of 12/15 lipoxygenase, clusterin and Timp-1 mRNAs were confirmed by real-time PCR. The pathology and gene expression changes are consistent with the hypothesis that IND may interfere with compensation mechanisms for osmotic stress in the papilla, since alteration in interstitial glycosaminoglycan (GAG) content is seen in RPN and other hyperosmolar states and since the targeted ICs are known to be responsible for maintenance of matrix & matrix GAGs. Finally, urine levels of haptoglobin, T-kininogen, and contrapsin-like protease inhibitor protein were altered, illustrating a good correlate to the GEP signature in a relevant, noninvasive biological matrix.

A METABONOMICS INVESTIGATION OF THE  
HEPTOTOXICITY OF VALPROIC ACID.

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Mice were injected subcutaneously with 60 mg/mL valproic acid in 0.9% sodium chloride and urine serum or liver tissue collected at 6, 12 and 24 hour timepoints. Valproic acid (VPA) has been employed for the treatment of epilepsy but has been shown to act as a hepatotoxin in some cases. Studies have proposed that the hepatotoxicity resulting from VPA treatment may result from alterations in branched-chain amino acid and fatty acid metabolism. NMR-based metabolomics was applied to investigate the changes in endogenous metabolites following dosing with VPA. Control samples were also collected at each timepoint for comparison. Principal component analysis of the urine samples showed a tight clustering of the control groups with looser clustering of the three timepoints from the dosed group. Bins associated with amino acids and glucose were responsible for the clustering noted. Discriminant analysis of the aqueous liver tissue samples was able to correctly classify the samples as control or dosed as well as by timepoint. Bins associated with amino acids and glucose were shown to be important in the development of a model for discriminant analysis.

POTENTIAL ROLE OF METHYLGUANIDINE FOUND  
IN METABONOMICS INVESTIGATION OF  
NEPHROTOXICITY.

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Metabonomics is a new technology for the investigation of drug toxicity performed in biofluids (e.g. urine) with analytical methods (e.g. NMR spectroscopy). Therefore metabonomics was applied for the investigation of nephrotoxicity. For this a metabonomics study was performed with PRP-X, a drug candidate that was terminated due to renal tubular dilatation/degeneration and hepatocellular hypertrophy in rats. In this study male sprague dawley rats were treated with 0, 10 and 200 mg/kg of RPR X for 5 days and urine was collected on day 1 and 5 postdose. Urines were analyzed by 1H-NMR spectroscopy and principal component analysis (PCA). In the PCA plot the urine NMR spectra from all rats were separated into 3 different groups, the first group dosed with 0 and 10 mg/kg, the second dosed with 200 mg on day 1 and the third group dosed with 200 mg/kg on day 5. Clear sepa-

ration along the x-axis was observed for all urine spectra from rats treated with 200 mg/kg compared to the 0 and 10 mg group. Responsible for this separation was a signal in the NMR spectra at 2.83 ppm elucidated as methylguanidine. Additional to this the urine spectra from rats treated with 200 mg/kg collected on day 1 were separated along the y-axis compared to the urine spectra collected on day 5. Responsible for this separation was a signal at 2.45 ppm and 3.01 ppm in the NMR spectra elucidated as 2-oxoglutarate. In the literature methylguanidine is described to be increased in renal and liver diseases and after proximal tubular injuries. It is reported that methylguanidine is formed from creatinine by reactive oxygen species. Therefore enhanced methylguanidine production is a result of oxidative stress in combination with creatinine accumulation. The results from this study indicate the potential of metabolomics as a powerful tool in toxicological studies.

PATTERN RECOGNITION OF NMR SPECTRA OF  
URINE FROM WISTAR RATS DOSED WITH LIVER AND  
RENAL TOXICANTS.

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A collaboration between Pfizer and the Center of Metabolomics Research at NCTR was initiated to study the ability of NMR in combination with pattern recognition techniques to rapidly detect toxicity in urine samples. Single doses of 4-aminophenol (150 mg/kg), puromycin aminonucleoside (150 mg/kg), Sodium Chromate (25 mg/kg), hexachlorobutadiene (200 mg/kg), Galactosamine (600 mg/kg), Allyl Alcohol (120 mg/kg), and thioacetamide (200 mg/kg) were administered to 4 adult male Wistar rats. Urine samples were collected daily in metabolism cages at Pfizer and monitored by 1D 1H NMR at NCTR. The NMR spectra of urine were binned using ACD's "intelligent binning" for statistical analysis. Two-dimensional principal component analysis (PCA) of the binned NMR spectra from urine on the second day after dosing was able to clearly differentiate rats dosed with renal toxins from rats dosed with hepatotoxins. Partial least squares-discriminant function (PLS-DF) models built from NMR spectra from day 2 urine samples were able to differentiate between renal toxicity, hepatotoxicity, and control samples.

DIBUTYLTIN EXPOSURE ALTERS CIRCULATING  
BLOOD GROWTH FACTOR LEVELS.

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Organotin compounds are commonly used as heat stabilizers in the manufacture of PVC plastics, in pesticides, and as preservatives for a wide variety of materials. Dibutyltin (DBT) is of particular interest due to its immunotoxic effects. In this study, we used ELISA assays to determine the effects of developmental dibutyltin dichloride exposure in Wistar rats on blood concentrations of several proteins including Nerve Growth Factor (NGF), Brain-Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3), Neurotrophin-4 (NT-4), Tumor Necrosis Factor Alpha (TNF $\alpha$ ), and Neuropeptide Y (NPY). These proteins are either growth factors or cytokines involved in development and inflammation, and may be useful biomarkers of effect. DBT effects were studied in pups of exposed dams (from GD6-20; gestational group) and in pups exposed directly (from PND3-21). DBT doses were given by gavage and included 0, 2.5 and 5.0 mg/kg three times per week. All pups were sacrificed at either PND21 or PND38. At PND21, gestationally exposed pups had an 11% decrease in body weight at the high-dose but no significant changes in body or brain weight at PND38. The directly dosed pups exhibited an 8% decrease in body weight at PND21 for both treatment groups, while at PND38 there was a 14% decrease in body weight and significant decreases in brain weight (7% in the low-dose and 13% in the high-dose groups). Blood from gestationally exposed high-dose pups showed increased protein concentrations of BDNF (at PND21) and decreased concentrations of NGF (at PND38). Blood from the directly dosed high-dose pups showed increased concentrations of NGF, NT-3, and NT-4 only at PND38. This study indicates DBT affects circulating levels of growth factors in blood following developmental exposure to DBT. This abstract does not necessarily reflect EPA policy.

EVALUATION OF CYTOCHROME P450 1A1 AND 1B1 IN  
HUMAN BLOOD LYMPHOCYTES AS BIOMARKERS OF  
EXPOSURE TO DIOXIN-LIKE COMPOUNDS.

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Cytochrome P450 1A1 (CYP1A1) and 1B1 (CYP1B1) are phase I enzymes the expression of which can be affected by many environmental compounds, including dioxins and dioxin-like compounds. Because CYP1A1 and CYP1B1 expression can

easily be determined in peripheral blood lymphocytes, it is often suggested as biomarker of exposure to these compounds. We investigated the variation in constitutive and induced ethoxresorufin-O-deethylase (EROD) activity and CYP1A1 and CYP1B1 gene expression in blood lymphocytes in a group of ten non-smoking females. Freshly isolated lymphocytes were exposed to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) or the dioxin-like polychlorinated biphenyl 126 (PCB126). All individuals showed a concentration-dependent increase of EROD activity by TCDD, which was significantly correlated with an increase in CYP1A1, but not CYP1B1 expression. The maximum induced EROD activity by 10 nM TCDD was very different among the individuals, but the EC50 values were about the same (0.8 ± 0.07 nM TCDD). PCB126 also caused a concentration-dependent increase of EROD activity, but was a 100-1000 times less potent than TCDD among the individuals. The allele frequencies for CYP1A1 MspI and CYP1B1 Val432Leu were also determined and reflected a normal Caucasian population, but the polymorphisms had no apparent effect on the expression and activity of these enzymes in this study. To further investigate the use of CYP1B1 as biomarker, it was applied to a large human population, exposed to PCBs in Slovakia. In this population, we found a large variation in CYP1B1 expression. And despite the high blood levels of PCBs and TEQs in the study population and the separation of the population into the CYP1B1 Val432Leu genotype groups, no significant correlation was observed between PCB levels and CYP1B1 mRNA levels in human lymphocytes. These studies indicate that it is unlikely that potential effects on CYP1A1 or CYP1B1 expression due to exposure to dioxins or dioxin-like compounds can be detected which makes its suitability as biomarkers of exposure questionable.

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MULTI-TISSUE CHARACTERIZATION OF RHYABDOMYOLYSIS FOR THE HIGH PROFILE FAILURE CERIVASTATIN.

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Gene-expression-based chemogenomic analysis of cerivastatin discriminated it from other HMG-CoA reductase inhibitors (i.e. statins). The ability to identify cerivastatin as distinct from other statins is critical since this compound was withdrawn from the market due to rhabdomyolysis and lethality. We have profiled 7 statins (lovastatin, simvastatin, cerivastatin, mevastatin, fluvastatin, atorvastatin and pravastatin) and close to 600 other compounds (3, 700 different dose-time treatments) in short, repeat dose studies in rats. In liver, kidney, and muscle, cerivastatin was differentiated based on its ability to perturb genes associated with hepatotoxicity, inflammatory response, and cytoskeleton. An in-depth analysis of genes perturbed, shows cerivastatin is the most potent inducer of inflammation in kidney and muscle among all compounds studied. Hierarchical clustering of liver expression data using genes correlated to ALT and AST revealed that cerivastatin clustered with a number of classic liver toxicants including methapyrilene, chloroform and 1-naphthylisothiocyanate. Perturbations in hepatic gene expression predict ammonia-induced toxicity due to repression of the urea cycle, possibly leading to secondary muscle fatigue or muscle damage, and a dysregulation of cytoskeleton genes. The dysregulation of cytoskeleton genes observed in liver is further exacerbated in muscle by the fact that key ECM and connective tissue proteins, including tenascin C, fibronectin, laminin, and collagen, are downregulated. The cytoskeleton dysregulation observed in muscle, combined with a gene expression pattern suggesting an altered excitation-contraction coupling due to an altered Ca(2+) buffering capacity, may explain the mechanism of myocyte necrosis that precedes rhabdomyolysis. To conclude, liver gene expression data in short-term rat studies discriminated cerivastatin from its peers and shed light on its risk of toxicity, while the muscle expression data confirmed the mechanistic information relevant to target organ toxicity.

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EVALUATION OF KIDNEY TOXICITY DETECTION METHODS: URINARY GSTS AND PROTEIN PROFILING BY SELDI-TOF-MS.

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Routine measures of serum and urinary biomarkers of renal injury are insensitive and nonspecific. In humans, clinical signs of renal damage usually appear only after severe renal injury has occurred. Thus, better predictive biomarkers for the early detection of kidney damage are clearly needed. The objective of this study was to compare the sensitivity and specificity of various detection methods to assess kidney injury in rats. Four known kidney toxicants (cisplatin, p-aminophenol, bromoethyamine and mercuric chloride), with varying mechanisms of toxicity, were studied. Eight male Sprague-Dawley rats were assigned to each of three treatment groups (control, low and high dose). After a single IP dose, urine samples were collected for eight days. Four animals per group were euthanized and necropsied at 48 and 168

hours. Histopathology examinations were conducted to provide pathological evidence of localized renal injury. Urinary volume, serum and urine chemistry,  $\alpha$  and  $\mu$  GST excretions, and protein expressions were measured at selected time points. Abundant proteins in urine samples were removed by affinity purification prior to running the samples through SELDI-TOF-MS. Data from the four toxicants suggest that GSTs are sensitive markers of tubular toxicity, but lack the ability to detect medullary toxicity (bromoethyamine). Serum markers often provided insensitive measurements relative to the extent of renal injury, whereas a simple measurement, such as urine volume, yielded a better indication of renal insult. SELDI-TOF-MS analysis demonstrated single or multiple protein patterns in treated groups' samples to indicate acute kidney injury. Resulting data suggest that multiple endpoint analyses of urinary markers are necessary to reliably assess acute renal injury in rats.

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CHARACTERIZATION OF EPOXIDE ADDUCTS OF POLYCYCLIC AROMATIC HYDROCARBONS (PAH) WITH HEMOGLOBIN (HB).

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We investigated the binding of selected PAH epoxides (benzo(a)pyrene, benzo(b)fluoranthene, dibenz(a, hanthracene, fluoranthene, benzo(ghi)perylene, and benz(a)anthracene) to Hb in order to determine the overall extent of binding kinetics of the PAH to Hb. The following experiments were carried out using mouse (C57BL/6 male) and human Hb *in vitro*. Packed red cells were resuspended in isotonic saline (2 mls) and 100  $\mu$ l aliquots were used in reactions. PAH epoxide stock solutions were dissolved in tetrahydrofuran (1 mg/ml) and 10  $\mu$ l PAH stock was added to reactions which were carried out at 37° for various times. Reactions were stopped by addition of ice cold water and globin precipitated by addition of Hb to acidified acetone. PAH epoxides were released by incubation with pronase and PAH tetrols extracted using of liquid and solid phase extraction and analyzed spectrophotometrically by HPLC. Similar kinetics were found in both mouse and human suggesting that similar nucleophilic sites of attack of the epoxide are present in both species. In addition, these results point out that the mouse model is appropriate for carrying out investigational studies using carcinogens which potentially can be applied to human biomarker studies. The results demonstrated that that those carcinogens that are weakly active had only a slight degree of binding to hemoglobin, while those that are stronger, such as BPDE, had a larger degree of protein binding. All of the epoxide metabolites yielded detectable levels of Hb adducts that were in proportion to the known carcinogenicity of the parent PAH. These results suggest that the formation of hemoglobin adducts to PAH carcinogens may serve as reliable biomarkers of exposure as well as carcinogenicity.

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EXHALATION OF CYTOKINES BY LABORATORY RODENTS.

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The pulmonary system is a major portal for the introduction of numerous agents. Identification and treatment of exposed individuals can be greatly aided by detailed information on the elicited pulmonary response. Exhaled breath has been used to assess the physiological state of humans by examining the levels of volatile compounds. More recent work has demonstrated the presence of cytokines in human exhaled breath. The present work focuses on the capture of proteins carried in the exhaled breath of laboratory rodents, as a means of determining the inflammatory status of the lung. Mice were exposed twice to endotoxin (E. coli) by intratracheal instillation and the exhaled breath was collected from a group of 50 animals 4 d following the first endotoxin treatment. Rats were exposed to bleomycin by intratracheal instillation and the exhaled breath was collected from a group of 50 animals at various times postexposure. To collect the breath the animals were placed in a modified nose-only Canon exposure tower and the breath was condensed, in cooled collection vessels, with an efficiency of 91 to 97%. Bronchoalveolar lavage (BAL) was performed on subgroups of animals to provide a portrait of the inflammatory status of the lung. The presence of cytokines was assessed using a bead-based immunoassay (Bio-Plex, Bio-Rad) specific for mouse (18 cytokine panel) or rat (9 cytokine panel). Pulmonary inflammation elicited by endotoxin or bleomycin treatment was readily detectable in BAL fluid as increased concentrations of total protein and numbers of inflammatory cells. Multiple cytokines were detected in the BAL fluid from both control and treated mice and rats. Similarly, the presence of cytokines was detectable in concentrated exhaled breath condensate (cEBC) of both rats and mice. In general the number of detectable cytokines were fewer in cEBC than in BAL fluid, although direct comparisons between the two methods cannot be made at this time. This work demonstrates that it is feasible to monitor the pulmonary inflammatory status of the most commonly used laboratory rodent models by non-invasively capturing exhaled cytokines.

THE INFLUENCE OF DIET ON ENDPOINTS  
TYPICALLY USED IN 13-WEEK TOXICITY STUDIES FOR  
RODENTS.

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One of the most important factors influencing the biological processes of animals is diet. For example, dietary factors can influence immune response, longevity, and responses to environmental insults. Because diet can have a profound effect, baseline values for various endpoints used in toxicity studies and creation of historical databases may be altered when diet is changed. This poster discusses changes in various endpoints between two diets fed to F344 rats and B6C3F1 mice in 13-week toxicity studies conducted by the National Toxicology Program. Until the mid 1990's the primary diet used in the NTP studies was NIH07. Because of health concerns associated with this diet, the NIH07 diet was replaced with the NTP2000 diet. Data from control animals for NTP 13-week toxicity studies were used to make comparisons between the NIH07 and NTP2000 diets. The endpoints evaluated include body and organ weights, hematology, and clinical chemistry variables. Standard parametric or nonparametric two group tests were applied to compare the endpoints. The comparison groups were stratified by animal sex, species, and diet. Diet differences were observed for several endpoints. For example, heart, kidney, liver and terminal body weights were lower and white blood cell counts were higher for the NTP 2000 diet in male rats compared to the NIH07 diet. These findings indicate that the diet is an important source of variation for several endpoints typically used in rodent toxicity studies and, therefore, should be considered when evaluating historical databases and determining reference values.

USE OF *IN VITRO* TECHNOLOGIES AND  
METABONOMICS IN THE STUDY OF  
PHOSPHOLIPIDOSIS.

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Phospholipidosis (PL) refers to the accumulation of phospholipids within cellular organelles. The presence of such lipid inclusions can present an issue for pharmaceutical drug development dependent upon the target organ(s) and the margin of safety. Thus, while not necessarily a 'show-stopper' event, ligands against certain drug targets have shown propensity to induce PL and it is advantageous to identify drug candidates least likely to induce this effect. Accordingly, we have developed a series of *in silico* and *in vitro* tools that allow ranking of molecules for physico-chemical properties that drive PL, and that guide *in vivo* testing in rodents. Animal studies conducted with a number of compounds encompassing several chemical classes indicated that pharmacokinetic parameters appeared to significantly influence PL induction; therefore, a facile *in vivo* screen was needed to further drive the SAR. Others have reported that phenylacetylglucine (PAG) can be used as a biomarker for PL. We have used <sup>1</sup>H-NMR to profile urine of rats treated with both known inducers of PL and compounds for which our *in silico* and *in vitro* data suggested a range of potential PL induction. We determined that PAG was closely associated with histologic evidence of PL. Although fasting was determined to also influence PAG excretion, statistical modeling (PLS-DA) allowed a clear distinction between non-compound and compound related effects. Logistic regression analysis of spectra obtained from an experiment in which rats were dosed with closely related analogs that induced a range of severity for PL allowed determination of a threshold ratio that could be used to predict the histologic findings. Current efforts are focused on developing chemometric models which encompass a greater number of metabolites and which improve the specificity of the prediction as compared to using only the PAG:creatinine ratio.

EVALUATION OF VITAMIN K AND VITAMIN K  
EPOXIDE AS BIOMARKERS OF COAGULOPATHY IN  
RAT SERUM UTILIZING HPLC.

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The assessment of circulating vitamin K and vitamin K epoxide status provides researchers with an earlier biomarker for coagulopathy compared to other standard measurements, such as prothrombin time in humans (*Am. J. Clin. Nut.* 1996, 64, 894-902). Vitamin K (phylloquinone) is a required co-factor for many of the proteins in the coagulation cascade and is critical for normal blood clotting. Some drug therapies can disrupt the vitamin K cycle resulting in accumulation of vitamin K epoxide with a concurrent drop in vitamin K. Use of these earlier biomarkers for coagulopathy in rats has not been investigated, but would be beneficial since rats are

commonly used for safety evaluation studies. The information presented includes a description of the liquid chromatography assay used to quantitate vitamin K and vitamin K epoxide in rat serum, an assessment of optimal study conditions necessary when collecting samples for vitamin K analysis, and the results of a preliminary study conducted to examine the anticoagulant effects of warfarin. The choice of commercial rat feed, the time of day for sample collection, and anticoagulant used were all found to affect vitamin K levels. The most critical factor is the time at which the sample is collected. Samples must be collected as close to the end of the 12-hour dark cycle as possible in non-fasted rats to ensure that vitamin K levels are measurable. Studies were also conducted to examine the levels of vitamin K and vitamin K epoxide in rats for which normal blood coagulation has been disrupted. Rats treated with warfarin for three days (0.1mg/kg, IP) were found to have significantly higher ratios of vitamin K epoxide to vitamin K (1.588) in comparison to control rats (0.293) that preceded changes in standard clinical coagulation biomarkers. Further research is warranted to determine the feasibility of using this ratio as an early biomarker of coagulopathy in rat serum for safety evaluation studies.

A MOLECULAR BASIS FOR THE PREDICTION OF  
RENAL TUBULAR INJURY BY DRUG SIGNATURES  
FOLLOWING SHORT-TERM COMPOUND  
TREATMENT.

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Using long-term drug-induced tubular nephrosis in the rat kidney as a model system, we used a robust and sparse support vector machine (SVM) algorithm to derive a valid signature containing only 35 genes for the prediction of late-onset kidney pathology after short-term compound administration. To better understand the biological basis of the classifier, an iterative approach of gene removal and signature re-derivation was used in order to identify all the genes that are necessary and sufficient to derive a signature. The iterative process was repeated until performance of the split sample cross validation dropped below a predetermined metric. An additional 151 genes, for a total of 186, were identified that are necessary to produce a valid classifier with an accuracy of at least 76%. The remaining 7292 genes on the microarray were tested using the SVM algorithm but were unable to produce a valid classifier. Examination of the 186 genes and their molecular and biological functions reveals many interconnected processes consistent with early nephropathy, including hypercholesterolemia, proteinuria, cellular stress, altered immune cell involvement and tubular regeneration. Many of these genes have previously been associated with nephrotoxicity or some form of renal injury. By comparison, other genes known to be responsive to nephrotoxicants in other more acute models of nephritic injury, were inaccurate for classification. Furthermore, putative biomarkers of kidney injury, such as Kim-1 or clusterin, did not accurately predict the future occurrence of toxicity, but rather reflected the actual occurrence of tissue injury. Considering the cellular heterogeneity of the kidney, it is likely that the observed changes in signature genes represent concurrent events in a complex, multi-cellular mechanism of toxicity. The multiple compound approach coupled with an iterative SVM data-mining illustrates the value of *in vivo* whole tissue mechanistic studies versus other methods which may not readily identify complex intercellular interactions.

BIOMARKERS THAT CHARACTERIZE FIBROSIS AND  
BILE DUCT HYPERPLASIA.

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We have developed a large library of Drug Signatures<sup>TM</sup>, biomarkers for toxicologic, pathologic and pharmacologic mechanisms of action, to improve compound selection during drug discovery. These biomarkers are derived from a database integrating ~15,000 gene expression microarray results from rats treated with >600 compounds with traditional measurements of toxicity, pathology, and pharmacology. In particular, signatures predictive of hepatic fibrosis or bile duct hyperplasia (BDH) were derived using gene expression data from experiments in which histologically-confirmed fibrosis and/or BDH occurred and from early time-point experiments which did not induce fibrosis or BDH but used doses that caused one or both after longer exposure. Both fibrosis and BDH signatures had >70% true positive rate and >98% true negative rate. While fibrosis-inducing treatments consistently caused BDH, the converse was not necessarily true. Preliminary evidence from a more sensitive histological method, however, suggested that some BDH-inducing treatments did induce incipient fibrosis. In addition, treatments which caused BDH but not fibrosis induced genes associated with fibrogenesis. These genes, which were also induced by treatments causing fibrosis, include Mmp9 and

Plod2. The fibrosis and BDH signatures share one high-impact gene, Osf-2, a cell adhesion molecule that acts as a tumor suppressor and which has not been previously associated with either fibrosis or BDH. Except for Mmp9, genes in the predictive fibrosis signature did not contain known fibrogenic genes, suggesting that the signature genes are differentially regulated very early in the fibrotic process. Our findings indicate that fibrosis is tightly correlated with BDH, suggesting a mechanistic connection between the two. Moreover, both the fibrosis and BDH signatures identify new roles for genes not previously associated with either toxicity. These mechanistic and predictive findings can hasten and improve decision making during drug discovery, saving time and effort.

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#### MULTI-ADDUCT ANALYSIS OF GLOBIN FROM MICE AND RATS AFTER INHALATION EXPOSURE TO 1, 3-BUTADIENE.

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Butadiene (BD) is an industrial chemical used in the production of synthetic rubber and is also found in gasoline and combustion products. BD is a multi-site carcinogen in rodents, with mice being the most sensitive species. BD is metabolized to several epoxides among which 1, 2, 3, 4-diepoxybutane (DEB) is the most mutagenic. Previous analysis of 1, 2, 3-trihydroxybutyl-valine (THB-Val) globin adducts suggested that most of them result from 3-buten-1, 2-diol (BD-diol) metabolism to epoxy-1, 2-butanediol (EBD), rather than from DEB. For specific examination of this metabolic step (BD to DEB), an immunoaffinity-capillary LC-MS/MS assay was developed for the analysis of the cyclic adduct N, N-(2, 3-dihydroxy-1, 4-butadiyl)valine (pyr-Val) and applied to globin samples from mice and rats exposed to BD through inhalation. Mice were exposed to 3, 6.25 or 1250 ppm BD for 10 days and rats were exposed to 3 or 62.5 ppm BD for 10 days, or to 1000 ppm for 90 days. The amounts of pyr-Val adducts were compared to 2-hydroxy-3-butenyl-valine (HB-Val), a biomarker for 1, 2-epoxy-3-butene (EB) and THB-Val, a biomarker for EBD, both measured by GC-MS/MS. This multi-adduct analysis provided new insight into species- and exposure-dependent differences in BD metabolism. In general, mice formed much higher amounts of pyr-Val than rats. The formation of HB-Val and pyr-Val was similar in mice exposed to 3 or 62.5 ppm BD, while HB-Val was 3-fold higher at 1250 ppm. In both species THB-Val adducts were much higher than HB-Val and pyr-Val. These data show that BD is primarily metabolized via the BD-diol pathway. In addition, mice are much more efficient at forming DEB adducts than rats, particularly at low exposures. The newly developed method should be readily adaptable to measurements of human globin samples from control and exposed humans. In general, the pyr-Val assay is a promising tool for understanding the formation of DEB associated with BD exposure.

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#### INTERACTIONS OF COMPLEX CHEMICAL MIXTURES FROM CONTAMINATED SEDIMENTS AT A SUPERFUND SITE.

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Because of multiple pathways of exposure and potential interactions of chemical mixtures, it is difficult to develop an accurate risk assessment for chemically contaminated sites. These include the Superfund sites. Risk characterization is the initial step towards ranking sites and evaluating appropriate remedial procedures. Chemical mixtures may alter toxicity through several different methods. Mixture interactions may alter transport across a membrane, induction of metabolizing enzymes or binding with critical macromolecules. The current study investigated potential interactions of mixtures of polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) extracted from sediments at a Superfund site. Sediment samples were collected from a reference station and five locations along a contaminated river in the Pacific Northwest. Dried sediments were extracted and analyzed for PAHs, semi-volatile organics and PCBs. Sediment extracts were analyzed *in vitro* using microbial genotoxicity bioassays and *in vivo* using female ICR mice. The extracts were either negative or (for one sample) weakly positive in the microbial bioassays. Animal studies indicated that the genotoxicity of sediment extracts was more strongly correlated with PAH content than with PCB concentration. When the sediment extracts were co-administered with benzo[a]pyrene (BAP) to mice, the complex mixtures appeared to inhibit the formation of BAP-DNA adducts. Application of increasing concentrations of sediment extract reduced the levels of DNA adducts attributed to the BAP-diol epoxide (Spot #4). Chemical analysis identified high molecular weight PCBs as the major contaminant. Total

PCB concentrations were generally at least 4-5 times greater than total PAH concentrations. These data suggest that chemical interactions reduced the potential genotoxicity of the complex mixtures extracted from the sediments.

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#### CORRELATION OF HEXENAL-DERIVED DNA BINDING WITH DETOXIFICATION AND DNA REPAIR STATUS IN CULTURED CELLS.

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Trans-2-hexenal (hexenal) is an  $\alpha$ ,  $\beta$ -unsaturated aldehyde that is used as a flavoring agent, and is naturally present in food. Hexenal, like other  $\alpha$ ,  $\beta$ -unsaturated aldehydes, exerts genotoxic effects *in vitro* without metabolic activation. Upon reaction with DNA, these aldehydes form 1,  $N^2$ -propanodeoxyguanosine adducts (PdG). Hexenal has not been evaluated for carcinogenic potential, although treatment of rodents with some other  $\alpha$ ,  $\beta$ -unsaturated aldehydes is associated with tumor formation. There is evidence to suggest that  $\alpha$ ,  $\beta$ -unsaturated aldehydes are detoxified via conjugation to glutathione (GSH), and that PdG are repaired by nucleotide excision repair (NER). We have recently developed an LC-ESI<sup>+</sup>-MS/MS method to measure the hexenal-derived PdG (Hex-PdG) in DNA. We hypothesize that Hex-PdG measurement in cultured cells could elucidate the roles of GSH conjugation and NER in the detoxification of hexenal and the repair of Hex-PdG, respectively. HeLa cells ( $\pm$  the glutathione depleting agent buthionine sulfoximine, BSO) have been selected to study the correlation between GSH depletion and Hex-PdG formation. The role of NER in the repair of Hex-PdG will be studied by comparison of Hex-PdG formation between isogenic NER proficient and NER deficient XPA-/- and XPC-/- cell lines. To date, HeLa cells have been exposed to increasing hexenal concentrations for 6 hours. The Hex-PdG dose-response was highly sub-linear, with Hex-PdG detectable at 30  $\mu$ M, an inflection point between 100-300  $\mu$ M, and continued increases of Hex-PdG to 1000  $\mu$ M hexenal. GSH concentrations were decreased by 45, 80 or 84% at 100, 300 or 600  $\mu$ M hexenal, respectively. Acute cytotoxicity did not occur at concentrations of up to 600  $\mu$ M hexenal. Thus, preliminary data suggests that the inflection point of the Hex-PdG dose-response occurs as GSH is being depleted. Upon their completion, these studies will provide further evidence on the roles of GSH and NER in the amelioration of the cytotoxic and genotoxic effects of hexenal.

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#### COMPARISON OF PLASMA AND URINE SAMPLES FROM ANIT-EXPOSED F344 RATS USING NMR SPECTROSCOPY.

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NMR metabonomic analysis was used to evaluate and compare the metabolic changes in urine and plasma caused by  $\beta$ -naphthylisothiocyanate (ANIT), a model cholestatic hepatotoxin. Male Fischer 344 rats were orally gavaged with 0, 0.01, 1.0, 20.0, 50.0 or 100 mg/kg of ANIT in corn oil. Urine was collected prior to dosing and daily for 4d post dose. Plasma was collected prior to dosing and at 24 h and 4d post dose, and clinical chemistry analyses were performed. Liver and kidney were removed at 4d post dose for histopathology. Urine and 4d plasma samples were analyzed by high resolution 600 MHz <sup>1</sup>H-NMR spectroscopy. The resulting spectra were processed and divided into 256 integral regions, and changes in the metabolic profile were assessed using principal component analysis to determine the dose-dependent biochemical variations caused by ANIT. After performing Fisher linear discriminant analysis, Receiver Operator Characteristic (ROC) curves were obtained for each dose group. Clinical chemistry analysis showed no changes at 20 mg/kg, recoverable changes at 50 mg/kg and persistently elevated liver enzyme levels at 100 mg/kg. Significant but mild liver pathological changes were detectable at 50 and 100 mg/kg. Urine NMR analyses for rats dosed with > 20 mg/kg ANIT showed a significant decrease in the tricarboxylic acid cycle metabolites, indicating a disruption in mitochondrial metabolism. ROC curves constructed from these data demonstrate the high predictive power of NMR-based metabonomics for ANIT-induced toxicity. Metabolic analyses of plasma and urine yielded complementary information. Some metabolites were detectable in both samples (lactate); however, plasma also revealed additional information not seen in urine NMR spectra, such as a significant decrease in plasma phenylalanine post-treatment. These data can be used to differentiate a recognizable pattern of metabolic profiles for injury caused by this cholestatic hepatotoxin.

## USE OF METABOLOMICS TO IDENTIFY BIOMARKERS OF BREVETOXIN EXPOSURE.

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Brevetoxins (PbTx) are toxins produced by the marine dinoflagellate *Karenia brevis*. Blooms of *K. brevis* occur along the Gulf Coast of the USA causing fish kills, marine mammal death and human health effects. Humans are exposed to PbTx by inhalation or by consumption of contaminated shellfish. Respiratory irritation seen in both normal and susceptible humans occurs at pM concentrations making quantification of exposure difficult. The goal of this work is to investigate use of metabolic profiles as indicators of PbTx exposure. Metabolomics was used to monitor urinary metabolites in mice after treatment with PbTx-3. Female mice were anesthetized and 0.1 ml PbTx-3 (5 ug/kg) was instilled into the lung. Mice were placed into metabolism cages and urine was collected at 24, 48 and 72 h. Control urine was obtained from a separate group of mice. Samples were collected on ice and stored at -80°C. Biochemical profiles were determined, in triplicate, on urine samples by LC/MS. Peaks in the LC/MS spectra were analyzed using proprietary algorithms. 4306 components were detected. Of these, 1019 were detected in all treatment groups. Cluster analysis was performed to see if replicates clustered together. Cluster analysis was performed using a pair-wise Pearson correlation for every pair of observations. Regression analysis was also performed on the log relative responses to find components that exhibit a linear or quadratic trend across time. 24 components were found with significant ( $p < 0.01$ ) quadratic trend across the 4 time points. In addition, 5 components were found with significant ( $p < 0.01$ ) linear trend. Compounds that did not show a trend were tested for a systematic difference between treated and pre-treated sample times and yielded 54 compounds with significant ( $p < 0.01$ ) differences. Of these, 23 are negative differences and 31 are positive. These data suggest that PbTx-3 produces unique metabolic changes in mice that can be identified using metabolomics.

## CAN LEVELS OF PLASMA TESTOSTERONE BE USED TO PREDICT MALE SEXUAL MATURITY IN RHESUS MONKEYS (MACACA MULATTA)?

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The Rhesus monkey is a species commonly used in non-clinical drug development. Much of non-clinical testing occurs in young animals. In order to assess the effects of drugs on the reproductive tract, sexually mature animals are required. Plasma testosterone concentration is sometimes used as a biomarker for the assessment of sexual development or sexual health in males from a variety of mammalian species. However, relating testosterone levels to sexual status in males is not simple in most species. The release of testosterone is pulsatile and therefore highly variable and often levels of the steroid are low in the plasma. An added complication with the Rhesus monkey is temporal variations in dominance, which may impact on testosterone secretion. This investigation was carried out to determine if plasma testosterone concentration, could determine sexual maturity *in vivo*. Plasma testosterone, testicular volume, testicular weight and bodyweight were recorded from 16 age matched Rhesus macaques. The testosterone values were recorded one week prior to termination. Terminal testicular volume and weight and terminal bodyweight were recorded at post mortem and were used as indicators of sexual status. Testosterone was compared with the terminal testicular volumes, the terminal testicular weights and the terminal bodyweight using principal components analysis. Sexual maturity was confirmed in these animals by histological examination of the testes. A strong correlation was found between testosterone values and all three indicators of sexual maturity. These data suggest that plasma testosterone has value as a biomarker of sexual maturity in the Rhesus monkey and that it may be used to monitor changes in sexual status *in vivo* as a result of sexual development or reaction to treatment.

## ADMINISTRATION OF A MEK INHIBITOR RESULTS IN TISSUE MINERALIZATION IN THE RAT DUE TO DYSREGULATION OF PHOSPHORUS AND CALCIUM HOMEOSTASIS.

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Upregulation of the Ras-Raf-MEK-MAPK cell pathway is present in neoplasia. PD325901 is a selective inhibitor of MEK, and currently in cancer clinical trials. In 2-week oral studies in rats, PD325901 produced ectopic mineralization of various soft tissues and vasculature, consistent with calcium-phosphorus deposition. However, similar lesions did not occur in dogs or monkeys, despite administration

of lethal doses, significantly higher plasma drug levels, and inhibition of MEK. Studies were conducted to investigate the time-course for lesion development and identify biomarkers of toxicity. Male rats received PD325901 by gavage at 1, 3, or 10 mg/kg; controls received vehicle. Five animals/group were necropsied on Days 2, 3, or 4 after 1, 2, or 3 doses, respectively, and histology, clinical laboratory tests, and measurement of phosphorylated MAPK (pMAPK) were conducted. Lung pMAPK was inhibited ≥81% at ≥1 mg/kg on all days, indicating pharmacologic activity of PD325901. On Day 2, serum phosphorus (P) and 1, 25-dihydroxyvitamin D (Vit D) were increased 33%-43% and 2- to 7-fold, respectively, at ≥1 mg/kg. Elevations in P and Vit D were also seen on Days 3 and 4. Despite decreases in albumin, serum total calcium (Ca) remained unchanged on Days 2 and 3, indicating increases in protein-free Ca. Tissue mineralization (stomach, heart, aorta, arteries) was first observed on Days 2, 3, and 4 at 10, 3, and 1 mg/kg, respectively, and was preceded by increases in P and Vit D. Necrosis of physis/metaphysis, and thickening of hypertrophic chondrocytes occurred at ≥1 mg/kg. In a second study, male rats received a single oral dose of PD325901 at 1, 3, or 10 mg/kg and serum chemistries evaluated for 1 week. Serum P and Ca were increased on Days 2 and 3, with recovery thereafter. In conclusion, PD325901 produces tissue mineralization in rats due to dysregulation of P and Ca homeostasis, likely due to elevated Vit D levels. Frequent monitoring of serum Ca and P in the Phase I cancer trial have not indicated risk for this toxicity.

## USE OF METABONOMIC ANALYSIS FOR BIOMARKERS OF MUSCULOSKELETAL SYNDROME IN RATS.

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Musculoskeletal syndrome (MSS) is characterized by joint pain and stiffness and microscopically by growth plate enlargement and soft tissue fibroplasia. MSS associated with matrix-metalloproteinase inhibitors (MMPI) has limited their use as therapeutic agents. Early non-invasive recognition of the potential to develop MSS, would provide insight into candidate identification for pharmaceutical development. The aim of this study was to explore the utility of metabolomic evaluation of rat urine to detect MSS during treatment with PD 200126, a broad spectrum MMPI. Male Sprague-Dawley rats were dosed with vehicle or 750mg/kg/day PD 200126 for 14 days. Urine was collected daily. Histopathological evidence indicative of MSS changes of mild to moderate severity in the femorotibial and tibiotarsal joints was seen in 75% of treated animals, which did not correlate to relevant clinical signs. NMR spectra from rats treated with PD200126 showed decreased levels of citrate, succinate, oxoglutarate, and hippurate, and increased levels of creatine. These spectral changes reverted to pretest values after dosing with PD200126 ceased and may prove useful for assessing the non-joint related toxicity of PD 200126. Supervised statistical methods were then used to determine if subtle NMR pattern changes could be related to the presence of MSS established by histopathology. Results show promise for using metabonomics as a non-invasive assessment tool for identifying MSS.

## INVESTIGATION OF A HEPATOTOXICITY SCREENING SYSTEM IN PRIMARY CELL CULTURE.

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Establishment of the *in vitro* hepatic toxicity screening system is demanded for earlier safety assessment. We investigated LDH release and mitochondrial respiration (WST-1) to detect cytotoxicity, morphological evaluation, and proteomics for estimating the proper biomarkers by using rat primary hepatocytes exposed to positive compounds known to induce hepatotoxicity. In LDH release, a dose-dependent increase was observed after exposure for 24 hours. Regarding WST-1, a dose-dependent reduction was observed after exposure for 6, and 24 hours to any of compounds. In the proteomics analysis, 31 candidate proteins were identified among the 103 showing altered expression spots. It is concluded that cytotoxicity was detected earlier by measuring WST-1 than by measuring LDH release because reduction of mitochondrial respiration is to be one of expressions of earlier toxicity in cellular function while the increase of LDH release can be shown only after failure of the cell membrane. Mitochondrial respiration ability may be a useful parameter of cytotoxicity for *in vitro* hepatotoxicity screening, as cytotoxicity can be detected in the early stage of exposure. To find out the toxicologically responsible biomarkers which correlate with the estimation of *in vivo*, precise correlation between *in vitro* and *in vivo* system is to estimate, and more comprehensive analysis of the 31 proteins identified are under investigation.

THE IDENTIFICATION OF POTENTIAL VASCULITIS BIOMARKERS TARGETED THROUGH SELDI SCREENING.

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Surfaced enhanced laser desorption ionization (SELDI) is a technique being utilized to screen *in vitro* and *in vivo* samples for changes in the protein profile as a way of discovering biomarkers of disease states. Proteins that show changes between control and disease states may then be targeted for identification by mass spectrometry. Plasma samples from animals challenged with compounds known to cause vasculitis and the media from an endothelial cell line challenged with hydrogen peroxide were profiled by SELDI. Differentially expressed proteins that correlated across *in vivo* and *in vitro* samples were targeted as priorities for protein identification by mass spectrometry. A protein of interest (molecular mass of 4952) in cell media and serum was purified and concentrated by molecular weight filtration and HPLC fractionation. Identification was accomplished by digesting the purified protein with trypsin and analyzing the data by mass spectrometry (MS and MS/MS) experiments and submitting the resulting peptide mass fingerprint and *de novo* sequences to a protein database search. Proteins were also digested by carboxypeptidase Y and analyzed by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) for C-terminal sequence information. The targeted protein in serum was identified by Ciphergen as inter- $\alpha$ -inhibitor H4 heavy chain. This identification was confirmed through peptide mass mapping, *de novo* sequencing and C-terminal sequencing. The protein in the *in vitro* sample with the same nominal molecular mass as the identified protein in serum has not yet been identified. However the carboxypeptidase Y derived C-terminal sequence information suggests that although the protein isolated from cell media has the same nominal molecular weight as the protein isolated from serum, the proteins are not the same.

ALANINE AMINOTRANSFERASE ACTIVITY IN THE FA2N-4 HEPATOCYTE CLONE.

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The aim of this study was to examine the effects of xenobiotics on common cytotoxicity endpoints vs. alanine aminotransferase (ALT) leakage from the Fa2N-4 human immortalized hepatocyte clone. Previous characterization of the Fa2N-4 cell line has included gene expression and drug metabolism endpoints. Yet, there has been no information in the literature regarding the leakage of the necrosis marker, ALT in response to toxicants in the Fa2N-4 cells. Elevated serum transaminases, especially ALT, are the single most important laboratory indicator of hepatic effects from early preclinical testing to the post market monitoring. Fa2N4 cells were dosed in multiwell plates with sodium phenobarbital, acetaminophen, tamoxifen, valproic acid, or staurosporine in the presence of vehicle. After 24 hours, the spent media was removed for lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and ALT analysis. Subsets of the samples reserved for ALT determination were analyzed in the absence/presence of cofactor supplement, pyridoxal 5'-phosphate (P-5'-P). The ALT reaction mixture in commercially available reagents lacks the cofactor P-5'-P and the possible effect of a deficiency encountered with a cell line was investigated within this study. In addition, the cells were evaluated for neutral red dye uptake, ATP depletion, and protein content. Results indicate that the addition of the cofactor to the reaction mixture produced no reproducible effect on ALT activities. Using ALT as a cytotoxicity marker, the rank order is as follows staurosporine>tamoxifen>acetaminophen>valproic acid>sodium phenobarbital. ALT leakage values corresponded to neutral red and/or ATP depletion results. In summary, addition of the P-5'-P cofactor at the tested concentrations did not increase the ALT responsiveness of the Fa2N-4 cells. The Fa2N-4 cell line was useful for ranking compounds for cytotoxicity, but the low levels of basal ALT leakage for the cell population on multiwell plates are lower than values seen with primary hepatocytes and not due to P-5'-P cofactor deficiencies.

TOXICOLOGIC AND PATHOLOGIC SURVEILLANCE OF SEARCH & RESCUE DOGS DEPLOYED TO THE WORLD TRADE CENTER, THE PENTAGON, AND THE STATEN ISLAND FRESH KILLS LANDFILL SITES.

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This was a prospective surveillance study to evaluate toxicant exposure, long-term mortality, and pathology in dogs utilized in search and rescue (SAR) efforts at the Sept. 11, 2001 terrorist attack sites. A total of 152 dogs were enrolled in the study.

Of these, 70 dogs deployed to the sites were used in the toxicology study and 51 non-deployed SAR dogs were enrolled as controls. Of the 70 deployed dogs, 13 were deployed at the Pentagon, 11 at Staten Island Fresh Kill, and 46 at the World Trade Center sites. Whole blood or blood plasma samples were collected between October 2001 and June 2002 for toxicological analyses. End-points included blood lead and mercury, plasma polychlorinated biphenyls (PCBs), and gas chromatography/mass spectrometric (GC/MS) analysis of blood plasma for presence of toxic organic compounds. A standardized protocol was used in all necropsies. Results indicated that two dogs deployed at the WTC tested positive for lead at 0.06 and 0.07 ppm. All plasma samples tested negative for PCBs at a 0.05 ppm detection limit. All plasma samples tested negative for toxic organic compounds by GC/MS. No statistically significant differences were found in blood mercury concentration between control dogs and deployed dogs for any of the three study sites. To date, 11 deployed dogs have died compared to 2 non-deployed control dogs. Causes of death included cardiomyopathy, neoplasia, enterotoxaemia, and unspecified respiratory disease. Some deployed dogs had mild to moderate anthracosis. These results indicate a higher mortality rate for deployed vs non-deployed dogs. However, the mortality rate in SAR dogs was not as high as had been anecdotal reported after the Oklahoma City Federal Building bombing. The study also showed that there was no significant exposure to monitored toxicants in dogs deployed for SAR at the sites.

SEQUENCE AND EXPRESSION ANALYSIS OF CANINE ALT1 AND ALT2.

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Alanine aminotransferase (ALT), also known as glutamate pyruvate transaminase (GPT), is a pyridoxal enzyme that reversibly catalyzes transamination between alanine and 2-oxoglutarate to form pyruvate and glutamate. Because of its relatively high and specific expression in liver, detection of ALT activity in the serum is considered a biomarker (or an indication) of potential damage to hepatocytes. In the rat, human, and mouse, it has recently been shown that there are two isoforms of ALT known as ALT1 and ALT2 each encoded by a different gene. The expression of these isoforms appears to vary with tissue type, and may also be species different. For example, in the human ALT2 is highly expressed in the brain, whereas in the mouse, it is less abundant. In preclinical rodent and clinical safety studies, some compounds are found to increase the level of serum ALT activity in the absence of corresponding hepatic necrosis. One possible explanation for this apparent anomaly is increased transcription of ALT1 and/or ALT2 in the liver; another is leakage or mobilization of ALT1 and/or ALT2 from extra hepatic tissues. To determine baseline expression of ALT1 and ALT2 in the dog, we have developed RNA-based assays that can specifically detect each isoform. cDNA sequences were determined based on *in silico* cloning from in-house, commercially available, and public domain databases. A putative full-length cDNA clone encoding ALT1 was assembled from EST and genomic fragments and its translated amino acid sequence found to be 90% identical to human ALT1. A partial clone for ALT2 was 94% identical to human ALT2. TaqMan (quantitative RT-PCR) assays were developed and used to measure expression in a variety of canine tissues. Results show that the level of ALT 1 expression is: liver > heart > kidney > fat > gastrocnemius. ALT 2 shows expression in the following order: gastrocnemius > fat > kidney > liver > heart. Relative expression of the isoforms is also tissue specific. For example, in liver, ALT1 mRNA expression is greater than ALT2 expression, whereas in fat and gastrocnemius, ALT2 is greater than ALT1.

AUTOMATED BIOCHEMICAL MEASUREMENTS OF ACYL-COA OXIDASE AND CARNITINE PALMITOYLTRANSFERASE FOR ASSESSMENT OF PPAR-INDUCED PEROXISOME PROLIFERATION IN CYNOMOLGUS MONKEYS.

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Peroxisome proliferator activated receptor (PPAR) agonists are currently being developed for a wide range of therapeutic indications including diabetes, dyslipidemia and obesity. PPAR preclinical safety assessment studies in rats and monkeys have been associated with increases in liver weight and proliferation of peroxisomes and mitochondria. PPAR associated enzyme activities are established markers of peroxisomal proliferation in rats but have not been extensively evaluated in monkeys. To help characterize PPAR-induced hepatic effects in cynomolgus monkeys, we automated and evaluated spectrophotometric biochemical assays for measuring two fatty acid  $\beta$ -oxidation enzymes in liver tissue homogenates from control and PPAR-treated monkeys. Peroxisomal enzyme acyl-CoA oxidase (ACOX) activity was assessed by measuring the cyanide-insensitive reduction of NAD in the presence of palmitoyl-CoA. Mitochondrial enzyme carnitine palmitoyltransferase (CPT) activity was assessed by measuring the carnitine-dependent release of CoA-SH from

palmitoyl-CoA. Assays were performed at 37°C on the Bayer ADVIA 1650 and normalized per mg protein. Intra-assay CV's were 1.7-6.5% for ACOX and 1.2-1.6% for CPT. Inter-assay CV's were 1.2-9.9% for ACOX and 1.6-6.2% for CPT. Linearity was demonstrated across the ranges of values encountered in each assay (ACOX:  $y = 0.9977x + 0.0022$ ,  $R^2 = 0.9998$ , CPT:  $y = 0.9970x + 0.0033$ ,  $R^2 = 0.9999$ ). Recoveries ranged from 100-102%. Enzyme activities were elevated 1.2 to 4.2-fold (ACOX) and 1.3 to 2.9-fold (CPT) in monkeys dosed for 14 days with a PPAR alpha/gamma agonist. Increases in liver weights (relative to body weight) were observed in mid and high dose monkeys. Peroxisomal proliferation was confirmed by transmission electron microscopy and morphometric analysis. Results suggest that increases in ACOX and CPT activities correlate with peroxisomal proliferation in monkeys.

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#### EVALUATING TOXICOLOGY AT A SYSTEMS LEVEL USING COHERENT ANALYSIS OF LARGE-SCALE DATA STREAMS.

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We have developed a novel systems biology approach for the study of liver injury based on the coherent analysis of genomic, metabolomic and histomorphometric data streams. We used this approach to study the effects of single oral doses (150-2000 mg/kg) of acetaminophen (APAP) on rat livers over a 48 hour period. At each sampling time (0, 6, 18, 24 and 48h), livers were snap frozen and processed for transcriptional analysis using Affymetrix RAE230 microarrays and metabolomic profiling using LC/MS. These experiments produced two large-scale data streams of 15, 923 transcripts and 549 endogenous small molecular weight biochemicals, which were combined with clinical chemistry data and histopathological classifications (normal, mild necrosis, medium necrosis and severe necrosis). Pattern recognition methods based on supervised machine learning algorithms were used to mine the data streams to discover sets of biochemical and transcriptional biomarkers that classified the samples by pathology end-point. Pathway analysis was then used to explore the possible range of biochemical mechanisms underlying the hepatotoxic effect. Classifiers identified using pattern recognition methods predicted liver histopathology with an accuracy of 94% measured objectively by 10-fold cross-validation testing. The classification accuracy for either transcriptional data and metabolomic data alone was lower than the performance of the data streams in combination. Automated pathway analysis using these data highlighted mechanisms that included phase I and II detoxification, oxidative stress, glutathione (GSH) metabolism, and amino acid metabolism. The biochemical changes associated with liver injury appeared more sensitive than traditional markers of liver injury, e.g. serum ALT and AST. This approach allows for detection of biochemical changes associated with liver injury at earlier times and lower doses than with conventional diagnostic endpoints.

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#### A COMPARISON OF THE SPECIFICITY AND SENSITIVITY OF TRADITIONAL BIOMARKERS OF NEPHROTOXICITY IN THE RAT WITH METABONOMIC AND PROTEOMIC METHODOLOGIES.

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There is currently great scientific interest concerning the possible advantages that proteomic and metabolomic technologies might have over traditional biomarkers of toxicity. Numerous papers promise great things from these technologies, however, there appears to be little hard evidence of their superiority over traditional techniques for assessing toxicity. This work evaluated the relative sensitivity and specificity of proteomic and metabolomic techniques compared to traditional methods for assessing xenobiotic-induced nephrotoxicity. A range of published studies with nephrotoxicants, where both old and new techniques were used for nephrotoxicity assessment, were evaluated. The data from each study were assessed and a comparison made of the relative sensitivity and specificity of the old and new methodologies. The data showed no consistent evidence that the novel methodologies were any more sensitive than traditional methods for assessing nephrotoxicity. However, this could be due to the relatively small number of published studies in which both old and new methodologies for nephrotoxicity assessment were used, and may not be the case with other forms of toxicity. In contrast, the novel methodologies were able to discriminate between the effects caused by different toxicants, both on the basis of different mechanisms of toxicity as well as on the basis of the specific location of the nephrotoxic lesion. In conclusion, despite high expectations for these new technologies, their benefit over traditional methodologies in terms of sensitivity remains to be demonstrated within the context of nephrotoxicity, although they can unlock mechanistic information. A great deal of validation work is

necessary before these techniques gain full acceptance by the regulatory authorities, however if they do achieve this hurdle then widespread use within regulatory toxicology studies is likely to become the norm.

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#### THE RELATIONSHIP OF TREATMENT-RELATED DECREASES IN SERUM ALKALINE PHOSPHATASE ACTIVITY WITH ALTERATIONS IN BODY WEIGHT FOR RATS IN 13-WEEK TOXICITY STUDIES.

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It is not uncommon for significant decreases in serum enzyme activities to occur in toxicity studies. Often these enzyme decreases are an enigma and not considered clinically or toxicologically relevant. There may be, however, underlying biological processes that can explain some of these changes. For example, in a series of studies conducted by the National Toxicology Program (NTP), alkaline phosphatase (ALP) activity demonstrated treatment-related decreases at a rate of about 39 to 56%, depending on the sex and study day. It has been suggested that decreases in ALP activity may be related to decreased food intake (anorexia or food avoidance) associated with chemical exposure. Since body weight can also be an indicator of nutritional status or food intake, assessing the relationship between ALP activity and body weight may be potentially useful in explaining the decreases in ALP activity. This poster will discuss the relationship of body weight and ALP as a function of treatment. Data from 13-week toxicity studies conducted by the National Toxicology Program are used to assess this relationship. Treatment-related decreases in body weight and serum ALP activity were evaluated using nonparametric trend tests and pairwise comparisons. Incidences of treatment-related body weight and ALP effects, individual and combined, are also reported. Results suggest a relationship between ALP and body weight and, thus, altered food intake may help to explain the decrease in ALP activity observed in toxicity studies.

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#### IDENTIFYING CANDIDATE BIOMARKERS OF VITAMIN D ANALOG - INDUCED RENAL MINERALIZATION USING A SYSTEMS BIOLOGY APPROACH.

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Vitamin D analogs such as calcitriol are used for the treatment of osteoporosis. The major side effect of this therapy is dose-limiting hypercalcemia and hypercalciuria with or without resultant nephrotoxicity. The particular type of nephrotoxicity that is commonly seen with vitamin D analogs is renal mineralization. The development of biomarkers for early detection of nephrotoxicity is necessary to help monitor patients on these drugs. To identify candidate biomarkers of vitamin D analog-induced renal mineralization, animals were dosed with a low dose and high dose (20 and 4000 ng/kg/day, respectively) of calcitriol to induce renal toxicity. Overall the high dose, 14 day male rats were hypercalcemic, having a serum calcium level that differed by greater than 2 mg percent from the control. They also had hypercalciuria, which was evident by the markedly increased urine calcium excretion levels from the controls. Other findings include: Decreased food consumption with concomitant decreased body weight gain; clinical observations of rough fur and hunched stance; acidic urine and an increase in n-acetyl- $\beta$ -D-glucosaminidase (NAG) concentration, a tubular enzyme. Tissues and body fluids from this study are also being characterized by a variety of other techniques, including genomics, proteomics, metabolomics, and image analysis. Results from these broad profiling techniques should help to identify specific genes, proteins, and metabolic intermediates and other biomolecules that can be further investigated and characterized as potential biomarkers for renal mineralization.

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#### EFFECT OF TESTICULAR EDEMA ON SPERMATOGENESIS IN TOXICANT-TREATED RAT TESTES.

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Radiation or toxicant exposure induces azoospermia in LBNF1 rats by blocking spermatogonial differentiation, even though stem spermatogonia persist. Intratesticular testosterone levels increase at week 4, followed by development of testicular edema, and a block in spermatogonial differentiation at week 6, indicating that elevated testosterone (T) may be responsible for testicular edema. Treatment of irradiated rats with GnRH antagonist (GnRH-ant) rapidly decreases

T levels and stimulates spermatogenic recovery after a 3-week delay. In this study, we test whether GnRH-ant treatment can reduce testicular edema and the relationship between T suppression, edema reduction, and spermatogenic recovery. Rat testes were irradiated with 6 Gy and at week 15, some rats were treated with GnRH-ant, exogenous T, or 17 $\beta$ -estradiol (E2) for 8 weeks. Rats were killed weekly, testicular interstitial fluid volume determined, T analyzed, and spermatogonial differentiation assessed by histology. In irradiated rats, the GnRH-ant treatment caused T to fall at week 1 of treatment, followed by interstitial fluid volume decrease at week 3, leading to recovery of spermatogenesis starting at week 4. Treatment with both GnRH-ant and T largely maintained T levels and interstitial fluid volume, and blocked GnRH-ant stimulated spermatogonial recovery, indicating that testicular edema is associated with increased T. Treatment with both GnRH-ant and E2 partially blocked the effect of GnRH-ant on T, but accelerated fluid volume decrease to 2 weeks, and spermatogenic recovery to 3 weeks, indicating that E2 may act directly, apart from T suppression, to promote fluid reabsorption. Since the time course and extent of testicular edema reversal by GnRH-ant and steroid treatment parallels the recovery of spermatogenesis, we suggest that steroid-mediated increases in testicular interstitial fluid can block spermatogenesis and decreasing fluid levels can induce recovery. Experiments are in progress to determine whether dibromochloropropane-induced spermatogonial arrest is similarly modulated.

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EFFECT OF ETHYLENE GLYCOL MONOETHYL ETHER ON SPERM MOTION IN RAT.

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Ethylene glycol monoethyl ether (EGEE) can cause testicular damage in experimental animals and man, resulting in decreased sperm count. In this study, we analyzed how EGEE affect sperm motion in rats after acute and subchronic treatment. EGEE was administered by oral gavage to adult male Sprague-Dawley rats at 100, 300 or 600 mg/kg/day, 6 times per week for 5 weeks. In another experiment, one dose at 300 or 1000 mg/kg was used. Twenty-four hours after the last treatment, sperm from cauda epididymis and spermatid was analyzed for changes in motion with a Hamilton-Thorne Sperm analyzer (HTM-IVOS). Subchronic treatment of EGEE resulted in decrease in motile sperm percentage and progressive motility in both epididymal and spermatid sperm dose-dependently. The values for the two parameters in highest dose group were less than 25% of that in controls. The velocity parameters (curvilinear velocity, average path velocity and straight line velocity) were also affected by EGEE. Unlike the subchronic treatment regimen, a single-dose of EGEE did not induce change in weight of the testes and epididymis. However, the percentages of motile sperm and progressively motile sperm were significantly decreased in the high-dose group. The proportion of sperm with rapid velocity was also 33% less than that of controls, whereas the percentage of static sperm was doubled. Interestingly, these changes occurring in the acute experiment were only observed in spermatid, while those in the epididymis were not evident. These results demonstrated that EGEE may not only induce testicular toxicity but also directly affect sperm motion.

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CHANGES OF GENE EXPRESSION IN THE EPIDIDYMIDES AND SPERM ACROSOME REACTION IN RATS TREATED WITH SULFASALAZINE FOR SEVERAL DAYS.

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Sulfasalazine (SASP) is known to affect fertility in mammals. We have reported that a decreased expression of CD59, membrane cofactor protein (MCP) and decay accelerating factor (DAF) genes may affect acrosome reaction and sperm motility in rats treated with SASP at 600 mg/kg for 28 days. In the present study, we investigated sperm motility with HTM-IVOS, acrosome reaction using FITC-concanavalin A lectin assay and the expression of the above mentioned genes by real-time RT-PCR. The assays were carried out after 1, 7 and 14 days of treatment. The acrosome reaction ratio decreased in a time-dependent manner (93.8, 82.6 and 59.1% of control on Days 1, 7- and 14 of treatment, respectively). The acrosome reaction ratio on Day 14 was the same as that of Day 28 of treatment. On the other hand, the expression of CD59, MCP and DAF genes decreased in the epididymides on Day 1 continued to decrease thereafter. It is known that many molecules such as CD59, MCP and DAF proteins, that are secreted from the epididymides participate in spermatozoa maturation. In rats, sperm maturation needs for about 14 days and it correlated with the date of the lowest acrosome reaction ratio. From these findings, we hypothesized that SASP reduces the expression of CD59, MCP and DAF as primary effects and continued treatment affects the maturation of sperm due to insufficient secretion of CD59, MCP and DAF proteins, which in the end reduces sperm motility and/or acrosome reaction.

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DEVELOPMENTAL TOXICOLOGY OF CJC-1131, A LONG-ACTING GLP-1 ANALOGUE.

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CJC-1131 is an anti-diabetic compound being developed for the control of type 2 diabetes and is currently in Phase II clinical trials. By applying the Drug Affinity Complex (DAC) technology to glucagon-like peptide-1 (GLP-1), the peptide selectively and covalently binds to circulating albumin after subcutaneous (SC) administration. The developmental toxicity of CJC-1131 was evaluated in pregnant Sprague-Dawley rats dosed SC with CJC-1131 from gestation day (DG) 7 to 17 either every other day (q2d) at 0, 0.1, 0.3, 1 and 3 mg/kg (pilot study) or daily at 0, 0.025, 0.2 and 1.5 mg/kg (main study). All CJC-1131 dosages with both dosing regimens caused dose-dependent reductions in maternal body weight gain and/or body weight losses and food consumption. Non dose-dependent decreases in fetal body weights occurred at all doses administered q2d whereas dose-dependent reductions in fetal body weights were observed with daily dosing of  $\geq$ 0.2 mg/kg/day. There were no changes observed during fetal gross external examination at doses as high as 3 mg/kg q2d in the pilot study. In the main study, postimplantation loss (i.e., early and late resorptions) was slightly increased, although within historical range, following daily dosing at 1.5 mg/kg/day. Reflecting the dose-dependent reductions in fetal body weight, reversible delays in skeletal development were noted at  $\geq$ 0.2 mg/kg/day and included reductions in average numbers of ossified caudal vertebrae, forelimb and hindlimb phalanges, and ossified hindlimb tarsals. Although within historical range and a common finding in rats of this strain, variations in vessel formation (absent innominate artery) were noted at 1.5 mg/kg/day. Despite some maternal toxicity of reduced food consumption and decreased body weight gain at 0.025 mg/kg/day, there was no effect on the fetal development and organogenesis at this dose level. Therefore, the developmental no-observable-effect-level (NOEL) for CJC-1131 in rats is considered to be 0.025 mg/kg/day.

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EFFECT OF IMPLANTED DEPLETED URANIUM (DU) ON MALE RAT REPRODUCTIVE SUCCESS, SPERM CONCENTRATION AND MOTION.

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DU projectiles are currently in use with the US military and possibly the armed forces of other countries. Therefore, battle injury from DU projectiles and shrapnel is a possibility. Removal of DU projectiles from the body may not always be possible because of their location in the body or their small size. It has been demonstrated that DU mobilizes and translocates to the gonads and several other tissues in rats implanted with DU alloy. In this study the effects of implanted DU pellets on sperm concentration, motility, and male reproductive success were evaluated in adult Sprague-Dawley rats implanted with 0, 12, or 20, 1 x 2 mm DU pellets. Twenty, 1 x 2 mm DU pellets (760 mg) of DU in a 500 gram rat is equal to approximately 0.2 pounds of DU in a 154 lbs (70 kg) person. The 0.2 pounds of DU in a 154 lbs person is equivalent to one-third of a 300 g, 30 mm APFSDS-T DU projectile. Urinary analysis at post-implantation day 30 indicated that the DU-implanted animals were excreting uranium in urine with urinary levels directly correlated with the implanted DU pellet dose. There was no effect of DU implantation on mating success when evaluated at 30-45 days and 120-145 days post-implantation. Velocity and motion of sperm isolated from rats treated with the positive control compound alpha-chlorhydrin were significantly reduced as compared with those for sperm isolated from sham surgery controls. When assessed at post-implantation day 150, the concentration and motion of sperm isolated from DU-implanted animals were not significantly different from sham surgery controls. Overall, the results of this study suggest that implantation of up to 20, 1 x 2 mm DU pellets in rats for approximately 21% of their adult lifespan does not have an adverse impact on male reproductive success or sperm motility or velocity.

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THE EFFECTS OF SUBACUTE ORAL EXPOSURE TO VINCLOZOLIN ON REPRODUCTIVE PARAMETERS IN PERIPUBERTAL AND POST-PUBERTAL BOARS.

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Although the antiandrogenic properties of vinclozolin (VCZ) and its metabolites are well-documented in rodents, little is known about the effects of VCZ on other mammals. Similarities in size and physiology, as well as genomic complexity, between humans and swine, make the boar a novel animal model for studying the potential effects of putative endocrine-disrupting compounds (EDCs). It was hypothesized that subacute oral exposure to VCZ would adversely affect reproductive

parameters, such as serum hormone concentrations, testicular and epididymal histology and sperm morphology and motility, in peripubertal and post-pubertal boars. Nine, 7-month-old and eight, 12 to 14-month-old boars were each divided into two groups which were orally exposed to either 0 or 100 mg VCZ/kg body weight for 14 days. Serum samples collected prior to and during the study were analyzed for concentrations of testosterone and estradiol. Following euthanasia, semen was collected from the cauda epididymis for computer-assisted analysis, and tissues were selected and prepared for histologic examination. Statistical analyses were performed using ANOVA and the general linear model (GLM) procedure. Testosterone concentrations increased initially and then decreased in VCZ-treated post-pubertal boars. Estradiol levels were higher in boars of both age groups dosed with VCZ. Histologic changes in the testes and epididymides, as well as abnormal sperm morphology and decreased sperm motility, were observed with administration of VCZ. Exposure to VCZ adversely affected various reproductive parameters in peripubertal and, in particular, post-pubertal boars. VCZ-associated histologic changes were more severe in swine than those reported in rodents administered the same dosage of VCZ. The boar shows promise as a sensitive comparative model for studying the effects of EDCs on male reproductive morphology and function.

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DELAYED PREPUTIAL SEPARATION (PPS) AND SP22 MEASUREMENT IN RATS ADMINISTERED BROMOCHLOROACETIC ACID (BCA) IN DRINKING WATER.

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Reproductive effects of BCA were determined in a dose range finding study (DRFS) and definitive two-generational study. Adult male and female CD<sup>®</sup>(SD) rats were administered BCA in drinking water for two weeks in the DRFS (10/sex/group) and ten weeks in the definitive study (25/sex/group) before mating. Females were dosed until the weaning of their litters. The F1 post-weanlings received BCA until acquiring puberty [PPS in males, vaginal patency (VP) in females] in the DRFS, and through mating to generate F2 offspring in the definitive study. The concentrations were 0, 50, 200, 400, 600, 800, and 1000 ppm BCA *ad libitum* in the DRFS and 0, 30, 300, and 600 ppm BCA in the definitive study. Age of male PPS was used to assess reproductive development. The males at  $\geq$  600 ppm in both studies, showed significant delay of PPS vs. the control males; At 600 ppm mean PPS delays were 2.2 days in the definitive study and 2.7 days in the DRFS; At 800 ppm, males had mean delays of 4.7 days and those males at 1000 ppm had delays of 5.2 days. Ages at acquisition were adjusted using body weight (BW) at acquisition as a covariant. In the DRFS, F1 females at  $\geq$  600 ppm had VP delays of 6.2 days; comparable delays were not seen in the definitive study. In the DRFS, the F0 females, males and F1 offspring at both weaning and puberty had dose-related decreases in BW. In the definitive study, BW was unaltered in F0 males, but was decreased in 600 ppm F0 females. The F1 males and females had decreased BW at 600 ppm at pnd 21 and as adults; the males also had decreased BW at 300 ppm. The sperm membrane protein SP 22 was determined in the caudal sperm of adult F0 and F1 males as a measure of adult reproductive competence and an important biomarker of fertility. Significant decreases in SP22 were seen with increasing BCA concentrations in both F0 and F1 males in the definitive study. This work was funded by a cooperative agreement from USEPA, RTP.

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USE OF 5 $\alpha$ -DIHYDROTESTOSTERONE (DHT)-EXPOSED UROGENITAL SINUSES (UGS) FROM FEMALE MICE TO INVESTIGATE INHIBITION OF PROSTATIC BUDDING CAUSED BY 2, 3, 7, 8-TETRACHLORODIBENZO-*P*-DIOXIN (TCDD).

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Mouse prostate development starts as projections (buds) of UGS basal epithelial cells into the surrounding mesenchyme on Gestation Day (GD) 16. *In utero* TCDD exposure blocks ventral bud formation and reduces dorsolateral bud number in male UGS. Prostatic budding is an androgen-dependent process, and only rudimentary UGS buds are evident in normal female UGS. TCDD appears to inhibit budding without affecting androgen signaling but the inhibitory mechanism remains unknown. Experiments were conducted to determine if DHT-exposed female UGSs can be a model system to investigate prostatic bud formation. This system allows androgen exposure times to be regulated. Pregnant mice were implanted with DHT-releasing pellets on GD 13, 14, 15, or 16. Each dam was then treated with single dose of 5  $\mu$ g/kg TCDD or vehicle 24, 48, 72, or 96 hours later, depending on how early pellet implantation occurred, such that treatment occurred no later than GD 17. UGSs were collected from female fetuses on GD 19 and observed by light microscopy. All DHT- and vehicle-exposed female UGSs displayed

characteristic male UGS development, including UGS enlargement, dorsal sulcus formation, and region-specific bud formation patterns. A male pattern UGS-urethral angle ( $\sim 100^\circ$ ) was also present when DHT exposure began before GD 16. These results demonstrate that exposure of female UGSs to DHT can closely replicate normal male UGS development. TCDD exposure on GD 16 or earlier blocked formation of all ventral and almost all dorsolateral buds regardless of when DHT exposure began. In males a similar time-course occurs and ventral budding is completely blocked by TCDD, but dorsal, lateral, and anterior budding is less vulnerable. The use of DHT-exposed female UGSs therefore provides a useful tool to investigate effects of TCDD on prostatic budding. The sex difference in region-specific sensitivity to budding inhibition by TCDD may facilitate elucidation of the mechanism by which TCDD inhibits prostatic budding. (Supported by NIH ES01332)

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IN VIVO EXPOSURE OF PREPUBERTAL RATS TO METHOXYCHLOR (M) INHIBITS *EX VIVO* LEYDIG CELL (LC) BASAL AND HUMAN CHORIONIC GONADOTROPIN (HCG)-STIMULATED TESTOSTERONE (T) FORMATION.

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M is pesticide which has been used as a replacement for DDT. Its active metabolite is reported to be 2, 2-bis(p-hydroxyphenyl)-1, 1, 1-trichloroethane (HPTE), and both M and HPTE exhibit weak estrogenic and/or antiandrogenic activities and have adverse reproductive effects on animals. In the current studies, prepubertal male rats (12 animals per treatment group) were gavaged once daily between 24-30 days of age with 0, 5, 40 or 200 mg/kg body weight of M in corn oil to evaluate whether LC from immature rats are sensitive to M. Animals were sacrificed  $\sim$ 24 h after the last exposure, and LC were isolated by density-gradient centrifugation of dispersed testes. *Ex vivo* LC T formation was measured after 4 h of incubation under basal conditions and following exposure to 10 mIU/ml hCG. In addition, because immature LC express high 5 $\alpha$ -reductase (5 $\alpha$ -R) activity, which causes the majority of synthesized T to be metabolized to dihydrotestosterone and its hydroxylated metabolites, a 5 $\alpha$ -R inhibitor was added to assess total T biosynthetic capacity. Final body weights of M-treated animals were no different than control; however, testes and seminal vesicle weights declined significantly to 81 and 69% of control, respectively, in animals exposed to 200 mg/kg M. *Ex vivo* LC T formation over 4 h under basal conditions declined significantly to 29% of control at the highest dose of M. A similar pattern of decline in T was observed when LC were incubated with a 5 $\alpha$ -R inhibitor, but T levels were  $\sim$ 30-fold higher. LC T formation following exposure to hCG for 4 h declined significantly to 34% of control in animals exposed to 200 mg/kg M, and a similar pattern of T decline was observed when LC were incubated with a 5 $\alpha$ -R inhibitor, except T levels were  $\sim$ 60-fold higher. Of interest, serum T, LH and FSH levels were unaffected by M. These results suggest that LC from immature rats are highly sensitive to the inhibitive effects of M.

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IN UTERO EXPOSURE TO 2, 3, 7, 8-TETRACHLORODIBENZO-*P*-DIOXIN ALTERS SONIC HEDGEHOG AND BONE MORPHOGENETIC PROTEIN 4 EXPRESSION IN THE DEVELOPING MOUSE UROGENITAL SINUS.

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2, 3, 7, 8-Tetrachlorodibenzo-*P*-dioxin (TCDD) inhibits mouse ventral prostate development through inhibition of epithelial bud formation in the fetal urogenital sinus (UGS). Prostate bud formation occurs as proliferating epithelial buds penetrate into the surrounding mesenchyme. Sonic hedgehog (Shh) in the UGS epithelium activates mesenchyme-mediated paracrine signaling to stimulate epithelial proliferation in emerging buds. Bone morphogenetic protein (Bmp4) expressed in the mesenchyme inhibits epithelial proliferation and budding is accompanied by clearing of Bmp4 expression at the sites of bud emergence. To determine if impaired ventral prostate bud formation following TCDD exposure is associated with disruption of mesenchymal-epithelial interactions involving these pathways, expression of *Shh* and *Bmp4* in male UGS of vehicle- and TCDD-exposed fetuses were compared by whole mount *in situ* hybridization. Pregnant dams were exposed to TCDD (5  $\mu$ g/kg, po) or vehicle on gestation day 13 (GD13), and male fetuses removed daily from GD14-GD19 for comparison of gene expression. *Shh* expression was uniformly distributed in UGS epithelium lining the urethra of both vehicle- and TCDD-exposed UGS up to GD17. Starting at GD18, concentrated expression in the nascent buds was evident in the dorsolateral region in both groups but was absent from the ventral region of TCDD-exposed UGS. Lack of Shh staining in this area was likely due to the absence of ventral bud formation in TCDD-ex-

posed mice. Initially, there was broad *Bmp4* expression in the vehicle-exposed UGS that progressively became restricted to areas surrounding the emerging buds from GD17 to birth. The ventral region of TCDD-exposed mice had no *Bmp4* expression, even though the canonical pattern of expression and re-distribution was present in other regions of the developing prostate. These findings suggest that inhibition of ventral prostate bud formation in the TCDD-exposed mouse fetus correlates with altered Shh and *Bmp4* signaling in the developing UGS. (Supported by NIH Grant ES 01332)

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### CRITICAL WINDOW OF MALE REPRODUCTIVE TRACT DEVELOPMENT IN RATS FOLLOWING GESTATIONAL EXPOSURE TO DI-N-BUTYL PHTHALATE.

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Gestational exposure to di-n-butyl phthalate (DBP), a ubiquitous environmental contaminant, has been shown to interfere with the development of the male reproductive tract by acting as an antiandrogen. This study was conducted to identify the critical days for the abnormal development of the male reproductive tract, specifically the testis and epididymis. Timed-pregnant Sprague-Dawley rats were dosed with DBP at 500 mg/kg/day on gestation day (GD) 14&15, 15&16, 16&17, 17&18, 18&19, or 19&20 (GD 0 = plug day). Anogenital distance (AGD) was measured on postnatal day (PND) 1, and 13; while nipple number was recorded on PND 13 only. After weaning males were allowed to mature to PND 90 at which time they were necropsied. Nipple number, and AGD were recorded and testes, epididymides, seminal vesicles, prostate gland, kidneys and liver weighed. Blood serum was collected and assayed for total testosterone concentration. There were no observable effects on litter size, sex ratio, or mortality of pups. Serum testosterone concentrations were not biologically affected in PND 90 DBP-treated offspring. Significant permanent reductions in AGD were seen in males exposed prenatally to DBP on GD 15&16 or GD 18&19. On PND 13 areolae were present in males exposed to DBP on GD 15&16, 16&17, 17&18, and 19&20, however significant permanent nipple retention occurred only in males after DBP exposure on GD 16&17. Exposure to DBP on only GD 17&18 elicited a significant reduction in epididymal weights; while exposure on only GD 16&17 caused a significant increase in the weights of the testes due to edema. Epididymal and testicular malformations were most prevalent after exposure to DBP on any gestational day. Epididymal malformations, characterized by agenesis of various regions, and small or flaccid testes were significantly increased in DBP exposed males only on GD 17& 18. These findings suggest that two-day DBP exposure is highly detrimental to the developing reproductive tract of the male fetus and the critical window for abnormal development is GD 16-18.

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### EFFECT OF DI(N-BUTYL) PHTHALATE ON MALE REPRODUCTIVE ORGAN DEVELOPMENT IN HYPOTHYROID RATS.

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Thyroid hormones play an important role in the growth and development of the male reproductive tissues, but their effects are not clearly understood. This study examined the effects of di(n-butyl) phthalate (DBP) on male reproductive organ growth and development in hypothyroid rats. Hypothyroidism was induced in pubertal male rats (4 weeks of age) by administering 0.1% propylthiouracil (PTU) in their drinking water for 30 days. DBP was administered to the hypothyroid (250, 500 or 750 mg/kg) and normal (750 mg/kg) rats by oral gavages for 30 days. The body weight of the PTU-treated hypothyroid rats was markedly lower than the control group. No significant changes in the testis, epididymides and adrenal weight were observed in the hypothyroid rats. However, DBP (750 mg/kg) significantly reduced the weights of the reproductive tracts in both the normal and hypothyroid rats. The total T3 and T4 serum level decreased, but the TSH level increased in the hypothyroid rats. Although the histomorphological examination showed a severe diffused Leydig cells hyperplasias in the DBP (750 mg/kg)-treated groups, these effect was mild in the DBP-treated hypothyroid rats. In order to investigate the metabolism of the sex steroid hormone, the steroidogenic factor-1 (SF-1) and StAR mRNA expressions levels were measured in the testis. The expression of SF-1 and StAR mRNA levels were significantly reduced in the PTU-treated hypothyroid rats. DBP (750 mg/kg) also significantly inhibited the expression of SF-1 and StAR in the testis of the normal rats, but SF-1 and StAR mRNA levels were slightly increased in the DBP-treated hypothyroid rats. In conclusion, the DBP-induced male reproductive organ alterations are weaker as a result of the induction of hypothyroidism than under normal conditions. The results suggest that an alteration in the thyroid hormone level can affect the male reproductive organ toxicity induced by DBP through a disturbance of the steroid hormone metabolism.

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### EFFECTS OF TCDD ON STEROIDOGENESIS OF RAT LEYDIG CELLS.

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Polychlorinated dibenzo-p-dioxins, such as 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) has been recognized as a highly potent developmental and reproductive toxicant. We have previously demonstrated effects of TCDD in modulating the expressions of rat Sertoli cell secretory products and markers for cell-cell interaction. In this study, we examined the direct biological effect of TCDD in primary culture of rat Leydig cells. Leydig cells were purified by percoll gradient centrifugation and the cell purity was determined by 3beta-hydroxysteroid dehydrogenase staining and testosterone induction assay. To examine TCDD induced biological consequences, we measured the changes in the secretion of progesterone and testosterone as well as transcripts levels of some selected steroidogenic enzymes (i.e. StAR, P450ssc, 3beta-HSD & CYP17alpha) in TCDD/hCG co-treated cells. Our results indicated that TCDD (0.2 or 2 ng/ml) treatments significantly suppressed hCG (5 or 10 ng/ml) induced testosterone secretion. The suppressive effect aligned with a reduction of progesterone secretion ( $P<0.05$ ) as well as a decrease in P450ssc mRNA expression ( $P<0.05$ ). Expressions of other steroidogenic enzymes (i.e. StAR, CYP17alpha and 3beta-HSD) were not significantly reduced. Interestingly, our data demonstrated that the TCDD-mediated suppressive effect cannot be overturned by a co-treatment with an AhR antagonist (PD98059). This observation indicated that the TCDD-elicited inhibitory effect was not directly mediated by AhR pathway. Moreover the inhibitory effect can be reversed by dbcAMP (2 mM) or high dose of hCG (50ng/ml) cotreatments. Taken together, our results implicated that direct TCDD exposure can interfere with the normal Leydig cell functions.

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### ESTROGEN RECEPTOR ALPHA IS NOT REQUIRED FOR NORMAL PROSTATIC BUD FORMATION OR FOR INHIBITION OF PROSTATIC BUD FORMATION BY 2, 3, 7, 8-TETRACHLORODIBENZO-P-DIOXIN (TCDD) IN MICE.

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A single maternal dose of TCDD on gestation day (GD) 13 impairs prostate development in C57BL/6J mice. Complete inhibition of prostatic bud formation in the ventral region of the prostatic anlage, the urogenital sinus (UGS), results in ventral prostate agenesis in adults. TCDD affects budding less severely in dorsolateral and anterior regions and causes smaller adult lobes with aberrant ductal structures. These effects of TCDD are mediated by the aryl hydrocarbon receptor (AhR) in the UGS mesenchyme (UGM), into which buds from the UGS epithelium (UGE) grow. The UGM is also the target tissue for prenatal androgens that drive prostate formation. Crosstalk between the UGM and UGE is therefore required for normal bud formation and for budding inhibition by TCDD. The AhR has been reported to interact with estrogen receptors alpha (ER $\alpha$ ) and beta (ER $\beta$ ), leading to transcription of estrogen-responsive genes in the absence of estrogen. ER $\alpha$  is predominantly expressed in UGM, the site of action of TCDD, whereas ER $\beta$  is predominantly expressed in UGE. In the current study, we utilized ER $\alpha$  knockout mice (KO) to assess the potential roles of ER $\alpha$  in mediating effects of TCDD on the UGS and in normal bud formation. ER $\alpha$  heterozygous (HT) males and females were mated overnight, and timed-pregnant dams were exposed to a single oral dose of 5  $\mu$ g/kg TCDD on GD 13. UGSs from male fetuses were collected on GD 18, UGM removed, and bud formation was visualized by scanning electron microscopy. No differences in position or appearance of buds in any region of the UGS were observed when vehicle-exposed ER $\alpha$  KO, HT, and wild-type (WT) male UGSs were compared. Likewise, effects of TCDD on bud formation appeared equivalent regardless of ER $\alpha$  genotype. Thus, ER $\alpha$  does not mediate inhibition of UGS budding by TCDD and is not required for normal prostatic bud formation. (NIH grant ES01332)

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### CHARACTERIZATION OF 2, 3, 7, 8-TETRACHLORODIBENZO-P-DIOXIN (TCDD) RESPONSIVENESS IN MOUSE UROGENITAL SINUS (UGS) USING ARYL HYDROCARBON RECEPTOR (AHR)-DEPENDENT LACZ MICE.

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Exposing C57BL/6J mice on gestation day (GD)13 to TCDD causes abnormal prostate development. The ventral region of the UGS is affected most severely; TCDD prevents formation of epithelial buds, leading to ventral prostate agenesis.

The critical window to inhibit budding in the ventral UGS is GD15-16. TCDD also alters bud formation in the dorsolateral region and has minimal effects on the anterior region of the UGS. These effects are mediated via AhR located in the UGS mesenchyme. RT-PCR shows that AhR mRNA is present ubiquitously in the UGS and is about twice as high in the ventral as in the dorsolateral region. It is unknown whether the AhR is activated by TCDD in a region-specific manner in the UGS. To determine whether region specificity of AhR activation can account for region-specific bud inhibition in the UGS, transgenic mice with an AhR-dependent *lacZ* reporter gene construct were examined. Male mice exposed *in utero* to a maternal dose of 5 µg TCDD/kg on GD13, 15 or 17 were sacrificed on GD14, 16 or 18. Two regions in all exposed UGS samples showed LacZ staining which increased in intensity as time of exposure increased. Staining was most evident in the ventral region, extended into the urethra in all samples and was weakly expressed in the dorsolateral region. Staining in the UGS and urethra was located specifically in the immediate periurethral region. A higher number of LacZ stained cells was found by flow cytometry in the ventral mesenchyme than in the dorsolateral mesenchyme. LacZ staining was not found in the UGS epithelium in any region. Control UGSs showed no staining, indicating little to no intrinsic AhR activity. These findings suggest that the loss of ventral buds is related to the presence of selectively activated AhR in the periurethral mesenchyme in the ventral UGS. (NIH grants ES01332, ES07026, ES01247 and ES09430).

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ANALYSIS OF CORTACTIN EXPRESSION IN THE ECTOPLASMIC SPECIALIZATION OF TESTES IN FLUTAMIDE TREATMENT MOUSE.

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The apical and basal ectoplasmic specialization (ES) is an actin-based (cell-cell) junctional structure in the Sertoli cell of the seminiferous epithelium, playing an important role in spermatogenesis. Recent reports showed that the apical ES was sensitive to exogenous chemicals, such as flutamide (FLUT), β-estradiol 3-benzoate and bisphenol A, inducing the deformation. Besides, cortactin is an actin-binding protein in the both ESs regulating the testicular dynamics associating with mediator proteins. The purpose of the present study is to evaluate whether the expression of cortactin becomes one of biomarkers of the adverse effect of the exogenous chemicals on spermatogenesis. ICR mice were subcutaneously injected with FLUT (0.012 µg/g body weight/day for 5 days, dissolved in corn oil), and were sacrificed on 6th day. Whole testis was processed for Western blot analysis. Localization of cortactin in the testis was detected by both immunohistochemistry and immunoelectron microscopy. Results obtained were: 1) the relative amount of cortactin in the treated testis were significantly decreased by 62%, 2) decreased cortactin expression in the apical ES of treated testis was confirmed by immunostaining, and 3) immunostaining of cortactin in the basal ES was obviously not affected by FLUT. Interestingly, the apical and basal ES responded differently to FLUT. These results suggest that cortactin may be one of biomarkers in the apical ES disruption caused by exogenous chemicals. Also, investigation of cortactin mediated dynamics in spermatogenesis may help studies in the field of testicular infertility and be a key to elucidate the mechanisms of the ES.

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CHARACTERIZATION OF THE GENE EXPRESSION PROFILE INDUCED BY ETHYLENE GLYCOL MONOMETHYL ETHER IN THE RAT TESTIS WITH DNA MICROARRAYS.

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Ethylene glycol monomethyl ether (EGME) is an organic solvent in paints, printing inks, thinners and photoresists. Its adverse effects on the male reproductive tract are well described and are characterized by testicular atrophy and impaired fertility. The most sensitive target cell is the primary spermatocyte in the dividing late pachytene stages. While the early lesion has been well characterized, the mechanism of toxicity is still poorly understood. In this study, we analyzed transcription profiling data generated with microarrays (Affymetrix RAE230A) to generate a mechanistic understanding of EGME-induced testicular toxicity. Testes were collected from male CD rats (n = 4), treated orally for 1 (Day 1) and 4 (Day 4) days with EGME at 200 mg/kg/day, and for 4 days with methoxyacetic acid (MAA), the major metabolite of EGME, at 200 mg/kg/day. Testes were collected for transcription profiling and histology. At Day 1, EGME induced apoptosis of spermatocytes in Stage XIV tubules, while at Day 4, changes were more extensive and extended to

Stages XII, II and V. MAA induced changes similar to those induced by EGME. Both compounds regulated the expression of a relatively low number of genes at both timepoints, and overall induced relatively similar gene expression profiles at Day 4, supporting MAA as being the major metabolite involved in EGME-induced testicular toxicity. Analysis of these gene expression changes provided some potential mechanisms for the toxicity, especially within the DNA damage and apoptotic pathways. Moreover, we identified several genes with a consistently altered expression pattern, providing a basis for the formulation of hypotheses. Collectively, these results indicate that transcription profiling can be used to gain a molecular understanding of testicular toxicity in the rat.

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THE USE OF CD-1 MICE FOR FERTILITY AND EARLY EMBRYONIC DEVELOPMENT (ICH-1 STUDIES).

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In circumstances where the rat may not be a suitable model, the mouse (*Mus musculus*) may be a substitute for fertility and early embryonic development studies (ICH-1). Standard dose routes (oral, inhalation, injection [subcutaneous, intermuscular and intravenous]) can be utilized with these mice. Male mice (Crl:CD®-1(ICR)BR) 7 weeks of age are dosed for 28 days prior to the placement for mating, during mating and following mating until terminal euthanasia. Female mice 8 weeks of age are treated in a similar manner for 14 days prior to mating, during mating and until Day 7 of gestation, inclusive. Estrous cycles of the females are monitored prior to mating by vaginal lavage. Mating is performed by pairing overnight and observation for the presence of vaginal plugs. The following standard parameters are typically measured: mortality, clinical observations, bodyweights, food consumption, estrous cycles, mating, gross pathological examination and organ weights. Male reproductive assessments, including epididymal sperm measures (motility, count and morphology) and testicular histopathology can be evaluated. Terminal examinations of the females include ovarian and uterine examinations on gestation Day 13. The reproductive parameters assessed show that this strain of mice has suitable fertility (fertility index 88-95%) and male and female reproductive characteristics for the use of group sizes of 20 to 25 mice per sex in an ICH-1 study.

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UNDERSTANDING HEPATIC VACUOLATION: GENE EXPRESSION ANALYSIS OF PHOSPHOLIPIDOSIS AND STEATOSIS IN RAT LIVER.

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Hepatocellular vacuolation, commonly observed in preclinical drug development, is a morphological indicator of possible phospholipidosis or steatosis. A more accurate classification of hepatic vacuolation relies on electron microscopy (EM) or special stains, such as Oil Red-O. In an effort to understand the molecular mechanism behind liver vacuolation, we used oligonucleotide microarray analysis to examine changes in gene expression profiles associated with these phenotypes. Amiodarone, which is known to cause phospholipidosis, and hydrazine, known to cause steatosis, were used as model compounds in this study. Sprague-Dawley rats were treated with these compounds and their liver samples harvested for gene expression profiling with RG\_U34A GeneChips (data from Gene Logic's Toxicology Express database). In addition to the gene expression data, clinical chemistry, hematology and histopathology data were collected and analyzed. We found that the expression profiles of amiodarone and hydrazine at early time points are similar, potentially indicating a common mechanism governing transcriptional responses leading to phospholipidosis or steatosis. A gene ontology (GO) and pathway analysis showed that stress, oxidative reaction, and fatty acid metabolism are the major theme among the changed genes. We were able to isolate a small subset of genes that differentiate expression profiles of phospholipidosis from steatosis and conducted the GO and pathway analysis on this subset of genes. The similarity between amiodarone and hydrazine may help to explain a previous report on steatosis induced by amiodarone, a drug more known for its ability to cause phospholipidosis. Mechanistic implications of relevant genes and pathways will be discussed, as will further characterization with other compounds that cause hepatic vacuolation.

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TOXICOGENOMIC STUDY OF HYCANTHONE, AN ANTITUMOR AGENT: MOLECULAR MECHANISMS OF HEPATOTOXICITY.

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Toxicogenomic studies have been conducted using thioxanthone antitumor agents with well-characterized target organ toxicity. Three have been tested in clinical trials; two have failed and one is in Phase I. Hycanthone (Etrenol®), caused dose-lim-

iting hepatotoxicity (increased ALT and AST) in mice and in humans in Phase I clinical trials for leukemia. The purpose of this study was to evaluate hycanthone-related gene expression in the context of conventional clinical and anatomic pathology endpoints. Hycanthone was administered i.p. to female CD-1BR mice for five days, at dosages of 50, 70, or 90 mg/kg. On Day 5, blood was collected for clinical pathology and livers were removed for histology and evaluation of gene expression using the Atlas<sup>TM</sup>Mouse Toxicology 1.2 arrays (Clontech). Statistically significant changes in gene expression were identified by analysis of variance (ANOVA). Hycanthone resulted in increased ALT and AST levels on Day 5 at 70 and 90 but not at 50 mg/kg. Histological effects were limited to capsular inflammation (due to i.p. administration) at all dosages. Changes in gene expression increased with dose. At 50 mg/kg, only genes involved in the acute phase response and genes with protease inhibitor activity were altered. At 70 or 90 mg/kg, gene changes occurred in pathways regulating: DNA damage and repair, the oxidative stress response, hydroxylase and isomerase, apoptosis, and the stress and inflammatory responses. The gene expression data suggest that the major pathways affected by hycanthone are consistent with the well-characterized cytotoxic action of thioxanthones and that DNA damage and oxidative stress are involved in thioxanthone-mediated liver cell injury and antitumor activity. The inflammatory/immune response is attributed to the effects of the drug on the liver surface after i.p. administration.

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### GENE EXPRESSION PROFILING IN RAT LIVER AND HEPATOCYTES TREATED WITH FIBRIC ACIDS: ANALYSIS OF THE DATA IN THE TOXICOGENOMICS PROJECT IN JAPAN.

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National Institute of Health Sciences and 17 pharmaceutical companies have started a five-year project, the Toxicogenomics Project in Japan (TGPJ) in 2002. The objective of TGPJ is to construct a large-scale database of over 150 drugs on rats and develop a system which will forecast the toxicity of new chemical in the early stage of drug development. In the present study, we examined gene expression profiles in rat liver and rat primary cultured hepatocytes treated with PPAR $\alpha$ -specific ligands, clofibrate, gemfibrozil and WY-14643, using Affymetrix GeneChip. Rats were treated orally with 3 dose levels of each compound and gene expression profiling was performed 3, 6, 9 and 24 hours after single dose, and 24 hours after 3, 7, 14 and 28-day repeated dose. Rat primary cultured hepatocytes were treated with 3 concentrations of the compounds and GeneChip analysis was performed 2, 8 and 24 hours after the exposure. In the liver of rats received repeated administration, we extracted genes which showed dose-dependent expression changes in common with the 3 compounds. These genes were categorized into lipid metabolism, transcription, signal transduction and stress response. A hierarchical clustering analysis of the expression data of 40 compounds in our database was performed by using the extracted gene set. It was found that the cluster containing clofibrate, gemfibrozil, WY-14643 also contained benzboromarone. The latter compound is for gout and structurally different from the peroxisome proliferators, but it was reported to be a PPAR $\alpha$  ligand. In TGPJ, this compound was found to cause hepatic hypertrophy as well as decrease in serum triglycerides, as observed in the fibrin acids. The expression profile of genes related to PPAR $\alpha$ -specific ligands showed a high similarity between *in vivo* and *in vitro* experiments. It was concluded that the extracted gene set could be a marker for PPAR $\alpha$ -specific ligands.

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### GENE EXPRESSION PROFILING IN RAT LIVER AND HEPATOCYTES TREATED WITH ETHIONINE - ANALYSIS OF THE DATA IN THE TOXICOGENOMICS PROJECT IN JAPAN -.

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National Institute of Health Sciences and 17 pharmaceutical companies have started a five-year project, the Toxicogenomics Project in Japan (TGPJ) in 2002. The objective of TGPJ is to construct a large-scale database of over 150 drugs on rats and develop a system which will forecast the toxicity of new chemical in the early stage of drug development. In the present study, we analyzed the effects of ethionine, a typical hepatotoxicant. Male SD rats were treated with ethionine (0, 25, 50 or 250 mg/kg, p.o., suspended in 0.5% methylcellulose) and sacrificed 3, 6, 9 and 24 hr after single administration, and 4, 8, 15 and 29 days after daily administration. Gene expression was evaluated in 3 out of 5 animals for each group by Affymetrix 230A GeneChip. Rat primary cultured hepatocytes were exposed to 0, 0.4, 2 and 10 mM of ethionine dissolved in 0.1% DMSO. Gene expression profile of the cell was examined 2, 8 and 24 hr after exposure using Affymetrix 230 2.0 GeneChip (n=2). Changes in gene expression (ratio to the control value) were sta-

tistically filtered. Repeated administration of ethionine decreased plasma cholesterol, triglycerides, and phospholipids. In histopathological examination, microvesicular steatosis was observed in the liver. The number of genes detected as modulated by the drug was dose-dependently increased and varied over time. Many of these changes were confirmed in rat primary hepatocytes. Therefore, our multi-dosage and multi-sampling point protocol was significant for data analysis. Among the modulated genes, the expression of sterol regulatory element binding factor-1 was decreased. This gene was suggested to be one of the early responsible genes for the effect of ethionine, since its down regulation was followed by the changes in other lipogenesis-related genes, such as apoprotein A-IV and lecithin-cholesterol acyltransferase. These results provide critical information regarding the mechanism of ethionine-induced hepatotoxicity.

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### GENE EXPRESSION ARRAY ANALYSIS OF MALE C3H AND C57BL/6 LIVER.

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C3H and C57Bl/6 mice are examples of strains of mice that are sensitive and resistant, respectively, to the spontaneous and chemical induction of liver tumors. In addition, these mice differ in their bone density and provide models for this endpoint. In order to understand the bases for the biological differences between C3H and C57Bl/6 mice, gene expression analysis was performed on livers from 4-week-old male mice. In this study, livers were harvested into RNALater and stored at -80° until further analysis. Long-oligonucleotide microarrays of 20, 000 mouse genes were used in 2-color hybridizations using a reference design. Correlation (R value) within the 2 groups ranged from 0.94-0.97. The background-subtracted raw data was normalized using locally weighted linear regression (LOWESS) in ArrayTrack, a database with software tools developed at the NCTR. Cluster analysis of the gene expression profiles showed a clear distinction between the C3H and C57Bl/6 mice. The Significance Analysis of Microarray (SAM) program was then used for analysis of significant gene expression changes between the two groups (1.4-fold and false discovery rate <0.05). The expression of more than 500 genes was found to be significantly altered and included genes involved in inter- and intra-cell signaling pathways, cholesterol metabolism, and lipid metabolism. In addition, there were differences in the expression of many of the cytochrome P450s and transcription factors. These results provide many candidate genes to explain the liver cancer susceptibility differences between these two strains.

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### REGULATION OF MOUSE HEPATIC TRANSPORTERS BY PERFLUORODECANOIC ACID (PFDA).

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Perfluorodecanoic acid (PFDA), also referred to as nonadecafluorodecanoic acid, is used as a corrosion inhibitor, lubricant, surfactant and wetting agent. In rodents, PFDA causes peroxisome proliferation, but is not a liver carcinogen like other peroxisome proliferators. The liver is thought to be the primary target organ of PFDA-induced toxicity due to efficient hepatic uptake and persistence in liver. Major hepatic transporters include uptake transporters, such as Na<sup>+</sup>/taurocholate cotransporting polypeptide (Ntcp) and organic anion transporting polypeptides (Oatps), as well as efflux transporters, including bile salt export pump (Bsep) and multidrug resistance-associated proteins (Mrps). Modulation of transporter gene expression may have profound effects on the toxicity of certain chemicals by altering their liver uptake and export. In the present study, we determined whether PFDA alters the mRNA expression of mouse hepatic transporters by using the branched DNA signal amplification (bDNA) assay. PFDA treatment markedly reduced the steady state mRNA expression of liver-predominant Oatp1a1, 1a4, 1b2, 2b1, and Ntcp by 99, 69, 93, 92, and 99%, respectively. Thus, there was a marked decrease in the expression of the transporters that are responsible for uptake of chemicals into liver. In contrast, PFDA increased mRNA expression of the Mrp transporter family that transports chemicals out of liver. PFDA increased the hepatic expression of Mrp2, 3, and 4 mRNA by 100, 200, and 3500%, respectively. However, PFDA decreased hepatic mRNA expression of Bsep, which exports bile acids into bile, by 80%. These data show that PFDA decreases the mRNA expression of the hepatic uptake transporters, Ntcp and Oatps, but increases the mRNA expression of the Mrp family of efflux transporters. In conclusion, there appears to be a coordinated down-regulation of uptake transporters, and up-regulation of efflux transporters. This response may decrease the liver burden of chemicals, and thus serves to protect the liver from toxicity. (Supported by NIH grant ES-09649, ES-09716, and ES-07079)

ANALYSIS OF BENZO(A)PYRENE-INDUCED GENE EXPRESSION CHANGES IN FISHER 344 RAT LIVER BY QUANTITATIVE REAL-TIME PCR.

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Benzo(a)pyrene (BaP), an environmental contaminant produced from diesel exhaust and organic matter combustion, is associated with a variety of adverse health effects, including cancer. In this study, we used Real-Time quantitative (Taqman) PCR (RT-PCR) to validate the expression of 6 target genes of interest cytochrome p450 1a1 and 1a2 (Cyp1a1, Cyp1a2), Glutathione-s-transferase (GST-yc2), Tumor Suppressor protein (p53), B-cell lymphoma (Bcl-2), Interleukin-1 beta (IL-1 beta) previously identified from our Affymetrix microarrays global transcript profiling experiment of BaP induced gene expression changes in Fisher 344 rat liver. These genes were selected because they showed 3-fold level of change in our Affymetrix experiments and also for their involvement in pathways regulated by BaP. Adult male F344 rats were exposed to BaP through the diet for either 2 weeks (0.1 or 0.01mg/g-diet) or 12-weeks (0.01, 0.1 or 1.0 mg BaP/g of diet). Real-Time PCR quantitative analysis of the target genes of interests revealed significant differences ( $p < 0.05$ ) between the control and treated groups. We also observed a temporal separation in the dose response of the genes selected. For instance, p53 was induced significantly over the control after 2-weeks of exposure, whereas Cyp1a1 and Cyp1a2 were strongly induced only after 12-weeks of exposure. On the other hand, both Gst-yc2 and IL-1 beta was induced after 2-weeks and higher dose-dependent inductions observed after 12-weeks of exposure. Furthermore, significant differences in average weight gain and feed consumption was observed between the high dose (1mg BaP/g of diet) and control groups after 12-weeks of exposure. Also, percent differences in tissue weights between the control and treated groups were observed.

ALTERATIONS OF GENE EXPRESSION IN FEMALE LUNG ADENOCARCINOMA CL5 CELLS BY MOTORCYCLE EXHAUST PARTICULATES AND BENZO(A)PYRENE.

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Motorcycle exhaust (ME) consists of a wide spectrum of toxicants including carcinogenic polycyclic aromatic hydrocarbons and volatile organic compounds. The major objective of the present studies was to investigate the ability of ME particulates (MEP) and benzo(a)pyrene to alter the expression profiles of genes important in metabolic activation and tumorigenesis, which may increase the risk of acquiring lung cancer. cDNA array studies were carried out using female lung adenocarcinoma CL5 cells treated with 100 mg/ml organic extracts of MEP for 6 hr. Gene expression changes were confirmed by real-time RT-PCR. The results showed that in metabolic genes, treatment with MEP extracts increased the mRNA levels of carcinogen-activation enzymes cytochrome P450 (CYP) 1A1 and CYP1B1. In oncogenes and tumor suppressor genes, MEP increased the expression of oncogene fra and tumor suppressor p21 and in contrast decreased the expression of tumor suppressor Rb. In cytokines and growth factors, MEP increased the expression of interleukin (IL)-1 $\alpha$ , IL-6, IL-11, fibroblast growth factor (FGF)-6, FGF-9, and vascular endothelial growth factor (VEGF)-D. Treatment of CL5 cells with 10 mM benzo(a)pyrene, a carcinogenic MEP constituent, for 6 hr increased the mRNA levels of CYP1A1, CYP1B1, fra, p21, IL-1 $\alpha$ , IL-6, IL-11, IL-15, FGF-6, FGF-9, and VEGF-D; but decreased Rb gene expression. These results show that MEP and benzo(a)pyrene have the ability to induce multiple genes in carcinogen-activation, inflammatory cytokine, and growth factor families of female lung adenocarcinoma cells and suggest that ME and gene interaction may present an environmental risk contributing to lung cancer.

EXPRESSION OF GENES INVOLVED IN POLYCYCLIC AROMATIC HYDROCARBONS (PAH) TOXICITY IN HEPG2 CULTURE CELLS.

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PAHs are complex mixtures produced by industrial processes. PAHs are carcinogenic to humans and display pleiotropic effects on gene expression. PAHs serve as substrates and inducers of phase I and II xenobiotic metabolism enzymes (e.g. CYP1A1, -1A2, -1B1, and GST). PAHs affect the metabolic rate and homeostasis and generate reactive intermediates that may act as final toxicants damaging human health. Therefore, due to the wide PAHs exposure and to their deleterious health effects, it is necessary to develop earlier, more specific and sensitive biomarkers to de-

tect their earliest alterations. Thus, the aim of this study is to evaluate the PAHs effects on gene expression in HepG2 cells, a human hepatic cell line, using a micro array approach. PAHs were extracted from soil samples collected in the neighborhood of coke ovens which contained benz[a]pyrene (BAP), benz[a]anthracene, and chrysene. The PAHs extracts were added to HepG2 cell cultures and the responsive genes detected. Cell viability was assessed by a MTT method. Gene expression was evaluated using cDNA microarray analysis using a 10K human genes library. About 1500 genes were shown to be regulated by the PAHs soil extract treatments, and the results were compared to those of BAP (positive control). The PAHs soil extract and BAP treatments up regulated 65 and 58% and down regulated 35 and 42% of the genes evaluated, respectively. These genes are involved in metabolic process such as cell cycle regulation, transcription, DNA replication and signal transduction. The PAHs effects on gene expression could be relevant tools as biomarkers of exposure. This approach is being investigated in our laboratory.

EFFECTS OF DIETS CONTAINING HIGH SOY PROTEIN AND ISOFLAVONES ON GENE EXPRESSION IN FEMALE RAT LIVERS.

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Phytoestrogens have been implicated both as agonists and antagonists of estrogen-dependent cancers. These naturally occurring compounds have been shown to have estrogenic properties. The major human dietary source of isoflavones is obtained from soy foods. In addition to the purported health benefits of phytoestrogens, possible concerns exist in their actions as endocrine disrupters. Particularly in populations where exposure is high, such as in infants consuming soy-based milk formulas. In this study, the effect of dietary soy and isoflavones on gene expression and protein levels in female rat liver was examined at days 28 and 70. Method: Rats were fed a controlled diet of isoflavone-free casein or one of two treatment diets replacing casein with either alcohol-washed soy protein or alcohol-washed soy protein plus Novasoy (isoflavone supplement) at a dose of 1250 mg/kg of diet. Results: Isoflavones are known estrogen mimics; as a result the effects isoflavones on known estrogen targets were assessed. Increases in estrogen receptor alpha/beta and progesterone receptor A (PR-A) (2-fold) were detected at day 28, while decreases in ER alpha and PR-A were observed by day 70. In order to investigate multiple gene targets, microarrays (containing 22K genes) were used. Data comparing the effects of control vs high isoflavone diets on female liver from day 28 show greater than 2-fold increases in gene expression in several metallothionein genes. Decreased gene expression (> 2-fold) was observed in genes involved in fatty acid metabolism. The list of genes includes fatty acid synthase, and fatty acid binding protein 5. Conclusion: Temporal expression of estrogen-responsive receptors was observed. In addition, metallothionein-related genes, which are known to respond to oxidative stress, were upregulated, suggesting a possible protective mechanism. Furthermore, downregulation of several genes involved in fatty acid metabolism may have serious consequences. Investigation into the changes in gene expression at day 70 and day 240 are on going.

A GENE SIGNATURE FOR BILE DUCT TOXINS.

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Hepatic bile duct cells (cholangiocytes) make up such a small percentage of the liver that their gene expression signatures are generally obscured by mRNA changes in the parenchymal cells, however bile duct damage is a common and sometimes serious side effect of novel compounds. Early prediction of bile duct damage is useful, and a gene expression signature should increase our understanding of this toxicity. Since bile duct networks are difficult to separate from other liver cells, we focused on the more easily dissected common duct as a surrogate for cholangiocytes. In addition to vehicle controls, rats were gavaged with the paradigm bile duct toxins (anaphthalisothiocyanate, ANIT, and dimethylaniline, DMA) or intrahepatic cholestasis inducers (ethinyl estradiol, EE, and erythromycin estolate, ERY), and common ducts and livers were removed 24 hours later for RNA preparation. Microarray analysis showed very different gene signature responses for common duct and liver to test compounds. ANIT and DMA had mild effects on gene expression in liver samples, and were best characterized as mild macrophage activators. EE produced a robust gene signature in liver characteristic of oxidative stress/reactive metabolites, as did ERY to a lesser extent. In contrast, gene expression responses were much stronger in the common duct than in the liver for ANIT and DMA with pronounced effects on mRNAs for cholesterol/ bile salt synthetic enzymes; EE and ERY had milder effects on common duct gene expression responses. We speculate that the gene changes observed in the common duct are adaptive and protective to the cells. Further study is required to investigate whether similar gene expression changes are occurring in the hepatic cholangiocytes.

CENTRAL ROLE OF LIVER RECEPTOR HOMOLOG-1 IN INDUCING LIVER-SPECIFIC GENES AND REGULATING LIPID METABOLISM IN KIDNEY OF 5/6 NEPHRECTOMIZED RATS.

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Liver differs from kidney in its high expression of enzymes and transporters controlling cholesterol, bile acid, and lipid metabolism (CBLM). In parallel, liver receptor homolog-1 (Lrh-1), an orphan nuclear receptor, is highly expressed in liver, but minimally expressed in kidney. Lrh-1 is critical in liver development, and can activate genes involved in CBLM, such as cytochrome P450 (Cyp) 7a1 and ATP-binding cassette (ABC) g5 and g8. Patients and experimental animals with chronic renal failure (CRF) have defects in CBLM. In CRF patients, blood and urine profiles of bile acids markedly change. The present study was aimed to investigate changes in renal expression of genes involved in CBLM during CRF and its molecular mechanism. Data show that in a CRF model of 5/6 nephrectomized rats, mRNA transcripts of Lrh-1 and 14 other liver-predominant genes (determined with the branched DNA signal amplification assay) were markedly induced in 4 of 7 female, and 1 of 7 male remnant kidneys with moderate injury. Renal induction of Lrh-1 correlated highly with renal induction of these 14 genes: Cyp2e1, 3a1, and 7a1, UDP-glucuronosyltransferase 2B2, sulfotransferase 20, Na<sup>+</sup>/taurocholate co-transporting polypeptide, bile salt export pump, organic anion transporting polypeptide 1, 2, and 4, Mdr2, Mrp2, as well as ABCG5 and g8. This is the first report of a correlation of Lrh-1 with ectopic expression of liver-specific genes. Moreover, induction of Lrh-1 in the four female remnant kidneys was associated with a complete prevention of renal triglyceride accumulation that occurred in the three female remnant kidneys lacking Lrh-1 induction. In summary, induction of Lrh-1 appears to confer to kidney the ability to express a large variety of liver-specific enzymes and transporters involved in CBLM. During CRF, renal induction of liver-specific enzymes and transporters may have profound effects on drug metabolism and disposition, thus affecting drug efficacy and toxicity. (Supported by NIH grants ES-09649 and ES-09716)

GENE EXPRESSION PROCESS IS A MAJOR INTRACELLULAR TARGET ASSOCIATED WITH A-NAPHTHYLISOTHIOCYANATE HEPATOTOXICITY.

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*a*-Naphthylisothiocyanate (ANIT) is a widely used model hepatotoxin for intrahepatic cholestasis. To elucidate the molecular basis for ANIT hepatotoxicity, DNA microarray analysis of liver tissues from rats 4 days after ANIT treatment (0.1 – 100 mg/kg) was performed. Statistical analysis showed that the expression levels of 195 genes were significantly changed after ANIT treatment. Hierarchical clustering analysis demonstrated that using the expression profiles of these genes as input data, animals could be grouped into clusters grossly corresponding to the dose of ANIT they received. Of the genes showing altered expression, 54 genes are related to the gene expression processes including transcriptional regulation, mRNA processing, translation, post-translational modification, protein folding, sorting and trafficking. Other biological processes affected by ANIT treatment include mitochondrial electron transport, transporter/carrier, cell adhesion and communication, signal transduction and metabolism. Self Organization Map Clustering analysis revealed co-regulation of genes in certain functional classes. For instance, genes related to mitochondrial electron transport were clustered in a group with a maximum response of down-regulation at low level exposure (0.5 mg/kg), while genes involved in transcriptional regulation/mRNA processing were clustered in a group showing a progressive decrease. In contrast, genes involved in translation, post-translational modification and protein sorting/trafficking were up-regulated. However, these genes almost evenly distributed into two clusters with a progressively increasing pattern and a biphasic pattern respectively. We are currently pursuing additional analysis addressing the biological pathways involved. These results should provide a better understanding of the molecular basis for ANIT hepatotoxicity and perhaps intrahepatic cholestasis in general.

GESTATIONAL EXPOSURE TO THE PHYTOESTROGEN GENISTEIN INFLUENCES EPIGENETIC GENE REGULATION IN MICE.

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Exposure to the phytoestrogen genistein has been linked to a variety of beneficial effects, including cancer chemoprevention (1) and decreased adipose deposition (2), as well as a number of negative outcomes including reduced reproductive

health (3). Since the epigenome is particularly susceptible to dysregulation during embryogenesis, we investigated the effect of gestational exposure to moderate levels of dietary genistein in viable yellow Agouti (Avy) mice. Female a/a dams were randomly assigned to receive soy-free diet or soy-free diet supplemented with 250 mg/kg diet of genistein two weeks prior to mating with Avy/a males and throughout gestation and lactation. Coat color phenotype and CpG methylation of the Avy intracisternal A particle (IAP) retrotransposon were assessed in day 21 Avy/a offspring. Bisulfite sequencing was used to quantify site-specific CpG methylation (4). Maternal supplementation with genistein shifted the coat color distribution of Avy/a offspring toward the brown (pseudoagouti) phenotype. Methylation analysis at nine Avy IAP CpG sites showed increased percentage of cells methylated in genistein exposed litters in comparison to controls. IAP methylation profiles in day 120 tissue derived from the endoderm (liver), mesoderm (kidney), and ectoderm (brain) were correlated to day 21 tail tissue suggesting that genistein acts early in embryonic development and that methylation effects are not transitory but persist into adulthood. These results suggest that early exposure to genistein affects adult phenotype via permanent alterations in the epigenome and may influence cancer chemoprevention and disease susceptibility. 1. Lamartiniere CA et al. *J Nutr*, 132: 552-558S, 2002. 2. Naaz A et al. *Endocrinology*, 144: 3315-3320, 2003. 3. Nagao T et al. *Reprod Toxicol*, 15: 399-411, 2001. 4. Grunau C et al. *Nucleic Acids Res*, 29, 2001.

USING RNA INTERFERENCE TO VALIDATE MECHANISMS OF TOXICITY.

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Small molecule inhibitors of the  $\gamma$ -secretase ( $\gamma$ -sec) complex have been shown to prevent the cleavage of amyloid precursor protein thus preventing the release and deposition of neurotoxic forms of  $\beta$ -amyloid in Alzheimer's disease. The reported *in vivo* toxic effects of  $\gamma$ -sec inhibitors in the gastrointestinal tract are associated with the dysregulation of Hes1 and Rath1 in the Notch1 differentiation pathway (Milano et al, 2004). Seeking further experimental support for Notch1 cleavage inhibition as the mechanism for toxicity mediated by  $\gamma$ -sec inhibitors, we employed RNA interference (RNAi) using a siRNA against Notch1. RNAi is a relatively new technology for negative genetic analysis that allows for sequence specific post-translational degradation of mRNA. A duodenal adenocarcinoma cell line, Htu80, expresses Notch1 and Hes1 and was chosen for our model system. Transfection conditions were optimized for 3 siRNAs targeted against Notch1. In two independent experiments, one of three siRNAs (Notch1\_C), showed  $\approx$ 65% gene knock down 48 hours post transfection. Notch1\_C was then used in two 3-day time course experiments that examined Notch1 expression 24, 48 and 72 hours post-transfection. Cells treated with medium containing transfection reagent for 72 hours in Experiment A were crenated and senescent by visual observation, indicating cytotoxicity. When transfection reagent was removed after 24 hours in Experiment B treated cultures were indistinguishable when compared to untreated controls. Data from these 2 experiments showed a similar expression pattern relative to the controls with 60% knockdown occurring at 48 and 72 hours. However, the data show no change in the expression of the Notch1 regulated transcriptional repressor, Hes1 in the 3-day time course experiment. This may be due to the duration of the time course or the degree of the silencing effect. While RNAi is a powerful method for negative genetic analysis, great consideration must be taken for the value of target analysis weighed against the investment of time. With experience, workflows may be streamlined for a less time intensive commitment.

CIGARETTE SMOKE CONDENSATE INHIBITS PROMOTER ACTIVITY OF LYSYL OXIDASE IN CULTURED LUNG FIBROBLASTS.

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Lysyl oxidase (LO) catalyzes the crosslinking of collagen and elastin stabilizing the lung extracellular matrix. LO deficiency is implicated in the pathogenesis of emphysema. Cigarette smoke is a major cause of emphysema development. Our previous studies have shown downregulation of LO mRNA in rat fetal lung fibroblasts (RFL6) exposed to cigarette smoking condensate (CSC). To further study CSC effects on LO gene expression, we isolated the 5'-flanking region of the LO gene from rat genomic DNA by PCR. DNA sequence inspection revealed a number of potential cis-elements such as CTF1, MTF1, HIF1 $\alpha$ , ARE, SP1, Oct-1, CdxA and AP2 existing in this LO promoter region, but the lack of a typical TATA box. The LO promoter region was restricted into the plasmid pGL3-basic upstream of the luciferase gene. Deletion analysis of the LO promoter showed that the proximal 1.6 kb upstream of the putative transcription start site (+1) was required for maximal expression of the reporter gene in transfected RFL6 cells. Two positive regulatory

segments, i.e., -124/-231 and -312/-518, and one negative regulatory segment, i.e., -232/-312, were identified. Transient transfection analysis demonstrated that although CSC-resistant cells derived from RFL6 cells exposed to graded doses of CSC displayed higher transfection efficiency these cells exhibited 60% lower luciferase activity in comparison to the parental control without CSC treatment. The 24-h pulse assay showed that CSC induced a biphasic response in the luciferase gene expression such that 20 $\mu$ g/ml of CSC enhanced LO promoter activity to 3.6-fold of the control, while 120 $\mu$ g/ml of CSC totally inhibited the luciferase gene expression. These results suggest that suppression of LO promoter activity by CSC at high doses is a key mechanism for downregulation of LO at the mRNA level while enhanced effects of CSC at low doses on LO promoter activity are under study (Supported by Philip Morris ERP & NIH grant R01-ES11340).

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#### REGULATION OF THE STEROIDOGENIC ACUTE REGULATORY PROTEIN (STAR) BY TRANSFORMING GROWTH FACTOR BETA (TGF-BETA).

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Exposure to environmental contaminants, including Roundup, Lindane, phthalates, and paper mill discharge, can significantly repress steroid production in mammals and in lower vertebrates such as fish. StAR is involved in the rate-limiting step in steroid production and is transcriptionally down regulated by toxin exposure. StAR transports cholesterol across the mitochondrial membrane for metabolism into steroids. We cloned the entire coding region of largemouth bass (LMB) StAR and used this sequence to develop a real-time PCR assay to quantify StAR mRNA levels in LMB ovarian follicle cultures. Exposure to dbcAMP and TGF-beta, two potent signaling molecules known to regulate mammalian steroidogenesis, also regulated StAR in bass. Dose response experiments at 0.25, 0.5, 0.75, and 1 mM dbcAMP show increased mRNA expression that saturates between 0.75 mM and 1 mM dbcAMP. TGF-beta, on the other hand, down regulates StAR mRNA by 2.3 fold after 14 hours. A polyclonal antibody specific to LMB StAR was developed to measure protein levels by western blot. To further analyze the regulation of LMB StAR, a 3 kb portion of the promoter was cloned. In silico analysis of this segment with other StAR promoters available in the database showed potential conserved regulatory sites that imply control by a wide range of transcription factors. The 3 kb promoter segment was transfected into Y-1 cells, a mouse adrenocortical cell line and tested with dbcAMP and TGF beta. The 3 kb and the 1.8 deletion constructs responded positively to dbcAMP but only the 1.8 kb construct was repressed by around 2-fold after 40 hr incubation with 10 ng/ml TGF-beta. Mutation of potential regulatory sites in the promoter, including estrogen response elements and COUP-TF sites are being tested for their role in TGF-beta signaling. Together, this data suggest that one way toxins may repress steroid synthesis, and more specifically StAR, is through TGF-beta signaling.

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#### COMPENSATORY CHANGES IN PHASE I, II, AND III ENZYMES IN MICE LACKING THE MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN 1 (MRP1/ABCC1).

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The multidrug resistance-associated protein-1 (MRP1/ABCC1) is a 190kDa membrane-bound glycoprotein that mediates cellular efflux of a variety of xenobiotics, typically as glucuronide, sulfate, or glutathione conjugates. We examined alterations in phase I, II, and III (transporter) activity or gene expression in the liver, kidney, colon, lungs, stomach, heart, and testes between FVB mice and mice that lacked the multidrug resistance associated protein 1 (mrp1) termed FVB/mrp1-/- mice. In the testes, 16a hydroxylation and 2a-hydroxylation activities were significantly upregulated in the FVB/mrp1-/- mice, which correspond to CYP2C11. In the lung, 15a hydroxylation and 16a hydroxylation were significantly reduced in the knockout animals, which correspond to CYP2A4 and CYP2C11, respectively. In the kidney, two unidentified hydroxylation activities and 2a hydroxylation were significantly upregulated. There were no differences in the liver, heart, or the stomach. Significant changes in phase II metabolizing enzymes included a 2.6-fold increase in hepatic sulfotransferase activity and 1.5-fold reduction in hepatic glucuronosyltransferase activity in the FVB/mrp1-/- mice. The expression of mrp2 and mrp5 was significantly increased in the liver, while mrp5 expression was reduced by 2-fold in the lungs in the FVB/mrp1-/- mice. There were no differences in transporter expression in the kidneys, testes, or stomach. Thus, mice lacking mrp1 have tissue-specific changes in levels of phase I, phase II, and phase III enzymes, presumably to compensate for the lack of mrp1.

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#### TIME COURSE OF HEPG2 GENE EXPRESSION FOLLOWING TREATMENT WITH THIOXANTHONE ANTITUMOR COMPOUNDS.

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Toxicogenomic studies have been conducted using thioxanthone compounds with well-characterized target organ toxicity. Three thioxanthones have been tested in clinical trials; two have failed and one is in Phase I. The present *in vitro* study compares the time course of gene expression in HepG2 cells for two thioxanthones, Hycanthone (Etrenol®) and SR33377. Hycanthone is hepatotoxic in humans and rodents, while SR33377 is not. HepG2 cells were exposed for 4 hrs (mimicking *in vivo* half-life) at concentrations resulting in 70-80% cell viability. After 4 hrs, compounds were removed and fresh media was added. Total RNA was extracted mid-incubation, at the end of the incubation, and 2, 8, 20 and 44 hours post-incubation. Atlas™ Human Toxicology II arrays (Clontech) were used to evaluate gene expression. Statistically significant changes in gene expression were identified by analysis of variance (ANOVA). Selected changes were confirmed by real-time RT-PCR. The fewest changes in gene expression occurred during the drug incubation period: 42 with Hycanthone and 26 with SR33377. The greatest number occurred at 2 hours with SR33377 (337) and 8 hours with Hycanthone (251). By 44 hours, gene expression had returned to control levels. Maximal gene expression preceded the peak in cytotoxicity (MTT). The major pathways affected by SR33377 and Hycanthone included genes regulating: DNA damage/repair, cell cycle progression, the oxidative stress response, immune response and induction of apoptosis. The primary difference in the expression profiles of the two compounds was prolonged gene expression changes observed with SR33377, most notable for genes involved in metabolism, the immune response and DNA damage/repair. Hycanthone and SR33377 are cytotoxic antitumor agents that both intercalate and bind DNA. The pathways affected by Hycanthone and SR33377 in HepG2 cells are consistent with this and suggest that DNA damage and oxidative stress are involved in thioxanthone-mediated cell injury and antitumor activity.

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#### ISOLATING (NEURO)TOXIC GENE EXPRESSION SIGNATURES.

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Toxicogenomic experiments present challenges in isolating phenotype-specific transcriptional changes. We use active and inactive isomers and other controls to address this issue. 1, 2-Diacetylbenzene (DAB), but not 1, 3-DAB, is (a) instantaneously protein-reactive, (b) chromogenic after a single systemic dose and, with repeated treatment, (c) able to induce axonopathy. Transcriptional changes were examined in brains of 12-wk-old male C57Bl/6 mice 1 hr-7 days after single intraperitoneal (i.p.) puncture (Mock) or i.p. treatment with Vehicle (2% acetone in saline), 50 mg/kg body weight 1, 3-DAB or 50 mg/kg 1, 2-DAB (a neuropathic dose if repeated). Gene expression studies employed fluorescently-tagged cRNAs hybridized to an Agilent mouse 60-mer oligonucleotide microarray with >22, 000 features. Data were subjected to Significant Analysis of Microarrays for  $q = 0.1$  and fold changes = or >1.5. At 1 hr post-treatment, gene expression with 1, 3-DAB vs. Vehicle (mostly down-regulation) differed from that of 1, 2-DAB vs. Vehicle (mostly up-regulation). Changes specific to the neurotoxic property (NP) of 1, 2-DAB were assessed by excluding genes co-modulated by Mock (few), Vehicle (some) and 1, 3-DAB (n=290 down- and 8 up-regulated features). The NP was associated with increased expression of only 28 genes encoding certain transcription factors/regulators and proteins linked to cell-cycle progression, synaptic function, and microtubule integrity, among others. The NP associated with a different set of genes at 6 hr, and overall modulation was markedly reduced by 24-48 hr post-treatment. These studies indicate the critical need for adequate controls (Mock, Vehicle, 1, 3-DAB) to isolate evolving gene expression signatures peculiar to the pathological mechanism initiated by 1, 2-DAB. Genes modulated by confounding factors (e.g. injection, vehicle, chemical class) must be excluded to identify toxic mechanisms. [Supported by NIEHS grants ES10338 & ES11384, and Oregon Workers Benefit Fund]

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#### PREDICTION OF TRANSCRIPTION FACTORS COMMONLY AFFECTED BY GLUTAMATE-CYSTEINE LIGASE EXPRESSION IN MICE EXPOSED TO ACETAMINOPHEN, CARBON TETRACHLORIDE OR TUMOR NECROSIS FACTOR.

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Glutathione is a major cellular antioxidant and free radical scavenger and its synthesis is rate limited by the activity of glutamate-cysteine ligase (GCL). We have created transgenic mice in which GCL can be induced using the progesterone an-

agonist mifepristone. Using DNA microarrays, we compared global gene expression in the livers of these mice after exposure to the hepatotoxins acetaminophen (APAP), carbon tetrachloride and tumor necrosis factor-alpha (TNF-a), all of which exert oxidative stress. Gene expression profiles were processed using GenMapp to retrieve gene ontology (GO) information, and gene expression patterns were analyzed for common up-stream promoter transcription response elements (TREs) using the program PAINT. The hepatotoxins induced stress response genes, inflammatory response genes and phase I and II metabolism genes. Induction of GCL transgenes modified gene expression profiles, especially in APAP and TNF-a treated mice. Analysis of the GO and TREs present in the promoters of up- or down-regulated genes revealed common themes of oxidative stress, inflammatory response and lipid metabolism. However, relatively few TREs were over represented ( $p<0.1$ ) in common among all three hepatotoxins. These included CAAT, Elk1, Ap4, CREBP, NGFI-C, c-ETS1, v-Maf, SREBP-1 and Brn-2. Genes whose expression after treatment was attenuated by GCL over expression had several TREs in common: v-Maf, Pax-3, NF-Y, SREBP-1, MYOGNF1, NGFI-C, Oct-1, Nkx2-5. This possibly indicates GCL status was an important factor associated with the genes expressed or repressed in common by these factors. We conclude that GCL status may be an important determinant for a number of gene expression responses common to all three hepatotoxins. Supported by NIH Grants 1P42ES04696, 1R01ES10849, 5U19ES011387, and 1P30ES07033.

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### MOLECULAR MECHANISMS OF PPARA-MEDIATED GENE EXPRESSION OF PXR.

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The Pregnan-X-Receptor (PXR) plays a central role in the transcriptional regulation of genes associated with compound metabolism, including CYP3A4, CYP2B6, GST-A2 and MDR1. Ligand promiscuity for activating PXR, combined with its large target gene set, allows PXR to act as a central regulator to changes in the fluxome, relating to both xenobiotics (many therapeutic agents are ligands for PXR), or endogenous chemicals (PXR target genes are involved in bile acid homeostasis, haem and cholesterol synthesis). PXR expression has been shown to be increased by several chemicals, and of particular interest are chemicals not traditionally associated with PXR target genes, such as the peroxisome proliferators. The aim of this study was to examine possible molecular mechanisms underlying this phenomenon. Bioinformatic analysis of 2.2kp of DNA immediately upstream of the PXR transcription start site, using MatInspector Professional to interrogate the Transfac 4.0 database identified putative binding sites for numerous factors. Next, this region was cloned into a SEAP reporter plasmid and a deletion series created (1.8kp, 1.5kp, 1.3kp, 1kp, 900bp, 600bp and 400bp anchored at the 3 end). Human hepatoma cells were transiently transfected and expression levels measured, to produce a map of positive and negative regulatory regions: Comparing this to the in silico assignments allows evidence-based predictions for the transcription factors involved in basal expression. A putative binding site for Peroxisome Proliferator Activated Receptor alpha (PPAR $\alpha$ ) was identified 1.3kb from the transcription start site, and shown to respond in a positive fashion to co-transfected PPAR $\alpha$  protein. In addition, electromobility shift analysis demonstrated binding of nuclear protein to this site and disruption of the site via mutagenesis resulted in both reduced binding of protein, and reduced activation in the reporter gene assay. In summary, we have used both in silico and *in vitro* approaches to characterise potential molecular mechanisms underlying PPAR $\alpha$ -mediated transcriptional activation of PXR expression.

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### DECREASED LONGEVITY AND ENHANCEMENT OF AGE-DEPENDENT LESIONS IN MICE LACKING THE NUCLEAR RECEPTOR PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ALPHA.

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The nuclear receptor peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ) is activated by peroxisome proliferators (PP), a large class of structurally diverse xenobiotic chemicals, hypolipidemic drugs and endogenous lipids. PPAR $\alpha$  alters the transcriptional programs of genes whose functions include lipid metabolism, inflammation, cell fate, and stress responses in liver, heart, kidney and skin. Many of these genes are also under control of PPAR $\alpha$  in the absence of exogenous peroxisome proliferator exposure. Mice which lack PPAR $\alpha$  (PPAR $\alpha$ -null mice) exhibit a number of defects in lipid metabolism and accumulate lipids in the liver. Here, we compared the age-dependent lesions in the liver, kidney and heart in PPAR $\alpha$ -null mice with those observed in wild-type SV129 mice, in the absence of exogenous chemical exposure. Groups of mice were sacrificed, at 6, 12, 18, 21, or 24 months of age, or allowed to age until moribund or found dead.

PPAR $\alpha$ -null mice had decreased longevity, due to a variety of causes. Statistically significant differences in the occurrence of a number of lesions between strains was observed. Hepatocellular carcinomas and multiple hepatocellular adenomas occurred in the livers of PPAR $\alpha$ -null mice but not wild type mice. Various non-neoplastic spontaneous aging lesions occurred at higher incidence, shorter latency, or increased severity in PPAR $\alpha$ -null mice compared with wild-type mice. In the liver, these included vacuolated hepatocytes and sinusoidal cells and mixed cell inflammation. The kidneys of PPAR $\alpha$ -null mice exhibited higher incidences and severities of cortical mineralization. Minimal myocardial mineralization occurred at a higher incidence in PPAR $\alpha$ -null mice. Our results imply that PPAR $\alpha$  delays the development of some spontaneous lesions associated with aging in the liver, kidney and heart of SV129 mice.

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### CONSTITUTIVE EXPRESSION OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ALPHA-REGULATED GENES IN DWARF MICE.

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Defects in growth hormone (GH) secretion or signaling in mice are associated with decreased body weights (dwarfism), increased longevity, increased resistance to stress and decreases in factors which contribute to cardiovascular disease and cancer. Peroxisome proliferators (PP) alter a subset of these changes in wild-type mice through activation of the nuclear receptor family member, PP-activated receptor alpha (PPAR $\alpha$ ). We tested the hypothesis that an overlap in the transcriptional programs between untreated dwarf mice and PP-treated wild-type mice underlies these similarities. Using transcript profiling, we observed a statistically significant overlap in the expression of genes differentially regulated in control Snell dwarf mice (Pit-1dw) compared to phenotypically normal heterozygote (+/dw) controls and those altered by the PP WY-14, 643 (WY) in +/dw mice. The genes included those involved in beta- and omega-oxidation of fatty acids (Acox1, Cyp4a10, Cyp4a14) and those involved in stress responses (the chaperonin, TCP1 epsilon) and cardiovascular disease (fibrinogen). The levels of some of these gene products were also altered in other dwarf mouse models including Ames, Little and GH receptor-null mice. The constitutive increases in PPAR $\alpha$ -regulated genes may be partly due to increased expression of PPAR $\alpha$  mRNA and protein as observed in the livers of control Snell dwarf mice. These results indicate that some of the beneficial effects associated with the dwarf phenotype may be due to constitutive activation of PPAR $\alpha$  and regulated genes.

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### PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR $\beta$ (PPAR $\beta$ ) REGULATES EXPRESSION OF CANCER-RELATED GENES IN LIVER.

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Previous studies in our laboratory using two chemically-induced liver toxicity models suggested that peroxisome proliferator activated receptor  $\beta$  (PPAR $\beta$ ) attenuates hepatotoxicity, and may function to prevent hepatocarcinogenesis. In this study, gene expression profiles of liver samples from wild-type and PPAR $\beta$ -null mice were examined, to identify potential PPAR $\beta$  target genes that are involved in liver toxicity, cell proliferation, and carcinogenesis. A cDNA-microarray analysis of 1706 genes was performed, and 47 genes were shown to be up-regulated while 43 genes were down-regulated in PPAR $\beta$ -null samples as compared to wild-type ( $p<0.05$ ). Subsequent northern blot analysis and/or real-time PCR was performed to confirm the expression changes detected by microarray analysis for some of the genes related to liver toxicity and carcinogenesis. E-cadherin, a tumor suppressor gene, is down-regulated by 2 fold in PPAR $\beta$ -null mice as compared to wild-type. Additionally, cathepsin E, which is highly expressed in multiple human cancers and is implicated in the promotion of metastasis, is up-regulated by 11.5 fold in PPAR $\beta$ -null livers as compared to the wild-type counterparts. Combined, these results indicate that PPAR $\beta$  modulates expression of critical cancer-related genes in the liver that could participate in the prevention of liver toxicity and carcinogenesis.

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### GENE EXPRESSION PATTERNS ASSOCIATED WITH FENOFIBRATE INDUCED MYOPATHY.

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Fenofibrate (FB) is a PPAR-alpha agonist that is used to treat dyslipidemia. Skeletal muscle myopathy is a rare, but serious adverse event reported in people taking FB. Myofiber damage may be a result of increased beta-oxidation leading to oxidative

stress. Generally, in the rat, the soleus depends on oxidative lipid metabolism and is composed of Type 1 fibers while the gastrocnemius depends on glycolytic metabolism and is composed of Type 2 fibers. The purpose of this study was to investigate gene expression changes in the soleus and gastrocnemius of rats given a myopathic dose of FB and to correlate these changes with clinical and anatomic endpoints. Following 10 days of treatment with 200 mg/kg/day FB, blood was collected for clinical chemistry and the soleus and gastrocnemius were collected for microscopic evaluation and gene expression analysis using Clontech Toxicology II arrays. Treatment-related myopathy was observed in the soleus but was not noted in the gastrocnemius. Increases in AST (3-fold) and ALT (2 fold) were observed; however there was no change in CK. In general, genes associated with oxidative stress, lipid synthesis and transport, and myofiber and collagen formation were more strongly upregulated in the soleus compared to the gastrocnemius. For example, metallothionein (MT)-1 and hemeoxygenase (HO)-1 were upregulated by 19.2- and 4.4-fold, respectively, in the soleus while in the gastrocnemius MT-1 was upregulated by 1.4-fold and there was no change observed in HO-1. Upregulation of genes associated with oxidative stress appear to be related to the myopathic response in the soleus. Genes associated with beta-oxidation were more strongly upregulated in the gastrocnemius and may suggest a shift from glucose utilization to lipids and beta-oxidation. The myopathic response observed in the soleus may be consistent with some clinical chemistry endpoints and with upregulation of genes associated with oxidative stress. However, the relationship of fiber-type sensitivity to damage remains unclear as a shift towards beta-oxidation was noted in the gastrocnemius without evidence of myopathy.

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### EVALUATION OF THE ACTIVITY OF THE PESTICIDE CYHALOFOP-BUTYL IN A PEROXISOME-PROLIFERATOR RECEPTOR- $\alpha$ REPORTER ASSAY.

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The potential of cyhalofop-butyl, a registered rice herbicide, to bind to and activate the peroxisome proliferator activated receptor-alpha (PPAR- $\alpha$ ) was evaluated *in vitro*, in a gene transactivation assay. The plasmids pSI-PPAR- $\alpha$  (containing the rat PPAR- $\alpha$  gene), pGL3-3XPPRE (containing 3 tandem repeats of the recognition sequence for the PPAR- $\alpha$ ), and pCMV- $\beta$ gal-SPORT, were transiently transfected into the rat hepatoma cell line H4IIE. The assay was validated by assessing the activity of a series of activating and non-activating PPAR- $\alpha$  ligands. WY14643 (EC50 ~250 nM) produced the maximal response and was the most potent of the test materials evaluated. Fenoprofen (EC50 ~7 uM), LY171883 (~5 uM) and arachidonic acid (~80 uM) exhibited similar maximal responses to WY14643, but at much higher concentrations. The plant-derived terpenoid geranylgeraniol (EC50 ~250 nM) and indomethacin (above 600  $\mu$ M) exhibited less-than-maximal responses in the assay. The negative control PPAR- $\gamma$  agonist ciglitazone and the phorbol ester, TPA, exhibited no significant activity in the assay. For the assessment of cyhalofop-butyl, two separate assays were conducted at eight concentrations ranging from 10 pM-1 mM. Cyhalofop-butyl exhibited a concentration-related increase in response, with maximal 2.8 and 3-fold increases >100  $\mu$ M, relative to vehicle control (DMSO). The concurrently run positive control, WY14643 (100 pM-1 mM) exhibited a maximal 8-fold induction in both assays relative to vehicle. The reproducible, concentration-related responses indicate that under the conditions of the study, cyhalofop-butyl binds to and activates the PPAR- $\alpha$ . This demonstrates a plausible mechanism whereby cyhalofop-butyl, though negative in both rat and mouse cancer studies, could produce peroxisome proliferator-associated tumors in rodent liver at excessive doses. However, hepatic PP induction is a physiological response associated primarily with rodents and generally not considered relevant to humans.

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### PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR $\beta$ (PPAR $\beta$ ) SELECTIVELY REGULATES KERATINOCTYE DIFFERENTIATION AND PROLIFERATION.

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Previous work has shown that peroxisome proliferator-activated receptor  $\beta$  (PPAR $\beta$ ) attenuates chemically-induced skin carcinogenesis, suggesting a critical role of PPAR $\beta$  in regulating keratinocyte cell proliferation and/or differentiation. In these studies, the functional role of PPAR $\beta$  in keratinocyte proliferation, differentiation, and inflammation was investigated using a highly specific PPAR $\beta$  ligand (GW0742) and a null mouse model. Differentiation induced by two classical methods of increasing culture medium Ca<sup>++</sup> level or TPA was not markedly different be-

tween wild-type and PPAR $\beta$ -null keratinocytes. However, GW0742 selectively induced mRNA markers of differentiation in primary keratinocytes and mouse skin, and this effect did not occur in the absence of PPAR $\beta$  expression. Treatment of cells with GW0742 inhibited cell proliferation in wild-type keratinocytes in a dose-dependent manner, whereas no influence on cell proliferation was observed in PPAR $\beta$ -null keratinocytes. In addition, myeloperoxidase (MPO), an enzyme marker of inflammation in skin, was significantly increased in TPA-treated mouse skin, and co-administration of GW0742 resulted in reduced activity in both wild-type and PPAR $\beta$ -null mice. Further analysis showed that GW0742 uncompetitively inhibits MPO activity. Combined, these results show that the antiproliferative role of PPAR $\beta$  in skin is due to its selective induction of differentiation and inhibition of cell proliferation, and that inhibition of inflammation induced by GW0742 is independent of PPAR $\beta$ . Supported by the National Cancer Institute (CA89607), J.M.P.

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### THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR $\beta$ (PPAR $\beta$ ) AGONIST GW0742 INHIBITS SKIN CARCINOGENESIS.

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The peroxisome proliferator-activated receptor  $\beta$  is ubiquitously expressed, and high expression levels are found in the skin. The biological roles of PPAR $\beta$  remain uncertain. Previous studies have shown that PPAR $\beta$ -null mice subjected to a two-stage chemical carcinogenesis bioassay exhibit greatly accelerated formation of papillomas in addition to a greater average number and size of papillomas, as compared to wild-type mice. These results suggest that PPAR $\beta$  attenuates cell proliferation and that activation of this receptor may inhibit skin cancer. In this study, PPAR $\beta$ -null and wild-type mice were subjected to a two-stage chemical carcinogenesis bioassay and topical treatment with a selective PPAR $\beta$  agonist (GW0742). The onset of papilloma formation was delayed in ligand treated wild-type mice, and this effect did not occur in PPAR $\beta$ -null mice. After 24 weeks of TPA and ligand treatment the average number of papillomas was decreased in ligand treated wild-type and PPAR $\beta$ -null mice as compared to controls. However, the average size of papillomas was smaller in ligand treated wild-type mice, and this effect was not observed in PPAR $\beta$ -null mice. These results support the hypothesis that PPAR $\beta$  attenuates proliferation and shows promising support that activation of this receptor may be used to inhibit skin cancer. Dose response studies with higher concentrations of ligand are currently underway. Supported by National Cancer Institute (CA89607), J.M.P.

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### TRANSCRIPTIONAL REGULATION OF TISSUE-SPECIFIC INHIBITOR OF MATRIX METALLOPROTEINASE 1 (TIMP1) BY PEROXISOME PROLIFERATOR-ACTIVATOR RECEPTOR $\beta$ (PPAR $\beta$ ).

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PPAR $\beta$ -null mice exhibit an early onset of skin tumor formation, increased skin tumor size and increased skin tumor multiplicity in response to a two-stage carcinogen bioassay (DMBA/TPA). Matrix metalloproteinases (MMPs) are overexpressed in many tumors and promote tumor progression, angiogenesis and metastasis through the degradation of extracellular matrix proteins. Tissue-specific inhibitor of matrix metalloproteinases (TIMPs) are the physiological inhibitors of MMPs, and overexpression of TIMPs have been associated with reduced metastasis and chemical carcinogenesis in skin. In these studies, the role of PPAR $\beta$  in the regulation of MMP9 and TIMP1 was examined. Zymogram analysis showed increased MMP9 activity for PPAR $\beta$ -null keratinocytes as compared to wild-type keratinocytes in response to TPA. Northern blot analysis showed increased TIMP1 mRNA expression in wild-type mice as compared to PPAR $\beta$ -null mice in response to TPA. A putative peroxisome proliferator response element (PPRE) was identified in the promoter region of TIMP1 and was subsequently cloned into a luciferase reporter vector. Co-transfection of the reporter vector into keratinocytes showed a dose-dependent increase in luciferase activity compared to controls following treatment with GW0742 in wild-type keratinocytes but not in PPAR $\beta$ -null keratinocytes. Results from these studies showed that PPAR $\beta$  can significantly regulate MMP9 activity, and that this may occur through direct regulation of an MMP9 in-

hibitor, TIMP1. Further, these results suggest that tumor progression and angiogenesis may be inhibited by ligand activation of PPAR $\beta$ . Supported by the National Cancer Institute (CA89607), J.M.P.

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TRANSCRIPTION PROFILING REVEALS HEPATIC HYPERTROPHY TO BE INDISTINGUISHABLE FROM THE PHARMACOLOGY OF PPAR $\alpha$  AGONISM IN THE RAT.

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Peroxisome proliferator activated receptor alpha (PPAR $\alpha$ ) agonism by xenobiotics in rodent models can result in hepatomegaly (a combination of hepatic peroxisome proliferation and hypertrophy) and tumor formation via poorly understood non-genotoxic mechanisms. Hepatomegaly is widely believed to be an on-target effect of PPAR $\alpha$  treatment but a systematic approach to characterizing this relationship has not been reported. Sprague-Dawley rats were treated p.o. for 2, 3, 4, 5, 6, 9 and 16 days with five nuclear hormone receptor agonists. Using compounds that target PPAR $\alpha$  (fenofibrate and Wy-14, 643), PPAR $\gamma$  (rosiglitazone), PPAR $\delta$  (bezafibrate, a pan agonist with PPAR $\alpha$ ,  $\gamma$  and  $\delta$  activities) and retinoic acid receptor (RAR) followed by transcription profiling of liver tissue, we designed a strategy of selection and deselection of ANOVA signature gene sets to characterize and rank the specificity of the transcriptional response for agonism of individual receptor classes. The modulation of 196 genes that correlated highly ( $p \leq 3.5 \times 10^{-6}$ ) with hepatic hypertrophy using a Monte Carlo simulation were found to be almost exclusively regulated by fenofibrate and Wy-14, 643 treatments but not by rosiglitazone or RAR treatments. Many of the 196 hypertrophy regulated genes are intimately involved at various stages of glucose and lipid metabolism, five of which have putative peroxisome proliferator response elements identified through sequencing. These data suggest that hepatic hypertrophy is indistinguishable from the pharmacological effects of PPAR $\alpha$  agonism in the rat.

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DISTRIBUTION OF COPPER (CU) AND ZINC (ZN) TRANSPORTER IN RAT CHOROID PLEXUS, BRAIN REGIONAL CAPILLARIES, AND CHOROIDAL Z310 CELLS BY REAL TIME RT-PCR.

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Cu and Zn are essential elements in living organisms. Evidence suggests that an altered brain homeostasis of Cu and Zn may contribute to the etiology of Alzheimer's disease. Both Cu and Zn in brain are regulated by transport mechanisms located at blood-brain barrier (BBB) and blood-cerebrospinal fluid (CSF) barrier (BCB). However, the distribution and abundance of related transporters in BCB and regional BBB were unknown. This study investigated the normal distribution of Cu and Zn transporters in choroid plexus (CP), capillaries of striatum, hippocampus, frontal cortex, and cerebellum in normal rats, and their expression in Z310 choroidal epithelial cells, by using quantitative real-time RT-PCR. Results showed that among five Cu transporters tested in brain capillaries, Ctrl1 and MTP1 mRNAs were most abundant in striatum, while DMT1 and ATP7B were highest in frontal cortex, and ATP7A in hippocampus capillaries. The CP contained Ctrl1 and ATP7B about 1.6-5.2 fold and 13-54 fold, respectively, higher than those of brain capillaries. Of seven Zn transporters, the DMT1 and ZnT1-4 mRNAs were greatest in frontal cortex capillary, whereas ZIP and PHT1 were highest in striatum. In comparison, the CP tissues had far more abundant ZnT1-4 mRNA levels, about 1.6-8.1 fold higher than those in frontal cortex capillaries. Moreover, the levels of ZIP and PHT1 were 1.3 and 17.2 fold, respectively, higher than those in striatum capillary. Z310 cells showed a generally decreased level of most Cu and Zn transporters compared to CP, except for ZnT2, which is higher in Z310 cells than in CP. Our data indicate that the transporters responsible for Cu and Zn movement in brain barriers appear to be unevenly distributed in brain regional BBB, with most active transport in brain cortex. The higher expression of Cu/Zn transporters in BCB than BBB suggests a critical role of BCB in maintaining Cu and Zn homeostasis in normal brain. (Supported by NIH/NIEHS ES08146)

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COPPER (CU) TRANSPORT AND TRANSPORTERS IN THE BLOOD-BRAIN BARRIER (BBB) AND BLOOD-CSF BARRIER (BCB).

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Cu is an essential metal involving in important enzymatic reactions. Deficient or excessive Cu in brain is reportedly associated with neurodegenerative diseases such as Menkes disease, Parkinson's disease, and Wilson disease. However, the mecha-

nisms whereby Cu is transported by brain barriers and the relative regional transport of Cu were unknown. This study tested the hypothesis that brain barriers play a major role in maintaining brain homeostasis of Cu. Uptake rates of Cu into brain capillary, parenchyma, cerebrospinal fluid (CSF), and choroid plexus (CP) were determined by *in situ* brain perfusion technique followed by capillary depletion. Levels of mRNAs encoding Ctrl1, DMT1 and ATP7B were assessed by real time RT-PCR. The perfusate contained 64Cu and a space marker, [<sup>14</sup>C]sucrose. Cu uptakes into brain were linear up to 120s. The unidirectional transport rate constants (Kin) for Cu into the CP and brain capillary were 1034 $\pm$ 369 and 319 $\pm$ 90  $\mu$ l/s/g, respectively, while Kin in CSF and parenchyma were much reduced, 0.8 $\pm$ 0.5 and 112 $\pm$ 29  $\mu$ l/s/g, respectively. A higher uptake by CP vs. much lower uptake by CSF indicates that cumulated Cu in CP is likely derived from blood rather than CSF. The uptake rate of Cu was about 3 fold more rapidly to capillaries than to parenchyma, supporting a significant role of BBB in transport of Cu into brain. Patterns of brain Cu uptake showed a regional difference. The Kin in regional brain exhibited in a descending order, hippocampus, midbrain, frontal cortex, striatum, and cerebellum, with cerebellum statistically significantly slower than other brain regions. The mRNA levels of Ctrl1 and DMT1 were about 3-5 fold higher in brain capillaries than those in parenchyma ( $p < 0.05$ ). The mRNA levels of Ctrl1 and ATP7B in the choroid plexus were 1.6-5.2 and 13-54 fold, respectively, higher than those in brain capillaries. Our data suggest that the choroids plexus has a high capacity in accumulating Cu from blood and Cu uptake into brain may be coordinated by Ctrl1/DMT1 at BBB and Ctrl1/ATP7B at BCB. (Supported by NIH/NIEHS ES08146)

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THE EFFECTS OF COPPER DIMETHYLDITHIOCARBAMATE (CDDC) ON RAT HIPPOCAMPAL ASTROCYTES.

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Health concerns over chromated copper arsenate (CCA) treated lumber has lead to the use of alternative chemicals for wood preservation. One such compound is copper dimethyldithiocarbamate (CDDC). Few toxicity studies have been performed on the effects of CDDC on neural cells. Copper has been known to accumulate in astrocytes resulting in oxidative stress, peroxidative damage and alterations in cytoskeletal structure. To determine the effects of CDDC, rat hippocampal astrocytes were exposed to various concentrations of CDDC (1.75 to 35  $\mu$ g/ml) for 1h washed in buffer and re-fed with complete media (Dulbecco's modified Eagle's media with 10% FBS) and allowed to recover for 24h. Cell viability was determined at the end of this time by trypan blue exclusion to be 77.2%, 66%, 55.3%, 27.5%, and 22.5% for 1.75, 3.5, 7.0, 14.0, and 35  $\mu$ g/ml, respectively. These results showed a significant decrease in viability relative to controls even at the lowest doses. Light and scanning electron microscopy confirmed the viability studies. Light microscopy showed cellular distortion including shorten processes and cell rounding. Granulation of the cytosol was observed and increased with increasing dose of toxicant. Scanning electron microscopy revealed alterations in the cell surface, including blebbing and fragmentation of processes. The higher doses of 14, and 35  $\mu$ g/ml resulted in severe morphological distortion. Nuclei often appeared raised, irregular and sometimes fragmented. Further investigations will study the effects of CDDC on the cytoskeleton particularly with reference to microtubular structure and copper concentration.

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MANGANESE EXPOSURE ALTERS IRON REGULATORY MECHANISMS AT BLOOD-CEREBROSPINAL FLUID BARRIER (BCB) AND SELECTED REGIONS OF BLOOD-BRAIN BARRIER (BBB) IN RATS.

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Previous *in vitro* data suggest that manganese (Mn) exposure increases the expression of mRNAs encoding transferrin receptor (TfR), which possess an iron (Fe) response element (IRE), by altering binding of iron regulatory protein-1 (IRP1) to TfR mRNA. The current study tested the hypothesis that *in vivo* exposure to Mn alters TfR expression at both BBB and BCB, leading to altered Fe transport at brain barriers. Male SD rats received daily oral gavages at doses of 5 or 15 mg Mn/kg as MnCl<sub>2</sub> for 30 days. Blood, cerebrospinal fluid (CSF) and choroids plexus were collected. Brain capillaries from striatum, hippocampus, frontal cortex, and cerebellum, were separated from parenchyma. Atomic absorption spectrophotometry revealed that the Fe concentration in controls was about 17-22 fold higher in choroid plexus than in other brain regions. Mn exposure resulted in a 67% decrease of serum Fe and an increased Fe in CSF (25%) and choroid plexus (67%) compared to control, while the concentrations of Mn and Fe in most brain regions tested did not change significantly. Capillary depletion followed by gel shift assay using S100

cytosolic extracts showed that binding of IRP1 to [32P] IRE-RNA probes was significantly enhanced in choroid plexus and capillaries of striatum, hippocampus, and frontal cortex ( $p < 0.01$ ). Quantitative real-time RT-PCR demonstrated increased levels of TfR mRNA in choroid plexus and capillaries of striatum and hippocampus ( $p < 0.05$ ), but not in frontal cortex and cerebellum capillaries, suggesting an up-regulation of TfR in BCB and selected regional BBB. The mRNA levels of ferritin, an Fe storage protein, were reduced by 87% in the choroid plexus and 34% in striatum capillary. Taken together, these data indicate that Mn, on the way to brain, alters Fe regulatory mechanisms at BCB and selected regions of BBB. This may underlie the distorted Fe homeostasis in the CSF. (Supported by NIH/NIEHS ES08146)

## 594 OVEREXPRESSION OF DMT1 IN THE CHOROID PLEXUS FOLLOWING MANGANESE (Mn) EXPOSURE.

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Divalent Metal Transporter 1 (DMT1), whose mRNA possesses a 3'-UTR stem-loop structure, has been identified in most organs and responsible for transport of various divalent metal ions. Previous work from this laboratory has shown that Mn exposure alters the function of iron regulatory protein (IRP) and increases iron (Fe) concentrations in blood-cerebrospinal fluid (CSF). This study aimed to test the hypothesis that Mn treatment, by acting on protein-mRNA binding between IRP and DMT1 mRNA, altered the expression of DMT1 in the choroid plexus (CP), where the blood-CSF barrier resides, leading to a compartmental shift of Fe from the blood to CSF. Western blot and real time PCR confirmed the presence of DMT1 in an immortalized choroidal epithelial Z310 cell line. Following *in vitro* exposure to Mn at 100  $\mu$ M for 24 and 48 hrs, the expression of DMT1 mRNA in Z310 cells was significantly increased by 45.4% ( $p < 0.05$ ) and 78.1% ( $p < 0.01$ ), respectively, as compared to controls. Accordingly, Western blot analysis revealed a significant increase of DMT1 protein concentrations at 48 hr after Mn exposure (100  $\mu$ M). When rats received, by oral gavage, 5 and 15 mg Mn/kg as MnCl<sub>2</sub> per day for 30 consecutive days, the levels of DMT1 mRNA in choroids plexus tissues were significantly increased by 258% and 305% ( $p < 0.05$ ), respectively. An electrophoretic mobility shift assay (EMSA), by using S100 cytosolic extracts from both *in vitro* cells and *in vivo* brain tissues, was conducted to investigate the effect of Mn exposure on the interaction between IRP and DMT1 mRNA. Results showed that Mn exposure increased binding of IRP to DMT1 mRNA in cultured choroidal Z310 cells, in animal CP, as well as in selected brain tissues. These data suggest that Mn appears to stabilize the binding of IRP to DMT1 mRNA, thereby increasing the expression of DMT1. The facilitated transport of Fe by DMT1 at the blood-CSF barrier may partly contribute to Mn-induced neurodegenerative Parkinsonism. (Supported by NIH/NIEHS ES08146)

## 595 IRON DEFICIENT AND MANGANESE ENHANCED DIETS ALTER METALS AND TRANSPORTERS IN THE DEVELOPING RAT BRAIN.

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Fe-deficiency is a prevalent nutritional disorder, affecting ~2 billion people, mostly pregnant and lactating women and children. Fe and Mn share similar transport mechanisms, competing for transport. In adults Mn toxicity leads to neurological disturbances, but little is known about developmental Mn toxicity. To study the interactions of Fe and Mn during brain development, pregnant Sprague-Dawley rats were fed one of four semi-purified diets from gestational day 7 until postnatal day (PN)21: control (35 Fe:10 Mn mg/kg diet), low Fe (ID; 3 Fe:10 Mn), high Mn (Mn; 35 Fe:100 Mn), or low Fe with high Mn (IDMn; 3 Fe:100 Mn). Control neonates were cross-fostered to experimental or control dams on PN4 and exposed to the diets via lactation until PN21. Hematological measurements confirmed Fe-deficiency (decreased Fe, hemoglobin; increased transferrin (Tf), total Fe binding capacity) in dams and pups fed "ID" or "IDMn" diets, while those fed "Mn" had some trends toward similar hematological changes. Western blot analysis revealed that both "ID" and "IDMn" increased expression of the metal transporters, Tf receptor and divalent metal transporter 1 (DMT1). Inductively coupled plasma mass spectrometry (ICP-MS) showed that all three experimental diets decreased brain Fe levels, while both Mn enhanced diets increased brain Mn levels. In addition, "ID" increased copper (Cu); "Mn" increased chromium (Cr); and "IDMn" increased Cr, Cu, cobalt (Co), zinc (Zn), and vanadium (V). Upregulated DMT1, a non-specific transporter, may be a route for increased metals in the brain following dietary manipulations. Because each of the metals affected by low Fe and/or high Mn are es-

sential metals for normal development and function, homeostatic disturbances may contribute to later consequences. Supported by NIEHS 10563 (MA) and 12768 (SJG)

## 596 INFLUENCE OF SUBACUTE MANGANESE SULFATE ON DOPAMINE AND N-METHYL-D-ASPARTATE RECEPTORS.

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The potential of manganese sulfate (MnSO<sub>4</sub>) to alter the dopamine (D<sub>2</sub> + D<sub>3</sub>) and N-methyl-D-aspartate (NMDA) receptor after fourteen days of daily gavage was studied in rats. Sprague-Dawley male rats were randomly given, by gavage, one of six liquid mixtures (suspended in 40 % corn starch; 10 % sucrose, and 12 % dextrinized corn starch) of manganese sulfate (MnSO<sub>4</sub>), negative control (40 % corn starch, 10 % sucrose, and 12 % dextrinized corn starch), or the positive control (6 mg/kg midazolam). The doses of the MnSO<sub>4</sub> were given to provide: 1.0, 10, 30, or 100 milligrams manganese/kg. Binding studies of the (D<sub>2</sub> + D<sub>3</sub>) receptor with raclopride were preformed in the basal ganglia. Binding studies of the NMDA receptor with CGP-39653 were preformed in the cerebellum, cerebral cortex, globus pallidus, and hippocampus. The receptor binding studies indicate that fourteen of daily gavage with 1 to 100 mg/kg MnSO<sub>4</sub> did not alter the affinity of the NMDA receptor or the maximum number of binding sites in the three of the brain areas examined (cerebellum, cerebral cortex, and globus pallidus). Only the affinity of the NMDA receptor in the hippocampus was altered by the 14-day oral exposure to 10 mg/kg MnSO<sub>4</sub>. In contrast, the affinity of (D<sub>2</sub> + D<sub>3</sub>) binding sites was not altered by any of the liquid mixtures containing manganese relative to the negative control given for fourteen days. The liquid mixtures containing the highest concentrations of manganese altered the maximum number of (D<sub>2</sub> + D<sub>3</sub>) binding sites. The receptor binding studies indicate that two weeks of daily gavage with 10 mg/kg MnSO<sub>4</sub> altered the affinity of the NMDA receptor in only one of the four brain regions examined, while higher oral concentrations of MnSO<sub>4</sub> altered the maximum number of (D<sub>2</sub> + D<sub>3</sub>) binding sites in the basal ganglia. (Supported by ATSDR 398948).

## 597 BIOLOGICAL MARKERS OF MN EXPOSURE: RELATIONSHIPS BETWEEN MN DOSE, PARTITIONING IN BLOOD, AND ACCUMULATION IN BRAIN.

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Elevated manganese (Mn) exposures are known to elicit a range of neurotoxic effects, including the well characterized manganism syndrome at very high exposures. However, the relationship between Mn exposure, the accumulation of a Mn body burden, and the emergence of neurotoxic effects is not very well defined, partly because of the absence of good biomarkers of exposure suitable for use in human studies. Here we investigated whether the nominal Mn dose metric (Mn dose per exposure event), as compared to the cumulative Mn dose metric (total Mn administered over the duration of exposure) was more predictive of resultant Mn levels in plasma, whole blood, and brain of Sprague-Dawley rats treated with Mn ip 3 times/wk at nominal doses of 0, 1.2, 4.8 and 9.6 mg/Kg over 5 wks, or 0, 1.2, and 4.8 mg/Kg over 15 wks. Also, to specifically investigate the partitioning of Mn in erythrocytes versus plasma in humans, we spiked fresh human whole blood with MnCl<sub>2</sub> to achieve [Mn] of 12, 25, and 50 ng Mn/mL (control ~7ng/mL), and separated plasma from the erythrocytes for analysis. In the animal study, the relative fraction (%) of Mn in plasma decreased in an exponential fashion from ~50% in controls (blood Mn 8ng/mL) to <10% in animals with blood Mn levels >50ng/mL. In contrast, in human blood, <10% of Mn in control samples was partitioned in plasma, whereas >70% of added Mn partitioned in plasma in blood spiked with Mn. In the animal study, blood and brain Mn levels showed the same dose dependent increase from 8 to 650ng/mL whole blood, and 1.5 to 8.3  $\mu$ g Mn/g brain (dw), regardless of the duration of treatment (5 or 15 wk). Blood vs brain Mn levels followed an asymptotic relationship with brain Mn leveling off at ~8  $\mu$ g Mn/g for blood Mn >400 ng/mL. The relationship between plasma Mn and brain Mn was less defined. These data substantiate the need for additional study into predictive biomarkers of Mn exposure and brain uptake that may be suitable for use in humans.

## 598 PROGRESSION OF NEUROCHEMICAL EFFECTS IN DIFFERENT BRAIN REGIONS AS A FUNCTION OF THE MAGNITUDE AND DURATION OF MANGANESE EXPOSURE.

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Manganese (Mn) is known to elicit symptoms resembling those of Parkinson's disease (PD) at high exposure levels, but its effects at low levels of exposure are uncertain. Because of the similarity of behavioral deficits at elevated Mn exposure to PD

symptoms, earlier Mn toxicity studies have proposed that striatal dopamine (DA) depletion, a hallmark of PD, is also produced by Mn, despite the observation in humans that Mn accumulates in the globus pallidus. To reconcile this, we have proposed the hypothesis that there is a progression of effects from the globus pallidus to striatum as a function of increasing magnitude of Mn dose and treatment duration (Gwiazda et al., *Neuro Toxicology*, 95:1-8, 2002). To test this, we administered Mn ip 3 times/wk to Sprague-Dawley rats at nominal doses of 0, 1.2, 4.8 and 9.6 mg/Kg over 5 wks, and 0, 1.2, 4.8 mg/Kg over 15 wks. We conducted a battery of motor tests, spontaneous motor activity (SMA) and rotarod measurements, evaluated brain, blood, and plasma Mn levels, and neurochemical levels in the striatum, globus pallidus, substantia nigra and motor regions of the thalamus. Mn treatment increased DA levels in the globus pallidus in animals receiving the highest Mn doses over both 5 and 15 wks, but had no effect on striatal or substantia nigra DA levels. Motor deficits measured as impairment in the balance beam and in hind limb hopping, and shorter latency to fall from the rotarod were observed at the highest dose at 5 weeks. No Mn effects were detected on SMA. Blood and brain Mn showed similar relative increases as a function of nominal dose at 5 and 15 wks, even though the cumulative Mn doses of 15 wks animals were three times higher than in animals exposed for 5 wks. These results suggest that 1) Across a wide range of Mn doses the globus pallidus is a more sensitive locus of Mn toxicity compared to the striatum, and 2) The magnitude of the Mn nominal dose is more important than exposure duration in bringing about an increase in Mn body burden and eliciting Mn toxicity

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### DETERMINATION OF DEPLETED URANIUM (DU) IN RATS FOLLOWING 6-MONTH EXPOSURE TO SURGICALLY IMPLANTED DU PELLETS.

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Depleted uranium (DU) is used to reinforce armor shielding and increase penetrability of military munitions. Although there is conflicting data, concern exists regarding the role of the metal in the etiology of Gulf War syndrome. We examined the potential of DU to accumulate in various brain regions following surgical implantation of metal pellets in rats. Male Sprague-Dawley rats were divided into five groups: non-surgical controls (NS), sham (SH, 0/20 DU pellets), low (LW, 4/20 DU pellets), medium (MD, 10/20 DU pellets) and high (HI, 20/20 DU pellets). Rats were weighed weekly as a measure of general health. No statistically significant changes in weight were observed among any of the groups for the duration of the experiment. At the conclusion of the study, animals were perfused with buffer to prevent contamination of brain samples with DU from blood. Brains were removed and dissected into six regions: cerebellum, brain stem, midbrain, hippocampus, striatum and cortex. Although there is dose-dependent trend toward greater DU accumulation among the various treatment groups, one-way ANOVA of preliminary data indicates that DU does not significantly accumulate in cortex (NS 1.849, SH 0.337, LW 0.658, MD 1.595, HI 2.542 ng DU/g wet tissue, n=3 for each group, p > 0.05) or hippocampus (NS 3.577, SH 2.829, LW 0.759, MD 1.346, HI 1.910 ng DU/g wet tissue, n=3 for each group, p > 0.05) compared to controls. Our data suggest that elevated brain-DU levels in non-perfused animals may not be due to tissue accumulation, but to DU present in the blood. Supported by Department of Defense, grant number DAMD17-01-1-0685.

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### NEUROLOGICAL EFFECTS OF ACUTE URANIUM EXPOSURE.

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We have previously shown that a single intraperitoneal exposure to depleted uranium increased regional brain uranium content. In the current study, we examined the neurological effects of a single intramuscular injection of 0, 0.1, 0.3, or 1 mg uranium/kg (as uranyl acetate, UA) in the presence and absence of stress. Stress treatments were applied for five days prior to injection and ceased just prior to injection. Animals that were stressed had four-fold higher plasma corticosterone levels at the time of uranium exposure ( $763 \pm 131$  vs.  $189 \pm 91$  ng/ml). Treatment with UA produced time and dose-dependent increases in serum and brain uranium levels, with the highest levels observed on day 3. Exposure to UA decreased ambulatory activity, forelimb grip strength, and weight gain, regardless of stress treatment. Rats treated with 1 mg/kg UA exhibited a 30% decrease in striatal dopamine content 3 days after dosing ( $59 \pm 6$  vs.  $41 \pm 5$  ng/mg tissue). The effect on dopamine was ameliorated by prior application of stress. No effect of DU or stress was ob-

served on levels of GABA, serotonin, norepinephrine, or GSH in the striatum, hippocampus, cerebellum, or cortex. These results indicate that single exposures to soluble uranium at doses as low as 0.1 mg/kg can have adverse neurological effects. However, uranium also produces renal toxicity (see Tobias et al., this meeting), so it is unclear if the neurological effects are a direct result of uranium or are secondary to renal injury. This work was supported by the US Army Medical Research and Materiel Command DAMD17-1-01-0775.

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### DIFFERENTIAL EFFECT OF LEAD (Pb) EXPOSURE ON THE N-METHYL-D-ASPARTATE (NMDA) INDUCED INWARD CURRENT IN DOPAMINE (DA)-CONTAINING NEURONS AND HIPPOCAMPAL CA1 NEURONS.

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Inorganic lead (Pb) exposure occurred either chronically *in vivo* or acutely *in vitro*, and then all electrophysiological experiments were conducted on rat midbrain or forebrain slices *in vitro* using the whole-cell patch-clamp recording technique. Newborn rat pups were exposed to inorganic lead (Pb) via the dam's milk during the first 10-17 days of postnatal life. The Pb-treated dams received 250 ppm lead acetate through their drinking water and controls received deionized water. Brain slices were also obtained from unexposed 2-week-old rat pups to examine the acute effects of Pb exposure *in vitro* for a short, 5-10 minute period, or a long, 2-6 hour period, before N-methyl-D-aspartate (NMDA) challenge. A population study design was used to compare the concentration-dependent effects of the NMDA-induced inward current in both the chronic and acute Pb-exposure models. In both the chronic *in vivo* and the acute *in vitro* Pb exposure models, the Pb concentrations were 0.01-10  $\mu$ M and the concentrations of the glutamatergic agonist, NMDA were 1-60  $\mu$ M. Neither the chronic *in vivo* nor the acute *in vitro* Pb exposure decreased the NMDA-induced currents elicited in nigral DA neurons. Under one specific condition, a 30  $\mu$ M challenge of NMDA in the presence of a long-acute Pb exposure to 3  $\mu$ M Pb resulted in a significantly augmented receptor current. Experiments were also conducted on nigral DA and hippocampal CA1 neurons, where each individual neuron acted as its own control. In these latter experiments, each neuron was first challenged with NMDA alone, then following short-acute perfusion with 3  $\mu$ M Pb, was re-challenged with NMDA in the continuing presence of Pb. The short-acute Pb exposure did not alter the magnitude of the NMDA-induced inward current in nigral DA neurons, but did significantly decrease the amplitude of the NMDA-induced inward current in hippocampal CA1 neurons. These results strongly suggest that Pb exposure has a differential impact on NMDA receptors that occur on different neuronal types.

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### ORAL EXPOSURE TO INORGANIC LEAD (Pb) IN RATS: BLOOD CONCENTRATIONS, BRAIN CONCENTRATIONS AND EFFECTS ON ACOUSTIC STARTLE.

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The present studies investigated the effects of chronic oral exposure to inorganic lead (Pb) in rats on Pb blood and brain concentrations and on the acoustic startle response and its modulation by pre-pulse inhibition (PPI) stimuli. Male SD rats were exposed to oral Pb exposure in drinking water for six weeks (Pb concentrations of 0, 25, 50, 100, 250 and 500 ppm Pb) or 12 weeks (0, 50, 250 ppm Pb). Subjects were tested for acoustic startle and the startle PPI before Pb treatments and at 3-week intervals. Startle test sessions were characterized by four different acoustic trials: (1) no stimulus (background activity), (2) Noise Alone (40 msec, 118 dB; 48 dB over background), (3) Prepulse Stimulus Alone (100 msec, 80 dB), (4) Pre-Pulse Stimulus + Noise (Pre-pulse Stimulus presented 100 msec prior to the 118 dB Noise). The startle trials were presented randomly using an average 30-second inter-stimulus interval. At the end of the Pb exposure period (6 weeks or 12 weeks), subjects were sacrificed and blood and brain Pb concentrations were determined. Pb concentrations in both blood (5-30  $\mu$ g Pb/deciliter) and brain tissue (50-400 ng Pb/gram brain) were linearly-related to the Pb exposure level. Pb concentrations in brain and blood were highly correlated within subjects. Two of the Pb treatments, 50 ppm and 500 ppm, reduced body weight gain when compared to controls. Exposure to 50 ppm Pb significantly reduced acoustic startle amplitude when compared to vehicle controls; this 50 ppm Pb exposure in drinking water resulted in blood Pb concentrations slightly below 10  $\mu$ g Pb/deciliter (the CDC threshold for toxicity). Presentation of the weak acoustic stimulus immediately prior to presentation of the full acoustic startle stimulus reduced the response to the full acoustic stimulus compared to control levels (the PPI effect); Pb exposure in drinking water for 6 or 12 weeks did not significantly affect this PPI effect. The present studies demonstrate that the acoustic startle response is a sensitive measure of Pb behavioral toxicity.

### LIPID PEROXIDATION AND DISTRIBUTION OF INORGANIC ARSENIC AND ITS METABOLITES IN RAT NERVOUS SYSTEM.

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In environmentally exposed humans to inorganic arsenic (iAs) it has been observed a deficit in attention, memory and learning abilities as well as peripheral neuropathies. Rodents exposed to iAs have been showed changes in several brain neurotransmitter systems and alterations in the locomotor activity. These effects have been associated to the presence of arsenic in cerebral tissue. In the present study we analyze the possible relationship between lipid peroxidation (LPO) and distribution of iAs and its metabolites (monomethyl arsenic, MMA and dimethyl arsenic, DMA) in several structures of the rat nervous system (brain, spinal cord, sciatic and sural nerves). Sodium arsenite diluted in water (10 mg/kg/day) was dosed to male Wistar rats (200-300 gr bw) during 30 days, by gavage. iAs and its metabolites concentrations were determined. Results indicated that DMA was the major iAs metabolite in nervous tissues (~95%) and it was higher in peripheral than in central nervous structures (sural nerve>sciatic nerve>spinal cord ≥brain). LPO, expressed by means of malondialdehyde content (MDA), was also observed in rat nervous tissues and it significantly differs from control group ( $p<0.0001$ ). In addition, MDA content show a close relationship with the arsenic concentration observed in all nervous tissues. Our results may indicated that the presence of DMA induce the formation of MDA and in consequence, the occurrence of oxidative stress in peripheral and central nervous tissues. In addition, our observations also illustrate the existance of a differential distribution of arsenic in peripheral nerves and spinal cord of the exposed rat.

### EFFECT OF INORGANIC ARSENIC EXPOSURE INITIATING FROM *IN UTERO* THROUGH AFTER BIRTH ON THE NEUROBEHAVIOR AND NEUROTRANSMITTERS IN THE BRAIN OF MOUSE OFFSPRING.

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It is known that chronic arsenic (As) exposure causes a wide variety of adverse health effects. Although there is a possibility of occurring damage on the central nervous system (CNS) of infant by As exposure during their premature period, a few was known. In this study, we investigated the effect of CNS function of the offspring from the dam exposed to As via drinking water after their growing period. Sodium arsenite solution at 50 ppm As was given as drinking water to couple mice (C57BL/6J, C3H and B6C3F1) for a week and then they were crossed. As was exposed to offspring through whole experimental period; via placenta, via breast-feeding, and via drinking water directly after weaning. We observed body weight, surface righting reflex, cliff avoidance, pivoting, walking, negative geotaxis, grasping reflex and grip strength to estimate growth as indicators of growth on offspring. Neurobehavioral tests; open-field test and Morris water maze test were operated on offspring at optimal age. We found no difference in growth indexes between As exposed offspring and control. Similar tendencies were also observed in neurobehavioral test. However high concentration of As were detected in the brain and liver of the offspring. Although biological exposure monitoring on As showed significant difference, we did not find the appreciable influences in the offspring. But we could not deny the possibility of faint appearing minor effects on CNS of offspring. So we analyzed neurotransmitter and their metabolisms in the brain from offspring by using HPLC and bio-technological ways. The results are helpful to understand the functional influences of CNS by chronic As exposure in the development period.

### NEUROBEHAVIORAL CHANGES IN METALLOTHIONEIN-NULL MICE PRENATALLY EXPOSED TO MERCURY VAPOR.

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We studied the neurobehavioral effects of prenatal exposure of MT-null and wild-type mice to elemental mercury vapor (Hg0). Pregnant mice of both strains were repeatedly exposed to Hg0 vapor at 0.50 and 0.56 mg/m<sup>3</sup> for 6 h per day until the 18th day of gestation. The behavioral effects were evaluated with locomotor activi-

ty in the open field, learning ability in the passive avoidance response and spatial learning ability in the Morris water maze at 12 weeks of age. Hg0-exposed MT-null mice showed a significant decrease in total locomotor activity in males, and a learning disability in the passive avoidance response and a retarded acquisition in the Morris water maze in females as compared with the control. In contrast, Hg0-exposed wild-type mice did not differ from controls in three behavioral measurements. The results indicate that MT-null mice would be more susceptible than wild-type mice to the behavioral neurotoxicity of prenatal Hg0 exposure. Mercury concentrations in the brain of both strains were slightly higher in the exposed group than in the control group, indicating the retention of residual mercury even 12 weeks after the cessation of the exposure. Brain concentrations of mercury were also significantly higher in the exposed-females than exposed-males in either strain. From these results, we suggest that the increased susceptibility of MT-null females to behavioral changes caused by prenatal Hg0 exposure is due to a greater retention of mercury and lack of MT-I, -II in the brain.

### MERCURY EXPOSURE OF MOTHERS AND NEWBORNS IN SURINAM: A PILOT STUDY.

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Aim: To study mercury levels in hair from mothers and newborns in Surinam. Methods: Hair and urine was collected from thirty-nine mothers of different ethnicity and hair from their newborns. The women delivered in a large hospital in the capital. Mercury analysis was performed. Results: Fourteen (36%) of the mothers had elevated Hg concentrations in the hair as compared with a reference US population. Thirty-one newborns (80%) presented with a higher mercury level in hair than their mothers. A positive correlation existed between the hair levels of the mothers and their newborns. Urine Hg concentrations were not elevated. Conclusion: Mercury accumulates during pregnancy in the unborn. Further study is needed to elucidate the background and consequences of this finding.

### THE EFFECTS OF TRIBUTYLTIN (TBT) CHLORIDE ON DOPAMINE METABOLISM IN BRAIN TISSUES OF FEMALE MICE AFTER SUBACUTE ORAL EXPOSURE.

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Tributyltin (TBT) compounds have been used as antifouling agents, and they are known as environmental pollutants and endocrine disruptors. One of target organs of TBT compounds is the central nervous system. We evaluated the neurotoxicity of TBT chloride in female mice following subacute oral exposure by determining the levels of neurotransmitters (norepinephrine, dopamine (DA), serotonin) and their metabolites (dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindoleacetic acid) in discrete brain regions using HPLC. Female BALB/c mice were exposed to 0, 25, 50, 125 or 250 ppm TBT chloride in their feed for one month. Following the treatment period, liver, kidney and spleen of each mouse were removed, and brain was dissected into six brain regions. The mean body weight of mice treated with 250 ppm TBT was significantly lower than those of the other groups. For relative organ weight, the mean relative liver or kidney weight of the high dose groups were significantly higher than the control or low dose groups. While, the mean relative spleen weight increased dose-dependently, and that of the 250 ppm group was significantly higher than those of other groups. HVA in the cerebrum of the 250 ppm group was significantly higher than other groups. DA in the medulla of the high dose-treated groups was significantly lower than that of the control. HVA/DA ratio in the medulla or midbrain of the 250 ppm group was significantly higher than that of control. Although there were differences of the effects of TBT on DA and DA metabolites among brain regions, TBT at the higher doses may increase DA turnover in brain of female mice.

### BLOOD BRAIN BARRIER (BBB) DISRUPTION AFTER VANADIUM INHALATION.

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Regulation of blood brain exchange is accomplished by ependymal cells which possess intercellular tight junctions. Loss of BBB function is an etiologic component of many neurological disorders. Vanadium (V) is an element widely distributed in the

environment and exerts potent toxic effects on a wide variety of biological systems. We reported previously that V produced dopaminergic cell death in substantia nigra and alterations in striatal neurons. Our aim was to investigate if V enters the SNC through the ventricles analysing the structure of the ependimal cells. CD-1 male mice 35g inhaled 0.02  $\mu\text{M}$  V2O5, one hour twice a week for 8 weeks. Mice were sacrificed after the 2nd, 4th, 6th, and 8th inhalation. The ventricle fragments were processed for scanning and transmission electron microscopy. The difference between the control and the exposed ependyma was the loss of cilia and the disruption between ependymal cells junctions. This damage might allow toxicants to easily access to underlying neuronal tissue releasing inflammatory mediators causing injury and neuronal death. The recognition of the mechanisms of BBB disruption would allow planning strategies to protect the brain from toxicants such as metals, which are related with neurotoxicity. Supported in part by DGAPA IN\_204304 and P.O.S Institute.

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CYTOTOXIC EFFECTS OF TELLURIUM TETRACHLORIDE AND (4-METHOXYPHENYL) TRICHLOROTELLURIUM ON RAT HIPPOCAMPAL ASTROCYTES.

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Tellurium is a rare metalloid that is used in the manufacturing of rubber, in photography, as a component of pesticides, and as a byproduct of copper processing. High concentrations of naturally occurring tellurium may be found in dairy products, nuts, fish and garlic, which accumulates tellurium from the soil. Tellurium has a similar electronic configuration to selenium, but does not have any known biological functions. Inorganic and organic tellurium compounds have been shown to be highly neurotoxic in rodents, causing peripheral demyelination. Chronic exposure to tellurium dioxide in rats, has been shown to cause tissue necrosis and decreased growth. Increased industrial use of this compound resulting in increased exposure may prove cause for concern. The purpose of this study was to investigate the cytotoxicity of two tellurium containing compounds on rat hippocampal astrocytes. Tellurium tetrachloride, and the organometalloid compound, 4-methoxyphenyl trichlorotellurium, were used to evaluate their cytotoxic effects. Rat hippocampal astrocytes were treated for 24h with the following concentrations of each compound: 500, 250, 125, 62.5, 31.25, 15.68, 7.81, 3.91, 1.95, 0.98 0.49 $\mu\text{M}$ . Viability was measured by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium (MTT) reduction based assay. Cells treated with tellurium tetrachloride showed a significant decrease in cell viability at all concentrations compared to control. Significant increases in cell viability were seen at the 62.5 $\mu\text{M}$  and lower concentrations when compared to the higher treatment groups. 4-methoxyphenyl trichlorotellurium showed markedly greater toxicity, with a significant reduction in cell viability seen at all concentrations except the 0.98 and 0.49 $\mu\text{M}$  doses. Light and scanning electron microscopy showed severe alterations in cell architecture. Loss of processes, cytoplasmic blebbing and fragmentation were observed.

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TMT-INDUCED NEUROGENESIS IN THE ADULT MOUSE HIPPOCAMPUS.

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Neurogenesis in the hippocampus of the mammalian brain continues from birth through adulthood and serves to maintain CNS function by compensating for cell loss due to aging or injury. The ability of a neurotoxic lesion to promote neurogenesis was examined in the hippocampus of adult mice given trimethyltin (TMT). C3H mice were dosed with bromodeoxyuridine (BrdU), a marker of cell proliferation, and 2.7 mg/kg TMT. Brains were removed for immunohistology on days 1-28 post-treatment. Onset of neurogenesis was observed within 48 hours after TMT exposure and continued at an accelerated rate for at least 4 days after TMT injury. Twenty-eight days after TMT exposure a significant number of BrdU+ cells remained visible in the hippocampal region, including the dentate gyrus and molecular layers. These cells also stained positive for the neuronal marker NeuN. TMT exposure did not appear to effect BrdU incorporation into cells in the ventricular zone (VZ), known to harbor stem cells and rapidly dividing neuronal precursors. To determine if the hippocampal BrdU+ cells were derived from the VZ, mice were injected i.c.v. with the carbocyanine dye DiI prior to TMT exposure. Twenty-eight days after TMT injection, DiI+ cells were detected in the hilus and granule cell layers of the dentate, and the molecular layer adjacent to the ventral arm of the dentate. Approximately 15% of the BrdU+ cells in the dentate gyrus co-labeled with DiI indicating a peri-ventricular origin. The DiI/BrdU co-labeled cells appeared to be GFAP— and may represent new neurons. These studies indicate that the regenerated cells in the chemically damaged hippocampus are likely daughter cells produced from nascent precursors within the hippocampus, as well as cells migrating from the VZ to the site of injury. (ES 11256, ES 10153, ES 05022)

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NEURONAL MICROTUBULE DAMAGE CAUSED BY *IN VITRO* EXPOSURE TO LEAD AND METHYLMERCURY.

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The heavy metal contaminants lead and methylmercury (MeHg) continue to pose human health risks, particularly to children. Numerous studies have described the cytotoxicity of either lead or methylmercury, but their interactive effects are poorly understood. This study examined the effect of joint exposures to lead and MeHg on the microtubule (MT) network, previously shown to be affected by the metals. Undifferentiated P19 embryonal carcinoma (EC) cells and their neuronal derivatives were treated with lead citrate (1.5, 3, 10, 50, and 100  $\mu\text{M}$ ) alone or in combination with methylmercury (1.5 and 3  $\mu\text{M}$ ). The microtubule network was stained for  $\alpha$ -tubulin or neuron specific  $\beta$ -III tubulin and examined by immunofluorescence. At 3  $\mu\text{M}$ , MeHg induced nearly complete MT disassembly in undifferentiated cells and loss of neurites and perikaryal MTs in EC derived neurons. At 1.5  $\mu\text{M}$  MeHg, partial MT disassembly was noted in undifferentiated cells, while neurites were retracted and perikaryal MTs were reduced in the neuronal derivatives. In contrast, MT injury was seen only at the 50 and 100  $\mu\text{M}$  lead concentrations. Joint exposures to lead and MeHg did not result in enhanced MT toxicity except at 1.5  $\mu\text{M}$  MeHg/100  $\mu\text{M}$  lead. Undifferentiated P19 cells were significantly more sensitive to MeHg and high concentrations of lead, presumably because of the high content of tyrosinated tubulin. Neuronal differentiation confers greater resistance to the metals as indicated by the increased stability of the MT network in D5 and 7 neurons. These observations suggest that lead does not significantly contribute to cytotoxicity caused by a joint exposure with methylmercury until high concentrations are reached. (Supported by NIH ES05022 and NIH/EPA ES011256)

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THE EFFECTS OF MANCOZEB ON THE MICROTUBULAR ARCHITECTURE OF RAT HIPPOCAMPAL ASTROCYTES.

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Mancozeb [Manganese-zinc ethylenebis(dithiocarbamate)] is a polymeric dithiocarbamate fungicide that has been used commercially for decades. It has recently been reported to severely alter olfactory neurophysiology. It has also been shown that manganese inhibits motor proteins that link membrane organelles along the secretory pathway to the cytoskeleton. Astrocytes play a central role in manganese (Mn) regulation in the CNS. Nevertheless, no adequate experimental studies on the effects of this divalent cation particularly in the form of a potential environmental toxicant on the cytoskeleton of astrocytes, has been reported. Rat hippocampal astrocytes maintained in Dulbecco's Modified Eagle's Medium with 10% Fetal Bovine Serum (FBS) were exposed to a range of concentrations of Mn-Zn dithiocarbamate for an hour at 37°C. Trypan blue exclusion was carried out to determine cell viability. After 1 hour of treatment a dose related effect with treatment was observed. Viabilities of 94.1%, 68.5%, 41.5%, 13.7% and 10.4% were seen with 5 $\mu\text{M}$ , 10 $\mu\text{M}$ , 12.5 $\mu\text{M}$ , 15 $\mu\text{M}$  and 20 $\mu\text{M}$  doses, respectively. The long term effect on astrocyte cytotoxicity/recovery was also investigated. Astrocytes were treated with low doses of Mancozeb for one hour (5 $\mu\text{M}$ , 7.5 $\mu\text{M}$ , 8.75 $\mu\text{M}$  and 10 $\mu\text{M}$ ) and then refed with complete medium and left to recover for 24 hours. Viabilities of 86.7%, 61.1%, 45.6% and 22.9% were seen. The 7.5 $\mu\text{M}$  dose of Mancozeb was chosen to study its effect on microtubular architecture in the astrocytes during this recovery period. Quantum dot biotin/streptavidin confocal microscopy directed against alpha-tubulin revealed severe alterations in microtubular architecture resulting from Mancozeb. These changes included condensation of tubulin with a loss of fibrillar structure, and a perinuclear loss of microtubular architecture. No effect on the marginal band was noted.

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NEUROPROTECTIVE EFFECTS OF MELATONIN ON NEUROBLASTOMA CELLS EXPOSED TO LEAD.

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The nervous system is the primary target for the low-levels of lead (Pb)-exposure and the developing brain appears to be especially vulnerable to Pb-neurotoxicity. Chronic low-levels of Pb-exposure cause growth retardation and intellectual impairment. In the present study the impact of low-levels of Pb on human SH-SY5Y neuroblastoma cells were assessed. The neuroblastoma cells were cultured in RPMI 1640 medium with 10% fetal-calf serum in a humidified air/5% CO<sub>2</sub> chamber at 37°C. The cells were exposed to Pb acetate (0.01-10  $\mu\text{M}$ ) for 48 hrs and proliferation was determined. The cell viability was determined using MTT reduction assay. Pb inhibited the proliferation of neuroblastoma cells in a concentration-dependent

manner. A 50% inhibition ( $IC_{50}$ ) in the proliferation of cells was observed typically at 5  $\mu$ M Pb. At 0-100  $\mu$ M melatonin, a metal chelating agent did not elicit any significant effect on growth and proliferation of these cells are exposed for 48 hrs. However, melatonin significantly reduced the inhibitory effect of Pb (at 5  $\mu$ M) on proliferation of these cells suggesting a protective effect against Pb-toxicity. The data also suggest that even low-concentrations of Pb can influence growth and development of brain leading to cognitive dysfunction in Pb-exposed children. These results further support our recent findings of detrimental effects of low-level Pb-exposure on neuronal dysfunction (This research work supported by NIGMS/NIH grant # GM 60853).

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#### BLOCKAGE OF INTERLEUKIN-6 SECRETION FROM ASTROGLIA BY LEAD: INVOLVEMENT OF GRP78.

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The involvement of lead (Pb), a developmental neurotoxicant, in neurologic disorders is consistent with both an astroglial role in neurologic disorders and Pb deposition in astroglia. However, the mechanism of Pb neurotoxicity is still not clear. Interleukin-6 (IL-6) is a cytokine, which is produced mainly by microglia and astroglia and plays a pleiotropic role including neuroprotection in the central nervous system (CNS). IL-6 secretion by astroglia is chaperoned by a 78 kD glucose-regulated protein (GRP78). Pb strongly binds to GRP78 and alters GRP78 compartmentalized redistribution. Thus, in this report we hypothesize that Pb can block IL-6 secretion from astroglia via binding to GRP78. In rat primary astroglial cultures, IL-6 secreted into the medium was stimulated by neuron-conditioned medium as detected by dot-blot assay. Pb at 1 to 10  $\mu$ M but not 50  $\mu$ M reduced IL-6 levels in the medium. This phenomenon was explained by the observation that the higher concentration of Pb induced IL-6 gene expression as assayed by RT-PCR. The reduction of IL-6 in the medium was in part contributed to by the impairment of IL-6 secretion, because IL-6 retention significantly increased in Pb-treated astroglia as detected by the fusion fluorescence protein image analysis. GRP78 depletion by approximately 35% with vector-based dsRNAi also significantly increased IL-6 retention in astroglia, supporting the involvement of GRP78 in IL-6 secretion. Co-localization of IL-6 with GRP78 in live astroglia, as determined by the image analysis of two differently colored fusion fluorescence proteins, further supports a chaperone role of GRP78 in IL-6 secretion. Therefore, these data indicate that Pb blocks IL-6 secretion and GRP78 probably mediates this process in astroglia.

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#### MANGANESE INDUCED APOPTOSIS IN SH-SY5Y HUMAN NEUROBLASTOMA CELLS.

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Excessive exposure to Manganese (Mn) has been shown to cause Manganism, a neurodegenerative disorder similar to Parkinson's disease. Although Mn toxicity has been studied for years, the mechanism by which Mn causes neurons to degenerate is still poorly understood. In recent years, the use of the manganese compound, MMT, as a gasoline additive has gained approval for use in the US. This could result in increased exposure to airborne manganese. Differentiated SH-SY5Y Human Neuroblastoma cells have been demonstrated to represent a valuable neuronal model to study the effects of neurotoxic drugs on cells in culture. Experiments conducted with this model, investigate the mode of cell death induced by Mn<sup>2+</sup>. To determine the effect of Mn<sup>2+</sup> on cell viability, SH-SY5Y cells were incubated with drug concentrations up to 500  $\mu$ M at 24, 48, and 72 hours, followed by morphological and biochemical analyses. The present study demonstrated that exposure to Mn<sup>2+</sup> caused loss of viability in differentiated SH-SY5Y cells at concentrations of the metal as low as 100  $\mu$ M. Morphological evaluation of Mn<sup>2+</sup> treated differentiated SH-SY5Y cells showed nuclear condensation and fragmentation as visualized by DAPI and *in situ* TUNEL staining at the following concentrations (1.953 and 62.5  $\mu$ M). These findings suggest that Mn-induced cell death occurs by apoptosis. The results of this study will help to elucidate the mechanism of Mn-induced cell death and allow for the design of neurotoxicity preventative strategies.

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#### THE MODULATION OF METALLOTHIONEIN-3 (MT-3) EXPRESSION IN MOUSE ASTROCYTES IN RESPONSE TO PROINFLAMMATORY MEDIATORS, SECOND MESSENGER ACTIVATORS, AND CELL STRESS.

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A common feature of many neurodegenerative diseases is the decreased expression of MT-3, a protein first identified as the growth inhibitory factor missing in Alzheimer's disease (AD). Due to this protein's neuroprotective properties (protec-

tion against  $\beta$ -amyloid toxicity and oxidative stress), the ability to increase the level of this protein during neurodegeneration could potentially slow the progression of the disease. Paradoxically, acute brain trauma such as stab wounds causes an increase in MT-3 expression several days after the insult. In an effort to identify factors that may be responsible for the modulation of MT-3 expression in the brain under the above pathological conditions, cultures of isolated mouse astrocytes were exposed to a series of candidate agents consisting of proinflammatory mediators [lipopolysaccharide and interleukin-1 $\beta$  (IL-1 $\beta$ ), cellular stress (sodium azide), general activators of second messenger pathways [tetradecanoyl phorbol acetate, dibutyryl-cyclic adenosine monophosphate (Bt<sub>2</sub>-cAMP), and calcium ionophore, (A23187)], a growth factor [epidermal growth factor (EGF)], and a known inducer of other metallothionein isoforms (zinc). The expression of MT-3 was monitored by real time PCR and normalized to the expression of glyceraldehyde phosphate dehydrogenase in exposures extending out to 8 days. Acute exposure to EGF and Bt<sub>2</sub>-cAMP (24 hrs) and sodium azide (4 hr exposure followed by 18 hr recovery) induced MT-3, 20-30%. During 8 day exposure to Bt<sub>2</sub>-cAMP, MT-3 was decreased 40%, whereas cells recovering from 4 hr of azide treatment still exhibited induced levels. Long-term exposure to zinc caused a 90% increase whereas at 24 hr no increase was noted. Exposure to proinflammatory mediators both acutely and chronically caused nearly a 50% decrease in MT-3 expression. Since chronic IL-1 $\beta$  exposure can down regulate MT-3, and sustained elevated levels of this cytokine have been found in AD lesions, this mediator may play a role in the modulation of MT-3 expression in neurodegenerative disease

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#### MANGANESE-INDUCED ALTERATIONS IN NF- $\kappa$ B-RELATED GENE EXPRESSION BY ACTIVATED MICROGLIA.

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The central nervous system is uniquely sensitive to inflammation and the brain microglia are a primary source of proinflammatory cytokines. In previous work we demonstrated that manganese and lipopolysaccharide in combination (Mn+LPS) potentiate microglial production of proinflammatory cytokines such as IL-1 $\beta$ , IL-6, and TNF- $\alpha$ . Microglial cells (N9) were exposed to up to 500  $\mu$ M MnCl<sub>2</sub> either by itself or combined with LPS (100 ng/ml). The Mn+LPS combination elicited dose-dependent cytokine production that was substantially greater than that induced by Mn or LPS alone. To determine the mechanism of Mn-induced potentiation of cytokine production, the early NF- $\kappa$ B-signaling pathway genes were examined by utilizing a pathway-specific gene array. N9 cells were exposed to Mn, LPS, or Mn+LPS for various time periods. At the end of exposure, RNA was isolated and cDNA synthesized to probe the gene array. In comparison to control cells, 1 hour exposure to Mn (250  $\mu$ M) and LPS (100 ng/ml) increased the mRNA expression for the TNF-receptor associated factor-1 (TRAF1) and GM-CSF. Both GM-CSF and TRAF-1 are known to promote cell growth while TRAF-1 may also induce proinflammatory cytokine synthesis by a NF- $\kappa$ B-dependent mechanism. Expression of the proinflammatory molecule complement component 3 (C3) was also increased following Mn+LPS exposure. By examining the time-dependent expression of these (and other) growth and inflammatory factors, we hope to elucidate the possible mechanism(s) for the Mn-induced proinflammatory cytokine production. (Supported by NIEHS ES11654).

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#### DIFFERENTIAL GENE EXPRESSION AT TWO DOSES OF METHYLMERCURY IN MOUSE CEREBELLA AS ANALYZED BY APOPTOSIS-SPECIFIC MICROARRAY.

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The neurotoxicant methylmercury (MeHg) is known to cause death of granule cells within the cerebellum. Although apoptosis has been implicated in this death, the specific apoptotic pathway is unknown. In this study, C57BL/6J mice at postnatal day (P) 34 were administered a total dose of 5.0 mg/kg or 1.0 mg/kg body weight methylmercury chloride via their food over 5 days. Cerebella from control mice and those exposed to MeHg were extracted on P50 following a 12 day latent period after final dosing. Genetic expression was analyzed in triplicate (12 mice total per treatment, 4 cerebella were pooled for each comparison) using apoptosis-gene-specific microarray analysis. Up-regulated genes (2+ fold compared to control) in the 5.0 mg/kg treated mice include: caspase-6, DR-6, Fasl, IL-15, IL-1ra, LIGHT, TRANCE and iNOS. Down-regulated genes include GAS2 and TNF R1. Gene alteration in the 1.0 mg/kg treated mice differed dramatically from those in the high dose group both in specificity and direction. The bulk of these genes was down-regulated (2+ fold) and include: A1, Apaf 1, Bax-a, Cyclin G1, NF- $\kappa$ B DNA binding subunit, RBBP4 and p53. While a total dose of 5.0 mg/kg MeHg results in primarily an up-regulation of genes involved in apoptosis, a total dose of 1.0 mg/kg

MeHg appears to have the opposite effect. In addition, the up-regulated genes observed in the 5.0 mg/kg treated mice are mainly cell surface receptor and immune associated, whereas the low-dose down-regulated genes are primarily mitochondrial and cell cycle related. It may be that significant cell death has already occurred in the 5.0 mg/kg treated cerebella and the analyzed cells are survivors of the high level MeHg insult. The cells analyzed at 1.0 mg MeHg may represent a different population that has not undergone cell death and is attempting to survive a lower MeHg insult.

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#### FORMATION OF REACTIVE OXYGEN SPECIES IN CEREBELLAR GRANULE CELLS OF MICE EXPOSED TO METHYLMERCURY.

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Methylmercury (MeHg) is a potent neurotoxicant that causes neuronal degeneration; neocortical and cerebellar granule neurons are particularly sensitive to MeHg toxicity. Studies using cultured granule cells exposed to MeHg demonstrate altered intracellular calcium ion homeostasis leading to increased intracellular calcium concentrations and cell death. Key gaps still exist concerning the specific mechanisms that cause granule cell death following *in vivo* MeHg exposure. Previously we found that mitochondrial membrane potential (MMP) was decreased in MeHg treated mouse cerebellar granule cells. Therefore, we postulate that *in vivo* MeHg exposure decreases MMP, alters mitochondrial function and potentiates reactive oxygen species (ROS) generation and that ROS species promote apoptosis. Male and female C57BL/6J mice at postnatal day (P) 29 were divided into control, 1.0 mg/kg and 5.0 mg/kg MeHg treatment groups and trained to eat moistened rodent chow for 5 days. At P34 mice were exposed to MeHg orally via moistened rodent chow. Cerebellar granule cells were acutely isolated at P50 and plated into chamber slides. Cells were loaded with 5-(and 6-) chloromethyl-2', 7'-dichlorodihydro fluorescein diacetate, acetyl ester (CM-H2DCFDA), a cell-permeant indicator for ROS that remains nonfluorescent until modified by intracellular esterases followed by oxidation occurring within the cell. Sequential time course analysis was performed; fluorescent images of granule cells from all three groups were acquired at 90-second intervals for 24 minutes. ROS species generated in granule cells from 1.0 mg/kg and 5.0 mg/kg MeHg treated mice were significantly higher than from controls at 24 minutes. Performance on footprint and vertical pole test is being analyzed to assess motor coordination. These observations suggest that *in vivo* MeHg exposure results in accumulation of ROS in cerebellar granule cells, which ultimately could promote apoptotic cell death and/or dysfunction of these cells. This work was supported in part by NIEHS (CERH) support to LCA (P30EF09106).

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#### METHYLMERCURY (MEHG)-INDUCED REACTIVE OXYGEN SPECIES (ROS) LEVELS ARE MODULATED BY INTRACELLULAR REDOX.

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Excessive free radical formation has been implicated in neurotoxic damage associated with MeHg exposure. The present study addresses the relationship between MeHg and cellular redox levels in neonatal rat cerebral astrocytes by employing laser scanning confocal microscopy (LSCM). We utilized three redox-sensitive fluorescent probes, CM-H2DCFDA (chloromethyl derivative of dichlorodihydrofluorescein diacetate), a probe for intracellular hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) detection; hydroethidine (HETH), a probe for superoxide anion (O<sub>2</sub><sup>-</sup>) detection; and CM-H2XRos (chloromethyl derivative of dihydro X-rosamine), a probe selective for mitochondrial ROS. Exposure of astrocytes to MeHg (10 uM; 30 min.) led to a significant (p<0.05) increase in mitochondrial as well as cytoplasmic ROS formation, and this effect was potentiated by pretreatment (24 hrs) with buthionine sulfoxane (BSO), a glutathione (GSH) depleter. Notably, mitochondria were the cellular organelles to exhibit the earliest increase in ROS production, preceding any other cellular site. Accordingly, mitochondria appear to play a prominent role in MeHg-induced injury. Treatment of astrocytes with MeHg (10 uM; 30 minutes) following pretreatment (24 hrs) with L-2-oxothiazolidine-4-carboxylic acid (OTC; an agent known to increase intracellular GSH levels) attenuated MeHg-induced ROS formation to levels statistically indistinguishable from controls. The free radical scavenger n-propyl gallate (n-PG) was equally effective in attenuating MeHg-induced ROS formation. In contrast catalase (H<sub>2</sub>O<sub>2</sub> scavenger) was ineffective in reducing MeHg-ROS formation in the astrocytes. Taken together, these studies suggest that (1) MeHg increases ROS formation; (2) GSH levels can modulate MeHg-induced ROS formation; (3) there is specificity for the effectiveness of antioxidants in protecting against MeHg-triggered oxidative stress. Supported by PHS grant NIEHS 07331.

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#### EFFECTS OF METHYLMERCURY ON MITOCHONDRIAL FUNCTION AND REACTIVE OXYGEN SPECIES FORMATION IN STRIATAL SYNAPTOSONES FROM RAT.

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Methylmercury (MeHg) is an environmental neurotoxicant. Several mechanisms have been suggested for its toxicity, including effects on calcium homeostasis, the cytoskeleton, mitochondrial function, formation of reactive oxygen species (ROS), and apoptosis and necrosis. The object of this study was to examine the relationship between ROS formation and mitochondrial function in isolated nerve terminals (synaptosomes) from the striatum of rats. We found that MeHg at concentrations of 0.5  $\mu$ M or higher decreased mitochondrial membrane potential ( $\Delta\Psi_m$ ), measured by the fluorescent dye JC-1 (Molecular Probes Inc.). Concentrations of MeHg of 1  $\mu$ M or greater lead to a total loss of  $\Delta\Psi_m$ , comparable to that seen after exposure to the mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP). Because MeHg can increase ROS formation in synaptosomes, and ROS can lead to mitochondrial dysfunction, we also measured ROS formation after MeHg exposure. MeHg increased ROS formation, measured by 2', 7'-dichlorofluorescein oxidation, in rat striatal synaptosomes. The effects were both time- and concentration dependent, but statistically significant effects were not evident unless MeHg concentrations were 10 times higher than those that reduced  $\Delta\Psi_m$ . ROS formation was almost completely abolished by treatment with the antioxidant Trolox. In contrast, Trolox had no effect in protecting against loss of  $\Delta\Psi_m$ . From these data we conclude that loss of  $\Delta\Psi_m$  is an initial event in MeHg toxicity in striatal synaptosomes. The loss of  $\Delta\Psi_m$  may, in turn, lead to other events involving opening of the mitochondrial permeability pore, release of mitochondrial calcium, and formation of ROS. Supported by NIEHS grant 1P01ES11263 and EPA grant R829390 to RFS.

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#### POTENTIATION OF MITOCHONDRIAL SUSCEPTIBILITY, OXIDATIVE STRESS AND NF-KB SIGNALING FOLLOWING CHEMICAL MIXTURE (LEAD AND ARSENIC)-INDUCED NEUROTOXICITY.

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Lead (Pb) and arsenic (As) as neurotoxic metals, can produce a cognitive deficits, central and peripheral neuropathy in human. The mechanism responsible for these effects on the central nervous system is not clear. Previous studies in animals and cell culture models show that both arsenic and lead can produce neuronal cell death that may be related to oxidative stress. In the present study, the vulnerability of the cultured dopaminergic neurons to Pb and As mixture by characterizing the cytotoxic interaction between Pb and As and then examined the underlying mechanism. In mesencephalic cells (dopaminergic cell lines), lead (0, 10, 20, 50 and 100 uM) and arsenic (0, 10, 20, 50, and 100 uM) produced minimal reactive oxygen species (ROS) dose dependently over 24 hr period. When cells were exposed concurrently to these metals, a burst of ROS was noted. To test the role of mitochondrial viability in response to Pb and As mixture, MTT up-take assay were done as a mitochondrial marker in cultured neurons. Cells that were exposed concurrently to Pb and As showed reduced MTT-up-take in dose dependent manner over the control and Pb and As alone exposed cells. Further, incubation of cells with Pb and As induced alteration in redox sensitive transcription factor, NF- $\kappa$ B activity and its significant change over the Pb and As alone have been analyzed and results will be discussed with cell death mechanism. This explains the neurochemical basis of the cells vulnerability to chemical mixture induced toxicity and determine if any antioxidants prevent cytotoxicity.

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#### EFFECTS OF GABA<sub>B</sub> RECEPTOR ACTIVATION & INHIBITION ON METHYLMERCURY-INDUCED ALTERATIONS OF INTRACELLULAR CA IN RAT CEREBELLAR GRANULE NEURONS.

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Acute exposure of cerebellar granule cells to methylmercury (MeHg) causes a biphasic increase in intracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) which contributes to delayed cell death. The pathways responsible for these effects are not yet known. The goal of this study was to determine whether G-protein-coupled GABA<sub>B</sub> receptors contribute to the MeHg induced increase in [Ca<sup>2+</sup>]<sub>i</sub> in rat cerebellar granule cells in culture. Baclofen and phaclofen were used to either inhibit or stimulate GABA<sub>B</sub> receptor function respectively. Single cell microfluorimetry using the Ca<sup>2+</sup> sensitive fluorophore fura-2 was used to examine the effects of MeHg on [Ca<sup>2+</sup>]<sub>i</sub>. Two specific measurements were taken: time-to-onset for increases in [Ca<sup>2+</sup>]<sub>i</sub> after exposure

to MeHg, and the ratio of the increase in fura-2 fluorescence induced by MeHg divided by the maximum increase in fluorescence following depolarization with 40mM KCl. Cells received one of three treatments: 30 min pre-treatment with 200 $\mu$ M baclofen followed by 0.5 $\mu$ M MeHg and 200 $\mu$ M baclofen, 30 min pre-treatment with 200  $\mu$ M phaclofen followed by 0.5 $\mu$ M MeHg and 200 $\mu$ M phaclofen, or 0.5  $\mu$ M MeHg. To assess cell viability and  $Ca^{2+}$  buffering ability, each cell dish received a 2 min depolarization with 40mM KCl 12 min before MeHg and/or GABA<sub>B</sub> receptor modulation treatment was administered. Both baclofen and phaclofen altered the fluorescence response to KCl-induced depolarization in the absence of MeHg. However, neither baclofen nor phaclofen altered the time-to-onset for MeHg-induced increases in fura-2 fluorescence for either the first or second phase compared to cells treated with MeHg alone. Baclofen and phaclofen, when compared to MeHg alone, also didn't alter the change in ratio fluorescence during either the first or second phase. Therefore, modulation of the GABA<sub>B</sub> receptor did not affect the time-to-onset for increases in  $[Ca^{2+}]_i$  induced by MeHg. The pathways affected by GABA<sub>B</sub> receptor modulation may not play a role in  $[Ca^{2+}]_i$  increases caused by MeHg in cerebellar granule cells. Supported by NIH grant R01-ES03299.

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**COMPARATIVE EFFECTS OF METHYLMERCURY (MEHG) ON INTRACELLULAR CA IN RAT CEREBELLAR GRANULE & CORTICAL NEURONS IN CULTURE.**

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Cerebellar granule cells are very sensitive to the neurotoxic actions of MeHg after both *in vivo* and *in vitro* exposure. Reasons for this selective vulnerability are unclear. Acute exposure of granule cells to MeHg increases  $[Ca^{2+}]_i$ , an effect which leads to delayed cell death. The goal of this study was to compare the sensitivity of granule cells and cortical neurons to MeHg-induced alterations in  $[Ca^{2+}]_i$ . Cortical neurons are not sensitive to MeHg exposure *in vivo*; we hypothesized that they would also be less sensitive to MeHg-induced increases in  $[Ca^{2+}]_i$ . Single cell micro-fluorimetry using the  $Ca^{2+}$  sensitive fluorophore fura-2 and neonatal rat cerebellar granule and cortical cells in culture were used to compare the effects of MeHg on  $[Ca^{2+}]_i$ . The time-to-onset for increases in  $[Ca^{2+}]_i$  after exposure to MeHg were measured. We tested the hypothesis that if cerebellar granule cells are more sensitive to MeHg than other less-sensitive neurons, then there will be a more prominent or earlier fura-2 response than with cortical cells. In both cell types, MeHg caused a biphasic increase in fura-2 fluorescence. Increasing the concentration of MeHg reduced the time to onset of this effect. At 0.5 $\mu$ M MeHg the 1st phase increase occurred in cortical cells at  $13.78 \pm 1.81$  min while in granule cells it occurred at  $6.22 \pm 1.01$  min. The 2nd phase increase in cortical cells at 0.5 $\mu$ M MeHg was seen at  $18.05 \pm 1.53$  min; in granule cells it was seen at  $8.08 \pm 1.0$  min. At 1.0 $\mu$ M MeHg, 1st and 2nd phases occurred at  $4.14 \pm 1.24$  and  $6.21 \pm 1.27$  min, respectively, in cortical cells. Corresponding values for granule cells were  $1.81 \pm 1.13$  and  $3.41 \pm 1.18$  min respectively. Thus there is a trend toward more rapidly occurring increases of fura-2 fluorescence with MeHg in granule cells than in cortical cells. Whether this will reflect fundamental differences in sensitivity of  $Ca^{2+}$  buffering or  $Ca^{2+}$  entry pathways between the two cell types is not yet clear. Supported by NIH grant R01ES03299, and a summer undergraduate research fellowship from the McNair SROP Program.

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**EFFECTS OF SEMI-CHRONIC METHYLMERCURY EXPOSURE ON INTRACELLULAR CALCIUM LEVELS IN RAT UNDIFFERENTIATED PC12 CELLS.**

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Methylmercury (MeHg) is an environmental neurotoxicant which disrupts intracellular Ca levels ( $[Ca^{2+}]_i$ ) when applied acutely to neurons. This effect occurs in two kinetically distinct phases: the first phase consists of release of  $Ca^{2+}$  from intracellular stores. The second phase is due to entry of  $Ca^{2+}$  into the cytoplasm from the extracellular fluid, perhaps via voltage-gated  $Ca^{2+}$  channels. The objective of the current study was to determine if semi-chronic exposure to MeHg of a neuron-like cell would alter  $[Ca^{2+}]_i$ . Single cell microfluorimetry using the  $Ca^{2+}$  indicator fura-2AM was used to examine effects of MeHg on  $[Ca^{2+}]_i$ . Undifferentiated, neuron-like rat pheochromocytoma (PC12) cells were treated for up to 72 hrs with 50 or 100 nM MeHg. Two specific measurements were made: baseline fluorescence representing resting levels of  $[Ca^{2+}]_i$ , and the response to membrane depolarization using KCl (40 mM). Resting levels of fura-2 fluorescence were not significantly altered by exposure to MeHg. PC12 cells typically respond to KCl depolarization with a distinct increase in  $[Ca^{2+}]_i$ . However, when PC12 cells exposed to MeHg were depolarized with KCl, the corresponding fluorescence response increased in

amplitude in a time-dependent manner at 24, 48 and 72 hrs when compared to control responses. The amplitude of this response was greater at all time points after 100 nM MeHg exposure than 50 nM exposure. Thus, semi-chronic exposure to low concentrations of MeHg causes a time- and concentration-dependent disruption of  $[Ca^{2+}]_i$  homeostasis during activity in PC12 cells, but doesn't appear to affect resting  $[Ca^{2+}]_i$ . (Supported by NIH grants 5T35RR017491-02 and R01ES03299).

**626**

**EFFECTS OF THIMEROSAL ON NGF SIGNAL TRANSDUCTION AND CELL DEATH IN NEUROBLASTOMA CELLS.**

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Signaling through neurotrophic receptors is necessary for differentiation and survival of the developing nervous system. The present study examined the effects of thimerosal on nerve growth factor (NGF) signal transduction and cell death in a human neuroblastoma cell line (SH-SY5Y cells). Following exposure to 100 ng/ml NGF and increasing concentrations of thimerosal, we measured the activation of TrkA, MAPK and specific isoforms of PKC (delta and epsilon). In controls, the activation of TrkA MAPK and both isoforms of PKC peaked after 5 minutes of exposure to NGF and then decreased but was still detectable at 60 minutes. Concurrent exposure to increasing concentrations of thimerosal and NGF resulted in a concentration-dependent decrease in TrkA, MAPK and PKC activation, with an EC50 of approximately 50 nM. Cell viability was assessed by the LDH assay. Following 24 hr exposure to increasing concentrations of thimerosal, the EC50 for cell death in the presence or absence of NGF was 596.3 nM and 38.7 nM, respectively. Following 48 hr exposure to increasing concentrations of thimerosal, the EC50 for cell death in the presence and absence of NGF was 104.7 nM and 4.35 nM, respectively. This suggests NGF provides protection against thimerosal cytotoxicity. To determine if apoptotic versus necrotic cell death was occurring, oligonucleosomal fragmented DNA was quantified by ELISA. Control levels of fragmented DNA were similar in both the presence and absence of NGF. In both, thimerosal caused increased levels of fragmented DNA (apoptosis) at 100 nM, which decreased at higher concentrations of thimerosal (necrosis). These data demonstrates that thimerosal could alter NGF-induced signaling in neurotrophin-treated cells at concentrations lower than those responsible for cell death.

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**METHYL MERCURY (MEHG) ALTERS IL-6 SIGNAL TRANSDUCTION IN MOUSE CEREBELLAR CELLS IN CULTURE.**

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MeHg is highly toxic to the developing CNS in humans, rodents, and primates, and its effects on neurocognitive and neuromotor function are persistent, ranging from subtle developmental delays to severe motor dysfunction and death. These effects have been associated with disruption of neuronal migration early in development, producing irreversible structural changes in specific brain regions including the cerebellum. We and others have shown that cytokines, of microglial origin, play a critical role in stimulating and supporting the movement of neurons during critical periods of development. We showed that *in vitro* exposure of murine cerebellar cultures to a non-cytotoxic dose (100nM) of MeHg inhibits neuronal migration and increases production of IL-6 and TNF-alpha and increases expression of IL1-beta mRNA. We also showed that administering anti IL-6 antibodies blocks the effects of MeHg on neuronal migration. Using the same *in vitro* cell system we utilized the Affymetrix ToxChip system to evaluate a broad range of molecular targets of MeHg in LPS-activated or non-activated organotypic cultures prepared from cerebella of 5 day old CD1 mice. For those transcripts altered by at least two-fold, the effect was confirmed by ELISA and Western blot analysis. MeHg exposure induced expression of several genes, including cytokines, chemokines, and cytokine receptors, including IL1-beta, IL6-alpha receptor, chemokine (CXC motif) ligand 2, and chemokine (CXC motif) as well as other genes involved in cytokine-dependent signaling and cell adhesion molecules N-CAM2 and V-CAM. IL6 protein was not modified; however MeHg increased levels of gp130, and IL-6Ralpha proteins. MeHg decreased phosphorylated STAT3, and CXCR4, and increased NCAM protein. These results are consistent with the hypothesis that MeHg may act on the IL-6 pathway, with effects on NCAM and CXCR4 as part of the molecular mechanisms of neuronal disruption induced by MeHg in early CNS development. This work was supported by Cure Autism Now and Heinz Family Foundations.

ACUTE EXPOSURE TO URANYL ION (UO<sub>2</sub>) *IN VITRO* DIMINISHES K<sub>+</sub>-STIMULATED GLUTAMATE/GABA RELEASE INDEPENDENT OF EXTRACELLULAR CALCIUM.

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Gulf War veterans retaining fragments of depleted uranium (DU) shrapnel have exhibited lowered performance on neurocognitive tests, and rats chronically exposed to DU have displayed apparent decreases in neuronal excitability. The goal of this study was to determine if acute exposure to divalent UO<sub>2</sub> *in vitro* alters calcium-independent glutamate/GABA release in a manner similar to that of other heavy metals (e.g., lead). A crude hippocampal P2 fraction was prepared from 2 month old Sprague-Dawley rats, and 0.8 mg synaptosomal protein loaded onto glass fiber filters. Synaptosomes were superfused with phosphate-free 25 mM HEPES (pH 7.4) saturated with O<sub>2</sub> and containing 200  $\mu$ M methoxyverapamil with Mg<sup>2+</sup> replacing Ca<sup>2+</sup>. Release was stimulated with brief perfusion of high K<sub>+</sub>-HEPES containing a glutamate reuptake blocker. Concentrations of UO<sub>2</sub> nitrate (0.01 - 316  $\mu$ M) were added to perfusion solutions, and 2-min fractions were collected beginning 45 min after exposure was initiated. Aliquots were derivatized and quantified by binary gradient liquid chromatography with fluorescence detection. In the absence of UO<sub>2</sub> and Ca<sup>2+</sup>, K<sub>+</sub> stimulation resulted in ~2-fold elevation of glutamate and GABA concentrations over baseline values. Perfusion of UO<sub>2</sub> in the absence of Ca<sup>2+</sup> diminished endogenous evoked transmitter release compared to responses in control tissue, resulting in an approximate IC<sub>50</sub> of 5  $\mu$ M. Thus, its inhibitory potency was similar to that observed in the presence of Ca<sup>2+</sup> (~2.6  $\mu$ M). Other heavy metals are well known to exhibit Ca<sup>2+</sup>-mimetic properties that are best observed under Ca<sup>2+</sup>-free conditions, but the actions of forms of uranium on exocytosis have not been identified. It is possible that acute exposure is insufficient to permit accumulation of intrasynaptosomal UO<sub>2</sub> concentrations. The use of permeabilized synaptosomes would be an approach to address this issue. (Supported by USAMRMC grant DAMD17-02-1-0212)

EFFECTS OF LOW-LEVEL MN EXPOSURE ON THE METABOLISM OF AMINO ACIDS, GABA, AND CELLULAR RESPIRATION IN GABAERGIC AF5 NEURAL CELLS.

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Chronic elevated manganese (Mn) exposure has been associated with neurological disorders, while the effects and mechanistic basis of lower exposures remains controversial. Human occupational and recent animal model studies suggest that elevated Mn may first affect GABAergic function in the basal ganglia of the brain, with comparatively little or no effect in the nigro-striatal dopaminergic system until higher exposures are reached. To investigate the mechanistic basis for low-level Mn effects on GABAergic cells, a rat striatal cell line (AF5) was treated with Mn, and compared to treatments with other agents of known mechanisms of toxicity (rotenone, paraquat, fluorocitrate, and gabaculine). Outcome measures included intracellular amino acid content, activity of selected enzymes, and mitochondrial function. Results thus far have shown that Mn affected cellular metabolism differently than the other compounds. Mn treatment caused 15 - 20% increases in cellular GABA and glutamate (GLU), and a 40% increase in cellular GLU released into the medium. Culture medium glutamine (GLN) consumption was increased to 180% of control, while alanine release into the medium was decreased to 75% of control with Mn treatment. In contrast, rotenone caused a 30% decrease in cellular GLU levels while cellular GABA levels were increased by 15%. Activity assays of the enzymes responsible for the synthesis and degradation of GABA, glutamate decarboxylase (GAD) and GABA-transaminase (GABA-T), revealed no direct effect of Mn. Assay of mitochondrial Complex II activity similarly revealed no direct effect of Mn. These data indicate that significant alterations in amino acid metabolism, presumably due to altered mitochondrial function, occur in AF5 neural cells during sub-lethal Mn exposure, and that these effects may account for increases in tissue GABA observed in animal models treated with Mn.

MN ALTERS CELLULAR COMT-1, SECRETORANIN II, AND UCH-LI LEVELS IN PC12 CELLS: IMPLICATIONS FOR MN NEUROTOXICITY.

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Elevated manganese (Mn) exposures have been shown to cause neurotoxicity in cell and animal models, and humans. Cell and animal model studies have reported dopaminergic systems as targets of Mn, though results have differed widely with

Mn dose. Human studies have identified elevated Mn (and iron) exposures as risk factors for Parkinsonism, and occupational studies have indicated that elevated Mn exposures produce a Manganism syndrome. In spite of these parallels, the mechanistic basis underlying Mn exposures as a risk factor for Parkinsonism remains unclear. To better understand this, we utilized a 2-D differential in-gel electrophoresis (DIGE) method to evaluate changes in protein expression patterns in a dopamine (DA) producing PC12 cell model. Cells were exposed to 100  $\mu$ M Mn for 24 hours, followed by assessment of cellular Mn levels, cytotoxicity (trypan blue, LDH, ATP, 8-isoprostanes), DA and metabolites, and changes in protein expression identified through the 2-D DIGE analyses. Cellular Mn accumulation increased ~100-fold with Mn exposure, with no measurable change in cell viability, LDH, ATP, or 8-isoprostanes. Cellular DA levels were reduced by a significant 20%, while DA metabolites were increased 45 to 145 % over controls. Cellular serotonin levels did not change. A total of 46 proteins (out of ~600) were shown to change with Mn exposure (25 increased, 21 decreased expression), and 30 of these were identified. Several proteins related to DA metabolism (COMT-1), packaging (secretorgranin-II), and protein degradation (UCH-LI) measurably changed in a pattern consistent with the observed changes in DA and metabolites: COMT-1 was increased 47%, secretorgranin-II (all seven detected isoforms) was decreased 40%, while an isoform of UCH-LI increased 90% in expression in Mn exposed cells. These results were confirmed by Western blot analyses. These data suggest that elevated Mn exposure may contribute to reduced packaging and elevated turnover of cellular DA, and thereby alter DAergic function without causing overt cytotoxicity.

MANGANESE INHIBITS THE BINDING OF THE COCAINE ANALOG [<sup>3</sup>H]-WIN 35, 428 TO THE DOPAMINE TRANSPORTER (DAT) FROM RAT STRIATUM.

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Exposure to high levels of manganese (Mn) causes a Parkinsonian-like syndrome in humans and non-human primates. The striatum and globus pallidus are basal ganglia structures that exhibit the highest Mn concentrations following exposure. We have previously shown a transient increase in [<sup>1</sup>C]-WIN 35, 428 binding to DAT in the primate striatum *in vivo* after acute Mn administration using positron emission tomography (Chen et al., *Toxicology Sciences* 78 supp: 75, 2004). We suggested that DAT in dopaminergic terminals is a target for Mn in the brain. To investigate the molecular mechanisms of acute Mn-induced dopaminergic dysfunction, we used an *in vitro* assay to measure the effect of Mn on the binding of [<sup>3</sup>H]-WIN 35, 428 to DAT from rat striatal membranes. We found that Mn inhibits [<sup>3</sup>H]-WIN 35, 428 binding to DAT with an inhibitory constant ( $K_i$ ) of  $2.0 \pm 0.3$   $\mu$ M (n=4). To determine the nature of the inhibition we performed saturation isotherms and Scatchard analysis of [<sup>3</sup>H]-WIN 35, 428 binding to DAT with or without 2 mM MnSO<sub>4</sub>. We found a significant decrease (30%, p<0.001) in the maximal number of binding sites ( $B_{max}$ ) in the presence of Mn ( $1270 \pm 127$  fmol/mg protein; n=4) relative to control ( $1819 \pm 161$  fmol/mg protein; n=4). No significant effect of Mn was measured on binding affinity ( $K_d$ ) (Mn =  $24.0 \pm 2.4$  nM, n=4; control =  $20.0 \pm 1.6$  nM, n=4; p>0.05). These *in vitro* findings suggest that the increase in DAT levels *in vivo* following acute Mn administration may be a compensatory response to its inhibitory action on the transporter. Further, Mn-induced DAT inhibition *in vivo* may increase extracellular dopamine levels leading to DA autoxidation, generation of reactive oxygen species and neuronal injury. [Supported by NIEHS ES10975 to TRG]

DIFFERENTIAL EFFECTS OF MANGANESE (MN) AND MPP<sup>+</sup> IN DIFFERENTIATED AND NON-DIFFERENTIATED PC12 CELLS.

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Mn and 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) are well-known neurotoxins which can cause Parkinson-like symptoms in humans and non-human primates by affecting dopaminergic neurons. Several studies have also suggested that the dopamine transporter (DAT) may play a role in the neurotoxicity of these compounds. Therefore, we addressed the characterization of DAT and the differential cytotoxicity of MnCl<sub>2</sub> and MPP<sup>+</sup> in two types of PC12 cells, differentiated and non-differentiated. PC12 (rat pheochromocytoma) cells have been used as an *in vitro* model cell line of dopamine-containing neurons. Cell death was measured using Alamar Blue assay and DAT-mediated cellular uptake was measured by a radiolabeled-based uptake assay in non-differentiated and differentiated PC-12 cells. The two types of PC12 cells exhibited different responses and sensitivity to MnCl<sub>2</sub> and MPP<sup>+</sup>. The dose-time response curves revealed that MnCl<sub>2</sub> was more effective in producing cytotoxic effects in non-differentiated PC12 cells with EC<sub>50</sub> values of

>10, 2 and 1.2 mM at 24, 48 and 72 hr, respectively. But, in contrast to  $MnCl_2$ ,  $MPP^+$  failed to cause cytotoxicity in non-differentiated PC12 cells over a concentration range from 0.001 to 1 mM for exposures up to 72 hr. On the other hand, the pattern of cytotoxicity was reverse in differentiated PC12 cells.  $MPP^+$  significantly decreased cell viability in differentiated cells with  $EC_{50}$  values of 0.25 and 0.20 mM at 48 hr and 72 hr, respectively, while the  $EC_{50}$  value of  $MnCl_2$  was > 8 mM at 72 hr. In uptake experiments, specific [ $^3H$ ]GBR12935 uptake in non-differentiated and differentiated PC12 cells was temperature sensitive and time dependent; however, rates of activity were different in the two cell types. These results provide support for the hypothesis that DAT-mediated uptake of Mn and  $MPP^+$  plays a role in the selective dopaminergic neurodegeneration characteristic of Parkinson's Disease that occurs following exposure to these compounds.

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### NEUROTOXIC EFFECT OF MANGANESE AND NEUROPROTECTIVE EFFECT OF COPPER IN A CELL CULTURE MODEL OF PRION DISEASES.

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Prion diseases are fatal neurodegenerative disorders increasingly recognized in both humans and animals. Normal prion proteins (PrPc) are abundantly expressed in the CNS, but the effect of environmental agents on the physiological function of prion proteins remains to be characterized. Because PrPc have high affinity for certain divalent cations, we characterized in the present study the effect of copper (Cu) and manganese (Mn) on prion-expressing neural cells and prion-knockout (KO) cells. Mn and Cu induced dramatically different effects; Mn was much more toxic than Cu. In general, PrPc-expressing cells were less susceptible to metal toxicity than prion-KO cells, suggesting that endogenous prion protein may protect against metal toxicity. Interestingly, Mn treatment depleted cellular glutathione (GSH) levels more significantly and in a dose-dependent manner in both cells, whereas Cu significantly increased GSH in prion cells. Mn treatment also activated the apoptotic cascade; including caspase-9 and 3, in a time-dependent manner in both cells. However, caspase activation was not observed after Cu treatment. Likewise, Mn-treated cells showed a dose-dependent increase in DNA fragmentation, which was blocked by the caspase inhibitor ZVAD and ZDEVD, while DNA fragmentation was not significantly increased in Cu-treated cells. Trace element analysis through Induction Coupled Plasma Mass Spectrometry (IC-PMS) revealed that prion cells have higher basal Mn levels and lower basal Cu levels consistent with Cu deficiency observed in certain prion diseases. Mn and Cu accumulation was proportional to the respective treatments, but with different rates of accumulation. Collectively, these results demonstrate that the essential metals Mn and Cu produce opposing effects - neurotoxicity vs. neuroprotection in neuronal cells - possibly by altering the ion handling mechanism and antioxidant capacity. This novel observation may have some functional implications in the pathogenesis and/or progression of prion diseases. [support : Iowa Healthy Livestock Initiative grant]

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### MECHANISM OF ARSENIC PERIPHERAL NEUROTOXICITY.

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**Introduction:** Peripheral arsenic neurotoxicity has been demonstrated clinically and in electrophysiological studies. The aim of this study is to investigate the mechanism of the peripheral neurotoxicity of As in rats, with special focus on interactions with axonal cytoskeletal proteins. **Methods:** Male Wistar rats (225-250g) were exposed to arsenite ( $NaAsO_3$ ) given as a single dose iv (0; 15 or 20 mg/kg) for 3, 6 or 9 hrs. Urine samples were collected for the duration of exposure namely 3, 6 or 9 hrs, whereafter the rats were sacrificed. Sciatic nerves (N. ischiadicus) were excised and cytoskeletal proteins were analysed with SDS-PAGE and Western blot. Arsenic in urine, blood and sciatic nerve was measured with AAS. **Results:** The amount of arsenic in urine of 15-mg/kg groups decreased in the 9-hour time course from 0.5 to 0.25 (mM As/mM creatinine). Blood arsenic content diminished from 2.3 to 1.58 mg/l in rats group receiving 15 mg/kg doses and reduction of 2.9 to 2.34 mg/l in rats group receiving 20 mg/kg doses. Arsenic was present in sciatic nerve but did not correlate with doses and/or duration of exposure. Biochemical analysis of N. ischiadicus revealed disappearance of neurofilament protein bands and fibroblasts in rats treated with the arsenite doses of 15 and 20 mg/kg. Few fibroblast protein bands disappeared in 20-mg/kg doses analyzed by SDS-PAGE. Some neurofilament proteins diminished in dose/effect manner measured in time and analyzed with western blot. **Discussion:** Arsenic affects the composition of cytoskeletal proteins in the rat sciatic nerve, especially the neurofilaments. The loss of these neurofilaments could explain the neurotoxicological effects.



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### TOXIC TORTS: TOXICOLOGISTS IN THE COURTROOM.

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Litigation involving exposure to chemicals is on the rise in the United States. This has created a new role for toxicologists. Toxicologists are often called upon to provide testimony (e.g. expert, fact) in a variety of legal situations (e.g. court, deposition, hearings, state, federal, defense, and plaintiff). However, explaining extremely complex and sometimes controversial scientific concepts and data to a non-scientist (e.g. judges, juries) can be challenging. Therefore, knowledge of skills necessary for effective clear testimony, attorney/client expectations, and potential ethical conflicts are critical prior to entering the court room. In addition, a toxicologist called upon to provide litigation support should be aware of the potential positive and negative career impacts. This sunrise session will provide a forum to discuss issues a scientist functioning as an expert witness may encounter as well as give information that will be valuable for scientist called upon for expert testimony.



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### ALTERED IRON HOMEOSTASIS (AIH) AS A BASIS FOR PULMONARY IMMUNOTOXICOLOGIC EFFECTS OF PARTICULATE MATTER.

M. Cohen. *Environmental Medicine, New York University, Tuxedo, NY.*

The scientific literature is replete with reports on the pulmonary toxicologic and immuno-toxicologic effects of particulate matter (PM). Although it has become increasingly accepted that the composition of PM is a major factor influencing biological effects, mechanisms to describe how composition might induce observed toxicities are mostly lacking. The altered iron homeostasis (AIH) theory postulates that specific components in PM induce alterations in the levels of free catalytically-active iron within the lungs as well as in iron availability to both lung epithelial and immune cells. These changes, in turn, impact upon local responses to infectious agents and allergens, as well as upon the release of cell products that might contribute to cardiopulmonary changes. The AIH theory not only provides a basis to explain how select PM constituents might induce these effects, but also how day-to-day or regional differences in the amounts of these components (relative to that of iron) may underlie the variability in reported health effects induced with equivalent doses of differing PM samples. Following introductory talks about the role of iron homeostasis in maintenance of immune cell functions and how components of PM may be selectively mobilized, this symposium will highlight specifically how AIH could be the basis for the observed alterations in allergic, immunologic, and cardiopulmonary responses after host exposures to PM.



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### THE ROLE OF IRON (FE) HOMEOSTASIS IN IMMUNE CELL FUNCTIONALITY.

C. L. Bowlus. *Department of Internal Medicine, University of California Davis Medical Center, Sacramento, CA.* Sponsor: M. Cohen.

This introductory talk will review the ways in which the maintenance of iron homeostasis is critical to both the normal and induced functionalities of lymphocytes and macrophages. The reciprocal regulation of iron metabolism and immune function occurs at many levels and macrophages are central to both processes. Within endosomal vesicles of macrophages, iron transport and antigen presentation pathways converge. Reactive oxygen species generated from iron may uniquely fragment autoantigens or expose cryptic epitopes. The iron transporter NRAMP1 is primarily expressed in macrophages and is critical for macrophage activation and differentiation as well as expression of tumor necrosis factor- $\alpha$ , interleukin-1 $\beta$  and MHC class II. In turn NRAMP 1 is regulated by interferon- $\gamma$ . In addition, alleles of NRAMP1 are associated with susceptibility to pulmonary infections and autoimmune disorders. Recently, a signaling role for iron has been recognized in the NF-kappa B activation of macrophages. Abnormalities in iron homeostasis can also affect lymphocytes. Iron deficiency is associated with impaired cell-mediated immunity, T cell proliferation and decreased numbers of circulating T cells, likely secondary to thymic atrophy. Conversely, the effects of transfusion iron overload on lymphocytes include decreases in CD4 T cells and T cell proliferation. Mutations in the MHC class I-like gene HFE results in hemochromatosis, which is associated with many T cell abnormalities including decreased CD8 T cells and p56lck activity. However, these differences have not been replicated in animal models. Future studies are needed to elucidate the precise pathways by which iron alters macrophage and lymphocyte functions and how alleles of genes central to iron metabolism influence immune function, particularly as they relate to the immunotoxicity of particulate matter.



## 638 MOBILIZATION OF METALS FROM PARTICLES: IMMUNOTOXICOLOGIC IMPLICATIONS.

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Particulate air pollution contains iron that may be involved in pathological effects after inhalation. Ambient particulate samples and a specific combustion source, coal fly ash (CFA), contain iron that can be mobilized from the particle *in vitro* and inside cultured human lung epithelial cells. The mobilized iron can then catalyze the formation of reactive oxygen species. The amount of iron mobilized from the combustion particulate CFA was dependent on the source of coal used to generate the fly ash and was greatest in the smallest size fraction collected from three different coal sources. In addition, the amount of iron mobilized from the CFA in solution by citrate correlated closely with the amount mobilized in human lung epithelial (A549) cells, as indicated by induction of the iron storage protein ferritin. The amount of the proinflammatory cytokine, interleukin (IL)-8, secreted in response to CFA treatment varied with the amount of iron mobilized intracellularly from the particles, with the greatest response to the smaller size fractions which released the largest amounts of iron. There was a direct relationship, above a threshold level of bioavailable iron, between the level of IL-8 and bioavailable iron in cells treated with CFA. Radical scavengers, e.g. tetramethyl thiourea and dimethyl sulfoxide, prevented the increased production of IL-8 by A549 cells treated with CFA, suggesting the role of a radical species in the induction of this inflammatory mediator. The mobilization of iron from CFA by citrate or within human lung epithelial cells, as well as the induction of IL-8, did not correlate with the total amount of iron in the particles. Instead, these measured values correlated directly with the amount of iron contained specifically in the aluminosilicate fraction of CFA. This work emphasizes the importance of determining the specific iron species responsible for the bioavailable iron in particles, since this could aid in identifying sources and/or combustion factors responsible for generation of bioavailable iron. (This work supported by the Health Effects Institute)



## 639 METALS, PARTICLES AND IMPACT UPON PULMONARY ALLERGIC RESPONSES.

*M. Gilmour. National Health and Environmental Effects Research Laboratory, USEPA, Research Triangle Park, Durham, NC.*

The increase in allergic asthma over the past few decades has prompted investigations into whether air pollution may affect either the incidence or severity of allergic lung disease. Population studies have demonstrated that as air pollution rises, symptoms, medication use and hospital admissions for asthma attacks increase, and this has been confirmed in a variety of animal models. There is less epidemiological evidence for air pollution exposure directly contributing to increased incidence of asthma, although recent studies are showing that proximity to highways is a risk factor for development of disease. Clinical experiments have demonstrated that diesel particles can potentiate *de novo* allergic immune responses in human volunteers, and these effects are amply supported by a large body of animal experiments. These effects have recently been associated with increased oxidative stress in the mitochondria of pulmonary epithelial cells. While the ability of metals themselves to act as immunological adjuvants has received less attention, a study comparing two towns in Germany found that the town of Hettstedt, with more metal-related industries and metals in the ambient air, had increased incidence of allergic disease than a nearby town Zerbst, which was largely agricultural and office-based with less metals in the air. Mice treated with filter extracts from Hettstedt had increased allergic immune responses after sensitization with ovalbumin allergen compared to Zerbst treated animals and there was also increased lung disease following antigen challenge. This presentation will describe the how metals potentiate allergic lung disease and discuss connections with altered iron homeostasis and oxidative stress. This abstract does not reflect EPA policy.



## 640 EFFECTS OF PARTICLES ON FE TRANSPORT AND THE IMMUNOTOXICOLOGIC OUTCOMES.

*A. J. Ghio. National Health and Environmental Effects Research Laboratory, USEPA, Research Triangle Park, NC.*

While Fe is essential for many aspects of cellular function, it can also generate oxygen-based free radicals that can result in significant injury to biological molecules. For this reason, Fe acquisition and distribution must be tightly regulated. Constant exposure to the atmosphere, however, results in significant exposure of the lungs to catalytically active Fe associated with particles. The same proteins that participate in the highly regulated Fe uptake in the gut are also employed in the lung. Iron transported intracellularly in the lungs can be sequestered within ferritin to prevent generation of oxidative stress. These proteins also expedite a release of ferritin from lung tissue to the lining fluid for clearance by the mucociliary pathway or to the

reticuloendothelial system for longterm storage. This is likely a major method for control of oxidative stress in the respiratory tract. We hypothesized that the protein responsible for Fe uptake in airway epithelial cells, divalent metal transporter 1 (DMT1), participates in detoxification of metals associated with particulate matter. Exposure of human airway epithelial cells to oil fly ash unexpectedly resulted in decrements in both DMT1 mRNA and protein expression. Similar decreases expressions were demonstrated after exposure of cells to vanadyl sulfate, a metal in high concentration in this specific ash. Pre-exposures of the cells to Fe and V were associated with an increased and a decreased uptake of metal, respectively. Pre-exposure of an animal model to Fe was associated with an elevated expression of DMT1, a more rapid transport of metal out of the lung, and diminished injury after particle exposure; in contrast, V pre-exposure decreased metal transport and increased injury from the particle. Exposure of Belgrade rats (deficient in functional DMT1) to the same particle resulted in a decreased transport of metal out of the lung and a greater injury relative to control rats. We conclude that DMT1 expression is associated with both metal transport in the lower respiratory tract and injury following exposure to a particle.



## 641 DO ALTERED FE STATUS-INDUCED EFFECTS ON TRANSCRIPTION FACTORS HAVE A ROLE IN PM-INDUCED PULMONARY/CARDIOVASCULAR DISEASES?

*K. Salnikow. Laboratory of Comparative Carcinogenesis, National Cancer Institute, Frederick, MD.*

At-risk populations, i.e., individuals with pre-existing hypertensive disease and/or atherosclerosis, as well as inflammatory and allergic responses, appear to have overtly negative responses to exposure to particulate matter (PM). However, both the mechanisms underlying these effects and the constituents in PM that might be causing these outcomes remain unclear. We hypothesized that the metals in PM - by targeting intracellular ascorbate - are altering the iron status (i.e., increasing the ratio of Fe[III]/Fe[II]) in lung cells. This leads to the inhibition of all iron-containing cellular hydroxylases followed by the loss of HIF-1α hydroxylation and hypoxia-like stress. The loss of ascorbate is deleterious for collagen proline hydroxylation and extracellular matrix assembly. Additionally, the inhibition of cellular hydroxylases causes impairment of assembly of all protein molecules that have collagen-like domains, including surfactants A and C and complement C1q. Thus, the depletion of cellular ascorbate by metals provides a link between metal exposure and a variety of respiratory and cardiovascular diseases and, maybe, to a carcinogenic process. Experimental data will be provided to show that metals like nickel, cobalt and a few select others indeed deplete intracellular ascorbate in human lung cells and activate HIF-1 transcription factor and the battery of hypoxia-inducible genes. In the absence of ascorbate, the iron in cellular hydroxylases is oxidized to iron[III] and can be reduced back to iron[II] only by ascorbate.



## 642 BEYOND LIVER TOXICOGENOMICS: GENE EXPRESSION BASED BIOMARKERS IN NON-HEPATIC TISSUES.

*K. L. Kolaja. Toxicology, Iconix Pharmaceuticals, Mountain View, CA.*

Toxicogenomics, the genome scale analysis of chemically induced changes in complex populations of mRNA to understand toxicity, has already dramatically impacted predictive and mechanistic toxicology. Gene expression based biomarkers can provide a precise and rapid assessment of toxicity or exposure, but most toxicogenomics efforts to date have been conducted in liver due to toxicological interest, technical ease of data creation, relative homogeneity of tissue sample, and historical inertia. Recently analysis of gene expression data in non-hepatic organs and tissues has started to gain momentum, and this session will include presentations of significant and promising examples ranging from toxicogenomic analyses of vascular, cardiac, kidney, neural and reproductive tissues treated with various drugs, chemicals, and toxicants. From these burgeoning studies, researchers are identifying biomarkers predictive of toxicity and indicative of various mechanisms and pharmacology in non-hepatic tissues.



## 643 MEASURING GENE EXPRESSION IN BLOOD VESSELS: THE CHALLENGES OF APPLYING TOXICOGENOMICS TO VASCULITIS.

*M. P. Lawton. Molecular and Investigative Toxicology, Pfizer, Inc., Groton, CT.*

Vasculitis is defined as an inflammation of blood vessels. In preclinical toxicology testing, drug-induced vasculitis has been observed with a number of structurally and pharmacologically diverse compounds. Although several mechanisms of vascular toxicity have been proposed, the exact mechanism(s) by these drugs damage

blood vessels is not known. In addition, there are no specific biomarkers that can be used preclinically or clinically to either predict or diagnose vasculitis; histopathology is currently the only method to detect vasculitis. To help address the need for mechanistic understanding and specific biomarkers we have been using gene expression profiling as a tool to characterize this lesion. Like most tissues, blood vessels are comprised of multiple cell types and can be found in close association with surrounding connective tissues such as fat, pancreas or lymph nodes. This complexity, along with the small size of most vessels, present unique challenges when generating and interpreting gene expression data from this tissue. Some of these challenges, along with the use of newer technologies to overcome them, such as laser capture microdissection, will be discussed in this presentation.

#### **644** GENE EXPRESSION PROFILING TO IDENTIFY BIOMARKERS OF REPRODUCTIVE TOXICITY.

**D. J. Dix.** *Office of Research and Development, USEPA, Research Triangle Park, NC.*

Testicular gene expression has been analyzed in rodents exposed to a variety of chemicals to identify biomarkers predictive or indicative of toxicity and impaired reproductive health. Using DNA microarrays we identified numerous biomarkers of reproductive toxicity in mouse and rat testes. In some cases these biomarkers were unique to the chemical or class of chemical exposures, while other biomarkers were associated with impaired reproductive function irrespective of exposure specifics. In order to extrapolate this animal research to considerations of human reproductive health, profiles of gene expression in human testis, sperm and steroidogenic cells were compared to profiles of rodent gene expression. Similarities between testis, sperm and cellular profiles; and rodent and human profiles, indicate the potential for using this type of toxicogenomic data for understanding chemical risks to human reproductive health. This abstract does not necessarily reflect EPA policy.

#### **645** EXPOSURE-INDUCED NEURODEGENERATION: A TOXICOGENOMICS CASE STUDY OF NEURONAL FUNCTION AND NEURODEGENERATIVE DISEASE.

**A. Brooks<sup>1,2</sup>, D. Cory-Slechta<sup>3</sup>, E. Richfeild<sup>3</sup> and M. Thiruchelvam<sup>3</sup>.**

<sup>1</sup>*Environmental Medicine, University of Rochester Medical Center, Rochester, NY,*

<sup>2</sup>*Functional Genomics Center, University of Rochester Medical Center, Rochester, NY*

*and<sup>3</sup>Environmental and Occupational Medicine, UMDNJ, Piscataway, NJ.*

The way in which chemical exposure affects molecular networks is critical for a complete biological understanding of behavioral, physiological, biochemical and clinical outcomes. To this end a variety of biomarkers technologies are being employed to help answer these questions. In addition, it is clear that the discovery of biomarkers at the level of gene, protein and the metabolite will be of paramount importance in assessing neural toxicity as a function of any environmental or chemical exposure. To this end, biomarker discovery from both neuronal tissue and peripheral blood will be essential in developing tools that can be used to accurately and efficiently predict the effects of a variety of exposures. Data from a model of idiopathic Parkinsons disease in addition to several models of exposure induced cognitive decline will be presented in this session. Correlations between behavior and gene expression profiles utilizing novel analysis approaches will be defined and illustrated. Lastly, a molecular model for data integration which ties together disparate data forms (i.e gene expression, protein expression and metabolite profiling) in the biomarker process will be presented.

#### **646** GENE EXPRESSION-BASED BIOMARKERS OF CARDIAC AND RENAL PHARMACO- AND TOXICOGENOMICS.

**K. L. Kolaja.** *Chemogenomics and Toxicology, Iconix Pharmaceuticals, Mountain View, CA.*

Chemogenomics is the study of pharmacology and toxicology using a combination of traditional drug development practices and new genomic tools. As a result of higher content genomic analyses, chemogenomics can be used to uncover previously unappreciated activities, good or bad, of a development candidate. We've created a database consisting of over 600 drugs, chemicals, and toxicants in short term rat studies to apply the strength of chemogenomics to drug development. Our approach has been to examine multiple end points across many tissues including liver, kidney and heart to create a broadly applicable drug development tool. This talk will describe the results of systematic mining of the database to create a library of gene expression based biomarkers (drug signatures), in the kidney and heart. In kidney, we've identified a biomarker that can predict compound-induced histopathology before it is detected using traditional approaches. In this example, we used *in vivo* kidney gene expression profiles from 64 nephrotoxic and non-

nephrotoxic compound treatments to derive a 35 gene signature to predict the future development of tubular nephrosis weeks before it appears histologically following short-term test compound administration. This kidney signature represents a validated genomic biomarker for the early prediction of compound-induced pathology. In heart, we've created over 70 drug signatures, which were used to better understand the off-target cardiotoxic mechanism of action of the anthracyclines. In addition, Signature Analysis, and Pathway and Gene Ontology Analysis indicated that the mechanism of action of the anthracycline drugs were consistent with oxidative stress, DNA damage, cell cycle arrest, inflammation and effects on cardiac electrophysiology. The above findings in heart and kidney confirm the utility of non-hepatic gene expression analysis as a means to create biomarkers for determining pharmacology, toxicology and mechanisms of action, which can be used at a very early stage in development and optimize selection of drug candidates.

#### **647** APPLICATION OF TOXICOGENOMICS BEYOND LIVER.

**Y. Yang, R. Ciurlionis, S. J. Abel, E. A. Blomme and J. F. Waring.** *R463, Abbott Laboratories, Abbott Park, IL.*

The application of gene expression analysis towards toxicology, also referred to as toxicogenomics, is rapidly being embraced by the pharmaceutical industry as a useful tool to identify safer drugs in a faster, more cost-effective manner. Almost all major pharmaceutical companies have devoted significant resources to develop and apply gene expression analysis towards toxicology. Toxicogenomics studies have so far mostly focused on the liver because of the homogeneous nature of this tissue and its importance in toxicology. At our laboratories, we have expanded our gene expression analysis studies to several other tissues, such as testis, bone marrow, spleen, kidney, and pancreas. The objectives of these studies include rank-ordering discovery compounds, guiding in dose selection for repeated-dose *in vivo* preclinical studies and generating a mechanistic understanding of toxicologic changes occurring in preclinical studies. This talk will highlight some applications as well as challenges when conducting toxicogenomics study using non-liver tissues.

#### **648** EMERGING ISSUES IN RISK ASSESSMENT AND RISK PERCEPTION OF NANOMATERIALS.

**J. S. Tsuji.** *Health Risk, Exponent, Bellevue, WA.*

Nanotechnology has been hailed as the new industrial revolution akin to silicon chip development in the last century. Nanomaterials are generally <100nm in size that potentially involve a variety of chemicals, shapes, and physicochemical properties that confer unique electrical, thermal, and imaging characteristics. Such variable characteristics have complex exposure and toxicological implications. Research supporting assessments of the health risks of nanomaterials, however, has greatly lagged technological development and nanomaterials are currently being used and proposed for various applications in technology, medicine, and consumer products with great potential for exposure, but relatively limited understanding of health risks. Little health risk information plays into public fears of nanotechnology. Like genetically modified organisms, the future of nanotechnology will depend on public acceptance of the risks versus benefits. Toxicity and exposure studies supporting health risk assessments of different nanomaterials are crucial to fill the current void in the knowledge of the potential effects of these products. This symposium presents recent findings for the components of characterizing health risks of nanomaterials. In addition to applications of available data for risk assessments of nanomaterials, risk perceptions by the public and by toxicology dogma, along with possible reasons for different interpretations of the recently published pulmonary toxicity studies will be presented. Methodologies and recent findings for characterizing aerosol exposures and the potential for dermal penetration are essential for assessing exposures to nanomaterials. Recent and ongoing research on various types of carbon nanotubes in mice indicates a potential for pulmonary toxicity. However, recent studies refute the common belief that inhalation toxicity always increases with smaller particle size. These recent toxicity and exposure data are beginning to shape risk assessments and safety evaluations that will be used to regulate nanomaterials in consumer products and provide assurance of health and environmental protection.

#### **649** CHARACTERIZING EXPOSURES TO NANOMATERIALS.

**A. D. Maynard.** *NIOSH, Cincinnati, OH. Sponsor: J. Tsuji.*

Characterizing exposures to particulate matter has always carried with it a unique set of challenges. Unlike bulk materials or gases and vapors, pertinent properties of particles extend beyond the chemistry of the material and encompass physical attributes such as shape and size. As available information on the toxicity of nanometer-scale particles and structures increases, it is apparent that we are being faced with a new set of challenges: Particle number, size, structure, surface area and sur-

face activity are indicated as potentially more relevant than mass concentration and bulk chemistry. As nanotechnology moves closer to widespread commercialization, new methods of characterizing relevant material attributes in toxicity studies and appropriately measuring exposure and dose, are required. Examples are given from two studies: On-line aerosol surface area measurements are becoming increasingly necessary when studying the toxicity of insoluble airborne nanoparticles. Diffusion charging, transmission electron microscopy and scanning mobility particle analysis have been compared for measuring aerosol surface area using particles of various sizes and morphologies. All three techniques give comparable results for particles smaller than 100 nm in diameter. For larger particles, some divergence between the three techniques is observable. Aerosol surface area alone is insufficient to characterize exposures where the bulk nanomaterial is heterogeneous. Research has shown that powders of unprocessed single walled carbon nanotubes (SWCNT) can release nanoscale particles into the air when agitated. The raw material in this case is a matrix of single walled carbon nanotubes, metal catalyst particles and compact carbonaceous material. To further explore the nature of nanoparticles generated while handling unprocessed SWCNT, the mass of single mobility diameter aerosol particles has been measured and effective particle density derived. Current results indicate that compact carbonaceous particles are primarily released into the air during agitation, suggesting that the nanotubes preferentially remain in the bulk material.

## 650 PULMONARY TOXICITY OF CARBON NANOTUBES IN MICE AND IMPLICATIONS FOR HUMAN RISK ASSESSMENT.

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The lungs of male B6C3F1 mice, held 7 or 90 days after intratracheal instillation of single-walled, carbon nanotubes, suspended in heat-inactivated serum and administered at doses of 0.1 and 0.5 mg/mouse, exhibited dose-dependent pulmonary granulomas and interstitial inflammation. Three carbon nanotube products, differing in their method of manufacture and the content of residual catalytic metals, were evaluated in this bioassay. In addition, serum (blank), carbon black (negative control), and quartz (positive control) were similarly administered to other groups of mice. We found that after 90 days quartz had induced a dose-dependent inflammation; however, no granulomas were found. In contrast, all 3 types of nanotubes induced pulmonary granulomas. One type of nanotube caused granulomas only at the highest dose, but the others caused granulomas at the lowest dose. If we assume that the pulmonary deposition of nanotubes is about 40%, that the 8-hour/day airborne concentration of respirable dust is 5 mg/m<sup>3</sup> (this is also the PEL for synthetic graphite), and that no significant clearance from the lung occurs, then we estimate that the lung burdens we delivered to the mice would be achieved in about 4 or 20 days for the 0.1 mg/mouse group and 0.5 mg/mouse group, respectively. This clearly suggests that, for the time being and until inhalation studies show that these materials have no effect on the lung, carbon nanotubes cannot be regarded as a relatively non-hazardous carbonaceous dust. In fact it appears prudent to treat the hazard from carbon nanotube dust similarly to the hazard from quartz dust. Further work will answer the important questions for human health assessment; these are as follows: Under what conditions are the nanotubes, which are produced and purified in industrial settings, respirable? How quickly are inhaled nanotubes cleared from the lungs and where are do they go? What are the long-term tissue responses to persisting nanotubes? How do the metal impurities influence the toxicity?

## 651 IMPACT OF EXPOSURES TO NANOPARTICLES ON RESPIRATORY HEALTH: PARTICLE SIZE MAY NOT BE MORE IMPORTANT THAN SURFACE CHARACTERISTICS.

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Several pulmonary toxicity studies in rats have demonstrated that nanoscale particles (defined as particles in the size range <100 nm) administered to the lung cause a greater inflammatory response when compared to microscale or macroscale particles of identical composition at equivalent mass concentrations. Contributing to the effects of nanoscale particles is their very high size-specific deposition when inhaled as singlet ultrafine particles rather than as aggregated particles. Some evidence suggests that inhaled nanoparticles, after deposition in the lung, largely escape alveolar macrophage surveillance and gain access to the pulmonary interstitium. In addition, *in vitro* studies indicate that nanoparticles are more toxic to pulmonary cells. However, some recent preliminary studies indicate that pulmonary exposure to nanoscale particulates may not always be more inflammmogenic compared to macro/microscale particles. For instance, preliminary studies comparing the effects of nano vs. fine-sized particles, have indicated that pulmonary exposures in rats to uncoated TiO<sub>2</sub> nanorods (200 nm lengths x 30 nm diameters) or TiO<sub>2</sub> nanodots (particle size < 30 nm) did not produce enhanced lung inflammation in rats when compared to fine-sized TiO<sub>2</sub> particle exposures (particle size ~ 270 nm). Similar results have been observed with nano and fine ZnO particles. Additional studies have compared lung toxicity in rats of uncoated nanoscale quartz particles (50 nm) vs. fine-sized quartz particles (particle size ~ 1.6  $\mu$ m). In pulmonary instillation studies,

at equivalent mass doses, the nanoquartz particles produced less intensive and sustained pulmonary inflammatory and cytotoxic responses when compared to the effects produced by the Min-U-Sil quartz particles. This result is intriguing since crystalline quartz silica particles are classified as a Category 1 human carcinogen by the International Agency for Research on Cancer (IARC). In summary, the preliminary findings from these three studies suggest that particle size is only one factor in determining pulmonary toxicity.

## 652 EVALUATION OF THE DERMAL PENETRATION OF NANOSCALE MATERIALS.

P. C. Howard. *Jefferson Labs, US Food & Drug Administration, Jefferson, AR.*

The skin is an important organ that has great surface area, provides a physical barrier, has immunologic and metabolic functions, and interacts with materials from the environment. Exposure of skin to ultraviolet light, for example, results in enzyme induction, the alteration of structural proteins and DNA, and the induction of cancer. The range of interactions of chemicals with the skin varies from those that do not penetrate the skin to others that penetrate very rapidly and elicit significant changes. Humans will be exposed to nanoscaled materials as the consequence of several natural and anthropogenic activities, including the incorporation of nanoscale materials into consumer products. What is not clear, but needs to be determined, is the effectiveness of the skin as a barrier to nanoscaled materials. For example, nanoscaled titanium dioxide and zinc oxide have been included recently in sunscreens as physical sunblock agents. Some studies have reported that nanoscale titanium dioxide can penetrate rodent and human skin; however, other reports have indicated nano- and micron-scaled titanium dioxide did not penetrate skin. The interpretation of the toxicity of dermally applied nanoscale materials relies on a thorough knowledge of the chemical and physical characteristics of the nanoscale material in the vehicle and on the skin. Ongoing research is evaluating the potential of, and the conditions under which, nanoscale materials can penetrate the skin and mediate effects such as phototoxicity. Issues central to the interpretation of dermal penetration include physical nature of the materials, the characterization of the test material, the vehicles used to deliver the nanoscale particles, the condition of the skin, and the methods used to assess penetration.

## 653 RISK ASSESSMENT AND SAFETY EVALUATION OF NANOMATERIALS IN CONSUMER PRODUCTS.

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Development of nanomaterials is rapidly transforming a variety of consumer products. With the increasing diversity of product applications, risk assessment screening adapted for new nanomaterials is critical to guide research and develop products that pose minimal health and environmental concerns, and to regulate potential risks. With more data, lifecycle risk assessments of products from manufacture to disposal can be conducted. Although research to support risk assessment and regulation lags behind product development, much of the current risk assessment framework can be adapted to screen materials for potential risks and to identify data needs. Screening tools may include QSAR analyses or *in vitro* tests to evaluate cellular absorption, effects on organelles, molecular changes, and stability of nanomaterial components. Although preliminary studies indicate that nanomaterials may be transported across cell membranes, be absorbed or retained in the lung, and penetrate skin, additional *in vivo* studies are necessary to continue addressing pharmacokinetic questions, including how the various nanomaterials are absorbed and transported, and whether they can penetrate blood-brain and placental barriers. Direct uptake to the brain via olfactory or trigeminal nerves is a concern based largely on rat studies, although potential compound and species differences are possible. Toxicity of nanomaterials is more complicated than smaller is more toxic, which is not always true; other factors such as chemistry, surface properties, and shape also affect toxicity. Exposure assessment considers aerosol potential, solubility, size, concentration, and tendency to agglomerate. Because of the diversity of products and target populations, carefully designed exposure scenarios with appropriate defaults are needed. Available research on ultrafine particles, metal fume, and fibers can help guide research and exposure assessments on nanomaterials. As the fields of nanotechnology and toxicology converge, creative approaches to risk assessment will be needed to ensure the safety of consumers.

## 654 NUCLEOCYTOPLASMIC TRAFFICKING IN MECHANISMS OF TOXICITY.

W. H. Watson<sup>1</sup> and G. H. Perdew<sup>2</sup>. <sup>1</sup>*Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD* and <sup>2</sup>*Center for Molecular Toxicology, Penn State University, University Park, PA.*

Transport of proteins across the nuclear membrane is tightly regulated by the nuclear pore, a multisubunit complex consisting of at least 30 proteins. There are few known examples of toxins or toxicants that act directly at the nuclear pore to affect

transport. However, import or export of specific proteins is a key component of cellular responses to a variety of toxicants. For example, many transcription factors are maintained as inactive cytoplasmic pools that can rapidly translocate to the nucleus upon exposure to toxicants and other stimuli. Interactions that anchor proteins in either compartment or target proteins to the nuclear pore may be disrupted (or enhanced) by toxicants or by specific posttranslational modifications such as phosphorylation or SUMO modification. An understanding of the factors that control the dynamic localization of proteins across the nuclear membrane is necessary for the definition of toxicological mechanisms and physiological pathways.

## 655 NUCLEOCYTOPLASMIC TRAFFICKING IN MECHANISMS OF TOXICITY.

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## 656 NUCLEAR PROTEIN TRANSPORT: A ROLE IN REGULATING CELLULAR RESPONSES TO STIMULI.

**A. H. Corbett, M. T. Harreman, T. M. Kline and A. E. Hodel.** *Biochemistry, Emory University School of Medicine, Atlanta, GA.* Sponsor: W. Watson.

All transport of macromolecules between the cytoplasm and the nucleus is mediated by the nuclear pore complex (NPC), which are large macromolecular channels embedded in the nuclear envelope. Although numerous proteins are synthesized in the cytoplasm and then routinely imported into the nucleus to perform their cellular function, there are also numerous examples of proteins that undergo regulated nuclear transport. For example, many transcription factors of toxicological relevance are maintained as inactive cytoplasmic pools that can be rapidly activated and imported into the nucleus upon exposure to toxicants and other stimuli. These regulated transport processes are mediated by soluble nuclear transport receptors that recognize and bind to cargo in the cytoplasm and then transport that cargo into the nucleus through NPCs. The best understood protein transport process is the import of proteins that contain a classical nuclear localization signal (NLS), but many other nuclear targeting signals also exist. Numerous studies have led to a rather detailed understanding of the molecular mechanism of transport receptor-mediated protein trafficking into and out of the nucleus. Interactions between transport receptors and cargoes are regulated by a small GTPase, Ran, which cycles between a nuclear RanGTP state and a cytoplasmic RanGDP state. This presentation will provide a basic overview of protein transport into and out of the nucleus as well as give an overview of some regulatory mechanisms. To illustrate one regulatory mechanism, results of our recent study that examined the molecular mechanism by which nuclear localization of a yeast transcription factor is regulated in a cell cycle-dependent manner will be presented. This study demonstrated that phosphorylation proximal to a classical NLS directly modulates binding to the transport receptor and hence import into the nucleus. These results could suggest a general mechanism by which post-translational modifications of proteins regulate their nuclear localization.

## 657 SPECIES-DEPENDENT DIFFERENCES IN AH RECEPTOR NUCLEOCYTOPLASMIC SHUTTLING PROPERTIES.

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A series of studies were initiated to determine the nucleo-cytoplasmic shuttling (NCPS) properties of the Ah receptor. The mouse Ah receptor (mAhR)-yellow fluorescent protein (YFP) undergoes dynamic NCPS in COS 1 cells and shuttling is inhibited by co-expression of XAP2. *In vitro* protein binding studies revealed that the presence of XAP2 in the mAhR complex inhibited importin beta binding to the

nuclear localization signal (NLS) of the mAhR. Differences in NCPS properties of mAhR-YFP and human Ah receptor (hAhR)-YFP in the presence or absence of XAP2 were determined in COS 1 cells. The mAhR-YFP localizes predominantly in the nucleus, whereas hAhR-YFP is cytoplasmic. Co-expression of XAP2 redistributes mAhR-YFP to the cytoplasm and inhibits NCPS, but has no effect on hAhR-YFP distribution or NCPS properties. XAP2 does not inhibit importin beta binding to the hAhR. XAP2-NLS, which contains a NLS sequence fused to the carboxy terminus, is capable of dragging a NLS mutant of hAhR-YFP into the nucleus, but not a NLS mutant of mAhR-YFP suggesting that only the human AhR moves into the nucleus with XAP2 in the receptor/hsp90 complex. Both the mouse and human AhR transcriptional activity is inhibited by XAP2. Mouse and human chimeric receptors with C-terminal half of the Ah receptor swapped between receptors were used to determine whether the divergent transactivation domains of these two receptors influences receptor properties found in the N-terminal half. Results revealed that the C-terminal half influenced NCPS properties of the AhR. Treatment with H2O2 to induce oxidant stress leads to rapid translocation of both the human and mouse Ah receptor into the nucleus, indicating that the shuttling properties of the AhR may be altered during disease processes.

## 658 SUMO MODIFICATION AND REGULATION OF NUCLEAR TRANSPORT.

**M. J. Matunis and H. Zhang.** *Biochemistry and Molecular Biology, Johns Hopkins University, Baltimore, MD.* Sponsor: W. Watson.

SUMOs are small ubiquitin-related proteins that are posttranslationally conjugated to other proteins, thereby regulating a wide range of cell functions. Several lines of evidence indicate that SUMO modification regulates the transport of proteins and/or RNAs between the nucleus and the cytoplasm. Of particular significance, enzymes that regulate SUMO modification and demodification localize to nuclear pore complexes (NPCs). We have found that the SUMO-specific protease SENP2 localizes to the nucleoplasmic filaments of the NPC. Interactions between SENP2 and the NPC, however, were found to be indirect. Using purified proteins, we found that SENP2 interacts directly with nuclear transport receptors that tether it to the NPC. Similar to transport receptors, SENP2 was also found to shuttle between the nucleus and the cytoplasm. Significantly, disruption of the SUMO modification pathway disrupted the nucleocytoplasmic shuttling of SENP2-interacting transport receptors. Our findings indicate that SUMO modification regulates the shuttling of nuclear transport receptors between the nucleus and the cytoplasm and implicate the SUMO-specific protease SENP2 as a direct player in this process.

## 659 TOXICANT-INDUCED NUCLEAR TRANSLOCATION OF THIOREDOXIN.

**W. H. Watson.** *Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD.*

Thioredoxin (Trx1) is a protein that plays a central role in protecting cells against oxidative damage. A wide variety of stimuli including peroxides, UV light and inflammatory cytokines cause Trx1 to move from the cytoplasm to the nucleus. Trx1 contains no recognizable nuclear localization or nuclear export signals, so it is unclear how the subcellular distribution of Trx1 is regulated. We have been investigating the effect of changes in the redox state of Trx1 itself on its localization within the cell. Trx1 contains five cysteines, two of which are in the active site and cycle between the reduced dithiol form and the oxidized disulfide form during catalysis. Under oxidative stress conditions a second disulfide between two of the non-active site cysteines was found, and this correlated with an increase in the amount of Trx1 in the nucleus. The amount of active Trx1 in the nucleus affected the expression of an NF- $\kappa$ B-dependent reporter gene. Because nuclear Trx1 affects the DNA-binding activity of redox-sensitive transcription factors such as NF- $\kappa$ B, the redistribution of Trx1 appears to be an important factor in regulating transcriptional responses to oxidative stress.

## 660 CURRENT STATUS AND FUTURE CONSIDERATIONS FOR THE DEVELOPMENT OF *IN VITRO* ALTERNATIVES TO THE DRAIZE RABBIT EYE TEST FOR ASSESSING OCULAR IRRITANTS.

**T. Hartung<sup>1</sup> and W. S. Stokes<sup>2</sup>.** <sup>1</sup>*ECVAM, Ispra, Italy* and <sup>2</sup>*NICEATM, NIEHS/NIH/DHHS, Research Triangle Park, NC.*

The Draize rabbit eye test has been used as the standard test method for assessing ocular irritation and corrosivity potential of chemicals and products for over 60 years. Largely due to the pain and discomfort associated with this test, there have been extensive efforts to develop and validate alternative test methods that would

partially or fully replace the current regulatory version of the Draize rabbit eye test. These efforts are also being driven by the 7th Amendment to the European Union Cosmetics Directive that will ban the use of animals for ocular testing by 2009 and pressure to minimize or avoid animal use for the European Union Chemicals Policy Initiative (REACH). This workshop will address scientific and regulatory considerations for developing and validating *in vitro* test methods that will refine, reduce, and replace the Draize rabbit eye test. ICCVAM, NICEATM, and ECVAM have recently initiated collaborations to assess the usefulness of currently available *in vitro* ocular toxicity methods and to review the state-of-the-science with regard to *in vitro* test methods. These efforts include an evaluation of several *in vitro* test methods for their ability to detect severe ocular irritants, an assessment of the performance characteristics of the *in vivo* rabbit eye test, and a review of the current status of methods for identifying nonirritants and mild to moderate irritants. Current and future challenges in validating alternative methods and approaches to evaluate ocular toxicity will be presented including the need for high quality reference data to assess test method performance.

## 661 CURRENT STATUS AND FUTURE CONSIDERATIONS FOR ALTERNATIVE METHODS TO REFINE, REDUCE, AND REPLACE ANIMAL USE FOR OCULAR SAFETY TESTING.

W. S. Stokes, NICEATM, NIEHS/NIH/DHHS, Research Triangle Park, NC.

The original and modified Draize rabbit eye tests have been used for over 60 years to identify potential acute ocular hazards of chemicals and products. Such testing has been highly effective in safeguarding human health by identifying and classifying potential ocular hazards. Nevertheless, public animal welfare concerns and a European law banning the use of this test for cosmetics testing no later than 2009 have led to significant efforts during the past 20 years to develop and validate *in vitro* tests to replace animals for ocular testing. While there is still no valid replacement for the Draize eye test, new testing guidelines now allow for the use of valid and accepted *in vitro* test methods in a tiered testing strategy. When animals are needed, such determinations can now be made with only 1-3 animals. The proposed tiered testing strategy provides for stepwise weight-of-evidence hazard decisions to be made based on existing data, structure activity/property relationship evaluations, *in vitro* data, and/or data from other *in vivo* testing. Topical anesthetics are also now permitted to reduce or avoid animal pain, provided that there is no interference with the outcome of the study. Future *in vitro* test methods and/or test batteries proposed to completely replace the *in vivo* test will require adequate validation and will need to accurately identify ocular irritants and corrosives, especially those that may cause permanent visual impairments. Appropriate reference chemicals with high quality *in vivo* data will be necessary to assess the performance of these new tests. Progress in refining, reducing, and replacing animal use for ocular toxicity is expected as tiered testing strategies are implemented, and existing and new mechanism-based test methods are validated and accepted.

## 662 THE PERFORMANCE CHARACTERISTICS OF THE *IN VIVO* RABBIT EYE TEST.

J. H. Haseman<sup>1</sup>, N. Y. Choksi<sup>2,3</sup>, C. Inhof<sup>2,3</sup>, J. Truax<sup>2,3</sup>, R. R. Tice<sup>2,3</sup> and W. S. Stokes<sup>3</sup>. <sup>1</sup>Consultant, NIEHS/NIH/DHHS, Research Triangle Park, NC, <sup>2</sup>ILS, Inc., Research Triangle Park, NC and <sup>3</sup>NICEATM, NIEHS/NIH/DHHS, Research Triangle Park, NC.

Alternative *in vitro* test methods proposed to substitute or replace an *in vivo* assay should provide equivalent or improved protection of human or animal health to gain regulatory and general acceptance. The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) is evaluating four *in vitro* ocular test methods as partial replacements for the detection of severe ocular irritants (i.e., those that induce or are likely to induce irreversible ocular damage). Integral to this evaluation and to the future evaluation of *in vitro* ocular toxicity test methods that are proposed as full replacements is an assessment of the performance characteristics of the current *in vivo* rabbit eye test. Ideally, this analysis would evaluate the ability of the rabbit eye test to correctly predict ocular toxicity in humans. However, due to the absence of adequate human data, estimating the likelihood of underpredicting a positive response in the *in vivo* rabbit eye test is the best approach for assessing the performance of this assay. Relevant *in vivo* rabbit eye test method data have been obtained from US Federal agencies and published literature. The underprediction rate for the *in vivo* rabbit eye test depends on the regulatory classification system used. For this analysis, the UN Globally Harmonized System of Classification and Labeling of Chemicals and the USEPA Ocular Toxicity Classification Scheme are the regulatory approaches being used to distinguish between nonirritants and various classes of ocular irritants. More than 500 chemicals were assigned to ocular irritation categories based on the observed responses in 3 to 6 animals. Based on this distribution of animal responses within each irritation clas-

sification level, the underprediction rate associated with a sequential testing strategy (maximum of three animals) was evaluated. The results and implications of these analyses will be presented and discussed. Supported by NIEHS contract N01-ES-35504.

## 663 PERFORMANCE OF BCOP, IRE, ICE, AND HET-CAM IN DETECTING SUBSTANCES THAT INDUCE SEVERE IRRITATION AND IRREVERSIBLE OCULAR DAMAGE.

N. Y. Choksi<sup>1,2</sup>, D. A. Allen<sup>1,2</sup>, C. Inhof<sup>1,2</sup>, J. Truax<sup>1,2</sup>, R. R. Tice<sup>1,2</sup> and W. S. Stokes<sup>2</sup>. <sup>1</sup>ILS, Inc., Research Triangle Park, NC and <sup>2</sup>NICEATM, NIEHS/NIH/DHHS, Research Triangle Park, NC.

Exposure of rabbit eyes to a test substance is one of the most common methods for assessing the ocular hazard potential of substances that are proposed to come in contact with or be placed near the eye. Concerns about animal welfare have led researchers to develop *in vitro* test methods as alternatives to the currently used *in vivo* rabbit eye test method. Recently, the USEPA (EPA) requested that NICEATM and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) evaluate four test methods; the Bovine Corneal Opacity and Permeability (BCOP) test; the Isolated Rabbit Eye (IRE) test; the Isolated Chicken Eye (ICE) test; and the Hens Egg Test-Chorioallantoic Membrane (HET-CAM) for their ability to detect severe ocular irritants, as defined by U. S. and international ocular irritancy classification systems. For this evaluation, substances that were classified as Category I ocular irritants according to the EPA classification system, as Category 1 ocular irritants according to the United Nations Globally Harmonized classification system, or as R41 ocular irritants according to the European Union classification system were defined as severe ocular irritants. The accuracy of each of the test methods for identifying severe ocular irritants, as defined by each of the identified regulatory classification systems, was evaluated. Based on the available data, this analysis indicated the extent that each of these *in vitro* test methods could be used to identify severe ocular irritants. Appropriate use of these *in vitro* methods to identify severe irritants prior to animal testing will reduce the likelihood of pain and suffering associated with subsequent ocular testing. Supported by NIEHS contract N01-ES-35504.

## 664 THE STATE OF THE SCIENCE ON *IN VITRO* TEST METHODS FOR DETECTING MILD TO MODERATE OCULAR IRRITANTS.

C. Eskes, T. Hartung and V. Zuang, ECVAM, IHCP, European Commission - DG JRC, Ispra, Varese, Italy. Sponsor: W. Stokes.

European chemicals and cosmetics legislation call for alternatives to animal testing, in particular in the areas of acute topical toxicity, such as eye irritation. Major validation studies in the 90's to replace the Draize test for eye irritation showed good reproducibility and reliability of some alternative methods but the range of criteria for injury and inflammation covered by the Draize rabbit eye test was found to be unlikely to be replaced by a single *in vitro* test. One of the major recommendation from experts in the field is to define test strategies for eye irritation testing, that utilizes the strengths of particular *in vitro* assay systems to address required ranges of irritation potential and/or chemical classes. In particular, the following methods appear to be promising for identifying mild to moderate ocular irritants: - Organotypic models: Chicken Enucleated Eye Test (CEET), Hen's egg test on the Chorio-Allantoic Membrane (HET-CAM), and CAM vascular assay (CAM-VA), - Human Corneal Epithelium (HCE) models: EpiOcular<sup>TM</sup> assay, and SkinEthic *in vitro* reconstituted HCE, - Cell-cytotoxicity based methods: Neutral Red Release (NRR) assay, and Red Blood Cell (RBC) haemolysis test for surfactants, - Cell-function based assays: Fluorescein Leakage (FL) and Silicon Microphysiometer (SM). Whereas the human corneal epithelium models have undergone (pre)validation, the organotypic models, cell cytotoxicity and cell function based assays, have undergone extensive evaluation studies. In parallel and in collaboration with ICCVAM's assessment of the organotypic models, ECVAM is currently evaluating the further models, based on the acquired data and on an in-depth review of the existing data. If validated, these methods might be able to identify mild to moderate irritants and could be part of test strategies allowing a reduction or replacement of animals used for eye irritation testing.

## 665 CHALLENGES IN THE VALIDATION OF ALTERNATIVE TEST METHODS TO EVALUATE OCULAR TOXICITY.

T. Hartung, C. Eskes and V. Zuang, European Centre for the Validation of Alternative Methods, European Commission DG JRC, Ispra, Varese, Italy. Sponsor: W. Stokes.

The current political environment in Europe strongly requests alternatives to animal testing, in particular in the areas of acute topical toxicity, such as eye irritation. Major validation and evaluation studies took place in the 90's to replace the Draize

test for eye irritation, but no single method was able to replace the Draize rabbit eye test. This was due to several factors including the limited quality of the existing *in vivo* data, the limitations of the animal and alternative test methods, and the fact that the range of criteria for injury and inflammation covered by the Draize rabbit eye test is unlikely to be replaced by a single *in vitro* test. Despite of the fact that no method has been validated according to the current criteria, several methods appear to be promising for replacing the animal test if used in combination with other alternative tests. In order to achieve the validation of alternatives that could fully replace the Draize rabbit eye test the following major challenges need to be addressed: - Make use of the existing data and apply a weight-of-evidence approach; - Acquire high quality *in vivo* data sets; - Define and validate test strategies; - Consider different regulatory schemes including the upcoming implementation of the Globally Harmonised System for classification; - Develop mechanistically based alternatives able to evaluate the persistence or reversibility of effects. ECVAM has established a Task Force and has organised a series of workshops involving international experts in the field in order to address those challenges; and is currently evaluating the different single promising methods in collaboration with ICCVAM-NICEATM. In addition, ECVAM is collaborating with the European Cosmetics Industry Trade Association (COLIPA), which entertains a research programme on eye irritation based on the mechanistic understanding of eye irritation and the possibility to assess irreversibility and reversibility of eye irritation effects.

## 666 MODE OF ACTION IN RELEVANCE OF RODENT LIVER TUMORS TO HUMAN CANCER RISK.

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The recent adoption of the IPCS mode of action paradigm (Sonich-Mullin et al., 2001, *Regul Toxicol Pharmacol* 34:146-152) coupled with the recent ILSI human relevance framework (Cohen et al., 2003, *Crit Rev Toxicol* 33:581-589) have led to an approach for the systematic analysis of data on modes of carcinogenic action of chemicals in experimental animals and its application to the assessment of human cancer risk assessment. Hazard identification and risk assessment paradigms depend on the presumption of similarity of rodents to humans, yet species-specific responses and high dose to low dose extrapolation plague the development of appropriate risk assessments. The first step in a mode of action analysis is to establish the key biochemical and cellular events, temporal occurrence, and dose response concordance relationships common to each mode of action of a chemical. The next step is to assess the biological plausibility and relevance to human cancer risk of the proposed mode(s) of action taking kinetic and dynamic factors into consideration. The identification of key events can be used to bridge species and dose differences. These discussions will be used to generate a minimal dataset necessary to establish selected modes of action. This mode of action framework has been previously applied to rodent liver tumors associated with exposure to peroxisome proliferators mediated through the alpha receptor (Klaunig et al., 2003, *Crit Rev Toxicol* 33:655-780). More recently, several additional modes of action for rodent liver cancer development have been described including cytotoxicity, P450 induction, hormone mediated and porphyrogenicity / metal overload. An understanding of the mode of action underlying cancer development in the liver will place rodent liver tumors into a more appropriate perspective when human risk assessment is performed.

## 667 THE PATHOGENESIS OF RODENT HEPATOCARCINOGENESIS: POTENTIAL APPLICATIONS TO HUMAN CANCER RISK.

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A variety of model systems of hepatocarcinogenesis in the rat and the mouse have been developed. Several in the rat have allowed the delineation and characterization of the stages of initiation, promotion, and progression as sequential processes in the pathogenesis of hepatocarcinogenesis. Identification of single putatively initiated hepatocytes in both rats and mice is possible by specific gene expression. Quantitation of such single initiated hepatocytes shows a linear dose response with chemical carcinogens in the rat. Clonal growth of about 1% of putatively initiated hepatocytes form focal lesions termed altered hepatic foci (AHF). The dose response of the development of AHF to several promoting agents, including phenobarbital, exhibits a sigmoidal response with a clear no-effect level and hormetic effect. Withdrawal of the promoting agent or fasting for five days results in a rapid loss of focal cells, which is primarily due to selective apoptosis. Re-administration of the promoting agent results in a rapid regrowth of the original number of AHF. Many, possibly all, promoting agents interact with specific receptors in target cells as the rate limiting and necessary step in their effect. Administration of a genotoxic carcinogen during the action of a promoting agent can result in expression of different genes in foci within AHF during the stage of progression. During the pro-

gression stage of chemical and genetic carcinogenesis in the rat, specific cytogenetic alterations occur. Epidemiologic studies indicate that the major causes of human neoplasia, tobacco abuse, diet, and reproductive and sexual behavior, involve tumor promotion as the principal pathway in their development. Thus, reversibility and threshold as well as hormetic effects of carcinogens should be taken into account in risk determination in the human.

## 668 FRAMEWORK FOR EVALUATING THE HUMAN RELEVANCE OF CARCINOGENIC MODES OF ACTION IN ANIMALS.

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Considerable effort has been made to evaluate the mode of action for specific chemicals causing cancer in rodents during the past several decades. However, the key question is the relevance of this mode of action to humans and what the implications of that mode of action (MOA) are for human risk assessment. A framework for such an analysis has recently been developed by an ILSI/RSI working group sponsored by EPA and Health Canada. The process is divided into three specific questions. The first is, Is the weight of evidence sufficient to establish the MOA in animals? This evaluation is performed based on the framework developed by the IPCS/EPA for determining animal mode of action. The measurable key events in the MOA are explicitly stated and evaluated. This is not only useful in formulating a mode of action, but clearly identifying any data gaps and uncertainties that remain. The second question is, Are key events in the animal MOA plausible in humans? This evaluation is based on a concordance analysis comparing the information known about the specific key events in both animals and humans. This evaluation is primarily a qualitative assessment. The third question is, Taking into account kinetic and dynamic factors, are key events in the animal MOA plausible in humans? This is a more quantitative analysis, and again relies on a concordance analysis between the animal model and humans. For both questions two and three, data may or may not be available for the specific chemical in humans, but broad knowledge of processes involved in humans, including anatomy, physiology, biochemistry, metabolism, etc., are critical. Based on the results of this analysis, a statement of the confidence in the analysis is made along with implications for carrying forward to the remainder of the risk assessment process. This human relevance framework is focused on hazard identification and evaluation. Several examples will be presented illustrating the process by which this framework can be applied

## 669 MODE OF ACTION AND HUMAN RELEVANCE OF PHENOBARBITAL-LIKE RODENT LIVER CARCINOGENS.

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Phenobarbital (PB) is the prototype of several rodent hepatocarcinogens (e.g. oxazepam, DDT) that induce tumors by a non-genotoxic mechanism. PB is negative in a wide range of genotoxicity tests and is not cytotoxic. A diagnostic effect of PB is induction of some P450 enzymes, particular of the CYP2B family. This is due to activation of nuclear receptors, particularly constitutive androstane receptor (CAR). However, whilst there is evidence that CAR plays a key role in the carcinogenicity of PB, it is uncertain if CYP induction is a surrogate for a wider pleiotropic response or if P450 itself plays a role, e.g. by generation of active oxygen species. Additional PB responses that are key in the tumorigenic effect include increased cell proliferation, inhibition of apoptosis, hypertrophy and development of altered hepatic foci. Hence, it is possible to identify a series of key events comprising a mode of action for PB. PB induces CYP enzymes in human liver, although it may act more through pregnane X receptor (PXR) than CAR. Nevertheless, human CAR is expressed and appears to be activatable by PB. Liver size is increased in humans treated with PB. Limited studies with human hepatocytes indicate that such cells are refractory to the hyperplastic and anti-apoptotic effects of PB. However, whilst the data for concordance analysis for PB are limited, there are convincing data showing that in patients receiving PB for many years, at doses producing plasma concentrations similar to those following a carcinogenic dose in rodents, there is no evidence of a carcinogenic effect. This is a situation where epidemiology data are useful in a human relevance framework. Once a robust mode of action has been established in rodents, and adequate human data show no evidence of a carcinogenic response, these data can be applied to chemicals sharing that mode of action.

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## MODE OF ACTION AND HUMAN RELEVANCE OF METAL OVERLOAD AND PORPHYRINOGENIC COMPOUNDS.

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Using the recently developed USEPA Framework for examining the mode of action of rodent carcinogens and their human relevance, the mode of action for metal overload (copper and iron) and porphyrinogenic chemical induced rodent hepatocellular cancer induction was examined. Iron and copper overload in the liver are seen in the human diseases hemochromatosis and Wilson's disease. Both iron and copper overload have been implicated as causal factors in both rodent and human hepatic cancer. Using the Framework Evaluation, our working group concluded that while copper and /or iron overload are associated with the development of rodent and human hepatic neoplasia, the scientific proof that either compound solely produces rodent or human liver cancer is lacking. It appears that additional toxicity to the liver resulting in hepatic damage are necessary for the induction of liver cancer by either copper or iron overload. We also evaluated the mode of action of porphyrinogenic compounds for hepatic rodent neoplasia. With the established porphyrinogenic agent, hexachlorobenzene, as a model, we concluded that porphyrinogenic compounds have a definable mode of action for rodent hepatic cancer that involves a chronic, dose and threshold dependent, persistent hepatocellular injury with resulting persistent compensatory hyperplasia. Using the USEPA Framework approach, our group concluded that for iron and copper metal overload, a definable mode of action for the formation of hepatic cancer in rodents by either iron or copper was not achievable based on the currently available scientific evidence. For porphyrinogenic compounds, with hexachlorobenzene used as an example, we concluded that the induction of rodent liver cancer followed a cytotoxic mode of action, and, as in the case of other cytotoxic hepatic carcinogens, the rodent mode of action was deemed possible for humans.

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## HORMONAL PERTURBATION AS A MODE OF ACTION FOR RODENT LIVER TUMORS.

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Hormonally active agents can contribute to the development of rodent liver tumors. In determining the mode of action of a non-genotoxic carcinogens, perturbation of hormonal regulation should be considered. Although the liver is not generally considered as a target tissue for hormones, it is quite hormonally responsive. Using estrogen as an example, rat liver is responsive to its carcinogenic action, while mouse liver is protected from cancer development. With respect to humans, the mode of action observed in rat; namely, hormonal perturbation is a plausible mechanism for primary liver cancer induction in the human. The key events in rodent liver carcinogenesis following exposure to estrogenic agents is perturbation of hormone level or function, altered cell proliferation to apoptosis balance, development of altered foci of cellular alteration. Use of the mode of action approach permits initial development of quantitative risk assessment that can be applied across species by use of the human relevance framework.

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## RODENT HEPATIC TUMORS: CYTOTOXICITY MODE OF ACTION.

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Cytotoxicity is a generally accepted mode of action (MOA) and has been defined for a number of nongenotoxic rodent carcinogens (e.g., formaldehyde-induced nasal tumors, chloroform-induced liver and kidney tumors, melamine-induced bladder tumors). In the liver, a cytotoxicant would produce protracted hepatocyte death leading to regenerative growth (persistent), allowing mutated cells to persist and proliferate, giving rise to preneoplastic foci and ultimately to tumors via further clonal expansion. Before a cytotoxic MOA can be defined, it is critical to ensure that other MOAs do not contribute significantly to hepatocarcinogenesis. For instance, it is important to ensure that DNA reactivity is not the source of the tumor findings. Furthermore, it is important to establish that there are parallel dose re-

sponses for the key events (i.e., cytotoxicity and proliferation) and tumors (not necessarily identical), as well as a specificity of the key events and the tumor response. Hepatotoxicity can be demonstrated with histopathology (necrosis versus apoptosis) with or without enzyme changes. Cell proliferation can be measured by BrdU labeling index and/or cell number and may need to be collected based on zonal distribution. Liver tumors formed as a result of sustained cytotoxicity and regenerative proliferation are considered relevant for evaluating human cancer risk. The dose-response relationship for a cytotoxic MOA is dependent on events which are nonlinear (or threshold).

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## NEUROIMAGING STRATEGIES FOR APPLICATION TO NEUROTOXICOLOGY AND RISK ASSESSMENT.

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Recent technological innovations now make it possible to apply many *in vivo* neuroimaging technologies such as positron emission tomography (PET) and magnetic resonance imaging (MRI) to small animals, including nonhuman primates, rats and mice. The availability of these new technologies coincides with progress in developing animal models of various developmental and neurodegenerative diseases and improvements in assessment protocols for identifying deficits in animals that correlate well with human deficits. The integration of neuroimaging techniques with traditional neurotoxicological assessments has the potential to enhance greatly the ability to relate behavioral, cognitive or motor dysfunction induced by a toxicant to structural and functional brain pathology. Changes in anatomy of soft and hard tissue, metabolism and gene expression can now be done in both a preclinical and a clinical setting using such technologies as Magnetic Resonance Imaging (MRI), Magnetic Resonance Spectroscopy (MRS), Positron Emission Tomography (PET), Computer Tomography Scanning (CT scan) and Visible and Infrared Spectral Imaging. This permits longitudinal studies of the same subject subsequently reducing the number of animals required for studies while providing definitive information as a basis of risk assessment. This workshop will be of interest to toxicologists in general and in particular to neurotoxicologists and risk assessors.

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## NEUROIMAGING: NEW APPROACHES FOR NEUROTOXICOLOGY.

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Recent technological innovations now make it possible to apply many *in vivo* neuroimaging technologies such as positron emission tomography (PET) and magnetic resonance imaging (MRI) to small animals, including nonhuman primates, rats and mice. Approaches have been delineated that promise the ability to assess apoptosis and gene expression in a longitudinal and noninvasive manner. Changes in anatomy of soft and hard tissue, metabolism, function and gene expression can now be done in both a preclinical and a clinical setting using such technologies as Magnetic Resonance Imaging (MRI), Magnetic Resonance Spectroscopy (MRSI), Positron Emission Tomography (PET), Computer Tomography Scanning (CT scan) and Visible and Infrared Spectral Imaging. This type of information is not readily accessible using conventional toxicological procedures and usually requires total destruction of the intrinsic structure of the sample of interest. Imaging provides an opportunity to provide much of this data in a nondestructive manner and presents the data in a three-dimensional format. The availability of these new technologies coincides with progress in developing animal models of various developmental and neurodegenerative diseases and improvements in assessment protocols for identifying deficits in animals that correlate well with human deficits. The integration of neuroimaging techniques with traditional neurotoxicological assessments has the potential to enhance greatly the ability to relate behavioral, cognitive or motor dysfunction induced by a toxicant to structural and functional brain pathology.

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## MAGNETIC RESONANCE IMAGING OF METALS IN THE BRAIN.

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High intensity signals in the basal ganglia on T1-weighted images are associated with increased deposition of iron, manganese, and copper at this site. There is growing interest in whether these changes in the magnetic resonance image (MRI) signal may be a useful biomarker of metal exposure. For example, the pallidal index (PI) calculated from the ratio of globus pallidus (GP) to subcortical frontal white matter signal intensity in the T1 MRI has been proposed as a biomarker of manganese exposure. To date, the PI has been used despite the paucity of data demonstrating any relationship between PI changes and brain manganese concentrations.

To address this data gap we exposed young male rhesus monkeys to manganese sulfate ( $MnSO_4$ ) at 0, 0.06, 0.3, and 1.5 mg  $Mn/m^3$  for 6 hr/day, 5 days/week, for 13 weeks. T1-weighted brain MRI studies (1.5 T) were performed on propofol anesthetized monkeys near the end of the 13-week  $MnSO_4$  inhalation. Brain manganese concentrations were determined shortly thereafter by graphite furnace atomic absorption spectrometry. Statistically significant linear relationships were found between GP manganese concentrations and T1 relaxation times and for the relationship between GP manganese concentrations and the PI. The calculation of the PI presumes that the white matter does not accumulate manganese; however, subchronic manganese exposure resulted in increased manganese concentrations in white matter tracts adjacent to the GP. These data show that T1 relaxation times and the PI are semi-quantitative biomarkers of manganese exposure and should prove useful for other paramagnetic metals as well.

## 676 MOLECULAR IMAGING OF CENTRAL NERVOUS SYSTEM BIOLOGY IN SMALL ANIMALS.

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Better mechanistic understanding of disease through mapping of the human and mouse genomes enables rethinking of human infirmity. For example, we may begin to associate disease states with their underlying genetic defects rather than with the organ system involved. That will enable more selective therapies in patients who are genetically predisposed to respond to them. Molecular imaging can briefly be defined as the remote sensing of cellular and molecular phenomena *in vivo*, i.e., in laboratory animals. Because one of the major goals of molecular imaging research is to interrogate gene expression non-invasively, it can impact greatly on the development of new therapies for a number of diseases. Most of molecular imaging research is undertaken in small animals, which provide a conduit between *in vitro* studies and human clinical imaging. We are fortunate to be able to manipulate small animals genetically, and to have increasingly better models of human disease. The ability to study those animals non-invasively and quantitatively with new, high-resolution imaging devices provides the most relevant milieu in which to find and examine new therapies or to understand the biochemical basis of disease. We will discuss molecular imaging broadly, but with an emphasis on applications within the central nervous system. We will compare and contrast the available imaging modalities and will provide specific examples including translation of molecular imaging research to the clinic and the motivation behind this emerging field in imaging science.

## 677 MRS TO ASSESS DEVELOPMENTAL NEUROTOXICITY.

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Drug exposure during development can have profound effects on brain development. However many women still use drugs of abuse while pregnant and many children and young adults are exposed to the first and second hand effects of drug use. Several drugs of abuse, including nicotine, cocaine, and methamphetamine, have been well studied in adult animal models and humans. With the increasing availability of brain imaging techniques, including magnetic resonance spectroscopy (MRS), the effects of these drugs on brain development are now also being studied in humans, with corresponding animal models. MRS can measure the levels of several major brain metabolites, including neuronal and glial markers, cell membrane turnover, cellular metabolism and energy, as well as some neurotransmitters. A review of MRS studies conducted in humans and animal models, *in vivo* and *ex vivo*, will be discussed, noting differences associated with type of drug exposure, age at exposure, length and dose of exposure, sex, and possible recovery after exposure to these drugs of abuse.

## 678 EARLY BIOMARKERS OF PARKINSON'S DISEASE AS DEFINED BY POSITRON EMISSION TOMOGRAPHY.

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Parkinson's disease (PD) is a neurodegenerative disorder that afflicts approximately 1% of the US population. We are using a non-human primate/MPTP model of PD to assess the progression of MPTP neurotoxicity. The goal is to measure the earliest changes in brain chemistry to identify an early biomarker of disease. Dopaminergic

phenotypic markers, including dopamine transporter (DAT) and vesicular monoamine transporter type 2 (VMAT-2) are being measured using PET with [<sup>11</sup>C]-WIN 35, 428 and [<sup>11</sup>C]-dihydrotetraabenazine, respectively. We also measure peripheral benzodiazepine receptor (PBR) levels as a marker of glial cell activation using [<sup>11</sup>C]-R-PK11195 PET. Finally, since MPTP is a potent inhibitor of complex I in mitochondria, complex I is being measured using [<sup>11</sup>C]-dihydrotetraabenazine PET. Our initial findings suggest that the loss of DAT and VMAT-2 in the striatum is present prior to behavioral manifestation of Parkinsonism. Decreased complex I level and increased glial cell activation is present early but is not selective to the striatum, the brain region affected in PD. These preliminary findings indicate that DAT and VMAT-2 may be useful markers for the early detection of PD. The finding that complex I level decreases in other brain structures besides the striatum suggests that other brain regions are also affected by MPTP administration. This is supported by the finding that the same brain regions exhibiting decreased levels of complex I also express increased PBR binding. These studies demonstrate the utility of *in vivo* imaging techniques such as PET in studying the progressive effects of neurotoxicant exposure on brain chemistry. We demonstrate the ability to measure multiple neuronal and glial markers in the same animal in a prospective fashion. This allows for each animal to serve as its own control and reduces the number of animals needed to obtain meaningful data. [Supported by MJ Fox Foundation]

## 679 EARLY EXPOSURE TO TCDD IMPAIR RAT BONE TISSUE COMPOSITION AND FUNCTION.

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Previously own studies in rats have shown that the dioxin TCDD and the dioxin-like PCB congener, PCB126, impair bone tissue composition and function. The purpose of this study was to investigate effects on rat bone caused by early exposure to TCDD. Pregnant dams were exposed to a single oral dose of 1  $\mu g/kg$  b.w. at gestation day 15. Control rats received the vehicle (corn oil). After weaning the pups were given either sucrose-rich diet (15% sucrose) and tap water containing 7% sucrose or normal rat powder feed and tap water. Offspring were killed at the age of 77 days. Peripheral quantitative computed tomography (pQCT) and biomechanical testing (3-point bending test) were performed on the excised tibia. Trabecular bone mineral density (BMD) was determined by metaphyseal scans of the proximal tibiae. Cortical parameters, including total and cortical bone mineral density, total and cortical cross-sectional area (CSA), were determined by diaphyseal scans. In the female rats a significant reduction of bone growth manifested as a reduced total BMD as well as total and cortical CSA were observed. The bone strength and the stiffness of the tibia were also reduced. Adding sucrose to the diet reduced the effects of TCDD. These findings were seen also after adjustment for effects on body weight. In the limited number of male offspring no significant effects were seen. In conclusion, our results show that a single oral dose of TCDD to rat dams during gestation impair bone tissue composition and function in the female offspring.

## 680 EXPRESSION OF EGFR AND ITS LIGANDS IN RESPONSE TO TCDD OR RETINOIC ACID IN EGF AND TGF $\alpha$ KO FETAL MOUSE PALATE.

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Epidermal growth factor (EGF) and transforming growth factor-alpha (TGF) are expressed in the developing palate and changes in patterns of expression are associated with cleft palate (CP) induction by retinoic acid (RA) and 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD). The incidence of CP is reduced in EGF knockout (KO) mice after exposure to TCDD or RA on gestation day (GD)12. TGF KO affected CP incidence only at high doses of TCDD or after exposure to RA on GD10. The teratogenic responses in embryos lacking EGF or TGF may be related to compensatory expression of other ligands for the EGF receptor (EGFR) in the target tissues. In the present study, EGFR, EGF, TGF, amphiregulin (AR), epiregulin (ER), heparin binding EGF (HB-EGF), and betacellulin (BTC) mRNA expression was evaluated by real time PCR. Pregnant mice were dosed with 100  $\mu g/kg$  RA or 24  $\mu g/kg$  TCDD on GD12 and total RNA was prepared from GD14 palates of wild type (WT), EGF KO, C57BL/6J, and TGF KO fetuses. Data are reported as cycle threshold for each strain, treatment and gene. EGFR and BTC expression were significantly elevated in both EGF KO and TGF KO palates. In the TGF KO the expression of EGF and HB-EGF was induced relative to levels in C57BL/6J. In response to either RA or TCDD, the WT palates had increased expression of EGF, HB-EGF and AR, compared to WT controls. In TGF KO, ex-

pression of ER was elevated by exposure to TCDD compared to control TGF KO. In EGF KO, TCDD or RA altered expression of EGFR or the ligands was not detected. In summary, the EGF and TGF KO palates show upregulation of EGFR and some of its ligands. Exposure to TCDD or RA also altered regulation of the ligands in the WT and TGF KO palates. Increased expression of EGFR and some of its ligands may represent compensation for the lack of expression of EGF and TGF in the KO and may facilitate palatogenesis in the KO. Absence of these responses in the RA and TCDD exposed EGF KO may be implicated in the reduced sensitivity for CP induction. This abstract does not necessarily reflect EPA policy.

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A ROBUST EXAMINATION OF EFFECTS OF TCDD ON THE DEVELOPING MALE REPRODUCTIVE SYSTEM.

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One of the most potent reported adverse effects of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) is on the developing male reproductive system, after exposure of the embryo/ foetus via dosing of the pregnant mother. This endpoint has been used as the basis of advisory limits for exposure to TCDD by the EU and UK [1]. We set out (1) to determine the foetal dose of TCDD associated with a toxic endpoint in a concurrent experiment; (2) to clarify inconsistency in the reported literature. Timed Wistar rats were exposed to control vehicle, 50, 200 or 1000 ng TCDD /kg bwt. on GD15. Dams were killed on GD16 and 21 for determination of TCDD concentration. ~20 dams per group were allowed to litter, and 5 males per litter retained after weaning. 25 animals per group were killed for reproductive assessment on PND 70. The remaining animals (~60) were assessed on PND120. TCDD at 1000 ng/kg caused a reduction in the number of pups born, and reduced the number of pups surviving to PND14. The male offspring in this group were lighter than control at all times although their weight gain exceeded controls, and had a delayed balano-preputial separation. The testes were slightly lighter than control in this group, and the seminology at PND70 showed a transient increase in abnormal sperm. There was no significant effect on epididymal sperm number at PND 70 or 120. There was no significant effect of TCDD at PND70 or 120 at lower dose levels. The foetal concentration of TCDD was ~ 0.02, 3, 9 and 50 ng/kg tissue in the 0, 50, 200 and 1000 ng/kg groups, respectively. This experiment has greater statistical power than previous investigations of TCDD developmental reproductive toxicity. A follow-up sub-chronic study will provide more robust data as a basis for further consideration of tolerable intake levels. This work was supported by a UK Food Standards Agency contract, T01034 1.

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COMPARISON OF MATRIGEL™ AND GELATIN SUBSTRATA FOR FEEDER-FREE CULTURE OF UNDIFFERENTIATED MOUSE EMBRYONIC STEM CELLS FOR TOXICITY TESTING.

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Murine embryonic stem (mES) cells have been used to evaluate cytotoxicity and developmental injury following exposure to embryotoxic agents. However, maintaining a homogeneous population of undifferentiated mES cells for this purpose has been complicated by the need for continuous co-culture with murine embryonic fibroblast (mEF) cells or limited passaging on plastic surfaces coated with gelatin. Here, we compare the synthetic basement membrane Matrigel™ with 0.1% gelatin substratum for feeder-free propagation of undifferentiated mES cells. Biomarkers of pluripotentiality, chromosome number, caspase-3 expression, and cardiomyocyte differentiation were monitored for mES cells cultured on Matrigel™ or 0.1% gelatin up to passage 7 (P<sub>7</sub>). Our results suggest that choice of substratum had no significant effect on population doubling time, cell viability, stage-specific embryonic antigen-1 (SSEA-1) expression, or early passage formation of beating cardiomyocytes (all  $P \geq 0.09$ ). In other comparisons, however, Matrigel™ supported significantly higher synthesis of alkaline phosphatase ( $7.7 \times 10^{-3} \pm 0.8$  vs  $6.6 \times 10^{-3} \pm 0.8$  units/liter/cell, respectively,  $P=0.01$ ), overall expression of activated caspase-3 following exposure to 5, 10, 50, 100 and 500 parts per billion (ppb) sodium arsenite ( $P < 0.0001$ ), and percent development to beating cardiomyocytes at P<sub>7</sub> ( $P=0.01$ ). Together, our findings suggest that Matrigel™ shows promise as a substrate for feeder-free propagation of undifferentiated mES cells for embryotoxicity endpoints.

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ALTERED TRANSCRIPTIONAL RESPONSES IN MOUSE EMBRYOS EXPOSED TO BISINDOLYLMALEIMIDE I (BIS I) IN WHOLE EMBRYO CULTURE.

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Protein kinase C (PKC) comprises a large family of serine/threonine kinases which are involved in a multitude of physiological processes regulating cell proliferation and differentiation. Bisindolylmaleimide I (Bis I), a specific PKC inhibitor, was utilized in this study to examine the transcriptional responses to PKC inhibition in mouse embryos during neurulation. CD-1 mouse embryos (3 to 6 somite stage) were exposed to 10µM Bis I for 0, 1, 3 or 6h using whole embryo culture. At these times 10-15 embryonic heads were removed and pooled for total RNA isolation. Biotinylated cRNA was prepared using GeneChip one-cycle target labeling and hybridized to GeneChip Mouse Genome 430 2.0 arrays (Affymetrix, Inc., Santa Clara, CA). Two-way ANOVA identified 3, 207 genes (of 34, 000 transcripts on the array) as being differentially expressed across dose and/or time. Pathway analysis of these genes identified numerous pathways as affected by the treatment with Bis I including apoptosis, Wnt signaling and oxidative phosphorylation. Gene Ontology (GO) analysis of molecular function identified an extensive list of genes associated with binding events and catalytic activity. Cluster analysis and linear models were used to further identify the transcriptional responses in mouse embryos exposed to Bis I and their association with embryotoxicity. *This is an abstract of a proposed presentation and does not necessarily reflect EPA policy. Funded by EPA/UNC Toxicology research program, training agreement CT 827206 with the Curriculum of Toxicology, University of North Carolina at Chapel Hill, North Carolina.*

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FOLATE PREVENTION OF OXIDATIVE STRESS IN FUMONISIN-INDUCED NEURAL TUBE DEFECTS.

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Fumonisins B1 (FB1) is a mycotoxin produced by *F. verticillioides*, a common contaminant of corn worldwide. An association between maternal FB1 exposure and increased risk for neural tube defects (NTDs) has recently been observed in human populations relying on corn as a dietary staple. FB1 disrupts sphingolipid biosynthesis by inhibiting ceramide synthase, resulting in elevated sphinganine levels, and depletion of downstream glycosphingolipids. We have previously demonstrated that early gestational exposure to FB1 results in NTDs in 80% of exposed inbred LMBC mouse fetuses. FB1 depletion of ganglioside GM1 altered plasma membrane microdomains, compromising folate uptake via the GPI-anchored folate receptor. Daily maternal folate supplementation reduced the incidence of NTDs, while replacement therapy with ganglioside GM1 restored folate uptake and rescued the phenotype. FB1 exposure has been shown to increase expression of inflammatory cytokines such as TNF $\alpha$ . Preliminary microarray data from FB1-exposed embryos indicate altered expression of sphingolipid and TNF $\alpha$  receptor superfamily genes, as well as numerous genes involved in modulating oxidative stress and nitric oxide production. Atypical expression of TNF $\alpha$  is seen in the developing neural tube following FB1 exposure, corresponding to positive TUNEL staining in the ventricular zone. The apoptotic cells also stain positive for iNOS and nitrotyrosine. Maternal folate supplementation reverses the nitrosylation, and prevents against excessive apoptosis in the neural tube. Folate is structurally similar to tetrahydrobiopterin (BH4), the endogenous co-factor for iNOS, and maternal administration of BH4 is effective in protecting against FB1-induced NTDs in our mouse model. Inadequate levels of BH4 result in uncoupling of iNOS, production of reactive oxygen species, and protein nitrosylation. Supplemental folate may functionally replace BH4 as a co-factor for iNOS in order to maintain redox homeostasis under conditions of oxidative stress.

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MATERNAL IMMUNE STIMULATION REDUCES DIABETES INDUCED CRANIOFACIAL MALFORMATIONS.

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Maternal diabetes can induce a number of developmental abnormalities in offspring of both laboratory animals and humans, including facial deformities, defects in neural tube closure, and alterations in cardiac, renal, optic and auricular development. The incidence of birth defects in newborns of diabetic women is approximately 3 to 5 times higher than among non-diabetics. In mice, non specific activation of the maternal immune system can reduce fetal abnormalities caused by

diverse etiologies, including diabetes induced neural tube defects. This study was conducted to determine the effects of non-specific maternal immune stimulation on diabetes induced craniofacial defects. Maternal immune function was stimulated prior to induction of hyperglycemia by one of three methods: 1. maternal footpad injection with FCA; or 2. maternal IP injection with GM-CSF; or 3. maternal IP injection with IFNg. Streptozocin (200 mg/kg IP) was then used to induce hyperglycemia (26-35 mmol blood glucose) in female ICR mice prior to breeding. Fetuses from 10-14 litters per treatment group, (over 100 fetuses per group) were collected at day 17 of gestation. Craniofacial defects were observed in fetuses from all groups, and consisted of severe clefting of the rostral maxillary region, reduced maxillary length, altered maxillary to mandibular ratio, and agnathia. Clearing and staining of the fetal heads revealed alterations in the frontal, parietal, interparietal, nasal, maxillary and premaxillary and mandibular bones. Maternal stimulation with IFNg and GM-CSF reduced the incidence and severity of facial deformities to a greater extent than did FCA. These exciting findings set the ground work for further studies into the mechanisms involved in the prevention of diabetes induced birth defects by maternal immune stimulation. Supported by NIH K01RR16241-01A1.

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ARSENIC TOXICITY: MOLECULAR AND DEVELOPMENTAL MECHANISMS IN MISCARRIAGE.

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**Rationale:** Human arsenic (As) exposure is linked to developmental defects in the nervous system and limbs, pregnancy complications and miscarriage. The goal of this project is to define the developmental and molecular mechanisms for how As exposure causes developmental defects and miscarriage. **Hypothesis:** As exposure *in utero* causes endothelial dysfunction leading to defective vasculogenesis that results in terogenesis and miscarriage. **Aims & Methods:** 1. Determine how As exposure affects fecundity, placentation and vascular morphogenesis. Timed-pregnant mice were exposed to sodium arsenite (As III; 150, 75, 37.5, 20, 10 ppm) in their drinking water. Pregnancy rate, birth rate, litter size, and pup/embryo weight were compared to controls. Embryos and placentas were examined for vascular abnormalities. Cultured quail embryos were exposed to AsIII and analyzed for alterations in vascular development. 2. Determine how As exposure causes endothelial dysfunction. Human microvascular endothelial cells were exposed to AsIII and analyzed for alterations in cell viability (MTT assay), proliferation (BrdU assay), apoptosis (TUNEL & caspase assays) and growth factor/cytokine expression (Luminex). **Results:** AsIII exposure causes miscarriage between E7.5 and E12.5, when the placenta and embryo become vascularized. Histology shows abnormal neovascularization in the embryo and placenta. The mouse results are consistent with the quail results; where the latter show abnormal yolk sac and embryonic neovascularization. As exposure experiments in microvascular endothelial cells (HMEC) reveal a loss of viability due to enhanced apoptosis and decreased proliferation; commensurate with altered VEGF and FGF-2 expression. **Conclusions:** These data suggest that As exposure causes angioblast and endothelial dysfunction during vascular development. ELISA and histological data suggest that these effects are likely due to changes in vascular morphogens/growth factors involving the VEGF regulatory pathway.

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SYNERGISTIC DEVELOPMENTAL TOXICITY OF POLYCYCLIC AROMATIC HYDROCARBONS: TOWARDS A MECHANISTIC UNDERSTANDING.

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Polyyclic aromatic hydrocarbons (PAHs) invariably occur in the environment as complex mixtures of non-substituted PAHs, alkyl-PAHs, and N-, S-, and O-ring substituted PAHs (such as carbazoles, thiophenes, and furans, respectively). Recent studies in our laboratories with real world mixtures derived from pollution events in marine and estuarine systems (the Exxon Valdez oil spill in AK and a wood treatment facility in the Elizabeth River, VA) revealed potent developmental toxicities in fish embryos. Developmental perturbations included head and tail deformities, and most pronounced were cardiovascular defects. Collectively these effects are consistent with the blue sac syndrome associated with embryo exposures to planar halogenated aromatic compounds, notably dioxin. These dioxin-mediated effects are associated with the binding of dioxin to the aryl hydrocarbon receptor (AHR), although mechanisms downstream of receptor binding remain unresolved. PAH mixtures include AHR agonists and we initially hypothesized that developmental effects observed were via this mechanism. However, aqueous exposures of rainbow trout embryos to a suite of PAHs with varying affinities for the AHR and potency

as CYP1A inducers failed to support a correlation between either binding or induction and toxicity. Other experiments with killifish embryos examined interactions between PAH-type AHR agonists (BNF and BaP) and a variety of CYP1A inhibitors (ANF, PBO, fluoranthene, carbazole and dibenzothiophene). In the majority of cases, we observed synergistic toxicity between AHR agonists and CYP1A inhibitors, which contrasts to the protective effect reported for CYP1A inhibition on the developmental toxicity of dioxin and PCB126. This observed synergy has important implications for accepted additive models of PAH toxicity. Current work is exploring mechanisms underlying these interactions, including alterations in PAH metabolism and gene expression, and oxidative stress.

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ARSENIC TRIOXIDE INHIBITS NUCLEAR EXPRESSION OF RXR AND BINDING OF RXR-CONTAINING HETERODIMERS.

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Recently, arsenic exposure has been linked to an increased risk for atherosclerosis and diabetes as well as cancer. We have previously shown that arsenic trioxide ( $As_2O_3$ ) inhibits the transactivation of those nuclear receptors that heterodimerize with RXR and control the expression of many genes, including those involved in cholesterol metabolism (via LXR) and glucose regulation (via PPAR). Here, we confirm that several downstream targets of nuclear receptors, such as ABCA1, CYP3A4, and vitamin D3 24-hydroxylase, are also inhibited by  $As_2O_3$ .  $As_2O_3$  treatment does not alter the levels of total RXR mRNA or protein, but we find RXR is a target of  $As_2O_3$ -induced phosphorylation. Through site-directed mutagenesis,  $As_2O_3$ -induced phosphorylation of RXR was linked to inhibition of nuclear receptor transactivation. Although total RXR levels are not altered,  $As_2O_3$  treatment decreases binding to retinoid response elements in a dose- and time-dependent manner. In addition, we see an increase in the cytoplasmic expression of RXR and concomitant decrease in nuclear expression. These data suggest a mechanism by which  $As_2O_3$  exposure might lead to the development of disease and even has implications to those leukemia patients being treated with  $As_2O_3$ .

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P53 MODULATES ARSENITE-INDUCED MITOTIC DISRUPTION AND ALTERED GENE EXPRESSION.

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Arsenic is both a carcinogen and an efficacious chemotherapeutic for acute promyelocytic leukemia. The underlying mechanism of arsenic-induced carcinogenesis or chemotherapy is unknown. Arsenic disrupts mitosis, induces mitotic delay, and alters chromosome segregation. Our data suggest p53 status is a determinant in response to mitotic disruption by arsenic. Arsenic-treated p53 deficient cells are prone to mitotic arrest associated apoptosis (MAAA). P53 wt cells are more resistant to MAAA and survive an aberrant, aneuploidogenic mitosis. To examine how p53 status impacts mitotic entry and exit in arsenic-treated cells, we used TR9-7 cells which express p53 from a tet-off regulated vector. TR9-7 cells expressing (p53+) or not expressing (p53-) p53 were synchronized in G2 and released with or without 5  $\mu$ M NaAsO<sub>2</sub>. Arsenite delayed mitotic entry independent of p53. Arsenite-treated p53+ cells exited mitosis normally, while treated p53- cells had delayed kinetics. To test if differential gene expression altered exit kinetics, microarray analysis was performed on mRNAs from G2 synchronized p53+ and p53- cells after 0 and 3 h arsenite exposure. Heme oxygenase and metallothionein, two oxidative stress response genes, were induced independent of p53 status. Inhibitor of DNA binding 1 (ID1) was induced 6.9-fold in p53+ cells and 4.3-fold in p53- cells. Immunoblotting confirmed induction that was more extensive and sustained in p53+ cells. As a dominant negative inhibitor of transcription, ID1 represses genes involved in cell cycle arrest. ID1 is elevated in many tumors and its overexpression immortalizes primary human keratinocytes and induces abnormal centrosome numbers. ID1 induction may play a role in the survival of arsenite-treated p53+ cells and contribute to the carcinogenicity of arsenic. (Support: USPHS grants R01ES011314, R01ES06460 F30ES013372, T32ES011564, R25CA44789, P30ES01247, Brown Cancer Center)

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P53 SUPPRESSION OF ARSENITE INDUCED MITOTIC ARREST IS MEDIATED BY P21.

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Arsenite is a chemotherapeutic for acute promyelocytic leukemia and may be efficacious in treatment of many forms of cancer depending on p53 pathway status. Therapeutic levels of NaAsO<sub>2</sub> (5  $\mu$ M) induce mitotic arrest associated apoptosis

(MAAA) in p53- cells. However, p53+ cells show delayed G2 exit and progress through M into G1. The purpose of this study was to examine the role of p53 in escaping arsenite induced mitotic arrest. TR9-7 cells expressing p53 from a tetracycline-off regulated vector were used as a model system. In this model, p21 correlated with p53 expression. Inactivation of cyclin B/CDC2 is necessary for mitotic exit and occurs by proteasomal degradation of cyclin B through anaphase promoting complex (APC) dependent ubiquitination. Arsenite treated p53- cells exhibit cyclin B stabilization and CDC2 phosphorylation on tyr15. Arsenite reduces CDC27 expression, a subunit of APC, independent of p53 expression. Remaining CDC27 becomes hyperphosphorylated, which may contribute to mitotic arrest. Wee1 and p21 activities are both p53 regulated and inhibit cyclin B/CDC2 by CDC2 tyr15 phosphorylation and direct binding, respectively. Activated p53 (ser15 phosphorylated) and p21 were induced by arsenite in p53+ cells. Arsenite induced cleavage of caspase 3 was higher and prolonged in p53- cells compared to p53+ cells. Therefore, p21 inactivation of cyclin B/CDC2 may allow mitotic exit and subsequent cell survival despite presence of stabilized cyclin B. CDC2 phosphorylation on tyr15, however, is not sufficient to allow escape from arsenite induced mitotic arrest. P21 siRNA knockdown caused p53+ cells to assume a p53- phenotype with an increase in mitotic index, cyclin B stabilization and CDC2 phosphorylation in response to arsenite treatment. We conclude that p53 mediated cyclin B/CDC2 inactivation and mitotic blockade release is not via Wee1 activation but via p21 induction and direct inhibition of CDC2. (Support: USPHS grants R01-ES011314, R01-ES06460, F30-ES013372, T32-ES011564, P30-ES01247)

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### ARSENIC EXPOSURE ACCELERATES ATHEROGENIC CHANGES IN APOE-/- MICE.

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Chronic arsenic ingestion causes atherosclerosis in humans. Acceleration of aortic lesion formation and alteration of vascular responsiveness in atherosclerosis prone ApoE-/- mice maintained on normal chow by fetal and post-natal arsenic exposure was examined. Mice were divided into 4 exposure groups: controls given tap water (T/T), transplacental gestational day 8-birth only exposure (A/T), 21 days post-natal to sacrifice exposure only (T/A), gestational plus post-natal exposure (A/A). Pregnant dams or weanling mice were given drinking water containing 85 mg/L NaAsO<sub>2</sub>. Fatty streak formation was examined throughout the aortic tree of mice sacrificed at age 10 weeks. Post-natal only arsenic-exposed mice displayed a 4- to 6-fold increase in lesion formation in the aortic sinus and arch compared to controls. Gestationally exposed mice showed no increase. Plasma cholesterol was unaltered by arsenic exposure suggesting that arsenic feeding exacerbates atherosclerotic lesion formation in ApoE-/- mice independent of plasma cholesterol concentration. Vasomotor function of the thoracic aorta from 10-12 week old animals was examined utilizing aortic rings to measure contractile force. Vessel contraction with either cumulative doses ( $10^{-9}$  -  $10^{-5}$  M) of phenylephrine (Phe) or buffer containing 80 mM KCl was unaltered by arsenic exposure. However, there were significant differences in the relaxation of arsenic exposed vessels induced with cumulative doses ( $10^{-9}$  -  $10^{-5}$  M) of acetylcholine following maximal contraction with  $10^{-5}$  M Phe, but not in the presence of sodium nitroprusside. Relaxation was reduced by 10-12% in T/A and A/T vessels and by 20% in A/A vessels compared with controls (T/T). These results indicate that nitric oxide availability becomes compromised in arsenic-treated vessels suggesting a likely defect in endothelial eNOS. We conclude that even brief arsenic exposures can induce atherosgenic changes in ApoE-/- mice independent of high fat diet. Supported by University of Louisville Center for Genetics and Molecular Medicine.

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### EXAMINING BIOMARKERS OF EXPOSURE AND PREPARATION TECHNIQUES FOR ARSENIC QUANTITATION IN BIOLOGICAL STUDIES.

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Accurate quantitation of any contaminant of interest is critical for toxicity and metabolism studies that support risk assessment. A first step in an arsenic exposure assessment study in Nevada quantified total arsenic (TAs) concentrations in biomarkers of exposure. Participants in this IRB approved study (N=95) were at least 45 years old, had lived in the area for more than 20 years, and were exposed to a wide range of arsenic concentrations in drinking water (3 ppb to 2100 ppb). Concentrations of arsenic in drinking water were determined for each subject by graphite furnace-atomic absorption spectrometry. Concentrations of TAs in urine, blood, and toenails were determined by hydride generation-atomic fluorescence spectrometry (HGAFS); toenail TAs was also determined by neutron activation

analysis (NAA). TAs concentrations in blood, urine, and toe nail samples ranged from below HGAFS detection to 0.03 ppm, 0.76 ppm, and 12 ppm, respectively. TAs in blood rarely exceeded the limit of detection. A good correlation was found between the TAs concentration in nails and in drinking water ( $r^2=0.46$ ,  $p<0.001$ ); a poorer correlation was found between TAs concentrations in urine and drinking water ( $r^2=0.19$ ,  $p<0.001$ ). Analyses of TAs in toenails by HGAFS and NAA yielded highly concordant estimates ( $r^2=0.84$ ,  $p<0.001$ ). These results suggest that toenails are a better biomarker of exposure than urine because the sequestration of As in toenails provides an integration of exposure over time that does not occur in urine. (This abstract does not reflect USEPA policy.)

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### BIOMONITORING OF A COMMUNITY FOR SOIL ARSENIC EXPOSURE.

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In response to community concerns over arsenic in soil from a pesticide manufacturing plant, a cross-sectional biomonitoring study was conducted of young children less than age 7, the age group most exposed to soil. Two first morning void samples from 77 children (47 percent of the population in this age range) were composited for analysis of total arsenic and urinary arsenic species related to ingestion of inorganic arsenic. Older ages (N=362) also participated, but were not targeted for the study. A subset of these older participants also provided toenail samples for total arsenic analysis. Soil (41 young children; soil arsenic <60 ppm), house dust (52 young children), and garden vegetable samples were collected and analyzed for arsenic. Speciated urinary arsenic levels were well below the reference level of 40  $\mu$ g/L denoting upper limit of background exposure and results for young children (GM=4.0, GSD=2.2; 0.89-17.7  $\mu$ g/L range) were similar to older participants (GM=3.8, GSD=1.9; 0.91-19.9  $\mu$ g/L range). Total arsenic levels in 26 participants exceeded the total arsenic reference level of 50  $\mu$ g/L; however, urinary species profiles indicated ingestion of dietary organic arsenic forms. Arsenic levels in all 67 toenails samples analyzed were below the reference level of 1 ppm, even though samples with higher arsenic levels showed evidence of external soil contamination. Correlations between speciated urinary arsenic levels and arsenic in soil ( $r=0.137$ ,  $p=0.39$ ) or house dust ( $r=0.049$ ,  $p=0.73$ ) were not significant for young children. Including older age groups did not change these results. Similarly, indirect indicators of soil exposure obtained from the geographic distribution of urinary levels within the community and from questionnaire responses (e.g., mouthing rates, eating garden vegetables, time spent outdoors) did not indicate increases in speciated or inorganic urinary arsenic levels due to arsenic in soil. In conclusion, no consistent evidence of increased arsenic exposure from soil was found for this community.

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### CARCINOGENIC EFFECTS OF CADMIUM CHLORIDE, CISPLATIN AND NICKEL SUBSULFIDE IN METALLOTHIONEIN-I/II DOUBLE KNOCKOUT MICE.

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Metallothionein (MT) is a high-affinity metal binding protein thought to mitigate the toxicity of various metals. MT may detoxicate a metal by direct binding or through action as an antioxidant for metals that generate reactive oxygen species. Cadmium, cisplatin and nickel have carcinogenic potential in humans and/or animals. Exactly how MT deficiency might impact the carcinogenic effects of these metals is unknown. Thus, groups (n = 25) of male MT-II/I double knockout (MT-null) or MT wild type (WT) mice received a single treatment with cadmium (1 or 5  $\mu$ mole Cd/kg, s.c.; as CdCl<sub>2</sub>), cisplatin (5 or 10 mg/kg, i.p.), or nickel (0.5 or 1.0 mg Ni<sub>3</sub>S<sub>2</sub>/site, i.m., into both hind legs), or were left untreated (control) and observed over the next 104 weeks. There were no differences in the incidence of spontaneous tumors in MT-null and WT mice. In cisplatin-treated MT-null mice a dose-related increase in hepatocellular tumors, including carcinoma (control 0%, 5 mg/kg 16.7%, 10 mg/kg 36.4%), occurred that was not seen in WT mice. Similarly, cadmium treated MT-null mice showed a dose-related increase in hepatocellular tumors (control 8.3%, 5  $\mu$ mole/kg 20.0%, 10  $\mu$ mole/kg 33.3%) that did not occur in WT mice. Nickel-treated MT-null mice developed lung adenocarcinomas not observed in WT mice, while pulmonary tumors were induced by cisplatin in both MT-null and WT mice. Lung tumors developed after cadmium treatment in WT mice but not in MT-null mice. Harderian gland adenocarcinomas were induced by cisplatin but only in MT-null mice. Nickel induced injection site fibrosarcomas in a dose-related fashion in both WT and MT-null mice. Overall, MT-null mice appear more sensitive to the carcinogenic effects of cadmium, cisplatin, and, to a lesser extent, nickel, but only in certain tissues. Thus, poor MT production may be a predisposing factor for cadmium, cisplatin and nickel carcinogenesis, at some, but not all, target sites.

## LEAD IS A RISK FACTOR FOR OSTEOARTHRITIS: MOLECULAR MECHANISMS.

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Evidence in the medical literature supports the notion that lead exposure is a risk factor for osteoarthritis. The observations are: i) sustained chronic exposure leads to joint pain, ii) lead in synovial fluid from bullet fragments induces a marked degeneration of the articular surface and iii) animal studies have documented that it is, in fact, the lead rather than other heavy metals or a mechanical impingement that causes the arthritis. Articular chondrocytes maintain a phenotype that allows for preservation of a normal matrix. A key regulator of the chondrocyte phenotype is TGFbeta. Interruption of TGFbeta signaling induces chondrocyte cloning, hypertrophy, mineralization and apoptosis. This change in cell behavior progresses to an arthritic pathology. Our data indicate that lead blocks the TGFbeta pathway in articular chondrocytes by blocking Smad phosphorylation and by up regulating Smurf levels. These effects cause a chronic but progressive degradation of the articular matrix. Methods Isolated articular chondrocytes were exposed to lead over a range of concentrations. TGFbeta signaling was measured by monitoring Smad activity with a specific reporter construct. Phosphorylated Smad and Smurf levels were measured with Western blots and RT-PCR. The knee joints of mice exposed to lead (blood level: 40 ug/dl, 12 weeks) were histologically evaluated for arthritic changes. Results: Lead significantly depressed TGFbeta signaling in articular chondrocytes at concentrations as low as 0.1  $\mu$ M. Phospho Smad levels were also markedly depressed. This effect was mediated by a reduction Smad kinase activity and an increase in Smurf function. The incidence of arthritic changes in untreated mice was 6%. The incidence in lead-treated mice was 40%. Discussion Our data provide evidence that the pathological features of osteoarthritis can be evoked in articular chondrocytes and animals by lead exposure. Isolated cells, when treated with low levels of lead are inhibited in TGFbeta signaling leading to osteoarthritis in an animal model.

## DISSOLUTION RATES OF NANOPARTICULATE METALLIC SPECIES IN ARTIFICIAL HUMAN SWEAT UNDER PHYSIOLOGICALLY RELEVANT CONDITIONS.

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The ability of nano-sized Al particles to improve the efficiency and power of combustion reactions when mixed with propellants and explosives has generated substantial interest and research over the past decade. As a result, a greater amount of nano-sized Al is being manufactured and the potential for human exposure to this material is increasing. One potentially significant route of exposure is dermal absorption. Given the extremely high surface area, nano-sized Al particles have the potential to dissolve in aqueous solution at a higher rate and produce greater numbers of Al ions on the skin surface than is generated by larger Al objects. As an initial investigation, dissolution rates of 150-nm and 20- $\mu$ m Al particles in artificial human sweat solutions were compared at biologically relevant temperature and pH levels. 1 mM suspensions were prepared in artificial human sweat adjusted to pHs 2.1, 4.5, and 6.9. The suspensions were placed in a 37°C water bath and agitated for 72 h. Sample aliquots were removed at 0, 3, 6, 12, 24, 48 and 72 h, filtered through a 0.02  $\mu$ m syringe filter and analyzed by ICP-AE. Additional materials, including MnO<sub>2</sub>, MoO<sub>3</sub> and SnO<sub>2</sub>, were also studied. For each material tested, dissolution rate was dramatically affected by pH, with rates in the pH 2.1 sweat solution more than ten times greater than dissolution rates in the pH 6.9 sweat. For workers exposed to powdered metals, dissolved species may prove to be a significant source of exposure.

## GENETIC TOXICOLOGY TESTING IN HTS.

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Many analytical tests have been reduced to simple high throughput screening assays. The battery of regulatory genetic toxicology tests do not lend themselves to HTS since they are not readily automated and require too much compound. There are some bacterial screening tests with good Ames prediction, but they lack key eukaryotic targets for known genetic toxins. More recently a validated yeast test has been introduced which like the bacterial tests is suitable for screening large number of compounds, but also provides chromatin and key enzyme targets (polymerases, topoisomerases etc). We reviewed data from both the battery of regulatory genetic toxicity tests, and the bacterial and yeast screening tests, published in the peer-reviewed literature and Internationally recognised databases. The data were used to compare the effectiveness of prediction of rodent carcinogenicity for the screening

and analytical tests, as well as an estimate of the effectiveness of the screening tests in predicting a positive result in the regulatory battery. In a previous study of 102 compounds (SOT2004: Kitching et al) a combination of bacterial and yeast tests was shown to predict 80% of rodent cancer data, and a similar figure was found in the same dataset for cancer prediction by the regulatory test battery. This wider study will include data from more than 250 compounds. Our principal conclusion from these data is that the tools now available for genotoxicity screening are now sufficiently well established to allow their routine use in both pharmaceutical screening and environmental testing. For the former, the data will be of real value in candidate choice (hits-to-leads) as well as lead optimisation programmes. For the latter, far more sampling should be possible so that full analytical resources need only be used where hazards are present.

## AMES II AND DEREK VERSUS THE STANDARD AMES PROTOCOL: A COMPARATIVE STUDY.

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The Ames II Assay is used as screening tool in early drug development due to the low amount of compound (5 mg) that is needed, the high throughput and its described overall predictivity for the Standard Ames Test. The purpose of this study was to compare the results of the Ames II Assay using 16 compounds representing 9 different DEREK alerts with results published for the Standard Ames Test. As the predictivity is not supposed to be 100 %, the study was performed to identify compound classes resulting in lower concordance between Ames II and the Standard Ames. In addition, the predictivity of DEREK for the outcome of the Ames II assay should be investigated. It could be demonstrated that 12 out of 16 compounds of different structural classes showed congruent results between the Ames II and standard Ames. 3 compounds were found to be negative in the Ames, but positive in the standard Ames (5-Amino-4-imidazolcarboxamide, N, N-Dimethylnitrosamine and Glutaraldehyde). All these compounds were detected in the standard protocol in the strains detecting base pair substitutions. One of the compounds (N-Hydroxymethylacrylamide) was found to be positive in the Ames II but not in the Standard Ames. We identified three classes of compounds for which further investigations should be conducted to improve the predictivity of the Ames II. In conjunction with DEREK analysis it seems to be possible to provide differentiated alerts for a mutagenic potential of a drug candidate.

## STUDY IN VITRO OF THE TUMORIGENIC ACTION OF ACRYLAMIDE ON THYROID CELLS.

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The main consequence of chronic administration of acrylamide to rats is the generation of thyroid tumors. There is no a priori reason for this relative specificity. The present work aimed at explaining the mechanism of the acrylamide effect. FRTL5 and PCC13 rat thyroid cell lines were used as model systems and primary human thyroid cell cultures for validation. As TSH acting through cyclic AMP is the main physiological growth control agent acting on the thyroid, the first hypothesis tested and rejected was that acrylamide could stimulate cAMP accumulation in these cells. The thyroid cell is one of the most active in the generation of H<sub>2</sub>O<sub>2</sub> which could act as growth or mutagenic signal. The second hypothesis rejected was that acrylamide would stimulate H<sub>2</sub>O<sub>2</sub> generation. A third hypothesis was that tumorigenesis resulted as in the case of chronic TSH or oncogenic stimulation: acrylamide would act as a mitogenic stimulant, by whatever mechanism, on these cells. As evaluated by FACS counting of cells having incorporated bromodeoxyuridine in their DNA, no such stimulation was observed *in vitro*. A fourth hypothesis was that acrylamide could induce DNA damage and consequently mutations in thyroid cells. To test this hypothesis cells were treated with acrylamide and DNA damage tested by the comet assay after permeabilization and chromatin dissociation. H<sub>2</sub>O<sub>2</sub> was used as positive control. Acrylamide at rather low concentrations (1 microg/ml) and after a relatively short time (one hour) indeed induced comet generation. This is not due to the induction of DNA double strand breaks as H<sub>2</sub>Ax phosphorylation, as evaluated by immunohistochemistry and Western blotting was negative. Thus, at this stage, the only effect of acrylamide discovered that could account for the generation of thyroid tumors is a DNA damage leading to mutagenesis.

## UNEXPECTED CLASTOGENICITY OF SOME PHARMACEUTICALS IS DUE TO DNA INTERCALATION.

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Rigorous genotoxicity testing of pharmaceuticals is mandated prior to FDA approval. Early pre-development genotoxicity screening prior to regulatory GLP genotoxicity testing is generally adequate to provide an indication of any complica-

tions that might be encountered in these later studies. Occasionally, however, positive responses are seen in the mouse lymphoma assay (MLA) and/or the *in vitro* chromosome aberration assay that were not predicted by early studies or by standard computational analysis of structural alerts (e.g., MCASE or DEREK) for clastogenicity. A previously published evaluation of marketed pharmaceuticals revealed nearly 70 such examples. Our previous studies have suggested that non-covalent drug/DNA interactions, notably DNA intercalation, may account for some of these unexpected positive results. In the present study, we investigate 30 commercially available pharmaceuticals previously shown to be clastogenic despite not being alerting for DNA intercalation ability in the cell-based V79 bleomycin amplification assay as well as by computational 3-D DNA docking studies. It is demonstrated that many, but not all, of these drugs appear to have intercalative activity which could explain their clastogenicity. These results are discussed with respect to mechanism of genotoxicity and the value of early predictivity of non-covalent DNA interactions.

## 701

### PHOSPHOTRIESTER ALKYLATION OF DNA BY MNU AND MMS.

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Recent *in vitro* data from our laboratory have shown that N-methyl-N-nitrosourea (MNU) and methyl methanesulfonate (MMS) yield different alkylation profiles for model proteins, although their alkylation patterns for nucleosides are similar. Alkylation of DNA bases by mutagenic/carcinogenic chemicals has been investigated for many years, however, much less information is available on alkylation of the deoxyribose-phosphate backbone of DNA, and the subsequent formation, biological fate, and significance of these phosphotriester (PTE) adducts. A new analytical methodology to quantify PTE adducts, using liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) was developed and applied to compare the formation of alkylated bases and PTE adducts in DNA. MNU and MMS were incubated with DNA from salmon sperm and calf thymus. The alkylated DNA samples were enzyme-digested to release nucleosides or dinucleotides, depending on the alkylation (phosphotriesters are resistant to nuclease P1). Samples were analyzed by LC/ESI-MS/MS. Results demonstrated that 24 hr treatment of calf thymus DNA with 10 mM MNU or MMS yielded different profiles *vis-à-vis* both base alkylation and PTE adduct formation. MNU treatment resulted in higher levels of methyl-deoxyguanosine adducts than MMS, while methyl-deoxyadenosine was higher following MMS treatment. Salmon sperm DNA treatment also resulted in similar, chemical-specific alkylation patterns. Both alkylating agents caused formation of PTE adducts, however MNU treatment resulted in an order of magnitude higher levels of the PTE alkylated dinucleotide Tp(Me)T than MMS. Preliminary data from cell cultures indicates that PTE adducts are also present following 4 hr exposures to MNU (treatment concentration range: 2-200  $\mu$ M). In light of their different biological effects, where MNU is considered a 'super mutagen' and MMS a 'super clastogen', this difference in formation of PTE adducts may play a role in the mechanism of mutagenicity/carcinogenicity of these alkylating agents.

## 702

### RECOGNITION AND REPAIR OF BUTADIENE EPOXIDE DNA ADDUCTS: INVOLVEMENT OF NUCLEOTIDE EXCISION REPAIR.

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Reactive epoxides, butadiene monoepoxide (BDO) and diepoxide (BDO2), are formed during the biotransformation of 1, 3-butadiene (BD). Both can react with DNA to form potentially mutagenic adducts. Mutations resulting from exposure to BD include single to multinucleotide deletions and point mutations (A/T?T/A transversions, A/T?G/C transitions). We examined the role that nucleotide excision repair (NER) has in recognizing and repairing BD-DNA adducts. Transgenic mice deficient in NER with a targeted knockout of the Xpc gene were exposed to BDO (300 mg/kg) and BDO2 (45 mg/kg) by intraperitoneal injection. Mice exposed to benzo-a-pyrene (BaP, 150 mg/kg) or cisplatin (6 mg/kg) were used as positive NER controls. Mutation induction was measured using the Hprt gene mutation assay. BaP significantly increased the frequency of Hprt mutations in Xpc knockout mice in support of the critical role this enzyme has in repairing bulky DNA adducts. The dose of cisplatin we chose proved to be acutely toxic to both Xpc proficient and deficient mice. BDO significantly increased the frequency of Hprt mutations as a function of Xpc status. In contrast, BDO2 did not increase the frequency of Hprt mutations. This apparent paradox may be explained by a reduced biologically effective dose reaching the target tissue (i.e. lymphocytes) because of the high reactivity

of the injected BDO2. We conclude that NER plays a role in recognizing and repair BD-induced DNA adducts. DNA sequence analysis of induced Hprt mutants as well as investigating other DNA repair pathways will elucidate the type(s) of mutations repaired by NER. Investigating BD-induced mutagenesis in additional mouse models of DNA repair deficiency will aid in understanding if other repair pathways are involved in repairing BD-induced DNA damage.

## 703

### BENZO[A]PYRENE-DNA ADDUCTS DERIVED FROM DIOL-EPOXIDE AND QUINONE PATHWAYS.

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Benzo[a]Pyrene (BaP), a well-known representative of polycyclic aromatic hydrocarbons (PAH), can be activated by cytochromes P450s (CYPs) and epoxide hydrolase to the dihydrodiol metabolite 7, 8-dihydroxy benzo[a]pyrene (BaP-D). BaP-D can be further metabolized by CYPs to 7, 8-dihydroxy-9, 10-epoxy-benzo[a]pyrene (BaP-DPE), which can covalently bind to DNA and form stable adducts. [<sup>15</sup>N] stable isotope-labeled (+/-)-anti-BaPDE- $N^6$ -dAdo and  $N^2$ -dGuo adduct standards were synthesized. Reverse phase HPLC method was developed for fast separation of all the adduct stereoisomers. The stereochemistry of these stereoisomers was determined by a combination of LC/MS, UV, and CD spectroscopy. (+/-)-anti-BaPDE was reacted with single-stranded and double-stranded calf thymus DNA *in vitro*. After successive enzyme digestion, the fully hydrolyzed adducts were analyzed by LC/MS/MS. The relative abundance of each adduct stereoisomer in the BaP-DPE-DNA reaction was quantified by using the isotope adduct standards. In both single-stranded and double-stranded DNA reaction, the tendency of BaP-DPE- $N^6$ -dGuo adduct formation is stronger than that of BaP-D- $N^6$ -dAdo adducts. (+)-trans-BaPDE- $N^6$ -dGuo, (-)-trans-BaPDE- $N^6$ -dGuo and (+)-cis-BaPDE- $N^6$ -dAdo are the major adduct stereoisomers. BaP-D can also be metabolized by aldo-ketose (AKR) to the corresponding quinone (BaP-7, 8-dione or BaPQ) in the quinone pathway. The adducts formed from the reaction of BaPQ and mononucleosides or DNA were studied by developed LC/MS/MS methods. The yield of adducts derived from diol-epoxide pathway and quinone pathway was compared. (Supported by NIH grant PO1 CA92537)

## 704

### SIGNALING PATHWAYS FOR DNA DAMAGE AND REPAIR, APOPTOSIS AND LYMPHOID PROGENITOR CELL SURVIVAL ARE DYSREGULATED BY N-ACETYL-L-CYSTEINE.

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Radiation induced free radicals induce DNA breaks and apoptosis of hematopoietic progenitor cells. N-acetyl-L-cysteine (NAC) scavenges free radicals and modulates free radical mediated signal transduction pathways. Thus, NAC may alter radiation induced DNA damage and repair. We have observed an increase in lymphoid tumors in 0-6 Gy gamma-irradiated wildtype and p53 haploinsufficient mice. The prevalence of lymphoma and loss of heterozygosity was increased by NAC in the diet. Specifically, an increase in the loss of the p53 wildtype allele was observed when NAC was added to the diet. To investigate, we irradiated mice after acclimation to a basal or basal plus 3% NAC diet. Three hours post-irradiation, mice were killed by CO<sub>2</sub> narcosis and bone marrow cells were removed and cultured or RNA isolation for gene expression analysis. Altered patterns of mRNA expression were observed in pathways that showed NAC suppression of Rb and NF- $\kappa$ B while inducing the transcription factor E2F1, Trp53 dependent Gadd45, Bax and Mdm2, but not p53. Bad, Bcl-w and caspases, but not Bax, Bcl-2, or Trail, expression was increased by NAC. Although the total number of cells/femur and total myeloid colony forming units (CFU)/femur were not significantly reduced, pre-B and pre-T CFU survival were greatly reduced. We speculate that high levels of intracellular NAC alters signaling pathways in pre-B and pre-T cells alters survival in radiation induced DNA damaged progenitor CFU in the bone marrow permitting selection and clonal amplification of pre-malignant lymphoid cells.

## 705

### PREVENTION OF ARSENIC CO-CARCINOGENESIS BY SELENIUM COMPOUNDS.

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Epidemiological studies show that inorganic arsenic (arsenite, arsenate) in drinking water increases skin, lung, bladder, and possibly other cancer risk in humans, but arsenite alone does not cause skin cancer in animals. Since arsenite is comutagenic and inhibits DNA repair, we hypothesized that arsenite is a cocarcinogen requiring a carcinogenic partner. This concept was tested in hairless mice. Mice given a suberythemic dose of solar UV (3 times a week) and 1.25-5 mg/l sodium arsenite in

drinking water for 26 weeks had a dose-related increase in skin cancers compared with mice given solar UV alone. The maximum cocarcinogenic effect occurred at 5 mg/l arsenite. Tumors arising in mice given UV arsenite appeared earlier and were larger and more invasive than those in mice given UV alone. Thus, arsenite may be able to partner with UV or other genotoxic insults to increase various cancers in humans. Selenium deficiency maybe one such insult. It has been suggested that in some parts of the world with high arsenic in the drinking water, the low Se levels in soil may exacerbate the arsenic toxicity and carcinogenicity. In HOS cells, organoselenium compounds blocked both spontaneous and arsenite-induced delayed mutagenesis. In the hairless mouse, the synthetic selenium compound p-XSC prevented the arsenite enhancement of solar UV-induced skin cancer, but had a much smaller effect on UV-induced skin cancer. Selenium is reported to counteract some of the effects of arsenic *in vivo* and *in vitro*. We suggest that the antimutagenic effects of selenium may occur via the antioxidant action of selenoproteins. The anticarcinogenic effect may have additional mechanisms that are specific to arsenic-selenium interactions, such as stimulation by selenium of arsenic excretion or other affects on arsenic metabolism.

**706**

CELLULAR DISTRIBUTION OF SELECTIVE AGE DEPENDENT TESTICULAR CYTOCHROME P450-DEPENDENT ALKOXYRESORUFIN O-DEALKYLATION IN SPRAGUE-DAWLEY RATS.

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This study examines the cellular localization and the constitutive cytochrome P450 (CYP) isoforms, which may play a role in 7-ethoxresorufin or 7-benzylxyresorufin O-dealkylase (EROD or BROD) activities in rat testes. In the absence of Leydig cells, neither EROD nor BROD activities are detectable in the testes, whereas enriched Leydig cells have a 4 to 5-fold increase in activities when compared to whole testes. Hepatic EROD and BROD activities in rodents have been associated with CYP1A1 and CYP1A2, but these isoforms were not immunochemically detectable in testicular microsomes. However, significant levels of CYP1B1 immunoactive protein was detectable. CYP1B1 mRNA was isolated from rat testes and expressed in human lymphoblastoid cells. The CYP1B1 recombinant cells expressed both BROD and EROD activity in relative amounts to testicular microsomes from young sexually-mature rats (approximately 3-fold more BROD than EROD). Preparations of recombinant cells expressing CYP2A1 were obtained commercially and found to express EROD, but not BROD activity. Testes from older rats expressed significant levels of CYP2A1 in addition to CYP1B1 and also expressed more EROD activity relative to BROD. CYP2A1 and CYP1B1 were the major CYP isoforms expressed in sexually mature Sprague-Dawley rat testes and appear to be responsible for the observed testicular alkoxyresorufin O-dealkylase activities. Knowledge of which CYP isoforms are expressed in testes from animal strains used in reproductive toxicology testing, in comparison to those expressed in human testes, is important for the evaluation of human risk to developmental toxicants, particularly those requiring metabolic activation.

**707**

ORAL ADMINISTRATION OF EGCG IS NON-TOXIC AND MODULATES CYP19 AND CYP2E1 IN THE FEMALE BALB/C MOUSE.

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Epigallocatechin gallate (EGCG) is a polyphenolic catechin that exhibits anti-carcinogenic properties in a variety of *in vitro* and *in vivo* cancer models. The exact mechanism responsible for the biological effect produced by EGCG has not been conclusively proven. We have previously postulated that changes in the metabolism of estradiol may be an important component of the action of EGCG as an inhibitor of breast cancer. In previous studies we demonstrated that EGCG modulates various CYP450 isoforms in the female mouse. However, EGCG (50 mg/kg, ip) also induced significant mortality and hepatotoxicity. Therefore, we have examined the ability of EGCG to modulate CYP450 isoforms and elicit hepatotoxicity following oral administration. Female BALB/c mice were administered EGCG (25 and 50 mg/kg) or saline (8 ml/kg) by oral gavage for 7 days. Ovarian and hepatic microsomes were prepared on day 8. Aromatase activity was determined in ovarian microsomes, while CYP3A, CYP1A and CYP2E1 catalytic activity and polypeptide levels were determined in hepatic microsomes. Hepatotoxicity was assessed by plasma ALT activity. The results demonstrated that EGCG was well tolerated by the mice as all mice survived the treatment regimen and there was no significant change in body weight, organ weight or hepatotoxicity elicited by EGCG. However, ovarian aromatase (CYP19) activity was decreased 31 and 46%, following 25 and 50 mg/kg of EGCG (CYP19 catalytic activity of 15 ± 2, 10 ± 1 and 8 ± 1 pmol/mg/h for saline, 25 mg/kg and 50 mg/kg EGCG, respectively. Additionally CYP2E1 catalytic activity and polypeptide levels were increased 2-fold following 50 mg/kg of EGCG (0.45 ± 0.05 and 0.91 ± 0.06 nmol/mg/min, for saline and 50

mg/kg of EGCG, respectively). However, no relevant changes in either CYP3A or CYP1A activity occurred. The results demonstrate that when EGCG is administered orally inhibition of aromatase is retained and importantly its lethality and hepatotoxic properties are lost. Therefore, EGCG when administered orally may have a future use as a non-toxic aromatase inhibitor.

**708**

MAJOR DETERMINANTS IN SEX DIFFERENCE ON DAILY RHYTHM OF HEPATIC P450 MONOOXYGENASE ACTIVITIES IN RATS.

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There is a sex difference in the daily rhythm of the hepatic cytochromes P450 (CYP) monooxygenase activities in rats. Briefly, the activities of CYP enzymes assessed by measurement of 7-alkoxycoumarin O-dealkylase (ACD) showed obvious daily fluctuations with high values during the dark period in males but not in females. Here we examined what kinds of CYP isoforms were greatly involved in the sex difference. Using an Affymetrix GeneChip system, Fourier transform was applied to analyze the daily fluctuation for each CYP probe. According to the results, many CYP probes showed obvious daily fluctuations with high values during the dark period in males but not in females. In particular, probes for CYP2B2, CYP2B15, CYP2D18 and especially CYP2E1 were extracted as the periodic probes specific to males. Moreover, the Western blot analysis for CYP2E1 was conducted to confirm the protein expression level. Consequently, the protein expression level of CYP2E1 at ZT18 was approximately 1.6 times as high as that at ZT6 in males. In contrast, there were no differences detected between at ZT6 and at ZT18 in females. Indeed, the CYP2E1 activity during the dark period was significantly higher than that during the light period in males. These results indicate that CYP2B2, CYP2B15, CYP2D18, and especially CYP2E1 might be the major determinants in the sex difference on the daily rhythm of hepatic ACD activities in rats.

**709**

INTERINDIVIDUAL VARIATION OF EXPRESSION LEVEL OF CYP3A4 AND ITS RELATED PHARMACOGENETIC GENES IN JAPANESE LIVER TISSUE.

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The CYP3A4 expression level analysis in Japanese liver mRNA was performed by the real-time reverse-transcription polymerase chain reaction method (RT-PCR) with TaqMan probe. The liver tissues were obtained from patients at Dokkyo University School of Medicine and Health Science Research Resources Bank (HSRRB) Japan after getting written informed consents. The interindividual variations of CYP3A4-related drug metabolizing enzyme, transporter, nuclear receptor and transcription factor genes were analyzed. The microsomal fractions were prepared from the same liver tissues and the protein levels of CYP3A4 and 3A5 were also investigated by Western blotting analysis. The expression level variation of CYP3A4 mRNA in 24 Japanese liver sample was correlated with CYP3A43, 2B6, 2C9, MRP2, OATP2, PXR and CAR mRNA ( $p < 0.0001$ ). As the result of the nuclear receptor mRNA, the level of PXR was correlated with CYP3A4, 3A43, 2B6, 2C9, MRP2, OATP2 and CAR and the level of CAR was correlated with CYP3A4, 3A43, 2B6, 2C9, PXR and HNF4A. The transcription factor HNF4A mRNA level was correlated with CYP2B6, 2C9 and CAR. The CYP3A5 levels of both mRNA and protein showed clear bimodal distribution: high and low expression groups. This expression level corresponds to the CYP3A5 allele frequency. These results showed that the expression level of CYP3A4 mRNA was correlated with not only the nuclear receptors but also the various drug metabolizing enzyme and transporter genes. The accumulated data on CYP3A4 expression might explain the difference of interindividual variation in the Japanese population.

**710**

USE OF NOVEL MULTIPLEXED QPCR ASSAYS TO ACCURATELY QUANTIFY HUMAN CYTOCHROME P450 EXPRESSION.

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A novel real-time qPCR system based on the activity of a DNAzyme (a catalytically active DNA sequence) was used for the quantification of three human cytochrome P450 transcripts including CYP1A2, CYP2B6, and CYP3A4. Quantifying a cy-

tochrome P450 target, a housekeeping gene, and a spiked synthetic target simultaneously in one triplex reaction generates more accurate and reproducible data than when each gene-specific assay is run separately. Use of triplex qPCR assays allows for accurate quantification of P450 expression that is both normalized to housekeeping gene expression and monitored for overall PCR efficiency via inclusion of a synthetic template in the assay. Additionally, use of a multiplex qPCR approach reduces experimental variation due to liquid handling errors, increases sample throughput, and conserves valuable experimental samples. Cytochrome P450 expression profiles were determined for induced and non-induced human primary hepatocytes. Rifampicin (RIF),  $\beta$ -naphthoflavone ( $\beta$ -NF), and phenobarbital (PB) were used as chemical inducers in a 72 hr induction study. There was a strong correlation between the level of induction of P450 CYP1A2, CYP2B6, and CYP3A4 as measured by traditional enzyme assays and real-time quantitative PCR. Likewise, in a RIF induction time course study there was a marked correlation between the level of induction of P450 CYP3A4 at each time point as measured by traditional enzyme assays and the quantitative PCR triplex assay. Triplex qPCR assays provide a robust, rapid, accurate, and reproducible method to measure the induction of cytochrome P450 expression in treated human primary hepatocytes.

## 711 USING P450-GLO<sup>TM</sup> LUMINESCENT ASSAYS TO SCREEN FOR XENOBIOTIC EFFECTS ON CYTOCHROMES P450.

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P450-Glo<sup>TM</sup> luminescence-based assays were used to characterize CYP450 enzyme inhibition, positive cooperativity and gene induction by xenobiotics. Inhibition and positive cooperativity were studied in recombinant CYP450 fractions and liver microsomes where the method was validated by testing compounds previously known to influence CYP450 activity. CYP450 gene regulation was studied in cultured rat and human hepatocytes where CYP450-Glo<sup>TM</sup> was used to measure changes in CYP450 enzyme activity as an end-point of gene expression. PXR, GR, AHR and PPAR $\alpha$ -dependent CYP450 gene inductions were observed and the CYP450 isoforms affected were inferred by sensitivity to isoform-selective inhibitors. Since the P450-Glo<sup>TM</sup> cell-based assay method left cells intact it was also possible to test for toxicity of the gene inducers by performing a luminescent cell viability assay subsequent to P450-Glo<sup>TM</sup>. The P450-Glo<sup>TM</sup> method measures CYP450 enzyme activity as light output from a luciferase reaction that is driven by the CYP450-catalyzed conversion of luciferase pro-substrates to D-luciferin. Luminogenic substrates for this method exist for human CYP450 isoforms CYP1A1, 1A2, 1B1, 2C8, 2C9, 2C19, 2D6, 2J2, 3A4, 3A7, 4A11, 4F3B, 4F12 and 19. The same substrates also react with rat isoforms including CYP1A1, 1A2, 2C6, 2C11, 3A1 and 4A. These homogenous assays were sensitive, simple to perform and readily configured in multi-well plate formats for rapid screening of multiple compounds against CYP450 activities.

## 712 DIOXIN AND DIET EFFECTS ON GROWTH AND P450 INDUCTION.

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Weight gain is retarded by 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD) and we attempted to rescue this wasting syndrome with a high fat diet. TCDD is a lipophilic toxin that is metabolized by CYP1A1, CYP1A2, and CYP1B1, and acts through the Ah Receptor (AhR). A lipophobic toxin, 7, 12-dimethylbenz[a]anthracene (DMBA), is also metabolized by CYP1A1, CYP1A2, and CYP1B1 and acts through the AhR. We examined the competitive inhibition of TCDD on DMBA induction of P450s. Pregnant mice were dosed with 1  $\mu$ g/kg of TCDD (or vehicle control) on gestational day 12.5. When pups were born, the cage was put on a high or low fat diet. Pups were weaned at post natal day (PND) 21. On PND 35, pups were dosed with 60 mg/kg DMBA on PND 31 to allow for gland involution, and sacrificed 24 hours later. Dams were given 60 mg/kg DMBA on PND 31 to allow for gland involution, and sacrificed 24 hours later. Livers were collected, and induction of CYP1A1, CYP1B1, and CYP2A1 was evaluated. Induction levels were different among groups.

## 713 DIOXIN INDUCTION OF CYP1A1 EXPRESSION IS SUPPRESSED IN HEPATITIS C VIRUS REPLICATING CELLS.

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Hepatitis C Virus (HCV) is a positive-stranded RNA virus that belongs to the Flaviviridae family. At least 400 million people worldwide are infected with the virus, and up to 85% of these people progress to chronic carriers, and can develop

cirrhosis and hepatocellular carcinoma. Although many processes are involved in the development of liver damage, the mechanism leading to cell injury is unclear. This study was initiated to test the hypothesis that chronic HCV infection modulates hepatic cytochrome P4501A1 (CYP1A) expression and function. CYP1A1 catalyzes the metabolism of numerous toxins to reactive intermediates, and is highly inducible by xenobiotics (e.g. polycyclic aromatic hydrocarbons and 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD; dioxin)) through the activation of the aryl hydrocarbon receptor (AhR). Using a human hepatoma cell model for HCV replication (Huh.8) for induction studies, we demonstrated that the induction in Huh.8 cells of CYP1A1 expression by TCDD, but not by  $\beta$ -naphthoflavone or 3-methylcholanthrene, was substantially diminished. TCDD exposure of Huh.8 cells resulted in greater than 50% suppression of CYP1A1 transcription compared to the parent cell line Huh-7; while protein levels and enzyme activities were further diminished. Time course and dose response experiments indicated suppression of TCDD-induced CYP1A1 mRNA in Huh.8 cells occurs as early as 6 hr following exposure and at all doses tested (0.1-10 nM). Nuclear AhR binding, as assessed by gel mobility shift assays, was reduced in extracts from TCDD-treated Huh.8 cells compared to Huh-7, although the decrease was considerably less than the magnitude of the transcription response. These findings demonstrate that TCDD-mediated AhR signaling is impaired in hepatocytes in which HCV is present, and that exposure of HCV-infected hepatocytes to xenobiotics can have the potential to perturb the cellular defense balance of the infected cell. Investigations into the molecular mechanisms responsible for these observations are ongoing.

## 714 DIFFERENTIAL REGULATION OF LIVER MITOCHONDRIAL CYTOCHROME P4501A1 (P450MT2) BY 3-METHYLCHOLANTHRENE IN MICE DEFICIENT IN THE CYTOCHROME P4501A2 GENE.

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Cytochrome P4501A (CYP1A) enzymes play important roles in the activation of carcinogens such as 3-methylcholanthrene (MC) to genotoxic metabolites. Recent studies have demonstrated that CYP1A1 is also present in the mitochondria. Mitochondrial CYP1A1 (450MT2) is targeted to mitochondria after proteolytic N-terminal cleavage of 32 amino acids from microsomal CYP1A1. The physiological significance of mitochondrial P450MT2 is not well understood. In this investigation, we tested the hypothesis that MC, which elicits a persistent induction of hepatic microsomal CYP1A1, would also elicit sustained induction of mitochondrial P450MT2 in wild type, but not CYP1A2-deficient mice. Eight week-old female wild-type (C57BL/6) mice or CYP1A2-null mice were treated with MC (100  $\mu$ mol/kg) or vehicle (corn oil), once daily for 4 days. Enzymatic activities [ethoxresorufin-O-deethylase (EROD)] and apoprotein contents of P450MT2 were determined in liver mitochondria of these animals at 1, 8, or 15 days after MC withdrawal. At each of the time points, MC-treated wild-type animals displayed a 5-6-fold induction of mitochondrial EROD (P450MT2) activities over those of control animals. On the other hand, exposure of CYP1A2-null animals to MC led to 8-10 fold induction of EROD activities at the 1 and 8 days, respectively. By 15 days, the elevation of P450MT2 expression in these animals declined to about 2-fold over control, suggesting that targeted CYP1A2 gene disruption alters the expression of mitochondrial P450MT2. Western blot experiments yielded data that correlated with those of CYP1A1 enzyme activities. Overall, the results were similar to those obtained with microsomal CYP1A1. Since persistent induction of P450MT2 in the mitochondria may lead to enhancement of oxidative stress and persistent damage of mtDNA, further studies on the mechanisms of persistent up-regulation of P450MT2 by MC would be of relevance to carcinogenesis mediated by polycyclic aromatic hydrocarbons. (Supported by NIH grant ES09132.)

## 715 ENDOGENOUS SUBSTRATES OF CYP1B1 WHICH MAY BE ENDOGENOUS LIGANDS OF THE ARYL HYDROCARBON RECEPTOR.

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Cytochromes P450 (CYP) catalyze many oxidative reactions for both xenobiotics and endogenous substrates. Altered CYP expression/function is implicated in a variety of human disease states including cancer. Frame-shift mutations in the human CYP1B1 gene have been linked to primary congenital glaucoma (PCG). Using a polyclonal antibody against human CYP1B1, we localized CYP1B1 protein primarily within the non-pigmented ciliary epithelium in human adult and fetal eyes, suggesting CYP1B1 in these tissues metabolizes a substrate that regulates development of the trabecular meshwork (generally considered the target tissue for PCG).

We also utilized an AhR-Luciferase reporter construct (responsive to Aryl Hydrocarbon Receptor (AhR) ligands) stably transfected into HepG2 cells to attempt to identify AhR ligands which may also be CYP1B1 substrates. Extracts from African Green Monkey CV-1 cells not expressing CYP1B1 were found to be highly active, while extracts from CV-1 cells stably transfected with human CYP1B1 were much less active. AhR activity was restored in a dose-dependent manner (0 - 750 nM) when 1B1-expressing CV-1 cells were grown in the presence of the CYP1B1 inhibitor 2, 4, 3', 5'-tetramethoxystilbene, suggesting CYP1B1 can metabolize an endogenous AhR ligand present in CV-1 cells. Tissue extracts from wild-type, AhR null and cyp1b1 null mouse heart, eye, liver, kidney and lung were tested for AhR activity. Highest activity was recovered from eye and heart of AhR null mice. Partial purification of mouse tissue extracts by HPLC generated AhR-active fractions. These results support the existence of endogenous AhR ligands that are also CYP1B1 substrates, thus constituting a regulatory feedback loop for CYP1B1.

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#### THE RESPONSE OF THE RAT HEPATIC P450 SYSTEM TO FIVE ARCHETYPAL HUMAN MICROSOMAL ENZYME INHIBITORS.

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Human enzyme inhibition following xenobiotic administration is an important phenomenon in drug development. Secondary effects of enzyme inhibition can include decreased catabolism of endogenous substrates or altered bioavailability of co-administered chemicals potentially leading to untoward drug-drug interactions. Although inhibition of the human P450 drug metabolizing isozymes is well documented, little is known in regard to the response of preclinical species to these inhibitors. The purpose of this study was to assess the effects of five human microsomal enzyme inhibitors on the cytochrome P450 system of the CD® rat liver. Male and female rats were dosed with ellipticine (ELP), yohimbine (YOH), 4-methylpyrazole (MET), 1-aminobenzotriazole (AMB), cimetidine (CIM), or the appropriate vehicle. Liver microsomal samples were prepared from all animals and assayed for the following general indicators of microsomal enzyme activity: cytochrome P450 content, NADPH cytochrome c reductase, and p-nitroanisole O-demethylase. In addition, samples were assayed for ethoxresorufin O-dealkylase, pentoxysresorufin O-dealkylase, methoxresorufin O-dealkylase, p-nitrophenol hydroxylase, ethylmorphine N-demethylase activity, and 3-[2-(N, N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarin demethylase activity. These enzymes were used as specific indicators of the isozymes CYP1A1, CYP2B, CYP1A2, CYP2E1, CYP3A and CYP2D2, respectively. Microsomes from a subset of animals were assayed by HPLC analysis for the metabolism of progesterone. Progesterone 21- and 2 $\alpha$ -hydroxylase are associated with CYP2C6 and male specific CYP2C11, respectively. While a reduction in enzyme activity was noted after dosing with CIM, MET and AMB, only AMB caused a reduction of activity in the majority of isozymes. In conclusion, these five xenobiotics affect the cytochrome P450 system of the CD® rat liver indicating the value of preclinical testing in rodents, but the specific isozyme response may differ from the published data for the human.

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#### EFFECTS OF DIESEL EXHAUST PARTICLES ON CYTOCHROME P450 ACTIVITIES IN PRIMARY CULTURES OF ADULT RAT HEPATOCYTES: A USEFUL INDICATOR FOR THE STUDY OF HEALTH RISK.

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Diesel exhaust particles (DEPs) are one of the major air pollutants in urban areas. The estimated health risk from air pollutants is considered to be very high. We investigated the effect of DEP extracts on health risk by measuring the activities of cytochrome P450 enzymes in the primary cultures of adult rat hepatocytes. For this examination, DEPs were fractionated into water-soluble and organic fractions. The organic portion was consecutively divided into fractions of crude extract, acidic, basic, aliphatics, aromatics, slightly polar compounds, moderately polar compounds and highly polar compounds. Hepatocytes were isolated and cultured in AB medium for up to 3 days. DEP extracts at 0.1, 1.0 and 10  $\mu$ g/ml in dimethyl sulfoxide were added directly to the medium from the initiation of culture. Treatment with fractions of acidic, basic, aliphatics, aromatics and slightly polar compounds produced significant dose- and time-dependent inductions of ethoxresorufin O-deethylase (EROD) activities. Treatment with crude extract maximally induced the EROD activity from the lowest dose at 72 hr after treatment. Treatment with fractions of acidic, aliphatics and aromatics compounds showed significant dose-dependent increases in benzoyloxyresorufin O-debenzylase (BROD) activities. In addition, treatment with fractions of basic and slightly polar

compounds induced the BROD activities from 1.0  $\mu$ g/ml at 72 hr after treatment. Treatment with fractions of water-soluble, moderate and high polar compounds did not alter these enzymes. Our results suggest that the activities of EROD and BROD in primary cultures of rat hepatocytes might be useful indicators for the study of health risk caused by air pollutants, such as DEPs. (Supported by a grant from the Ministry of Environment, Korea).

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#### IN VITRO EVALUATION OF HISTAMINE AND ITS MAJOR METABOLITES AS INHIBITORS OR INDUCERS OF CYTOCHOME P450 EXPRESSION IN HUMAN LIVER.

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Histamine dihydrochloride (Ceplene™, 2-(1H-imidazol-4-yl)ethanamine) is a synthetic analog of histamine, which is being tested as a subcutaneous formulation in cancer patients. Histamine seems likely to play a role in gene function and cell growth regulation via certain CYP450 enzymes. We have investigated the ability of histamine and its major metabolites, N-methylhistamine (NMH) and imidazoleacetic acid (IAA) to inhibit or induce the major CYP450 enzymes in humans. In order to examine their ability to act as direct inhibitors, each test article (TA) was added together with the marker substrates to human liver microsomes. The TA concentrations were 0, 0.1, 0.3, 1.0, 3.0, 10, 30, and 100  $\mu$ M. Each marker substrate concentration was approximately equal to its respective Km. The following CYP450 enzymes were evaluated: 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4/5 and 4A11. In the induction protocol, human hepatocytes were treated with vehicle, positive control inducers,  $\beta$ -naphthoflavone (33  $\mu$ M) as a strong inducer of CYP1A2 and rifampin (20  $\mu$ M) as a strong inducer of CYP3A4 or one of 3 concentrations of each TA (1.0, 10, and 100  $\mu$ M). The results of IC50 values for histamine, IAA or NMH indicate little or no inhibition of the CYP450 enzymes examined. In addition, the results indicate that treatment of cultured human hepatocytes with histamine, NMH, and IAA did not cause an increase in 7-ethoxyresorufin O-dealkylation (CYP1A2 activity) and testosterone 6 $\beta$ -hydroxylation activity (CYP3A4/5 activity) in human hepatocytes. As expected, treatment of cultured human hepatocytes for 3 consecutive days caused appropriate increases in CYP1A2 and CYP3A4/5 activity in hepatocytes treated with  $\beta$ -naphthoflavone and rifampin (6.5- and 9.0-fold, respectively). In conclusion, these results provide no evidence that histamine has the potential to inhibit or induce CYP450 enzymatic activities in human liver suggesting that histamine is unlikely to interact with the metabolism of CYP450 substrates.

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#### COMPARATIVE STUDIES OF CYTOCHROME P4503A4 INDUCTION RESPONSE IN PRIMARY CULTURES OF CRYOPRESERVED AND FRESH HUMAN HEPATOCYTES.

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One important and well-established *in vitro* system for determining cytochrome P450 (CYP) induction, a major cause of drug-drug interactions, is fresh primary human hepatocyte cultures. However, the availability of donor hepatocytes is limited and unpredictable and, therefore, the use of cryopreserved hepatocytes is increasing. This study examined whether cryopreserved hepatocytes exhibit similar induction responses of CYP3A4, a predominant isoform of P450 family, to that of fresh hepatocytes. A prototypical CYP3A4 inducer rifampicin (RIF) and five new molecule entities (NMEs) were tested in one cryopreserved and two fresh hepatocyte cultures. The preparations were incubated with RIF (20  $\mu$ M) or varying concentrations of the test compound for 3 days. After treatment, CYP3A4 activity was determined by measuring the formation of 6-beta hydroxytestosterone *in situ*. The cells were then lysed and CYP3A4 mRNA levels were determined using one-step real-time RT-PCR. The results of CYP3A4 activity and mRNA levels revealed that the CYP3A4 induction response to RIF and the test compounds was consistent in both cryopreserved and fresh human hepatocyte cultures in this limited donor set. RIF treatment resulted in an average of 92 +/- 34 fold induction in CYP3A4 activity in fresh hepatocytes and 31 +/- 7 fold induction in cryopreserved hepatocytes. CYP3A4 mRNA levels increased concurrently to 192 +/- 147 fold in fresh hepatocytes and 23 +/- 6 fold in cryopreserved hepatocytes. Moreover, four out of five test compounds showed induction responses in both CYP3A4 activity and mRNA levels, which were consistent between the fresh and cryopreserved preparations. Our results demonstrate that cryopreserved hepatocytes are a comparable alternative to fresh hepatocytes for testing the *in vitro* P450 induction potentials of NMEs.

## REGULATIONS OF HUMAN CYP3A4 PROXIMAL AND XREM PROMOTER ACTIVITIES IN HEPA I AND HEPG2 CELLS.

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In order to gain the insight of the molecular mechanism of CYP3A4 gene expression, study has been undertaken to investigate the regulation of CYP3A4 gene expression by proximal promoter in human hepatoma HepG2 cells. HepG2 cells were transfected with a plasmid pCYP3A4-Luc containing 1kb of the CYP3A4 proximal promoter region (-863 to +64 bp) in front of a reporter gene, luciferase, in the presence or absence of pSAP-SXR. In HepG2 cells, CYP3A4 inducers, such as rifampicin, PCN and RU486 showed minimal stimulation of CYP3A4 proximal promoter activity in the absence of SXR and histone deacetylase (HDAC) inhibitors. 4-Dimethylamino-N-[4-(2-hydroxycarbamoylvinyl)benzyl]benzamide (IN2001), a new class HDAC inhibitor significantly increased CYP3A4 proximal promoter activity over untreated control cells and rifampicin concomitant treatment with IN2001 increased further CYP3A4 proximal promoter activity that was stimulated by IN2001. The results of this study demonstrated that both acetylated histone and SXR are essential to increase of CYP3A4 proximal promoter activity by CYP3A4 inducers such as PCN, rifampicin, and RU486. In Hepa-I cells, CYP3A4 inducers and estradiol increased modestly the luciferase activity when TSA was co-treated, but this increment was not enhanced by SXR cotransfection. Also, effects of HNFs and ER on CYP3A4-XREM-proximal promoter activity were investigated in HepG2 cells and results would be discussed. Taken together, these results indicated that the inhibition of histone deacetylation was required to SXR-mediated increase in CYP3A4 proximal promoter region when rifampicin, or PCN was treated. Also this data suggested that HDAC inhibitors seemed to facilitate the CYP3A4 proximal promoter to be activated by chemicals. [This research has been supported by biochallenger program MOST Korea.]

ROLE OF NF- $\kappa$ B IN REGULATION OF PXR TRANSCRIPTIONAL ACTIVITY: A MECHANISM FOR THE SUPPRESSION OF CYTOCHROME P450 3A4 BY INFLAMMATORY AGENTS.

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Cytochrome P450 3A4 (CYP3A4) is the predominant isoform of cytochromes P450 in adult human liver. It is responsible for the metabolism of more than 50% of current prescription drugs. Many physiological and pathological factors have effects on CYP3A4 gene expression. It is a long-standing observation that inflammatory conditions decrease drug metabolism capacity in human and experimental animals. CYP3A4 expression levels are known to be suppressed in human and rodent hepatocytes by the inflammatory agents such as endotoxin, TNF- $\alpha$  and IL-1 $\beta$ . However the mechanism of the suppression remains poorly understood. In the current study, utilizing a human PXR-driven reporter system, we investigate the mechanism of suppression of PXR-mediated CYP3A4 expression. We demonstrated that LPS, dsRNA and TNF- $\alpha$  down-regulated the PXR-driven reporter gene. We observed that in HepG2 cells overexpression of p65, the transcriptionally active component of NF- $\kappa$ B which is activated in hepatocytes by inflammatory agents, has the same suppressive effect on PXR-mediated CYP3A4 expression as LPS, dsRNA and TNF- $\alpha$ . These suppressive effects could be reversed by the dominant super repressor I $\kappa$ B $\alpha$  as well as pyrrolidine dithiocarbamate, which is a known chemical inhibitor of NF- $\kappa$ B. Mammalian one-hybrid and two-hybrid assay show that there is functional interaction between PXR and p65. Our results strongly suggest that NF- $\kappa$ B activation plays an important role in mediating the suppression of CYP3A4 gene expression. (Supported in part by NIEHS Grants ES09859, ES09106 and American Heart Association Grant 0355131Y.)

DOWN-REGULATION OF HEPATIC CYTOCHROME P450S IN ENDOTOXEMIC MICE IS INDEPENDENT OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR ALPHA (PPAR $\alpha$ ).

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Inflammatory agents such as bacterial lipopolysaccharide (LPS) down-regulate the expression of many cytochrome P450 mRNAs and proteins. Previous studies in LPS-treated PPAR $\alpha$  mice indicated that some effects on P450 mRNAs were attenuated or blocked in PPAR $\alpha$  knockout mice (analyzed by Northern blotting), implicating PPAR $\alpha$  in the down-regulation of P450s during inflammation. To confirm these findings, adult female wildtype and PPAR $\alpha$  knockout mice were administered either saline or 1 mg/kg LPS, and hepatic and renal mRNA and protein ex-

pression of four P450 isoforms were examined by quantitative reverse transcriptase PCR and western blotting 16 hours later. mRNA expression of the proinflammatory cytokines interleukin (IL)-1 $\beta$ , IL-6 and tumor necrosis factor alpha (TNF $\alpha$ ), and the acute phase proteins  $\alpha$ 1-acid glycoprotein and fibrinogen were also investigated. LPS administration significantly decreased the hepatic expression of CYP2A5 (each reduced to 7% of control), 2C29 (46 and 25%), 3A11 (17 and 15%), and 4A10 (each 19%) in both PPAR $\alpha$  wildtype and knockout mice, respectively. LPS also down-regulated hepatic P450 proteins detected of both strains using rat P450 2C, 3A and 4A antibodies, and these results correlated with effects on mRNA. Treatment with LPS significantly increased renal CYP4A10 mRNA expression (224% of control) in PPAR $\alpha$  wildtype mice and 2C29 expression (226% of control) in PPAR $\alpha$  knockout mice, with little effect on renal CYP2A5 and 3A11 expression. LPS significantly increased mRNA expression of IL-1 $\beta$ , IL-6, and TNF $\alpha$  in both wildtype (380, 712, and 2615%) and PPAR $\alpha$ -deficient (1013, 378, and 928%) mice. Expression of  $\alpha$ 1-acid glycoprotein and fibrinogen were also significantly increased after LPS treatment in wildtype (652 and 745%) and knockout (397 and 355%) mice, respectively. Because decreases in hepatic P450 mRNA expression were essentially identical in both wildtype and knockout mice, these data indicate that down-regulation of P450s during inflammation is PPAR $\alpha$  independent. (Supported by NIH Grant GM46897)

## GENERATION AND CHARACTERIZATION OF A TRANSGENIC MOUSE MODEL WITH HEPATIC EXPRESSION OF HUMAN CYP2A6.

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CYP2A6 is a human cytochrome P450 enzyme active in the metabolic activation of numerous xenobiotic compounds, including procarcinogens. CYP2A6, which is polymorphic, is mainly expressed in the liver, and it is believed to play an important role in systemic clearance of nicotine. The aim of this study was to prepare and characterize a CYP2A6-transgenic mouse model, a model that should be valuable for studying the *in vivo* function of this polymorphic human enzyme in drug metabolism and chemical toxicity. The transgene construct contained the mouse transthyretin promoter/enhancer, a full-length CYP2A6 cDNA, and a downstream neomycin resistance gene for positive selection in embryonic stem cells. Liver-specific expression of transgenic CYP2A6 was confirmed by immunoblotting and RNA-PCR. The transgenic mouse, originally generated on a mixed C57BL/6 and 129/Sv genetic background, has been backcrossed to the C57BL/6 strain for seven generations. This was necessary because the orthologous mouse Cyp2a5 genes of the two parental mouse strains have structural and regulatory differences that would confound the analysis of the transgenic CYP2A6. Hepatic microsomes from homozygous transgenic mice (on a C57BL/6 background) had significantly higher activities than that of wild-type controls toward coumarin and nicotine. For example, with 0.05 mM nicotine, microsomes from the transgenic mice were >2-fold more active than those of either C57BL/6 or 129/Sv mice in the formation of cotinine. Experiments are currently under way to determine the *in vivo* capacity of transgenic CYP2A6 to activate various drugs and other xenobiotic substrates, including nicotine and coumarin. Preliminary studies with coumarin indicated that, following intraperitoneal administration of a single dose (150 mg/kg), coumarin was eliminated at a higher rate in the transgenic mice than in the WT strains, a finding that is consistent with the reported role of CYP2A6 as the major coumarin hydroxylase in human liver. (Supported in part by NIH grant ES07462)

## AFFINITY LABELING OF SER-57 IN RAT CYTOCHROME P450C24A1 (CYP24A1) OF THE VITAMIN D PATHWAY: MUTATION AND CHARACTERIZATION.

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Cytochrome P450C24A1 or CYP24A1 is a multifunctional regulatory enzyme that mediates homeostasis within the vitamin D endocrine system. CYP24A1, an inner-mitochondrial membrane hemoprotein, regulates vitamin D hormone levels by removing 25(OH)D3 from the circulation and excess 1, 25(OH)2D3 from kidney and target tissues. The development of safe and efficacious vitamin D-based therapeutics will require a detailed understanding of the structure-and-function of CYP24A1. In the current study, binding of substrate to recombinant rat CYP24A1 was investigated using a <sup>3</sup>H-radiolabeled 3 $\beta$ -25(OH)D3 affinity analog. MS/MS analysis of labeled sample identified Ser-57 as the only residue covalently modified by the affinity probe. Site-directed mutagenesis was conducted to explore the role

of Ser-57 in substrate-binding and catalytic function. A neutral S57A mutant displayed impaired binding of the endogenous substrate 1, 25(OH)D3 and altered metabolism of the pro-hormone 25-OH-D3. In contrast, a charged S57D mutant strongly enhanced binding affinity and side-chain oxidation of endogenous vitamin D substrates. The S57D mutant also displays enhanced stability and altered biophysical properties compared to wild-type enzyme. Our results suggest that Ser-57 mediates substrate interactions via hydrogen bonding with the A-ring of vitamin D substrates. However, because the affinity probe fails to induce substrate-induced spectral perturbations of the enzyme's heme center, it is unclear if Ser-57 is an active site residue. A computer-based homology model of rat CYP24A1 suggests that Ser-57 resides on the enzyme's surface, near the mouth of the substrate-access channel, where it may coordinate substrate recognition and accessibility. We hypothesize that Ser-57 is key contact residue within a hydrophobic, peripheral binding site that mediates the high-affinity-binding of vitamin D substrates.

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THE USE OF THE MECHANISM BASED INHIBITOR 2-ETHYNYLNAPHTHALENE (2EN) TO PROBE THE ACTIVE SITE OF CYP2B4.

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Mechanism-based inhibitors are metabolically converted by an enzyme to reactive metabolites that covalently bind and inactivate that enzyme. 2-Ethylnaphthalene (2EN) has been reported to be a mechanism-based inhibitor of CYP2B4. However, there are two components to the inhibition, (1) the inactivation of CYP2B4 by 2EN, which requires prior metabolism and covalent binding of the 2EN metabolite, and (2) an inhibitory component that does not require prior metabolism of the inhibitor. The goal of this study is to characterize both inhibitory components of 2EN with different CYP2B4 substrates. CYP2B4 dependent metabolism of p-nitroanisole (PNA), 7-ethoxycoumarin (7-EC), 7-ethoxy-4-trifluoromethylcoumarin (7-EFC), benzphetamine (BZP), 7-pentoxyresorufin (7-PR), 7-benzyloxyresorufin (7-BR), and testosterone were examined in the absence and presence of 2EN. Preincubation of the reconstituted system with 2EN led to a time dependent inactivation of each of the CYP2B4-dependent activities examined, with the Kinact being similar with each of the substrates used. However, the ability of 2EN to reversibly inhibit CYP2B4-dependent activities was dependent on the substrate employed. Interestingly, the degree of reversible inhibition was roughly related to the molecular size of the substrate. With a small substrate such as PNA, which contains a single aromatic ring, only a small degree of inhibition (about 3%) was observed. Larger substrates exhibited a larger degree of reversible inhibition. In conclusion, the inhibition of cytochrome P450 2B4 by 2EN was substrate-dependent and the degree of reversible inhibition seemed related to the molecular size of substrate. The results are consistent with both 2EN and the smaller substrates being able to simultaneously fit within the CYP2B4 active site. As the size of the substrate is increased, the ability of both the substrate and 2EN to fit into the site is more restricted, leading to greater inhibition of substrate metabolism.

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INHIBITION OF THE HUMAN LIVER MICROSOMAL AND HUMAN CYTOCHROME P450 1A2 AND 3A4 METABOLISM OF ESTRADIOL BY DEPLOYMENT-RELATED CHEMICALS.

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Cytochrome P450 enzymes (CYPs) are major catalysts involved in metabolism of xenobiotics and endogenous substrates such as estradiol (E2). It has previously been shown that E2 is predominantly metabolized by CYP1A2 and 3A4 with 2-hydroxyestradiol (2-OHE2) being the major E2 metabolite. The purpose of this study was to examine possible effects of deployment-related chemicals on the metabolism of E2. Use of phenotyped human liver microsomes (HLM), 6 male and 11 female, demonstrated that individuals with high levels of CYP1A2 and 3A4, regardless of gender, have the greatest potential to metabolize E2. Preincubation of human liver microsomes (HLM) with a variety of ligands, including pesticides, resulted in varying levels of inhibition of E2 metabolism. The greatest inhibition was observed with organophosphorus compounds, including chlorpyrifos (CPS) and fonofos, with up to 80% inhibition observed for 2-OHE2 production. Carbaryl, a carbamate pesticide, and naphthalene, a jet fuel component, inhibited ca. 40% of E2 metabolism. Preincubation of CYP3A4 with CPS, fonofos, deltamethrin, or permethrin under the same conditions resulted in 94, 87, 58, and 37% inhibition of E2 to 2-OHE2, respectively. The Ki values indicated that fonofos and CPS were the most potent inhibitors of E2 metabolism to 2-OHE2 and inhibited E2 metabolism noncompetitively. Preincubation of CYP1A2 with CPS, fonofos, carbaryl, or naphthalene resulted in 96, 59, 84, and 87% inhibition of E2 to 2-OHE2, respec-

tively. The Ki values indicated that CPS, naphthalene, 1-naphthol, carbaryl, 4-hydroxy carbaryl were the most potent inhibitors of E2 metabolism to 2-OHE2 followed by carbaryl methylol and fonofos. (This research is supported by US Army Medical Research and Material Command Agreement DAMAD 17-00-2-0008)

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CORRELATIONS BETWEEN ACTIVITIES OF CYPs 2E1/1A2 AND HISTOLOGIC FIBROSIS IN HCV PATIENTS.

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Liver injury in HCV very likely involves increased hepatic oxidative stress. Hepatic cytochromes P450 could represent a source of excess oxidative stress. We report results of the activities of two P450 isoforms, CYPs 2E1 and 1A2, in untreated HCV patients whose liver biopsies were ranked for degree of histologic injury. METHODS: Chlorzoxazone (CZX) and theophylline (TH) oral clearances were determined as markers of CYP2E1 and 1A2 respectively in 41 HCV patients. Portal (P) and lobular (L) inflammation and fibrosis (F) score were graded according to Ludwig. The histologic score (HS) was formed by adding P+L+2xF. Correlations were made between histologic score (HS) and CYP 2E1 and 1A2 activities, plasma hyaluronic acid (HA), procollagen propeptide III (PPIII), and TGF $\beta$ 1. RESULTS: CYP 2E1 activities for HCV patients with mild, moderate and severe histologic injury were 4.9 $\pm$ 2 (n=14), 5.5 $\pm$ 3 (n=16) and 6.4 $\pm$ 4 (n=11) respectively with correlation coefficient r=0.211. For 1A2 all patients were additionally divided into two groups – non-smokers and smokers. There were no differences between mild, moderate and severe groups for non-smokers. In smokers however the activities were 0.46 $\pm$ 0.1, 0.77 $\pm$ 0.1 and 0.63 $\pm$ 0.2 (n=9, 8, 5) with r=0.240. For HA, PPIII and TGF $\beta$ 1 r=0.272, 0.206 and -0.311 respectively. CONCLUSIONS: As expected, HS is associated with increased markers of endothelial dysfunction (HA) and fibrosis (PPIII). Surprisingly, TGF $\beta$ 1 values were negatively associated. With regard to hepatic P450s, our results suggest that greater degrees of histologic injury (HS weighted to emphasize fibrosis stage) correlate with increased activities of CYP2E1 and, in smokers, of CYP1A2. This may be because these P450s contribute to increased hepatic oxidative stress. Whether decreasing the activities of these P450s will be of benefit to HCV patients will require further study. Acknowledgments: This work was supported by NIH Grant RO1 AA12852-01, GCRC Grants MO1 RR02602, MO1 RR00073.

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ROLE OF CYTOCHROME P450 2E1 (CYP2E1) IN TRICHLOROETHYLENE (TCE) METABOLISM AND DISPOSITION: COMPARATIVE STUDIES USING CYP2E1-/- AND WILD-TYPE MICE.

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TCE is a known rodent carcinogen and was classified as probable human carcinogen. Metabolism of TCE occurs via glutathione conjugation and CYPs-dependent oxidation. While a number of CYPs were implicated, CYP2E1 is considered the primary high affinity enzyme responsible for TCE metabolism. The objective of this work is to assess the role of CYP2E1 in the metabolism and disposition of TCE using CYP2E1-null (KO) vs. wild type (WT) mice. Mice were administered 1, 2-<sup>14</sup>C-TCE by gavage at 1000 mg/kg and placed in glass metabolism cages that allowed for the collection of expired air, urine, and feces. At 24 after dosing, animals were euthanized and blood and tissues were collected and frozen for analysis. Exhalation of TCE derived <sup>14</sup>CO<sub>2</sub> accounted for 17-18% of dose, and was not significantly different in KO Vs. WT mice. Elimination of TCE-derived organic volatiles in the expired air accounted for 23 and 49% of the administered dose in WT and KO mice, respectively. In contrast, excretion of TCE-derived radioactivity in the urine was approximately 17 and 40% of the administered dose in KO and WT mice, respectively. Pretreatment of mice with 1-aminobenzotriazole (ABT), a universal CYPs inhibitor, prior to TCE administration significantly inhibited <sup>14</sup>CO<sub>2</sub> exhalation to 2-3% of the administered dose, and increased the exhalation of TCE-derived organic volatiles to 70-77% of the dose in mice of both genotypes. Preliminary analysis suggested that parent TCE is the primary constituent of the expired organic volatiles in ABT pretreated mice. Urinary excretion in ABT-pretreated mice was significantly decreased in KO and WT mice and accounted for approximately 4 and 12% of the dose, respectively. In conclusion, these data suggest that while CYP2E1 plays an important role in TCE metabolism and disposition, other CYPs play an important role as well. Additional studies are in progress to compare the identity of TCE metabolites in the urine and expired air of KO and WT mice in order to quantify the contribution of various CYPs to TCE metabolism.

CYTOCHROME P450 2E1 (CYP2E1) INDUCTION BY PYRIDAZINE PRODUCES QUALITATIVE AND QUANTITATIVE CHANGES IN THE METABOLISM OF TRICHLOROETHYLENE TO POTENTIALLY CARCINOGENIC METABOLITES.

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CYP2E1, an isozyme of cytochrome P450, is expressed mainly in the liver. It is inducible by a variety of xenobiotic compounds such as ethanol, acetone and aspirin. CYP2E1 catalyzes the oxidation of many small and hydrophobic volatile organic chemicals (VOCs), including trichloroethylene (TCE). It is generally accepted that CYP2E1 induction will result in increased production of metabolites from TCE, and an attendant increase in cancer risk. The objective of the current study was to investigate the influence of CYP2E1 induction by pyridazine (PZ) on TCE oxidation as well as its subsequent effects on downstream metabolites that have been implicated in hepatocarcinogenesis. Groups of 6 male Sprague-Dawley rats of 175 g body weight were gavaged with a series of doses of TCE up to 200 mg/kg. Other groups of 6 animals were induced with PZ (200 mg/kg, ip) for 3 days before being challenged with TCE 24 hrs after the last PZ dose. Serial blood samples were collected for up to 24 or 48 hrs via an indwelling carotid artery catheter. TCE and its major metabolites were analyzed in the headspace of the micro blood samples by gas chromatography. TCE Cmax values were substantially lower in the induced animals, reflecting increased first-pass hepatic elimination of TCE. The PZ-dosed animals had higher trichloroethanol (TCOH) AUCs, but markedly lower trichloroacetic acid (TCA) AUCs, when compared to those of uninduced rats. Dichloroacetic acid (DCA) levels, which were barely detectable blood in uninduced rats, were prominent in induced groups, suggesting increased conversion of TCA to DCA. Whereas, chloral hydrate (CH) levels were unaffected by PZ, but TCOH formation appeared to be favored over TCA. Additional work will be needed to determine whether CYP2E1 induction is of consequence with very low TCE exposure levels.

PROTECTIVE EFFECT OF DIALLYL SULFONE AGAINST LUNG MUTAGENESIS INDUCED BY VINYL CARBAMATE.

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Alliin, a major component in garlic, is converted by the enzyme allinase to organosulfur compounds including diallyl sulfide. Diallyl sulfide is a competitive inhibitor of CYP2E1 and undergoes sequential oxidation to yield diallyl sulfoxide and diallyl sulfone (DASO<sub>2</sub>). DASO<sub>2</sub> is further oxidized by CYP2E1 to diallyl monooxide, which mediates the autocatalytic destruction of CYP2E1. In this study, we tested the hypothesis that DASO<sub>2</sub> protects against vinyl carbamate (VC)-induced *in vivo* mutagenesis. VC is a potent lung carcinogen that is also metabolized by CYP2E1 to an epoxide, a metabolite that is believed to be the ultimate mutagenic and carcinogenic species. Our studies used Big Blue® transgenic mice and the lambda (λ) cII positive selection system that detects mutations in the λ cII transgene by infecting an hfr strain (*E. coli* G1250); only phages with cII mutations lyse at low temperatures (24°C) while all infected bacterial cells lyse at 37°C, allowing determination of mutant frequency. Eight-week old F<sub>1</sub> mice (BigBlue® x A/J) were gavaged with 200 mg/kg of DASO<sub>2</sub>, treated with 60 mg/kg VC (i.p.) 2 h later, and sacrificed four weeks after VC treatment. A 3-fold significant reduction (P < 0.01) in mutant frequency in the VC-treated mice (mean ± SEM; 11.23 ± 2.3 per 10<sup>5</sup> plaques) versus DASO<sub>2</sub>/VC-treated mice (3.82 ± 1.0 per 10<sup>5</sup> plaques) was observed. Pretreatment with DASO<sub>2</sub> completely eliminated VC-induced mutations since DASO<sub>2</sub>/VC mice had nearly identical mutant frequencies as control mice (3.7 ± 1.3 per 10<sup>5</sup> plaques). Mice treated with DASO<sub>2</sub> alone had a 1.8-fold reduction in mutant frequency (2.04 ± 0.6 per 10<sup>5</sup> plaques) versus control mice; although not statistically different, these data suggested some degree of protection against spontaneous mutations. The inactivation of CYP2E1 by DASO<sub>2</sub> oxidation most likely prevented or decreased the formation of the mutagenic VC-epoxide *in vivo*. These results supported the hypothesis that DASO<sub>2</sub> protects against VC-induced mutagenesis. (Supported by NCIC Grant No. 014061).

ROLE OF TOXICOKINETICS IN THE BIOACTIVATION-MEDIATED LIVER INJURY OF THIOACETAMIDE IN *AD LIBITUM* FED AND DIET RESTRICTED RATS.

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Thioacetamide (TA) is bioactivated by CYP2E1 to TA sulfoxide (TASO), and further to highly reactive sulf dioxide (TASO<sub>2</sub>), that initiates hepatic necrosis by covalent binding. Studies showed that bioactivation-mediated injury of TA does not

obey dose-response. Despite higher bioactivation-mediated (Stage I) injury of TA due to induction of CYP2E1, moderate diet restriction (DR) protects rats from a normally lethal dose. TA causes disproportionately higher increase in liver injury after a low dose (50 mg/kg) compared to a 12-fold higher lethal dose (600 mg/kg) in the DR rats. Low dose produces a 6-fold higher injury whereas the high dose produces a delayed and mere 2.5-fold higher liver injury compared to the *ad libitum* (AL) fed rats. The present objective was to determine if lack of dose response in AL and DR rats between low and lethal doses could be explained by saturable toxicokinetics of TA. Rats maintained on 35% DR for 21 days and AL regimen were given 50 or 600 mg TA/kg ip. TA and TASO were quantified in plasma, liver, and urine. With increasing doses, the half-lives of TA and TASO increased, revealing zero order kinetics for TA metabolism. At higher dose, covalent binding of <sup>14</sup>C-TA to liver macromolecules was lower indicating that bioactivation of TASO to TASO<sub>2</sub> is inhibited and consequently, urinary excretion of TASO increased. These results explain the inverse dose response for Stage I injury of TA. Microsomal metabolism of TA over a concentration range (0.01-10 mM) showed saturation of TA conversion to TASO at and above 0.5 mM TA concentration, equaling the *in vivo* plasma and liver levels found at the high dose. Incubation with CYP2E1 supersomes confirmed saturation of TA metabolism to TASO. Low dose showed two-fold higher covalent binding in DR, explaining higher Stage I liver injury in DR rats vs AL rats. These findings suggest saturation of CYP2E1 at the first step (TA to TASO) and substrate inhibition at the second step (TASO to TASO<sub>2</sub>) of TA bioactivation in the AL and DR rats. (Supported by ES09870)

CYP2E1 OXIDATION MEDIATES THE SPERM TOXICITY OF 1-BROMOPROPANE IN MICE.

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1-Bromopropane (1-BrP) has been demonstrated to induce dose and time dependent reproductive organ toxicity and reduce sperm motility in rodents. The contribution of CYP2E1 metabolism to 1-BrP induced reproductive toxicity has now been investigated using wild type (WT) and CYP2E1-/- mice. Sperm motility was determined following inhalation exposure (conc.) to universally <sup>13</sup>C-labeled 1-BrP. In inhalation studies (800 ppm), the half-life of elimination of 1-BrP from the chamber was significantly greater in CYP2E1 -/- mice relative to WT (3.2 vs. 1.3 hr). Despite a 170% increase in 1-BrP exposure and a 250% increase in 1-BrP mean residence time in CYP2E1-/- vs. WT mice, sperm motility in CYP2E1-/- mice did not change relative to control. This suggests that CYP2E1-mediated oxidation is responsible for 1-BrP induced sperm toxicity. In these same animals, urinary metabolites were identified by LC-MS/MS and one and two-dimensional <sup>13</sup>C-NMR. Regardless of route or dose, the mercapturic acid and of 1-bromo-2-hydroxypropane (2OHBrP) was the major urinary metabolite (>80%) and direct GSH conjugation of 1-BrP was insignificant in WT mice. In CYP2E1 -/- mice, 2OHBrP-GSH conjugate levels were decreased with concurrent increases in direct conjugation of parent compound. This NMR data showed that the ratio of direct conjugation to 2-hydroxylation increased 5 fold in CYP2E1 -/- mice relative to WT. Both 1-BrP and 2OHBrP inhibited WT mouse sperm motility *in vitro*. However, only 2OHBrP reduced the motility of sperm obtained from CYP2E1-/- mice *in vitro*, suggesting conversion of parent to 2OHBrP within the spermatozoa themselves is critical to reduction of motility. Overall, these data suggest that reduction in sperm motility may be mediated by the CYP2E1-dependent metabolism of 1-bromopropane to 1-bromo-2-hydroxypropane.

HEPATIC METABOLISM OF AROMATIC AMINE HAIR DYE COMPONENTS AS COMPARED WITH THAT OF 2-AMINOFLUORENE.

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p-Phenylenediamine (PPD) and p-toluenediamine (PTD) are widely used ingredients in permanent hair dyes; however, little has been published on their hepatic cytochrome P450 (CYP)-mediated oxidation. N-hydroxylation by CYPs is regarded as a key step in the activation of arylamines that leads to the development of bladder cancer. Although most epidemiology studies evaluating the relationship between personal use of hair dyes and bladder cancer do not show a significant association, it has been reported that the risk of bladder cancer was increased in women who were frequent users of permanent hair dyes (1, 2). The purpose of the present study was to determine whether PPD and PTD are metabolised by human hepatic CYPs to form N-hydroxyamines. The metabolism of <sup>14</sup>C-PPD and <sup>14</sup>C-PTD was evaluated using intact human hepatocytes, human liver microsomes, and heterologously expressed human CYPs. Analyses were by LC and LC-MS/MS. p-Phenylenediamine was N-acetylated by human hepatocytes to form N-acetyl-PPD

and N, N-diacyl-PPD. There was no evidence for the formation of mono-oxygenated metabolites of 14C-PPD or 14C-PTD by hepatocytes, microsomes or recombinant CYPs, or for enzyme-mediated covalent binding to microsomal protein. In contrast, experiments with 2-aminofluorene (2-AF) showed evidence of CYP-mediated metabolism to at least four different hydroxylated metabolites. The lack of evidence for hepatic CYP-mediated metabolism of PPD and PTD is inconsistent with the hypothesis that these compounds play a causal role in the development of bladder cancer in users of permanent hair dyes via a mode of action involving hepatic metabolism to N-hydroxyarylamines. 1. Gago-Dominguez et al (2001). Use of permanent hair dyes and bladder-cancer risk. *Int. J. Cancer* 91, 575-579. 2. Gago-Dominguez et al (2003). Permanent hair dyes and bladder cancer: risk modification by cytochrome P4501A2 and N-acetyltransferases 1 and 2. *Carcinogenesis* 24, 483-489.

### 734 DECHLORINATION OF PCB IUPAC #101 BY HUMAN CYP 2B6 TRANSFECTED INSECT CELL MICROSOMES.

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Identification of specific human CYP isoform metabolism of PCBs is important to our understanding of PCB kinetics and health effects in humans. PCB IUPAC #101 is a non-coplanar PCB for which oxidative metabolism has been well characterized in the literature. Non-coplanar PCBs are the most abundant congeners found in human and animal tissues. PCB 101 was an abundant congener in original Aroclor mixtures and is found in numerous environmental matrices. It has been described as a rapidly metabolized PCB congener in mammals. Low or undetectable concentrations of PCB 101 are found in most human serum samples. The CYP families involved in PCB metabolism of non-coplanar congeners are CYP 3A and CYP 2B found primarily in liver. The CYP 3A family metabolizes a large number and variety of xenobiotics and endogenous substrates while the CYP 2B family has a limited substrate spectrum and is found in low baseline concentrations. Because of human CYP 3A's lack of substrate specificity, human CYP 2B6 mediated metabolism of PCBs was chosen for initial investigation. Because PCB 101's metabolism has been well characterized it was chosen as the initial congener to be studied by human CYPs in-vitro. Reaction mixtures containing Gentest super-somes (CYP2B6 + b5), PCB 101 (reaction concentrations 12-122 nM) and a NADPH regenerating system were incubated for various times at 37°C under ambient atmospheric and low oxygen saturation conditions. No hydroxylated products were identified in our studies, however, significant concentrations of dechlorination products, eg, PCB 70, were identified under the low oxygen saturation conditions. Reductive dechlorination has not been described as a major mechanism of PCB metabolism in humans. However, these data show that CYP enzyme mediated dechlorination may occur in humans under low oxygen conditions. Dechlorination of PCBs requires further investigation to better understand their impact on biomarker, risk assessment, pharmacokinetic, and human health effects studies of PCBs.

### 735 REGIO-SPECIFIC ( $\omega$ TO $\omega$ -6) FATTY ACID (LAURIC, MYRISTIC AND PALMITIC) HYDROXYLATION IN HUMAN POOLED LIVER AND RECOMBINANT MICROSOMES.

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Most studies of lauric acid hydroxylation have focused on only two hydroxylation products,  $\omega$  and  $\omega$ -1. Modifications of an HPLC method (Lemaire et al., 1992) to include GC-MS (Buhler et al., 1997) have allowed more sub-terminal hydroxylation products including  $\omega$ -2 to  $\omega$ -6 to be quantified. Further modifications now allow the measurement of not only lauric acid hydroxylation but also the hydroxylation of myristic and palmitic acids (Holmes et al., 2004). We have used a modification of the method to allow measurement of not only  $\omega$  and  $\omega$ -1 but also  $\omega$ -2 to  $\omega$ -6 sub-terminal hydroxylation products of lauric, myristic and palmitic fatty acids. Previously we have shown regio-specific lauric acid hydroxylation in human pooled liver and recombinant microsomes. Preliminary evidence indicated significant lauric acid sub-terminal hydroxylation, specifically  $\omega$ -2,  $\omega$ -5 and  $\omega$ -6 by both human liver male and female pooled microsomes, with only female microsomes catalyzing  $\omega$ -1 while products of  $\omega$ -3,  $\omega$ -5, and  $\omega$ -6 were produced by human recombinant CYP4A11. Interestingly, CYP2B6 produced significant lauric acid sub-terminal hydroxylation at the  $\omega$ -1,  $\omega$ -2, and  $\omega$ -3 positions with high  $\omega$ -6 activity. Additionally the source of the recombinant enzyme and the source of the cytochrome P450 reductase influenced the lauric acid hydroxylation pattern by CYP2E1. The sub-terminal hydroxylation of myristic and palmitic acids has not previously been reported. There is potential for sub-terminal hydroxylation products to act as second messengers in cellular signal transduction or to interfere with steroid biotransformation. These hypotheses will be investigated in future research.

### 736 ROLE OF CYTOCHROME P450 3A IN

### HEPATOTOXICITY OF THE CHLOROACETANILIDE HERBICIDE ALACHLOR.

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Alachlor [2-chloro-2', 6'-diethyl-*N*-(methoxymethyl)acetanilide] a chloroacetanilide herbicide is used in agriculture for pre-emergent weed control of corn and soybeans. Previous studies have shown that alachlor is acutely hepatotoxic to rats at doses > 400mg/kg and suggest a role for CYP3A1/2. Also, human CYP3A4 catalyzes alachlor *N*-dealkylation to 2-chloro-*N*-(2, 6-diethylphenyl)acetamide (CDEPA; Coleman et al. *Chem.Biol.Interactions*, 122:27-39, 1999), the putative rate limited precursor to formation of a reactive metabolite, diethylbenzoquinoneimine (DEBQI). Therefore, the objectives of this study were to examine the role of CYP3A in alachlor hepatotoxicity and to determine the toxicity of the intermediate metabolites CDEPA and 2, 6-diethylaniline (DEA). HepG2 human hepatoblastoma cells were chosen as a model system since these cells express constitutive and dexamethasone-inducible CYP3A7 and can be further induced by dexamethasone (DEX) to express CYP3A4 (Krusekopf et al. 2003. *Eur. J. Pharmacology* 466:7-12). Release of lactate dehydrogenase into the culture medium was used as the endpoint for cytotoxicity. In our studies, we observed that alachlor hepatotoxicity (24hr, 0-800 $\mu$ M) was time-dependent and unaffected by pretreatment with 25 $\mu$ M DEX (EC50s $\mu$ M): mean  $\pm$  SEM = 253  $\pm$  7.8 vs. 249.8  $\pm$  14.7 for - vs. + DEX. Further triacetyloleandomycin a CYP3A4 inhibitor, and triazolam a CYP3A7 inhibitor, did not alter alachlor toxicity. The alachlor metabolite DEA was not toxic, while toxicity of CDEPA was evident only in the presence of an arylamidase inhibitor, PMSF. Collectively, these data do not support hepatotoxicity of alachlor initiated by the CYP3A4/7 isoforms through a pathway including DEA as an intermediate. Support: Johnston Science Foundation and Louisiana Board of Regents.

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### INTERINDIVIDUAL VARIABILITY IN P450-MEDIATED METABOLISM OF 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE(NNK) IN HUMAN WHOLE PERIPHERAL LUNG MICROSOMES.

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NNK is a potent tobacco-specific carcinogen that is believed to play an important role in lung cancer induction in humans. The objective of this study was to determine if interindividual variability in pulmonary NNK metabolism could be attributed to differences in P450 contributions and whether these differences are influenced by P450 genetic polymorphisms. Human whole peripheral lung microsomes were incubated with [<sup>5</sup>-<sup>3</sup>H]NNK and NNK metabolism was assessed by HPLC with radiometric detection. The keto-reduced form of NNK, NNAL, was the major metabolite, with production ranging from 0.0087 to 0.443 %/mg protein/min. Total NNK bioactivation ranged from 1.67 $\times$ 10<sup>-3</sup> to 6.33 $\times$ 10<sup>-3</sup> % total  $\alpha$ -hydroxylation/mg protein/min. Total NNK detoxification by N-oxidation ranged from <6.67 $\times$ 10<sup>-4</sup> to 6.00 $\times$ 10<sup>-3</sup> % total N-oxidation/mg protein/min. Consistent with our previous observations, subjects could be classified as either high (8 subjects) or low (8 subjects) bioactivators (5.00 $\times$ 10<sup>-4</sup>-9.23 $\times$ 10<sup>-4</sup> and 1.93 $\times$ 10<sup>-3</sup> $\pm$ 1.01 $\times$ 10<sup>-3</sup> % total  $\alpha$ -hydroxylation/mg protein/min, n=16, P< 0.05). Similarly, for detoxification, subjects could be grouped into high (4 subjects) and low (12 subjects) categories (4.25 $\times$ 10<sup>-3</sup> $\pm$ 1.66 $\times$ 10<sup>-3</sup> and 6.94 $\times$ 10<sup>-4</sup> $\pm$ 5.01 $\times$ 10<sup>-4</sup> % total N-oxidation/mg protein/min, n=16, P< 0.05). Subjects (n=84) were genotyped for the CYP2A13 Arg257Cys polymorphism and NNK metabolism for the one variant (Arg/Cys) was similar to that for other subjects. Distribution of subjects between bioactivation categories is not consistent with reported frequencies of established P450 genetic polymorphisms, suggesting environmental contributions to the variability in NNK metabolism. Identification of high and low categories for bioactivation and detoxification may be useful for predicting susceptibility of individuals to NNK-induced carcinogenesis. (Supported by CIHR Grant No.MOP-10382).

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### COMPARATIVE METABOLISM OF AFLATOXIN B1 (AFB1) BY ZEBRAFISH, RAINBOW TROUT AND HUMAN CYTOCHROMES P450.

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The zebrafish is one of the non-mammalian models used to investigate mechanisms of AFB1-induced carcinogenesis. To understand mechanisms of AFB1-induced carcinogenesis in zebrafish, the key enzymes involved in the activation and detoxification of AFB1 has to be identified in this fish model. The present work was carried out to determine the ability of cDNA-expressed zebrafish P450s to metabolize

AFB1 *in vitro*. The enzyme activities of three recombinant zebrafish P450s (zCYP2K6, zCYP1A and zCYP3A65) were compared with those of the rainbow trout (tCYP2K1) and human P450s (hCYP1A1, hCYP1A2 and hCYP3A4) that are known to metabolize AFB1. The oxidation of AFB1 mediated by the various P450s was evaluated by examining the formation of AFB1 epoxide (as a GSH conjugate), AFM1, AFQ1, DNA adducts and protein adducts. Trout CYP2K1 was 3 times more active than the zebrafish ortholog, zCYP2K6, in catalyzing the formation of AFB1-GSH conjugate, DNA adducts and protein adducts. However, zCYP2K6 was 4.4 times more active than hCYP1A1 and was as active as hCYP1A2 in forming the AFB1-GSH conjugate. Zebrafish CYP1A was comparable to hCYP1A1 in forming the AFB1-GSH conjugate. Human CYP3A4 was 12 times more active than zCYP2K6 and 4.7 times more active than tCYP2K1 in mediating the formation of the GSH conjugate. Furthermore, the rate of formation of the AFB1-GSH conjugate mediated by hCYP3A4 was 54-fold and 14-fold higher than with hCYP1A1 and hCYP1A2, respectively. Zebrafish CYP3A65 was inactive toward the formation of AFB1-GSH conjugate. Human CYP3A4 oxidized AFB1 to AFQ1 whereas the zebrafish ortholog, zCYP3A65, did not form AFQ1. Human CYP1A1 and hCYP1A2 catalyzed the formation of AFM1 but zCYP1A, zCYP2K6, zCYP3A65, and tCYP2K1 produced little or no AFM1. These findings indicate that zebrafish P450s were less efficient than trout CYP2K1 and human CYP3A4 in forming the AFB1-8, 9-epoxide which might partially explain the relative insensitivity of zebrafish compared to trout to AFB1-induced carcinogenesis. (Supported by NIH grants Nos. ES 00210 and ES 11587).

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#### EXPRESSION AND CHARACTERIZATION OF CHANNEL CATFISH CYP 2X1.

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Previous studies of channel catfish identified a novel cDNA encoding the cytochrome P450 isoform, CYP2X1. To better characterize CYP2X1, the protein was expressed in Sf9 cells and examined for catalytic ability. A pFastBac<sup>TM</sup> 1 donor plasmid, containing the CYP2X1 reading frame, was transferred to the bacmid DNA (Baculovirus genome) at the Tn7 site-specific transposition in DH10Bac competent cells. Isolated microsomes from Sf9 cells demonstrated a maximum CO reduced spectrum at 450nm, and exhibited a band at approximately 57 kD on SDS-PAGE. CYP2X1 catalyzed Benzphetamine demethylase activity at 0.790  $\mu$ M/min/ $\mu$ M of CYP2X1. However, enzymatic activity was not observed following incubation with p-Nitrophenol, Fenthion or Testosterone. Studies are currently underway to evaluate alkoxy resorufins as substrates. These results indicate CYP2X1 displays activities consistent with other piscine CYP2 isoforms.

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#### CHARACTERIZATION AND COMPARISON OF METHODS FOR VESICULAR RECONSTITUTION OF CYTOCHROME P450 2B4 AND NADPH P450 REDUCTASE.

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The gel filtration and cholate dialysis methods for vesicular preparations of liver phosphatidylcholine (PC), cytochrome P450 2B4 (P450), and P450 reductase were compared with respect to protein incorporation and catalytic activity. FPLC size-exclusion chromatography on a Superose 6 column (MW cutoff 5, 000, 000) showed that the majority of P450 (>90%) eluted in the void volume (indicating vesicular incorporation). However, a significant proportion of the reductase (>50%) was not incorporated in the PC vesicles under typical reconstitution conditions (> 1 mg PC and 0.5% cholate (w/v)). The proportion of reductase incorporation was increased by lowering the lipid to detergent ratio used in the solubilization steps of the methods. The vesicular preparations derived from the gel filtration method were significantly more active than reconstitutions where the proteins were preincubated with sonicated dilauryl phosphatidylcholine (DLPC) or PC. The rates of benzphetamine demethylation from these preparations were 3 to 4 fold higher, respectively, than with the reconstituted systems prepared using sonicated DLPC. Although the rates of benzphetamine metabolism using the vesicular samples prepared by cholate dialysis also tended to be higher than those catalyzed by the non-vesicular DLPC reconstitutions, the results were more variable, and in some cases rates catalyzed by the cholate dialysis were similar to those of the DLPC controls. These high rates of metabolism were attained despite the fact that the reductase to P450 ratios in the CD and GF preparations were lower (due to the < 60% incorporation of reductase) than those of the sonicated reconstituted system. These results suggest that the CD and GF methods produced a more catalytically effective incorporation of P450 and reductase into the liposomes, than with the more conventional reconstitutions with DLPC.

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#### QUANTITATIVE DOSE-RESPONSE EFFECTS OF ACETYLAMINOFLUORENE HEPATOCARCINOGENESIS IN RATS.

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We have conducted a series of dose-response studies using 2-acetylaminofluorene (AAF) in order to delineate liver tumor initiating effects and their practical thresholds at very low doses. AAF was delivered 3 times per week for up to 16 weeks intragastrically in carboxymethylcellulose (CMC) to F344 male rats. The individual doses were 0 (group I), 0.0026 (II), 0.026 (III), 0.87 (IV), 2.61 mg/kg (V). The following parameters were measured at 4, 8, 12 and 16 weeks: DNA adducts in 108 nucleotides (nts) by 32P-postlabeling; the hepatocellular replicating fraction (RF) as percent proliferating cell nuclear antigen-positive (PCNA+) hepatocytes; glutathione S-transferase-placental type-positive (GST-P+) foci per cm<sup>2</sup> of liver tissue (HAF/cm<sup>2</sup>). At 16 weeks, the values for DNA adducts were 0.4 (group I, CMC), below the limit of quantitation (II), 0.6 (III), 6 (IV), and 21 (V) in 108 nts. The values of RF were 1.4 (I), 1.6 (II), 1.8 (III), 1.7 (IV) and 8.2 (V). Lastly, the values of HAF/cm<sup>2</sup> were 1.1 (I), 1.5 (II), 1.3 (III), 0.6 (IV), and 10.4 (V). The DNA adduct values of groups IV and V were statistically significant compared to control values. These values indicate that the most sensitive parameter to be RF proliferation was evident only in group V, as was the increase in preneoplastic HAF/cm<sup>2</sup>. In previous studies, the cumulative dose of group IV was below the threshold of promotable (with 24 weeks of Phenobarbital) hepatocellular neoplasia, whereas the cumulative dose of group V was above the threshold of promotable neoplasia, yielding an incidence of 100% neoplasms. Thus, 42 mg/kg (group IV) could be considered a practical threshold for promotable hepatocellular neoplasia, despite the occurrence of significant DNA adducts per 108 nts. Moreover, the DNA adduct values of group III were 10 times lower than the values of group IV (cumulative dose of 1.25 mg/kg). These findings indicate that hepatocellular integrity, including repair capability, and proliferation are pivotal in chemical carcinogenesis.

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#### INFLUENCE OF STRAIN AND DIET ON HEPATOCARCINOGENICITY OF N-BUTYL-N-(4-HYDROXYBUTYL)NITROSAMINE (BBN) IN RATS.

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While BBN is widely used as a specific initiator of urinary bladder carcinogenesis for initiation/promotion (I/P) bioassays, our previous investigation clearly demonstrated that it also can act as an initiator of hepatocarcinogenesis. Formalin-fixed and paraffin-embedded liver tissues from two I/P studies were cut, immunohistochemically stained for glutathione S-transferase placental form (GST-P), and quantitatively analyzed. In experiment 1, male Sprague-Dawley (SD)/cShi strain rats (highly sensitive to bladder carcinogenesis by BBN) exhibiting spontaneous hydronephrosis and hydroureter and SD/gShi rats (insensitive), with a genetic predisposition for small testes, were treated with 0.05% BBN in the drinking water for 4 weeks and then housed for up to week 36, with or without exposure to bladder tumor promoters. Slightly higher quantitative values for small GST-P positive (GST-P+) foci were found in SD/gShi (8.57/cm<sup>2</sup>) compared to SD/cShi rats (2.48/cm<sup>2</sup>). In experiment 2, F344 and Lewis rats, subjected to a urinary bladder I/P study, were maintained with MF diets or CA-1 diets for 36 weeks. Higher quantitative values of GST-P+ hepatocytic foci (more than 1 mm in diameter) were found in Lewis rats (9.51/cm<sup>2</sup> for MF diet; 7.95/cm<sup>2</sup> for CA-1 diet) compared to the F344 strain (2.82/cm<sup>2</sup> for MF diet; 1.30/cm<sup>2</sup> for CA-1 diet). Slightly higher values were also noted in rats receiving the MF rather than the CA-1 diet in both strains of rats. No modifying effects on GST-P+ foci were apparent in rats receiving bladder tumor promoters in either experiment. Thus, it was confirmed that the selective bladder carcinogen BBN also acts as a liver carcinogen. The results, from quantitative analysis of small GST-P foci as end point marker lesions, indicate that liver tumor modifying potential of test chemicals can be evaluated in the I/P protocol for urinary bladder carcinogenesis using any strain of rat.

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#### THIAMETHOXAM INDUCED MOUSE LIVER TUMORS AND THEIR RELEVANCE TO HUMANS 1. MODE OF ACTION STUDIES IN THE MOUSE.

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Thiamethoxam, a neonicotinoid insecticide, which is not mutagenic either *in vitro* or *in vivo*, caused an increased incidence of liver tumors in mice when fed in the diet for 18 months at concentrations in the range 500 to 2500 ppm. A number of dietary studies of up to 50 weeks duration have been conducted in order to identify

the mode of action for the development of the liver tumors seen at the end of the cancer bioassay. Both thiamethoxam and its major metabolites have been tested in these studies. Over the duration of a 50 week thiamethoxam dietary feeding study in mice, the earliest change, within one week, is a marked reduction (by up to 40%) in plasma cholesterol. This was followed 10 weeks later by evidence of liver toxicity including single cell necrosis and an increase in apoptosis. After 20 weeks there was a significant increase in hepatic cell replication rates. All of these changes persisted from the time they were first observed until the end of the study at 50 weeks. They occurred in a dose dependent manner and were only observed at doses (500, 1250, 2500ppm) where liver tumors were increased in the cancer bioassay. There was a clear no-effect level of 200ppm. The changes seen in this study are consistent with the development of liver cancer in mice and form the basis of the mode of action. When the major metabolites of thiamethoxam, CGA322704, CGA265307 and CGA330050 were tested in dietary feeding studies of up to 20 weeks duration, only metabolite CGA330050 induced the same changes in the liver as those seen with thiamethoxam. It was concluded that thiamethoxam is hepatotoxic and hepatocarcinogenic as a result of its metabolism to CGA330050. Metabolite CGA265307 was also shown to be an inhibitor of inducible nitric oxide synthase and to increase the hepatotoxicity of carbon tetrachloride. It is proposed that CGA265307, through its effects on nitric oxide synthase, exacerbates the toxicity of CGA330050 in thiamethoxam treated mice.

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THIAMETHOXAM INDUCED MOUSE LIVER TUMORS AND THEIR RELEVANCE TO HUMANS 2. SPECIES DIFFERENCES IN RESPONSE.

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Thiamethoxam is a neonicotinoid insecticide that is not a mutagen, but it caused a significant increase in liver cancer in mice, but not rats, in chronic dietary feeding studies. Previous studies in mice have characterised a carcinogenicity mode of action which involved depletion of plasma cholesterol, cell death, both as single cell necrosis and as apoptosis, and sustained increases in cell replication rates. In a dietary feeding study, female rats were exposed to thiamethoxam at 0, 1000 and 3000ppm for 50 weeks, a study design directly comparable to the mouse study in which the mode of action changes were characterised. In rats, thiamethoxam had no adverse effects on either the biochemistry or histopathology of the liver at any time point. Cell replication rates were not increased, in fact they were significantly decreased at several time points. The lack of effect on the rat liver is entirely consistent with the lack of liver tumor formation in the 2 year cancer bioassay. Comparisons of the metabolism of thiamethoxam in rats and mice have shown that concentrations of the parent chemical were no lower in rat blood than in mouse blood in both single dose and dietary studies, strongly indicating that thiamethoxam itself is unlikely to play a role in the development of liver tumors. In contrast, the concentrations of the two metabolites, CGA265307 and CGA330050, shown to play a role in the development of liver damage in the mouse, were 140 (CGA265307) and 15 (CGA330050) fold lower in rats than in mice following either a single oral dose, or dietary administration of thiamethoxam for up to 50 weeks. Comparisons of the major metabolic pathways of thiamethoxam *in vitro* using mouse, rat and human liver fractions have shown that metabolic rates in humans are even lower than those in the rat, suggesting that thiamethoxam is unlikely to pose a hazard to humans exposed to this chemical at the low concentrations arising from its use as an insecticide.

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THIAMETHOXAM INDUCED MOUSE LIVER TUMORS AND THEIR RELEVANCE TO HUMANS 3. WEIGHT OF EVIDENCE EVALUATION.

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Thiamethoxam was shown to increase the incidence of mouse liver tumors in an 18 month study; however, thiamethoxam was not hepatocarcinogenic in rats. Thiamethoxam is not genotoxic, and, given the late life generation of mouse liver tumors, suggests a time-related progression of key hepatic events that leads to the tumors. These key events were identified in a series of studies of up to 50 weeks that showed the time-dependent evolution of relatively mild liver dysfunction within 10 weeks of dosing, followed by frank signs of hepatotoxicity after 20 weeks, leading to cellular attrition and regenerative hyperplasia. Metabolite CGA330050 was identified as generating the mild hepatic toxicity, and metabolite CGA265307 exacerbated the initial toxicity by inhibiting inducible nitric oxide synthase. This combination of metabolite-generated hepatotoxicity and increase in cell replication rates is postulated as the mode of action for thiamethoxam-related mouse liver tumors. The relevance of these mouse-specific tumors to human health was assessed by using the framework and decision logic developed by ILSI-RSI. The postulated

mode of action was tested against the Hill criteria and found to fulfill the comprehensive requirements of strength, consistency, specificity, temporality, dose-response, and the collective criteria of being a plausible mode of action that fits with known and similar modes of action. Whereas the postulated mode of action could theoretically operate in human liver, quantitation of the key metabolites *in vivo* and *in vitro* showed that mice, but not rats or humans, generate sufficient amounts of these metabolites to initiate the hepatic toxicity and consequent tumors. Indeed, rats fed 3000ppm thiamethoxam for a lifetime did not develop hepatotoxicity or tumors. In conclusion, the coherence and extent of the database clearly demonstrates the mode of action for mouse liver tumorigenesis and also allows for the conclusion that thiamethoxam does not pose a carcinogenic risk to humans.

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ESTIMATION OF A NO OBSERVED EFFECT LEVEL FOR 4, 4'-OXYDIANILINE, A GENOTOXIC LIVER CARCINOGEN, IN A 16-WEEK FEEDING STUDY USING MALE F344 RATS.

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We have already reported the presence of practical no observed effect levels for the genotoxic hepatocarcinogens, 2-acetylaminofluorene and 2, 4-diaminotoluene. In the present study the carcinogenic potential of 4, 4'-oxydianiline (ODA) at very low doses was evaluated in male F344 rats. Groups of 90 or 30 animals (21-days old at the commencement) received 0 (as control), 0.1, 1, 10, 25, 50, 100 and 200 ppm ODA in their diet for 16 weeks. Liver weights were measured and quantitative values for glutathione S-transferase placental form (GST-P) positive hepatocytic foci, considered as preneoplastic lesions, were analyzed. During the dosing period no adverse effects were found in terms of survival, clinical signs, body weights and blood biochemistry in any of the treated groups. Statistically significant elevation of relative liver weights was found in the 200 ppm group, but not in animals fed diet containing 100 ppm or less. GST-P positive foci, consisting of 2 or more cells were significantly increased at doses of 10 ppm and above, but values for 0.1 and 1 ppm demonstrated no differences from the controls. These results suggest that 1 ppm (average intake; 0.0068 mg/kg/day) is a practical no-observed effect level for hepatocarcinogenesis by ODA.

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MUTAGENICITY OF PYRROLIZIDINE ALKALOIDS IN RAT LIVER.

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Plants containing pyrrolizidine alkaloids (PAs) are found world-wide and have the potential to poison livestock, wildlife, and humans. PAs are rodent carcinogens and induce mutations in a number of *in vitro* systems. However, the mutagenicity of PAs has not been evaluated in tumor target tissue. In this study, we determined the mutagenicity of riddelliine, a widely studied PA, and comfrey, a perennial plant containing more than 9 different PAs in rat liver, the target tissue for their carcinogenicity. Groups of 6 transgenic Big Blue rats were treated for 12 weeks either by gavage with 0.1 to 1 mg/kg riddelliine or by feeding a diet containing 2% comfrey root. The animals were sacrificed one day after the last treatment and liver DNA was isolated for analysis of mutations in the transgenic cII gene. A significant dose-dependent increase in MF was found for riddelliine treatment, increasing from 30 x 10-6 in the control animals to 47, 55, and 103 x 10-6 in the 0.1, 0.3, and 1.0 mg/kg dose groups, respectively. The MF for rats fed with comfrey was 146 x 10-6, a 5-fold increase over the control ( $p < 0.001$ ). Molecular analysis of the mutants indicated that there were statistically significant differences between the mutational spectra of the control rats and the rats treated with riddelliine and comfrey, while the riddelliine and comfrey spectra were quite similar. G:C > T:A transversion was the major type of mutation in rats treated with PAs (35% of riddelliine mutants and 42% of comfrey mutants), whereas 55% of mutations from control rats were G:C > A:T transitions. In addition, mutations from the PA-treated rats included an unusually high frequency (8% for riddelliine, 17% for comfrey) of GG > TT and GG > AT tandem base substitutions, while none of these mutations were in the control spectrum. These results indicate that PAs are genotoxic carcinogens in rat liver and that different types of PAs produced a similar and unique mutational spectrum.

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EXPERIMENTAL STUDY ON CARCINOGENESIS INDUCED BY CYANOBACTERIAL MICROCYSTINS AND ORGANIC EXTRACTS OF TAP WATER.

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Conventional drinking water treatment processes are not sufficient to remove Cyanobacterial Microcystins (MCs), and chronic exposure to MCs via drinking water may have tumour-promotion effect. Certain disinfections by-products

(DBPs) in drinking water were found to be mutagenic. Coexistence of DBPs and MCs in drinking water may lead to potential synergistic effects during carcinogenesis. In order to investigate the synergistic effects of MCs and organic extractions of water (OEWS) and its potential mechanism during carcinogenesis, a two-stage hepatocarcinogenesis rat model (Solt-Farber Model) was established using OEWS and MCs applied as the initiator and promoter, respectively. The histopathological changes in liver were observed, and expressions of GSTPi and other genes involved in MAPKs signals pathway were detected, and DNA binding activity of AP-1 and GEPI binding activity of nuclei proteins were analyzed by EMSA as well. The results showed that OEWS and MCs could induce hyperplastic and precancerous foci as well as nodule in livers of rats. MCs alone could up-regulate the expressions of PELK-1, c-jun, and c-fos, and enhance DNA binding activity of AP-1 and GEPI binding activity of nuclei protein in hepatocytes of rats. Further promoted up-regulation of related gene expressions were also seen in this study when rats were initiated with OEWS before the administration of MCs ( $p<0.05$ ). It was also indicated that OEWS alone could induce expressions of GSTPi mRNA and protein in livers. While compared to OEWS, MCs alone could not induce the expression of GSTPi, but could promote the expressions of GSTPi mRNA and protein induced by OEWS ( $p<0.05$ ). The results suggested that MCs possessed tumor-promoting activity while OEWS were mutagenic and possessed initiation activity. MCs and OEWS have synergistic effects during experimental hepatocarcinogenesis in rats. Inhibiting of protein phosphates activity and up-regulating of MAPKs signals pathway may be potential tumour promotion mechanism of MCs during carcinogenesis.

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#### INHIBITION OF GAP JUNCTIONAL INTERCELLULAR COMMUNICATION BY CHLOROHYDROXYFURANONES IN WB-F344 CELLS.

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Chlorohydroxyfuranones (CHFs) are mutagenic by-products in chlorinated drinking water. The CHFs MCA (3, 4-dichloro-5-hydroxy-2(5H)-furanone), CMCF (3-chloro-4-(chloromethyl)-5-hydroxy-2(5H)-furanone), and MCF (3-chloro-4-methyl-5-hydroxy-2(5H)-furanone) have been shown to be genotoxic *in vitro* in bacteria and in mammalian cells. In a two-stage cell transformation assay MCA and MCF promote transformation foci formation. All three CHFs inhibit gap junctional intercellular communication (GJIC) in mouse Balb/c 3T3 fibroblasts. Inhibition of GJIC is supposed to be one mechanism in tumor-promotion. In the present study, the effects of MCA, CMCF, and MCF on GJIC were measured in rat WB-F344 liver cells using the scrape loading dye transfer technique. The cells were exposed to MCA (10, 20, 40, and 80  $\mu$ M), CMCF (6.25, 12.5, 25, and 50  $\mu$ M), and MCF (50, 150, 450, and 1350  $\mu$ M) for 1 hour and 12 hours. All three CHFs inhibited GJIC up to 67-73%, in a dose-dependent fashion, in the order of potency CMCF~MCA>MCF. The inhibition by MCA and MCF augmented during the 12 hours exposure period, whereas CMCF inhibited GJIC to the same extent at both exposure times. After 1 hour exposure, the GJIC recovered slowly in CHF-free medium. These results indicate that all these three CHFs inhibit GJIC in rat liver cells *in vitro*.

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#### $\alpha$ 2-MACROGLOBULIN: A NOVEL CYTOCHEMICAL MARKER CHARACTERIZING PRENEOPLASTIC AND NEOPLASTIC RAT LIVER LESIONS NEGATIVE FOR HITHERTO ESTABLISHED CYTOCHEMICAL MARKERS.

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In risk assessment of compounds targeting the liver one problem has been that well established cytochemical markers of preneoplastic lesions such as glutathione S-transferase (GST-P) are inadequate to detect some types of altered foci. In the present study, we tried to identify a novel marker characteristic for rat hepatocellular preneoplastic and neoplastic lesions, undetectable by well established cytochemical markers. GST-P negative hepatocellular altered foci (HAF), hepatocellular adenomas (HCAs), hepatocellular carcinomas (HCCs) were generated by two initiation-promotion models with N-nitrosodiethylamine (NDEN) and peroxisome proliferators, Wy-14, 643 and clofibrate. Total RNAs isolated from laser-microdissected GST-P negative HAF (amphophilic cell foci) and adjacent normal tissues were applied to microarray analysis. As a result, 5 upregulated genes were identified, and further detailed examinations of the gene demonstrating most fluctuation, i.e., that for  $\alpha$ 2-macroglobulin ( $\alpha$ 2M) were performed. In RT-PCR,  $\alpha$ 2M mRNA was overexpressed not only in GST-P negative HAF but also in GST-P negative HCAs and HCCs. *In situ* hybridization showed accumulation of  $\alpha$ 2M mRNA to be evenly

distributed within GST-P negative HAF (predominantly amphophilic cell foci). Distinctive immunohistochemical staining for  $\alpha$ 2M could be consistently demonstrated in GST-P negative HAF, HCAs and HCCs induced not only by peroxisome proliferators but also NDEN alone. Moreover quantitative analysis of the number of  $\alpha$ 2M mRNA positive foci revealed that  $\alpha$ 2M was a sensitive marker for detection of GST-P negative HAF as compared to conventional histopathological examination. Thus our findings suggest that  $\alpha$ 2M is an important novel cytochemical marker to identify hepatocellular preneoplastic lesions, undetectable by established cytochemical markers and is tightly linked to rat hepatocarcinogenesis.

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#### MECHANISMS OF 2-BUTOXYETHANOL CARCINOGENESIS.

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Chronic exposure to 2-butoxyethanol increases liver hemangiosarcomas in mice. We proposed that the production of liver hemangiosarcomas result from Kupffer cell activation, secondary to hemolysis from 2-butoxyethanol exposure. The present studies examined the effect of 2-butoxyethanol and its metabolites, 2-butoxacetaldehyde and 2-butoxyacetic acid, along with the contribution of the Kupffer cell, on mouse endothelial cell DNA damage (measured by the Comet Assay). 2-Butoxyethanol, 2-butoxyacetic acid (1-10mM), or 2-butoxyacetaldehyde (0.1-1.0mM) did not induce an increase in endothelial cell DNA Damage over control after 2, 4, or 24 hrs of exposure. The effect of hemolyzed RBCs on the induction of endothelial cell DNA damage was also examined and produced an increase in damage after 4 hrs of treatment. Similarly, ferrous sulfate (0.1-1.0 $\mu$ M; 2-24 hrs) and hydrogen peroxide (50-100 $\mu$ M; 1-4 hrs) induced DNA damage in the endothelial cells. Our proposed mode of action argues that Kupffer Cell activation plays a central role in the development of the 2-butoxyethanol induced hemangiomas. To address this, the effect of 2-butoxyethanol, 2-butoxyacetic acid and/or hemolyzed RBCs on macrophage activation was studied. 2-Butoxyethanol and 2-butoxyacetic acid did not activate macrophages (TNF- $\alpha$  release) after 4 hrs treatment. However, hemolyzed RBCs (10 x 10 $^6$ ) increased TNF- $\alpha$  concentrations. The effect of activated macrophages on endothelial cell DNA damage and cell proliferation were also studied. Co-culture of endothelial cells with activated macrophages resulted in increased endothelial cell DNA Damage after 4 or 24 hrs of treatment. Activated macrophages also increased endothelial cell DNA synthesis after 24 hrs compared to control. These data suggest that iron from hemolyzed RBCs and/or products from Kupffer cell activation (possibly ROS), lead to the production of DNA damage in endothelial cells. In addition, the increases in cytokines from the activated Kupffer cells may stimulate endothelial cell proliferation, contributing to the induction of hemangiosarcomas.

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#### EVIDENCE FOR THE INVOLVEMENT OF THE KUPFFER CELL IN HEPATIC CARCINOGENESIS.

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The induction of hepatocellular neoplasia in rodents is a multistep process involving a series of cellular modifications. Historically, studies have focused on the hepatocyte as the target cell of chemical carcinogens, however, recent studies are emerging that suggest a role for non-parenchymal cells, specifically Kupffer cells, as mediators for the induction of cell proliferation by tumor promoters. The present studies further defined the role of the Kupffer in hepatocarcinogenesis through examining the effect of Kupffer cell modulation on hepatocellular growth. Kupffer cell depletion was achieved using liposomal delivery of clodronate. Initial studies demonstrated that clodronate liposome treatment reduced the number of Kupffer cells and inhibited lipopolysaccharide-mediated TNF $\alpha$  production. LPS (0.25 mg/kg i.p.; 1x or 2x/week) in male B6C3F1 mice resulted in an increase the number of Kupffer cells and DNA synthesis. Co-treatment with clodronate liposomes decreased the number of Kupffer cells to levels below control values in all groups. Kupffer cell depletion produced a 50% reduction in the basal level of DNA synthesis in liver, and prevented LPS-induced DNA synthesis (~80% reduction). Hepatic focal lesions were produced in B6C3F1 mice using diethylnitrosamine. Following preneoplastic lesion development, LPS (0.25 mg/kg i.p., 1x/week) for 7 or 28 days increased the relative number of hepatic focal lesions (~ 2.7-fold compared to control), as well as an increase in focal lesion volume (3.5-fold increase). In addition, LPS produced a 3-fold increase in focal lesion DNA synthesis. Inactivation of Kupffer cells reduced the number, volume and DNA synthesis in diethylnitrosamine-induced hepatic focal lesions. These data provide evidence supporting the involvement of the Kupffer cell in hepatic carcinogenesis, and suggest that this cell type may function at the tumor promotion stage of the cancer process.

MECHANISTIC ANALYSIS OF CARCINOGENIC AND NONCARCINOGENIC LIVER TOXICANTS USING TOXICOGENOMIC DATA DERIVED FROM RAT LIVER OR PRIMARY RAT HEPATOCYTES.

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The ability to rapidly identify agents that have the potential to induce carcinogenicity is critical for selecting safe drugs. The current study was designed to identify the similarities and differences at the gene expression level for a panel of agents that induce carcinogenicity via genotoxic or nongenotoxic mechanisms. Specifically, DNA damage response, cell proliferation/cell death, and DNA repair genes will be evaluated in both the rat liver and primary rat hepatocyte model systems using Affymetrix RGU34 Gene Chip® set. In addition, the study will include two negative controls. The two negative controls are Rosiglitazone, which is pharmacologically active in the liver and it is currently being examined as a chemopreventive agent and Acetaminophen, which induces liver damage via necrosis and is not considered to be a carcinogen. Acetaminophen is interesting from the perspective that there are elevations in genes associated with DNA repair such as BTG2, PCNA, and GADD45a. Furthermore, there were elevations in numerous p53 target genes such as cyclin G1, mdm2, and BTG2 following exposure to acetaminophen, similar to the response observed following the administration of genotoxic carcinogens such as ethionine. However, acetaminophen lacked a robust gene expression response for DNA repair genes such as MGMT and APEX1 that are routinely overexpressed following treatment with many genotoxic carcinogens. Interestingly, samples exposed to rosiglitazone lack both the DNA damage and DNA repair response. Is the overexpression of p53-regulated genes enough information to assume that DNA damage has been induced or should other genes associated specifically with DNA repair be dysregulated in combination with the p53-regulated gene sets? The results from this analysis will shed light on similarities and differences in the gene expression responses correlated with carcinogenicity for genotoxic, nongenotoxic carcinogens and negative control compounds.

DEVELOPMENT OF A GENE SIGNATURE THAT DISCRIMINATES BETWEEN GENOTOXIC AND NON-GENOTOXIC COMPOUNDS.

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Efforts are underway to develop multiple gene signatures of toxicity including hepatotoxicity, nephrotoxicity, oxidative stress, inflammation response and carcinogenicity. This study describes the efforts and results to develop a gene signature that identifies carcinogens and classifies them as genotoxic or non-genotoxic. Moreover, this assay is being developed to characterize compounds as genotoxic or non-genotoxic using *in vivo* or *in vitro* platforms. Primary rat hepatocytes in culture, C9 liver cells, and Sprague-Dawley rats were treated with a set of genotoxic or non-genotoxic compounds including clofibrate, fenofibrate, estradiol (non-genotoxic) and 2-acetylaminofluorine, benzo[*a*pyrene, methyl methanesulfonate, mitocycin C, cyclophosphamide, and cisplatin (genotoxic). After treatment for 7 days in the live rats and 24 hours in the hepatocytes and C9 cells in culture, RNA was isolated from the livers of the rats, and the cells in culture and expression of selected genes was measured relative to the expression of control genes. Genes related to xenobiotic metabolism, proliferation, apoptosis, and DNA damage were part of the panel of genes whose expression was measured. Statistically significant correlations were found in rats and cells treated with the same compounds. The data support the use of gene expression in conjunction with *in vitro* platforms as a replacement for *in vivo* platforms in some early screens. Successful cross platform carcinogenic gene signature development and utilization suggests genotoxicity or nongenotoxicity of a compound determined *in vitro* would correlate with its potential to have the same toxicity *in vivo*. Finally, understanding the functions of genes whose expression changes *in vivo* and *in vitro* are similar in response to the same perturbations should be helpful in determining for future studies which physiological responses observed *in vitro* systems are relevant *in vivo*.

A GENE EXPRESSION SIGNATURE FOR NON-GENOTOXIC CARCINOGENS.

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There are a number of rapid, predictive assays for DNA damaging carcinogens, but about half of tumors in rodents are due to non-genotoxic carcinogens, and these compounds are not so easily predicted, with definitive results requiring two years of high dose compound administration. Unexpected discovery that a compound is a

non-genotoxic carcinogen can be a devastating, expensive failure. While establishing a database of liver gene expression responses to over 100 paradigm hepatotoxins, many of the compounds were noted to be non-genotoxic carcinogens, and a gene expression signature for this type of toxicity was determined using data collected 24 hours after compound administration. Although liver is not always the target organ for tumor development, this tissue frequently shows broad gene expression changes to non-genotoxic carcinogens; many of these changes are probably adaptive to prevent hepatic tumor formation. Two classes of hepatotoxins, PPAR $\alpha$  agonists and macrophage activators, produce strong gene expression signatures that overwhelm the responses of other compounds in the database. Both classes of compounds lead to characteristic liver tumors (peroxisome proliferators induce adenomas, macrophage activators lead to carcinomas), but were removed from the database before characterizing other non-genotoxic carcinogens. Gene selection methods were as previously published. Briefly, compounds identified in the literature as non-genotoxic carcinogens were split into training (65%) and testing (35%) sets, and training set compounds were compared to known non-carcinogens to select the best distinguishing genes. Accuracy of prediction was tested using testing compounds; other novel compounds are presently being tested. Some of the best genes for distinguishing non-genotoxic carcinogens from other compounds confirmed literature reports (for example, Ddb1 - damage-specific DNA binding protein 1), but many were surprises (for example, G6pt1 - glucose-6-phosphatase).

EFFECTS OF 3-METHYLCHOLANTHRENE ON HEPATIC GENE EXPRESSION PATTERNS IN RATS USING CDNA MICROARRAY ANALYSES.

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3-Methylcholanthrene (MC), a polycyclic aromatic hydrocarbon (PAH), is one of the most potent carcinogens known to date. In experimental animals, MC induces the expression of cytochrome P4501A1 (CYP1A1) and CYP1A2, which play important roles in the bioactivation of MC to genotoxic metabolites. In this study, we tested the hypothesis that treatment of rats with MC would lead to persistent induction of genes encoding CYP1A1/1A2, phase II enzymes, as well as other genes that may play a role in MC-mediated carcinogenesis. Adult female Sprague-Dawley rats were treated with MC (93  $\mu$ mol/kg), i.p., once daily for 4 days, and gene expression patterns were investigated using hepatic RNA isolated from animals at 1, 15, and 28 days after MC withdrawal. Gene expression was monitored by hybridization of the RNA with a chip containing 4608 unique clones from liver-derived EST libraries fortified with clones of known liver genes representing ~4000 genes. Following hybridization, the chips were scanned using Agilent Microarray scanner, and the data were analyzed using Agilent Feature Extraction. Several Ah receptor-regulated genes encoding phase I (CYP1A1, 1A2, 1B1) and phase II (glutathione-S-transferase- $\alpha$ , NAD(P)H:quinone reductase, epoxide hydrolase, UDP-glucuronosyltransferase, and aldehyde dehydrogenase) enzymes were persistently induced (3-13-fold) by MC for 15-28 days. The induction of these drug metabolism genes correlated well with enzymatic, protein and mRNA studies. MC also elicited sustained induction (5-7-fold) of acute phase genes, i.e. haptoglobin and alpha-1-acid glycoprotein (AGP) for up to 28 days, and this was accompanied by sustained liver damage in the MC-exposed rats. Elevation of acute phase proteins is observed in many cancerous tissues. Thus, determining the mechanisms of persistent induction of the above-mentioned genes might lead to new strategies in the prevention/treatment of cancers induced by chemical carcinogens. (Supported in part by NIH grant ES009132 to BM and DOD grant BC021962 to SK.)

GENE EXPRESSION PROFILING IDENTIFIES GENE CHANGES INDICATIVE OF POTENTIAL EARLY NEOPLASTIC LIVER LESIONS CAUSED BY THE RODENT HEPATOCARCINOGEN METHYLEUGENOL.

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Methyleugenol is a potent rodent carcinogen used widely as a fragrance and flavoring additive that caused liver tumors in both rats and mice in the 2 year NTP bioassay. To determine if gene expression profiling can be used to identify indicators of preneoplastic lesions, we examined hepatic gene expression in male Fisher rats following exposure to methyleugenol and in hepatocellular carcinomas obtained from rats in the methyleugenol NTP study. Methyleugenol was administered by gavage for 1 to 14 days, with hepatic gene expression measured at various times during the treatment regimen, as well as 6 months after the last dose. Hepatic gene expression of treated samples or tumors was measured with the Agilent rat 60-mer oligonucleotide microarray compared to time matched controls. Comparison of gene expression changes during high dose methyleugenol treatment with hepatocellular carcinomas revealed significant overlap of differentially expressed genes. Surprisingly, two of the gene expression profiles generated from livers 6 months

after low dose methyleugenol treatment were markedly similar to those obtained from hepatocellular carcinomas, as determined by hierarchical clustering. Histological analysis of liver samples 6 months after methyleugenol treatment showed a dose-dependent increase in the number of animals with subtle GST-pi positive foci. Preliminary analysis of additional 6 month liver samples from animals exposed to intermediate doses of methyleugenol suggests gene expression alterations are present and persist for 6 months after the last exposure that reflect the potential preneoplastic lesions. These gene expression alterations should provide insight into the mechanisms of hepatocarcinogenesis induced by methyleugenol.

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#### GENE REGULATION PATTERNS IN RAINBOW TROUT AFLATOXIN B<sub>1</sub>-INDUCED HEPATOCELLULAR CARCINOMA COMPARED TO NORMAL ADJACENT LIVER.

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Hepatocellular carcinoma (HCC) is one of the most common malignancies in humans worldwide particularly in Southeast Asia, Japan and Africa, although incidence of HCC in the United States is rising and exhibits the fastest increase among solid tumors. The rainbow trout has proven an excellent model for the study of aflatoxin B<sub>1</sub> (AFB<sub>1</sub>)-induced human hepatocarcinogenesis and chemoprevention because it exhibits mechanisms of metabolism, DNA adduction, oncogene activation and pathology similar to mammalian models. Recently, a number of studies have examined differential gene expression in HCC to identify genes responsible for tumor formation and to further understand the underlying molecular mechanism for disease. In this study, we examined, for the first time, gene expression by microarray in the trout tumor model. Rainbow trout embryos were initiated with 50 ppb AFB<sub>1</sub> and then tumors were isolated from 13 month old juvenile fish following our standard tumor protocol. Global gene expression was determined in HCCs compared to normal adjacent liver utilizing a rainbow trout oligonucleotide array containing approximately 1500 genes for toxicology, comparative immunology, carcinogenesis and stress physiology. We observed distinct gene regulation patterns in HCC compared to adjacent normal tissue including upregulation of genes important for cell cycle control, transcription and cytoskeleton formation and down regulation of genes involved in detoxification and drug metabolism, lipid metabolism and retinol metabolism. These gene classes have previously been observed to be important in the regulation of HCC in human and rodent models. Overall, these findings may lead to a better understanding of the mechanism of AFB<sub>1</sub>-induced hepatocarcinogenesis in the trout model and to identify conserved genes important in carcinogenesis across species. Supported by NIH grants ES03850, ES07060, ES00210 and CA90890.

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#### TOXICOGENOMIC ANALYSIS OF NUCLEAR RECEPTOR-MEDIATED AND -INDEPENDENT RESPONSES TO PEROXISOME PROLIFERATORS.

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Use of microarray technology is a rapid and efficient method for profiling gene expression changes that can be used to elucidate the mode of action of toxicants. Advances in microarray data analysis now allow for construction of detailed molecular maps displaying pathways associated with a specific toxic response. These capabilities can be useful when studying agents that are suspected to have similar modes of action. In the case of some nongenotoxic carcinogens, there appears to be a large degree of similarity in the mode of action which involves activation of a nuclear receptor, and induction of genes associated with metabolism, cell proliferation, DNA damage and oxidative stress. In this study, we investigated peroxisome proliferator-induced response in mouse liver, which is mediated by nuclear receptor PPAR-alpha. The current body of knowledge surrounding these compounds reports an increase in oxidants, induction of mitogenic cytokines in Kupffer cells, increased cell proliferation, and PPAR-alpha dependent molecular events. PPAR-alpha null mice, NADPH oxidase-deficient mice (p47 phox-null) and wild-type C57BL/6J mice were fed WY-14, 643 (0.5% w/w) for up to 5 weeks. Liver tissue was used to measure gene expression levels and phenotypic response. A map of microarray gene expression data for treated wild-type and p47 phox null mice revealed an induction of metabolism, cell proliferation and DNA repair pathways. This response was not observed in PPAR-alpha null mice. Phenotypic anchoring of gene expression data was conducted by measuring oxidant production and induction of lipid metabolism and other genes. We show that while PPAR-alpha is required for these processes, the Kupffer cell-mediated responses are diminished after weeks of peroxisome proliferator treatment. (Supported by ES11391, ES11660).

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#### COMPARATIVE EFFECTS OF TRICHLORACETIC ACID ON RODENT AND HUMAN HEPATOCYTES: ROLE OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR $\alpha$ (PPAR $\alpha$ ).

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Trichloroethylene (TCE) and trichloroacetic acid (TCA), a metabolite of TCE, belong to the peroxisome proliferator class of chemicals and induce liver tumors in mice following chronic exposure. The carcinogenic effect of peroxisome proliferators is believed to involve agonist binding to the peroxisome proliferator activated receptor  $\alpha$  (PPAR $\alpha$ ). Whether TCE and TCA induce hepatic cancer through this mode of action was evaluated in the present studies. Induction of DNA synthesis and apoptosis by TCA was examined in hepatocytes from B6C3F1, PPAR $\alpha$  knockout and 129/Sv wildtype mouse strains. TCA (0.1 - 5.0 mM) produced a concentration-related increase DNA synthesis in both B6C3F1 and 129/Sv hepatocytes at 24, 48 and 72 hrs. In hepatocytes from PPAR $\alpha$  knock mice, TCA failed to increase DNA synthesis at any time point examined. In human hepatocytes, TCA decreased DNA synthesis. Apoptosis was increased by 2.5 and 5.0 mM TCA (-2-fold) in B6C3F1 hepatocytes after 48 and 72 hrs and by 5.0 mM TCA (1.5-2.5-fold) in 129/Sv hepatocytes at 48 and 72 hr exposure. No changes in apoptosis were seen in PPAR $\alpha$  null or human hepatocytes. In addition, peroxisomal  $\beta$  oxidation, a measure of peroxisome proliferation was increased by TCA in hepatocytes from B6C3F1 (- 2-4-fold over control), and 129/Sv (-2-fold) mice, whereas no induction was seen in hepatocytes from PPAR $\alpha$  null mice or in human hepatocytes. In a 7 day *in vivo* study, TCA (0.5 and 2.0 g/L) and TCE (250 and 1000 mg/kg) increased DNA synthesis (2.0-5.7-fold) and peroxisomal  $\beta$  oxidation in the 129/Sv mouse, while no changes in these endpoints were seen in the PPAR $\alpha$  null mice. Neither TCA nor TCE altered levels of apoptosis in either strain of mice. These data demonstrate that (1) human hepatocytes are refractory to the induction of DNA synthesis and apoptosis by TCA and that (2) the PPAR $\alpha$  is required for the induction of DNA synthesis observed following TCA and TCE exposure.

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#### THE EFFECTS OF AMMONIUM PERFLUOROOCTANOATE (APFO) ON THE TRANSCRIPTIONAL PROFILE OF PANCREAS AND LIVER OF MALE RATS.

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APFO is a PPAR ligand which has been shown to induce pancreatic acinar cell, liver and testicular tumours in rats. The mechanism(s) of pancreatic carcinogenesis is poorly understood but evidence suggests that direct genotoxicity is not involved. In order to investigate pancreatic gene expression changes that may be associated with pancreatic carcinogenesis 6 male Sprague-Dawley rats (6-7 weeks old) were fed APFO in the diet at a concentration of 300 ppm for 4 weeks and transcriptional profiling using a rat cDNA array containing 14, 000 genes was performed in pancreas and liver. 577 and 617 APFO treatment-regulated genes were identified in the pancreas and liver respectively. 267 pancreas genes and 294 liver genes were up-regulated and 310 pancreas and 323 liver genes were down-regulated. In the liver many of the transcriptionally induced genes were found to be regulated by the transcription factor PPAR alpha. In the pancreas, a marked up-regulation of phosphoenolpyruvate carboxykinase (PEPCK), a PPAR gamma-regulated gene in adipose tissue, was observed. Groups of genes involved in MAP kinase and Notch signaling, together with target genes for these signaling pathways, were also up-regulated. Gene expression changes which could lead to metabolic acidosis and perturbations in glutamine metabolism and apoptosis were also observed. Examining the broad transcriptional effects of APFO in the liver generated a profile of gene expression changes indicative of increases in hepatic beta-oxidation and fat metabolism. This observation is consistent with the compound acting as a PPAR alpha agonist and peroxisome proliferator. By contrast gene expression changes observed in the pancreas were quite different and suggested possible effects on gluconeogenesis and glutamine metabolism. The profile of regulated genes associated with carcinogenesis were indicative of metabolic acidosis, oxidative stress and endogenous processes of DNA damage against a background of increased mitogenesis. These studies were funded by Plastics Europe Fluoropolymers Committee.

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#### EFFECT OF 6MONT'S ADMINISTRATION OF HEPATIC TUMOR PROMOTERS ON IGF SIGNALING PATHWAY EXPRESSION IN THE RAT LIVER.

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The aim of this study is to determine the integrity of the insulin and insulin-like growth factor signaling pathway in the rat liver following 6 months exposure to the hepatic tumor promoting agents, tamoxifen, mestranol and phenobarbital. Female

Spague-Dawley rats were administered the promoting agent admixed into the basal diet (Phenobarbital at 500 mg/kg diet; mestranol at 0.2 mg/kg diet; and tamoxifen at 250 and 500 mg/kg diet) and fed to the rats for six months prior to sacrifice. The expression levels of 28 genes related to IGF signaling pathway were analyzed by real time PCR. For each treatment, an N=5 samples was analyzed. Expressions of IGFBP1 and IGFBP5 were increased, while IGFBP2 was decreased by mestranol and tamoxifen administration. However, phenobarbital administration resulted in a decreased expression of only IGFBP2. Receptor genes, IGF-II receptor (IGF-IIIR), Insulin receptor (InR) and Insulin receptor-related receptor (IRR), were increased in all cases. Glycogen synthase kinase-3 (GSK3), which mediates cell growth, was also increased in all cases. Specifically, GSK3 beta was increased 10 fold by phenobarbital, while about 2 fold change was induced by the others, indicating that different regulatory system might exist. In order to determine the relative changes in the IR and TR pathways, IGF-I and SPOT14 expression was monitored and the results showed decrease of SPOT14 by tamoxifen treatment. In conclusion, these tumor promoters may alter the expression of receptors in the insulin signaling pathway and the downstream gene, GSK3, required for cell proliferation.

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#### DIFFERENTIAL CHANGES OF GENE EXPRESSION PROFILES IN MALE LONG-EVANS CINNAMON AND F344 RATS BY THE ADMINISTRATION OF N-NITROSOMORPHOLINE.

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The Long-Evance Cinnamon (LEC) rat is an inbred strain with a mutation at the ATP7b gene leading to hepatic copper accumulation and spontaneous hepatitis. Additionally the LEC rat develops a high incidence of spontaneous liver tumor and is highly susceptible to chemical carcinogens, but the comprehensive molecular response to hepatocarcinogens remain largely obscure. In this study we analyzed the gene expression profiles by the administration of a hepatocarcinogen, N-nitrosomorpholine (NM), in LEC rats before hepatitis compared with F344 rats. Male LEC and F344 rats, 3 each at 5 weeks old, were orally administrated a daily dose of NM (10 mg/kg/day) or vehicle for 4 weeks, and were sacrificed. According to serological and liver histological examinations, NM induced moderate hepatocellular damage in both LEC and F344 rats at similar magnitudes. Gene expression profiles were analyzed by using GeneChip® RAE230A. It revealed clearly different profiles between vehicle-treated LEC and F344 rats, 32 genes being higher, while 42 genes were lower, expressed in the former rather than in the latter. By the treatment of NM, several genes were up or downregulated in both rats. Whereas the expression of 15 and 2 genes increased and decreased, respectively, in both rats, the expression of 7 and 19 genes increased and decreased, only in LEC rats, and the expression of 33 and 28 genes increased and decreased only in F344 rats. From the results of comprehensive gene expression analysis of LEC rat liver, typical apoptosis-related genes were found to be induced by NM. Particularly, the expression levels of GADD45, caspase3 and p21 activated kinase1 genes were remarkably up regulated in NM-treated LEC rats liver. These results suggested that NM causes different molecular responses in the liver of LEC rats before inducing various serological or histological responses, which may be one of the mechanisms underlying the high susceptibility toward chemical carcinogenesis.

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#### DOWN-REGULATION OF E-CADHERIN BY THE INTEGRIN-LINKED KINASE PATHWAY (ILK) IN HEPATOCYTES: A POSSIBLE MECHANISM FOR HEXACHLOROBENZENE-INDUCED TUMOR PROMOTION.

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The ILK pathway is overexpressed in tumors of various tissues. We have previously shown an activation of the ILK pathway in the liver of female rats given hexachlorobenzene (HCB), an environmental epigenetic carcinogen. This was accompanied by a down-regulation of E-cadherin, an adhering junction protein, and connexin32 (Cx32), a gap junction protein. The aim of this study was to demonstrate the role of the ILK pathway in the regulation of adhering and gap junctions in liver cells. MH<sub>1</sub>C<sub>1</sub> rat hepatocytes exposed to HCB for 7 days showed increased levels of the inactive form of GSK3β, i.e. phospho-GSK3β, an important messenger of ILK activation, and a decrease in Cx32, as observed *in vivo* exposed rats. Two different GSK3β specific inhibitors were used to confirm the role of the ILK pathway in E-cadherin and Cx32 regulation. Both inhibitors (kenpaulone 5 and 10 μM, SB-415286 125 and 150 nM) markedly reduced GSK3β activity and caused an approximately 50% decrease in E-cadherin mRNA levels, whereas Cx32 mRNA levels were unchanged. This suggests that in MH<sub>1</sub>C<sub>1</sub> cells, Cx32 expression is not modulated by GSK3β, but depends on either another messenger of the ILK pathway,

such as Akt, or another pathway. Stable transfections of MH<sub>1</sub>C<sub>1</sub> cells with Cx32 expression vector to increase Cx32 expression levels resulted in a marked increased E-cadherin mRNA levels thereby suggesting a link between Cx32 and E-cadherin regulation. Together, these data demonstrate that in rat hepatocytes E-cadherin is down-regulated by the ILK pathway via GSK3β inhibition and up-regulated by Cx32 overexpression. Clearly, both Cx32 and E-cadherin are regulated differently in hepatocytes suggesting that HCB may modulate several signalling pathways in the promotion of hepatocarcinogenesis. (Supported by The Canadian Liver Foundation, NSERC, and the Fondation Armand-Frappier).

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#### TOXICOGENOMIC APPROACH FOR PREDICTION OF HEPATOCARCINOGENS USING RAT HEPATOMA CELLS AND COMPARISON OF GENE EXPRESSION PATTERNS WITH CHEMICAL-TREATED RAT AND HUMAN HEPATOMA CELLS.

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The long-term rodent bioassay is the standard method to predict carcinogenic hazard of chemicals for human beings. However, this assay takes a long time and high costs. Our aim has been to develop a rapid and reliable prediction method for carcinogenicity based on microarray technology in cultured cells. We selected 39 chemicals that have been well characterized for carcinogenicity. Rat hepatoma cells (MH1C1) and human hepatoma cells (HepG2) were treated with the chemicals for 3 days. A set of genes for prediction of carcinogenicity were selected with statistical methods using a support vector machine (SVM). As a test set, six external samples underwent prediction of carcinogenicity based on the results of the SVM analysis and comparison with actual findings. Twenty-four of 39 chemicals were compared for expression responses between MH1C1 and HepG2 in ortholog genes. As the result of cross-validation, the predictive ratio for carcinogenicity was over 80%. With the six test set samples, the results predicted by SVM were reasonable. In contrast to MH1C1, the pattern of gene expression induced by chemicals was very different in the human (HepG2) case. These results indicate that toxicogenomic approach on hepatoma cells can be useful for carcinogenicity. With interspecies analysis, careful consideration is needed for the application of rat data to the human situation. Acknowledgement: This work was sponsored by NEDO(New Energy and Industrial Technology Development Organization)

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#### EVALUATION OF A VACCINE DELIVERY SYSTEM FOR RECOMBINANT ANTHRAX PROTECTIVE ANTIGEN IN RABBITS.

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Vaccine candidates such as recombinant proteins and synthetic peptides often lack the intrinsic adjuvant activity of traditional vaccines. The safety and adjuvant activity of aluminum compounds and new adjuvants also remain questionable. We developed a vaccine delivery system (F127/CpG) using Pluronic® F127 for sustained release and a CpG motif as adjuvant. F127/CpG demonstrated safe and efficacious delivery of recombinant anthrax protective antigen (rPA) to mice with improved responses compared to aluminum hydroxide (alum). Measures of safety and efficacy of rPA/F127/CpG were presently assessed in rabbits to determine if the beneficial characteristics of F127/CpG were maintained in a second and more sensitive animal used for inhalation anthrax studies. Rabbits received a primary (week 0) and booster (week 4) SC injection of 6.25 μg rPA in alum, F127/CpG/alum, or F127/CpG. Potential adverse effects were measured by following weight gain, clinical chemistries, and blood cell differentials for 8 weeks. Protective immunity was assessed by measuring biologically active antibody to rPA via a toxin neutralization assay. Injection of rPA/F127/CpG caused no overt adverse effects when compared to rPA/alum, rPA/F127/CpG/alum, or vehicles. Rabbits in all groups had similar gains in body weights. Clinical chemistries and blood cell differentials did not vary among the rPA or vehicle groups and were within normal ranges. At 8 weeks, relative levels of neutralizing antibodies to anthrax lethal toxin were 209, 167, and 153 for rPA/alum, rPA/F127/CpG/alum, and rPA/F127/CpG and did not differ significantly among groups. These data demonstrate that injection of rPA in F127/CpG is well tolerated and induces an effective immune response in rabbits as in mice. In contrast to mice, the efficacy of rPA/F127/CpG immunization in rabbits is not significantly improved compared to rPA/alum. This may be due to inherent differences in immune systems between species and the antigen dose tested. (Supported by NIH grant #1R43 A/52891-01)

HISTOLOGICAL RESPONSE OF RAT ORGANS TO REPEATED BLAST OVERPRESSURE EXPOSURE. EFFECTS OF NUMBER OF EXPOSURES AND TIME AFTER EXPOSURE.

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Blast overpressure (BOP) is the sharp rise in ambient pressure from explosive detonation, firing of high-powered weapons or from terrorist bombings and accidental occupational explosions. Exposure to BOP can cause auditory and nonauditory injury such as hearing loss, lung contusion and other manifestations of blunt trauma. We have previously evaluated and scored lung injury from 1 BOP exposure. To further assess the health risks of BOP, we examined the histological response to repeated BOP exposures in 5 organs. Anesthetized rats were exposed from 1-6 consecutive BOP exposures (- 62 kPa) at 3 min interval then euthanized after 5 min, 1, 6, and 24 h. After each exposure, the lungs were fixed inflated at 25-cm water pressure with 10% formaline-phosphate buffer. Heart, kidneys, small intestines, and brain were also extracted and fixed. All organs were stained with hematoxylin/eosin and examined microscopically. The results indicated minimal cumulative effect from repeated BOP exposure, although animals exposed to 6 blasts exhibited slightly greater damage to the affected organs. The lungs displayed the greatest changes showing mild to moderate focal and multifocal hemorrhage and congestion that continued to increase moderately up to 24 h even with a single exposure. The small intestines had mild multifocal hemorrhage and congestion, the brains displayed minimal focal to multifocal hemorrhage, and the hearts had focal to diffuse congestion and hemorrhage. The kidneys were the least affected displaying only slight congestion. We conclude that BOP-induced injury increases only slightly after repeated exposure, but moderately beyond 1 h. These observations have occupational and military implications re-emphasizing the need for protection from any BOP exposure, and stressing the importance of injury management within the first hour after exposure to minimize further risk of multiple organ failure.

DEVELOPMENT OF A MICROBIAL RISK ASSESSMENT METHODS COMPENDIUM AND ASSESSMENT OF SECONDARY DATA U.

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The threat of a microbial terrorist attack on buildings and drinking water systems has elevated the urgency for a method to assess and mitigate the possible human health impact of exposure to deliberate biological contamination. A critical need for achieving this objective is the development of microbial risk assessment methodology that leverages and extends aspects of recent work to incident based biothreat assessment. Therefore, a compendium of relevant studies and methods for microbial risk assessment has been developed based on literature and technical reports published from 1994 to 2004. The objectives of this effort were to identify, compile and evaluate existing studies for exposure assessment, dose-response assessment, or risk characterization for microbial agents that might subsequently be extended to support decision making process to decontaminate buildings and drinking water systems. Comprehensive literature searches were conducted of commercial electronic data bases and selected web sites for government agencies and international organizations. More than sixteen hundred titles were evaluated, including published manuscripts, books, and reports subjected to various levels of peer-review. The poster provides a tutorial example for assessing the applicability of secondary data largely in the absence of sufficient primary sources using data utility criteria. In addition, the poster will address data gaps and potential solutions for modeling airborne, waterborne, foodborne, and dermal hazards of concern for indoor air and drinking water distribution systems. The state of the science reflected in the microbial risk assessment methods compendium is the foundation for further development and testing of novel methods in this rapidly evolving field.

MICROARRAY ANALYSIS OF RAW264.7 CELLS EXPOSED TO BACILLUS ANTHRACIS- EVIDENCE OF VOLLUM 1B-INDUCED MACROPHAGE APOPTOSIS.

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*Bacillus anthracis* is the causative agent of anthrax disease. Inhalational anthrax is the most severe form of the disease and the lungs are the likely route of exposure during a terrorist attack. Following deposition of *B. anthracis* spores, alveolar macrophages (Mφ) phagocytose the bacteria and transport them to the draining

lymph system. En route, the spores germinate into vegetative bacteria, become metabolically active, secrete several known toxins and ultimately kill the host Mφ. The mechanisms by which *B. anthracis* spores evade destruction by the Mφ defenses and escape from them into the circulation remain unknown. Defining these mechanisms will lead to a better understanding of the infection stage of anthrax and may contribute to novel therapies for the disease. To this end, we have developed and applied microarray techniques to an *in vitro* Mφ-*B. anthracis* spore model using RAW264.7 Mφs infected with the virulent Vollum 1B strain (V1B) of *B. anthracis*. In this model, RAW Mφs are 50% viable 7 hours after phagocytosis of V1B spores and 100% dead at 15 hours. The mechanism of RAW cell death remains unknown, but was reversed by the addition of anti-lethal factor antibody, suggesting the V1B-derived toxin mediated, in part, RAW cell death. Microarrays were conducted 2 hours after the addition of V1B spores. Categories of genes increased by V1B in Mφs include those involved in cytoskeletal rearrangements, metabolism, gene transcription and reactive oxygen species generation. Additionally, several genes identified are directly linked to apoptotic pathways: Rhot-1, Rhot-2, LIMK2, Card10 and Rai 3. These observations suggest V1B germinates in Mφs, expresses lethal toxin in sufficient quantity to kill the Mφ and the mechanism of Mφ death is via an apoptotic pathway.

DECONTAMINATION OF BACILLUS ANTHRACIS SPORES ON INDOOR BUILDING SURFACES USING FORMALDEHYDE GAS.

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The intentional release of *Bacillus anthracis* spores in the mail prompted extensive remediation and clean-up efforts. The USEPA (EPA) has recently established the Building Decontamination Technology Center to test the performance of technologies intended to decontaminate biological agents from indoor building surfaces. This study assessed laboratory-scale decontamination efficacy of *B. anthracis* Ames spores with the surrogates *B. subtilis* and *Geobacillus stearothermophilus* deposited on porous and non-porous indoor surface materials. *B. anthracis*, *B. subtilis*, and *G. stearothermophilus* spores ( $1 \times 10^8$  CFU) were dried on seven types of indoor surfaces (carpet, wood, painted concrete, glass, laminate, galvanized metal, and painted wallboard) and exposed to 1100 ppm formaldehyde gas for 10 hours. Formaldehyde exposure significantly decreased viable *B. anthracis*, *B. subtilis*, and *G. stearothermophilus* spores on all test materials. The log reduction in viable spores across all seven test materials ranged from  $\geq 5.2$  to  $\geq 7.9$ , 6.0 to  $\geq 8.0$ , and 5.7 to  $\geq 7.6$  for *B. anthracis*, *B. subtilis*, and *G. stearothermophilus*, respectively. Significant differences in spore log reduction were observed between *B. anthracis* and both surrogates. Microorganisms from commercial biological indicators and spore strips having spore loads of approximately  $1 \times 10^6$  CFU were evaluated in parallel as a qualitative decontamination assessment. These biological indicators and spore strips were partially inactivated ( $\geq 50\%$  for all exposures) by formaldehyde. These results provide information for correlating the decontamination of virulent *B. anthracis* spores with surrogates on indoor surfaces using formaldehyde gas. Supported by the USEPA National Homeland Security Research Center.

DECONTAMINATION OF BACILLUS ANTHRACIS SPORES ON INDOOR BUILDING SURFACES USING HYDROGEN PEROXIDE GAS.

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In 2001, mailed *Bacillus anthracis* spores contaminated mail processing and distribution centers and the Hart Senate Office Building, which led to extensive remediation and clean-up efforts. The USEPA (EPA) responded to US homeland security concerns by establishing the Building Decontamination Technology Center to test the performance of technologies intended to decontaminate biological agents from indoor building surfaces. This study assessed laboratory-scale decontamination efficacy of *B. anthracis* Ames spores with the surrogates *B. subtilis* and *Geobacillus stearothermophilus* deposited on porous and non-porous indoor surface materials. *B. anthracis*, *B. subtilis*, and *G. stearothermophilus* spores ( $1 \times 10^8$  CFU) were dried on seven types of indoor surfaces (carpet, wood, painted concrete, glass, laminate, galvanized metal, and painted wallboard) and exposed to  $\geq 1000$  p.p.m. hydrogen peroxide gas for 20 minutes. Hydrogen peroxide exposure significantly decreased viable *B. anthracis*, *B. subtilis*, and *G. stearothermophilus* spores on all test materials except for *G. stearothermophilus* on carpet. The log reduction in viable spores across all seven test materials ranged from 3.0 to  $\geq 7.9$ , 1.6 to  $\geq 7.7$ , and 0.8 to 6.0 for *B. anthracis*, *B. subtilis*, and *G. stearothermophilus*, respectively. Significant differences in spore log reduction were observed between *B. anthracis* and both surrogates. Microorganisms from commercial biological indicators and spore strips (having spore loads of approximately  $1 \times 10^6$  CFU), evaluated in parallel as a qualitative decontamination assessment, were inactivated by hydrogen peroxide exposure. These

results provide new information for correlating the decontamination of virulent *B. anthracis* spores with surrogates on indoor surfaces using hydrogen peroxide gas. Supported by the USEPA National Homeland Security Research Center.

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#### A METHOD FOR THE SIMULTANEOUS MEASUREMENT OF SPECIFIC IgGS TO FIVE CDC SELECT BIOTERRORISM AGENTS IN SERUM.

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The Centers for Disease Control and Prevention has classified several agents which may be used in a bioterrorism attack as select agents. They are classified as such based on ease of dissemination, mortality/morbidity rate, and potential for social disruption. A subset of these agents include *Bacillus anthracis* (Ba), *Yersinia pestis* (Yp), *Francisella tularensis* (Ft), ricin toxin (RT) and staphylococcus enterotoxin B (SEB). Exposure/infection with these agents has been shown to cause the production of specific serum IgG antibodies. Comparison of pre- and post-incident IgG antibody levels has been shown to be a useful method to biologically monitor decontamination and clean-up workers for potential exposure to bioterrorism agents (*Occ Environ Med*. 61: 703-708, 2004). We describe a fluorescent covalent microsphere immunoassay (FCMIA) to measure specific IgG antibodies to Ba (protective antigen [PA] and lethal factor [LF]), Yp (F1 and V antigens), Ft, RT and SEB simultaneously in human Ba vaccinee sera which had been fortified with animal select agent specific IgG antibodies. Intra- and interassay coefficients of variation were <15% (N=3). There were no significant differences (P>0.70) in dilution curves when the assays were performed individually vs. multiplexed. When the observed vs. expected dilution curves were compared, highly linear relationships were observed (mean  $r^2=0.985 +/- 0.006$  [SD], P<0.001). Finally, the curves yielded linear responses for most analytes upon serum dilution from  $1 \times 10^2$  to  $1 \times 10^5$ . These data indicate that multiplexed FCMIA is a method to measure specific IgGs in serum to CDC select agents and may be of value in screening either decontamination workers or the general population for exposures/infections to these agents.

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#### GENERATION, METHODOLOGY AND ANALYSIS OF VX VAPOR.

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Low vapor concentrations of VX were generated to assess dosages at which threshold clinical signs (e.g., miosis) occur as a result of whole-body exposures in rodents. Saturated VX vapor streams were generated by directing nitrogen carrier gas through a glass vessel (multi-pass saturator cell) containing liquid VX. The saturator cell body was submerged in a water bath to help maintain a constant temperature. VX vapor concentration was manipulated through controlling the water bath temperature and the nitrogen gas flow. In order to facilitate calibration of saturator cell output, data was collected to validate previously published data on VX vapor pressure. Unlike other agents, it was also observed that passivation, or coating of the chamber was critical before analytical concentration determinations could be made. Furthermore, a constant flow through the chamber was necessary in order to maintain the passivation. Concentration uniformity was confirmed at several locations in the chamber to assure an even distribution. Steps were taken to verify that the agent generated was vapor with no measurable signs of aerosol. Analytical methodology was developed to verify generated concentrations. Performing inhalation vapor exposures of low volatility materials also introduced potential contamination hazards to personnel handling exposed animals, or performing routine operation with the chamber. Test methods have been developed to address these additional hazards created and how to safely generate these agents. In order to address residual contamination of animals and cages, swab samples were collected following exposure and analyzed for the presence/absence of the generated material. In addition, contaminated animal carcasses were dipped in a solvent to extract any residual VX, and the extract analyzed by large volume liquid injection/gas chromatography. As higher concentrations will be generated in support of additional studies, these contamination issues will become of greater importance.

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#### CORRELATING GENE EXPRESSION PROFILES WITH THERAPEUTIC EFFICACY OF POTENTIAL VESICANT MEDICAL COUNTERMEASURES.

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Sulfur mustard (SM) is a potent alkylating agent. We are developing medical countermeasures to reduce the injury caused by SM exposure. Screening in the mouse ear vesicant model has identified three effective compounds: dimercaprol (British

anti-lewisite), indomethacin, and octyl homovanillamide (OHV). To identify gene expression changes that correlate with compound efficacy we used oligonucleotide microarrays to compare gene expression profiles in vehicle-exposed skin, SM-exposed skin, and skin pretreated with each compound before SM exposure. Mice were topically exposed on the inner surface of the right ear to SM alone or pretreated for 15 min with one of the compounds and then exposed to SM. Left ears were vehicle-exposed. Ear tissue was harvested 24 hr later for ear weight determination (an endpoint indicating compound efficacy). RNA was extracted from the tissues and used to generate oligonucleotide microarray probes. Principal component analysis of the gene expression data revealed partitioning of the samples based on drug treatment and SM exposure. Vehicle-exposed mouse ears clustered away from the other treatment groups. SM-exposed mouse ears pretreated with dimercaprol or OHV clustered more closely with vehicle-exposed ears, while SM-exposed mouse ears pretreated with indomethacin clustered more closely with SM-exposed ears. This clustering of the samples is supported by the ear weight data, in which the indomethacin group has ear weights closer to the SM-exposed group, whereas the dimercaprol and OHV groups have ear weights closer to the vehicle-exposed group. Correlation coefficients were calculated for each gene based on the correlation between gene expression level and ear weight. These data provide the basis for understanding what gene expression changes are important in the development of effective SM medical countermeasures.

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#### FLUORIDE ION REGENERATION OF CYCLOSARIN (GF) FROM MINIPIG TISSUE AND FLUIDS FOLLOWING WHOLE BODY GF MIOSIS LEVEL VAPOR EXPOSURE.

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Recent developments to improve nerve agent biomarker techniques include methods for measuring fluoride regenerated Sarin (GB) in blood and tissue. Our efforts extend the fluoride ion regeneration method to be able to determine cyclosarin (GF) in red blood cell and tissue of minipig blood samples after whole body exposure to GF at miosis levels. Blood samples were taken serially before, during, and after whole body GF exposure from the minipig via venous catheter allowing agent exposure profiles to be generated. After processing the samples with fluoride ion and extracting with C-18 solid phase extraction cartridges the ethyl acetate extract was analyzed by GC/MS. The GC/MS method utilized an autoinjector, a large volume injector port (LVI), positive ion ammonia chemical ionization detection in the SIM mode, and a 2H11-GF stable isotope internal standard. Results indicated that the method range was 10-1000 pg on column. The detection limit was 3 pg of GF on column despite the complexity of the red blood cell/tissue matrix. Conditions that needed to be optimized for the LVI included injection volume, initial temperature, pressure, and flow rate. The regenerated GF (R-GF) profiles differ greatly from the regenerated GB (R-GB) profiles in the minipig at similar exposure levels. The onset of the appearance of R-GF in the blood seems to be delayed and maximum levels are reached at much later times as compared to GB exposures. Quantities of R-GF were recovered from minipig blood during the first several hours of inhalation exposure that were one-fifth to one-tenth of R-GB recovered at equimolar exposure concentrations and equal exposure duration times.

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#### CHOLINESTERASE AND SARIN (GB) REGENERATION PROFILE FOLLOWING LOW LEVEL GB IN GUINEA PIGS: USEFULNESS FOR PBPK MODELING.

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Physiologically based pharmacokinetic (PBPK) modeling may be a useful risk assessment tool for interspecies extrapolation and prediction of response to chemical warfare nerve agent (CWNA) exposure. The current study was performed to integrate CWNA toxicity data across different routes of exposure in guinea pigs. Animals were given a single subcutaneous (sc) dose of GB (0.1 & 0.4 LD50). Arterial blood was drawn from the right carotid artery at time intervals between 1 min and 4 hr post-exposure. Blood levels of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), and blood and tissue levels of regenerated sarin (rGB) were assayed. AChE and BuChE were significantly inhibited 1 min following 0.4LD50 GB, and remained inhibited at 4 hr, with the greatest level of inhibition

at 40 min. Following 0.1 LD50 GB, AChE was decreased at 40 and 60 min, while BuChE was not affected. In plasma, rGB reached maximal levels 20 min after 0.1 and 0.4 LD50 GB and gradually declined over 4 hr. In red blood cells (RBC), rGB reached maximal levels 10 min after 0.4 LD50 GB exposure and remained elevated over the course of 4 hr; however, RBC rGB was not as great as plasma rGB. After 0.1 LD50 GB exposure, rGB in RBC was not significantly elevated. These findings suggest that both plasma and RBC measures of rGB and cholinesterase indicate low level exposure, but that plasma rGB and RBC AChE may be more sensitive measures of extremely low level GB exposure. In the 2-4 hr after low level GB exposure, the highest levels of rGB were in lung and kidney. A future goal is to compare the pharmacokinetics of low dose GB in guinea pigs exposed by acute inhalation with our current measures following sc exposure. PBPK modeling will then be used to extrapolate these findings to different routes of administration and to compare similar exposures in other species.

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## EFFECT OF TOPICAL IODINE TREATMENT ON EARLY SULFUR-MUSTARD-INDUCED CUTANEOUS CHANGES AND EPIDERMAL-CELL PROLIFERATION.

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Exposure of skin to sulfur mustard (SM), a potent chemical-warfare and strong alkylating agent, results in severe damage. Topical iodine treatment reduces SM-induced skin lesions. The aims of the present study were to test the effect of topical iodine on SM-induced skin toxicity at an early stage after exposure and to analyze the effect of SM followed by iodine treatment on cellular proliferation. The treatment groups included SM + iodine, SM + iodine vehicle, and SM only. Control groups received iodine only or iodine vehicle only. One animal with 6 separate application sites was used for each type of treatment. One microliter of SM was applied on 6 sites on the back of the animal. Iodine or vehicle was applied 20 min later for a duration of 2 hours. One hour before the end of the experiment, 150 mg/kg bromodeoxyuridine (BRDU) was injected intraperitoneally. Animals were sacrificed 5 hours after SM exposure. Histopathology showed that only iodine significantly reduced dermal infiltration of neutrophils induced by SM. Visualization of cellular proliferation was accomplished by BRDU staining and blind labeling index (LI) counting in epidermal cells along a fixed unit of length. No significant difference occurred between the groups of SM + iodine and SM + vehicle or SM only. We concluded that iodine did not reverse the significantly-reduced LI parameters induced by SM. The results indicate that iodine exerts its protective activity predominantly by its anti-inflammatory activity, not through a primary effect on epidermal-cell proliferation. (This study was supported by the US Army Medical Research and Material Command under Cooperative Agreement DAMD17-03-2-0013 of the Binational Science Foundation Research Project 2001186.)

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## PRETREATMENT OF HUMAN EPIDERMAL KERATINOCYTES WITH D, L SULFORAPHENE PROTECTS AGAINST SULFUR MUSTARD CYTOTOXICITY.

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Sulfur mustard (SM) is a powerful cytotoxic agent in addition to being a potent vesicating agent, mutagen, and carcinogen. This insidious chemical agent reacts with glutathione (GSH) and forms GSH conjugates which appear to be excreted through the mercapturic acid pathways in mammals. The question of whether glutathione-S- transferases (GST) are involved in the formation of these conjugates remains unresolved. In previous studies, ethacrynic acid (EAA), a putative inhibitor of GST and Oltipraz (OLT), a known inducer of GST were ineffective in modulating this enzyme in HEK so this hypothesis could not be tested. Higher levels of intracellular GSH appeared to be responsible for the resistance of EAA-pretreated HEK to SM. A more efficient inducer of GST was needed to test whether this enzyme could be used to modify cytotoxicity following SM exposure. D, L- sulforaphene (DLS), a compound from broccoli extract known to be a potent inducer of GST was tested for GST induction in HEK. GST levels increased optimally (40%) in 24-well plates of HEK within 4 hours using 0.5 µg DLS/ well over a 48-hour incubation period. When the drug was removed by washing, and the pre-treated cells were challenged with 0-200 µM SM, there was an approximate 10-15% increase in survival at 24 hours compared to non-pretreated controls. This protective effect due to increased levels of GST was overcome at 300 µM SM, where there was no difference in survival between pretreated and non-pretreated controls. Glutathione levels were also assessed and showed no increase at 4 hours in HEK with DLS pretreatment. Higher levels of glutathione appear not to be responsible for this protection against SM as shown with EAA. More efficient compounds that can increase glutathione-S-transferase levels in target cells may be helpful in designing a medical pretreatment countermeasure against sulfur mustard.

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## THE MATRIX METALLOPROTEINASE INHIBITOR GM 1489 REDUCES MMP-9 ACTIVITY AFTER SULFUR MUSTARD EXPOSURE *IN VIVO*.

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Bis(2-chloroethyl)sulfide (sulfur mustard, SM) is a chemical warfare agent that penetrates the skin rapidly, causing extensive blistering within several hours. The damage includes separation of the dermis from the epidermis, potentially as a result of collagen degradation in the basement membrane zone by matrix metalloproteinases (MMPs). We hypothesize that skin damage is increased by upregulation of the MMPs following mustard-induced inflammation. MMP-2 (gelatinase A) and MMP-9 (gelatinase B) in the absence or presence of MMP inhibitors were investigated to better assess their role in SM-induced skin injury. Mouse ears were treated with 80 µg of liquid SM and ear specimens collected 24, 72, and 168 hours after exposure. Specimens were examined for the expression of MMP-2 and MMP-9 by histology, real-time PCR, gelatinase activity assays, and Western blot analysis. SM exposure increased mRNA levels for MMP-9 by 2-fold, 8-fold, and 21-fold for 24, 72, and 168 hours, respectively, when compared to the control untreated ears. There were no observable changes in the MMP-2 mRNA levels between treated and controls. A time-related increase in overall gelatinase activity was observed in SM treated ears. Western blot analysis confirmed the increased gelatinase activity was due to MMP-9. Pretreatment with the MMP inhibitor GM 1489 N-[(2R)-2-4-methylpentanoyl]-L-tryptophan-(S)-methyl-benzylamide resulted in a 50% reduction of MMP-9 mRNA at 72 and 168 hours after SM treatment when compared to samples without pretreatment with MMP inhibitor. These studies demonstrate that the MMP inhibitor GM 1489 is a promising candidate as an effective pharmacological countermeasure against SM injury. \*This work is supported by the US Army Medical Research and Materiel Command under Contract No. DAMD17-02-C-0091

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## THE EFFECT OF SULFUR MUSTARD EXPOSURE ON THE PERCENT, SIZE AND DENSITY OF HUMAN T CELLS (CD3+).

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Isolated human leukocytes from normal human volunteers were exposed to 0, 10, or 100 µM sulfur mustard (SM) at 1, 4, and 24 hrs. The effects of the SM on the percentage, size, and density of the human T cells were determined by flow cytometry and analyzed using the FlowJo program (Tree Star Inc., Stanford U, CA). The results showed a time- dependent decrease in the percent of T cells among exposed cells. At 1 hr post-SM exposure, there was no discernable change in the CD3+ cells. By 4 hrs post-SM exposure, there was a concentration-dependent decrease in percent of CD3+ cells; but no shift in the peak channel of the forward scatter pattern. At 24 hrs post-SM exposure, there was both a large decrease in the percent of CD3+ cells and a shift to the left in the peak channel. The concentration-dependent shift to the left of the peak channel in the SM-exposed cells indicated a decrease not only in the percent of CD3+ cells but also in the size of the remaining T cells. When the side scatter parameter was studied at 1, 4, and 24 hrs post-SM exposure, there was no difference of the SM-exposed T cells at either the 1 or 4 hr time points. At the 24 hr time point, there was a SM-induced, concentration-dependent shift to the right indicating an increase in cell density. When lymphocytes are exposed to SM, T cells become smaller and denser in a concentration-dependent manner between 4 and 24 hrs after exposure. These changes are consistent with the hypothesis that SM-exposed lymphocytes die by an apoptotic mechanism.

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## OBSERVATIONS ON THE INTERACTION OF SULFUR MUSTARD WITH CYTOCHROME P450.

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The potential for interactions at the level of metabolism among chemicals encountered during military deployment is an area that has gained interest since the advent of Gulf War Syndrome. Sulfur mustard is one of the compounds of concern. However, the characteristics of the interaction of sulfur mustard with cytochrome

P450 have received little attention. We tested p-nitroanisole-O-demethylase activity using liver microsomes (0.5 mg microsomal protein/ml) from male CD1 mice induced with phenobarbital as our enzyme source. The complete incubation mixture included NADP and the standard NADPH regenerating system. In the presence of 3.4 mM sulfur mustard we observed a 55% reduction in the uninhibited reaction rate, indicating sulfur mustard interaction with the microsomal oxidative drug metabolizing system at some level. We followed this up with an experiment to determine whether sulfur mustard was interacting with NADPH-cytochrome P450 reductase or with cytochrome P450 itself. We assayed human NADPH-cytochrome P450 reductase using bovine heart cytochrome c as an electron acceptor in place of the cytochrome P450. The system was further simplified by the use of NADPH and omission of the NADPH regenerating system. We followed cytochrome c reduction spectrophotometrically by measuring the increase in OD at 550 nm. The inclusion of 2.4 mM sulfur mustard yielded a 30% drop in the rate of cytochrome c reduction. We conclude that sulfur mustard interacts with NADPH-cytochrome P450 reductase in a manner that reduces the rate of substrate oxidation by the P450. If this effect generalizes to all CYP isoforms, as might be expected, an overall reduction in the rate of oxidative drug and xenobiotic metabolism could be possible in anyone exposed to sulfur mustard.

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PUTATIVE ROLE OF PLATELET ACTIVATING FACTOR (PAF) ANALOGS IN CELL CYCLE ABBERRATIONS IN HUMAN ENDOTHELIAL CELLS *IN VITRO*.

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Our laboratory studies the mechanism of toxicity of the chemical blister agent sulfur mustard (SM) for the purpose of developing medical countermeasures to this chemical threat agent. SM is a cytotoxic alkylating agent with mutagenic and vesicant properties. A major feature of SM tissue injury is inflammation. Platelet activating factor (PAF), one of the inflammatory mediators potentially involved in the cell death cascades induced by SM, was studied for its effect on human pulmonary artery endothelial cells (HPAEC). PAF is known to effect pulmonary vasculature. We evaluated the effect of several PAF analogs on cell growth and cell cycle via incorporation of 5-bromo-2-deoxyuridine (BrdU). PAF C-16 is a naturally occurring phospholipid produced upon stimulation through two distinct pathways known as the 'remodeling' and 'de novo' pathways. It is a potent mediator of neutrophil migration, reactive oxygen species production, and IL-6 secretion. PAF C-16 (10 uM) caused greater than 20% increased cell death and lysis at 24 hrs, with the remaining cells exhibiting reduced cytoplasm. 2-Thio PAF C-16 (10 uM) caused greater than 30% cell death at 24 hrs after exposure. PAF C-16 and azelaoloy PAF increased S-phase from 24% in controls to 56% and 52% respectively. They also decreased G1 from 62% in controls to below 49% and eliminated G2, suggesting a block at or near the S/G2 interface. Several other PAF analogs did not cause increased cell death, altered morphology or changes in cell cycle. PAF analogs are potent stimulators of inflammatory response which in our laboratory caused G2 cell cycle blockade and cellular toxicity in the absence of SM. The resemblance of the morphological and cell cycle changes seen with PAF to those seen with SM suggest that these mediators may play a key role in the pathology following SM exposure.

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ORAL DOSING WITH N-ACETYL-L-CYSTEINE SIGNIFICANTLY INCREASES GLUTATHIONE LEVELS AND GLUTATHIONE-S-TRANSFERASE ACTIVITY IN RAT SKIN.

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Sulfur mustard (2, 2-dichlorodiethyl sulfide, HD), a vesicant, remains a major chemical threat to troops and civilians; yet no specific medical countermeasures are currently available. Phases of dermal HD toxicity range from erythema to blistering and ultimately necrosis, however the mechanism of HD-induced damage remains unclear. Glutathione (GSH), a free-radical scavenger in cells which is decreased following HD exposure, may be involved. N-acetyl-L-cysteine (NAC), a synthetic cysteine derivative, has been shown to not only increase GSH levels in animals and humans but also reduce the toxicity of certain chemicals. To determine if NAC could be a potential prophylaxis for HD exposure, adult Sprague-Dawley rats were orally dosed with 600 or 1200 mg/kg NAC containing 6  $\mu$ Ci/g  $^{14}$ C-NAC or water (control vehicle). Significant levels of  $^{14}$ C were detected in rat skin 30 minutes after a single oral dose of  $^{14}$ C-NAC (600 or 1200 mg/kg) and levels remained elevated for up to 6 hrs. Skin GSH levels and glutathione-S-transferase activity were significantly increased in rats following daily oral NAC administration (600 or 1200 mg/kg) for 30 days. Gene expression analysis is ongoing to determine if GSH associated genes were significantly affected by repeat-oral NAC exposure. Since NAC distributes to the skin and increases GSH, daily oral administration may prevent GSH depletion and subsequent tissue damage following HD exposure.

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TOPICAL IODINE SUPPRESSES SULFUR-MUSTARD-INDUCED TUMOR NECROSIS FACTOR-ALPHA INDUCTION IN MOUSE-EAR MODEL.

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Sulfur mustard (SM), also termed mustard gas, is a potent vesicant that elicits an inflammatory response upon exposure of the skin. Evaluation of mouse ear 3 hours after exposure to 0.318 mg SM revealed acute inflammatory-cell aggregates in the vascular beds accompanied by strongly TNF-alpha-positive neutrophils as measured by immunohistochemistry. Eight hours after SM exposure, this phenomenon became intensified and associated with infiltration into the adjacent dermis. In ear skin topically treated with iodine, however, no inflammatory cells were observed 3 hours after SM exposure; 8 hours postexposure, blood vessels contained very few TNF-alpha-positive inflammatory cells. Administration of 1 and 2 micrograms per 30 gram body weight anti-TNF-alpha antibodies significantly reduced ear edema by 49% and 30%, respectively. These findings were corroborated by quantitative analysis of the histological findings. Iodine pretreatment of peritoneal mouse neutrophils elicited a concentration-dependent reduction in the oxidative burst of the activated cells. Iodine also scavenged hydroxyl radicals generated by glucose oxidase in a concentration-dependent manner. Since TNF-alpha may be induced by oxygen radicals, iodine-induced protection against SM might result from hydroxyl-radical scavenging by iodine and its suppression of the oxidative burst of activated neutrophils. The combination of iodine and anti-TNF-alpha antibodies might constitute a new approach for treatment of SM-exposed individuals. (This study was supported by US Army Medical Research and Material Command under Cooperative Agreement DAMD17-03-2-0013 and the Binational Science Foundation Research Project 2001186).

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DEVELOPMENT OF INTERSPECIES AND MULTI-EXPOSURE ROUTE DOSE METRICS FOR SARIN VIA PHYSIOLOGICALLY BASED PHARMACOKINETIC/PHARMACODYNAMIC MODELLING.

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One of the significant challenges for the relevant application of animal models for studies of chemical warfare agent (CWA) toxicology is to produce actual risk estimates of human response through conversion of pharmacokinetic and pharmacodynamic results. The majority of mechanistic animal studies with CWAs have been at near lethal to supra-lethal doses, by dosing routes (i.e. subcutaneous, intramuscular) that are less applicable to likely human exposure scenarios (i.e. inhalation, dermal), and in animal models that are compromised. This confounds the direct application of these kinds of data to operational risk management processes. One quantitative approach to extrapolate animal study results relevant to human exposure scenarios and best estimate responses is the use of physiologically based pharmacokinetic/pharmacodynamic (PBPK/PD) models. This approach is to simulate potential human responses from CWA exposures through development of mathematical constructs, which incorporate physiologically specific characteristics of both the test species and humans. Using a previously developed PBPK/PD model of sarin inhalation pharmacokinetics and pharmacodynamics in the Gottingen miniature swine, we parameterized the model for the guinea pig and simulated new experimental data with previously published results from studies using subcutaneous, intravenous and inhalation dosing routes. These simulations permitted comparison of the results from CWA studies by different study exposure routes using different species, provided the basis for utilizing extensive data resources for sarin and will be used to extrapolate these PBPK/PD model results to predict human dose-response effects.

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A PBPK REGIONAL MODEL FOR NERVE AGENTS IN THE BRAIN.

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Traditional chemical warfare (nerve) agents, such as sarin, soman and VX, are organophosphorous compounds which bind to acetylcholine esterase (AChE) enzyme, resulting in inhibition of hydrolysis of acetylcholine. Accumulation of acetylcholine at the nerve junction causes continuous stimulation, resulting in observable effects such as miosis, increased tracheobronchial secretions, tremor and convul-

sion. Extended low-level exposures are believed to lead to more subtle mental and behavioral consequences, which may depend on the dose to particular brain regions (dose-response). Physiologically-based pharmacokinetic or toxicokinetic (PBPK/TK) models are accepted tools for data integration and cross-validation of diverse studies. Previous related PBPK models include one by Kim et al for 2, 4-D, which incorporates membrane-limited transfer into brain regions, and transfer from brain regions to cerebrospinal fluid (CSF). The present models are based on a PBPK model for nerve agents developed in our laboratory, extended to incorporate regional brain blood flows, regional flow-limited brain uptake, tissue volumes and CSF flow. Regional brain activity increases regional cerebral blood flow, altering the regional distribution of lipophilic agents and toxins such as nerve agents, thereby altering the response. Limited experimental data has been collected in rat, minipigs, guinea pig and humans, and includes regional AChE inhibition measurements in the frontal, occipital, parietal and temporal cortex, as well as the optic nerve and olfactory bulb in the minipig. Regional brain blood flows and tissue volumes for animals and humans have been collected from the literature. Consistent and defendable data, suitably incorporated into this PBPK/TK modeling framework, will significantly reduce errors currently embedded in various estimates of toxicity, and will provide a consistent basis for extrapolating and integrating experimental data and information on health effects with potential performance decrements resulting from low-level exposure scenarios.

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SARIN (GB) REGENERATION AND CHOLINESTERASE PROFILES FOLLOWING INHALATION OF GB IN GUINEA PIGS: APPLICATION TO PBPK MODELING.

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A risk assessment tool for interspecies extrapolation and prediction of response to chemical warfare nerve agent (CWNA) exposure is physiologically based pharmacokinetic (PBPK) modeling. This study was performed to integrate CWNA toxicity data using the inhalation route of exposure in guinea pigs (GP). After the 60 min GB LC<sub>50</sub> was determined (239 mg·min/m<sup>3</sup>), GP were exposed to GB vapor (0.1 & 0.4 LC<sub>50</sub>) in a modified whole body inhalation chamber that allowed serial collection of blood from the left carotid artery from as many as eight animals during the 60-min exposure. Arterial blood was also collected at various time intervals post-exposure (up to 24 hrs). Mixed blood was collected at 7 & 14 days post-exposure. Blood gas and other physiological parameters were measured using i-STAT. The onset of miosis (within 6-min) was measured using IR technology while the animals were exposed. Blood levels of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE)(pre- & post-exposure, during exposure), and blood and tissue levels of regenerated sarin (rGB) were assayed. At the GB concentrations tested, rGB levels were higher in plasma than RBC, but RBC rGB was still measurable while plasma rGB was barely detectable 7 days post-exposure. Post-exposure, high levels of rGB were found in the eyes, lung and kidney. The results suggest that plasma and RBC rGB and cholinesterase are useful indicators of low level exposure. These results combined with data from the subcutaneous route of exposure will be used to refine existing PBPK models and to compare with similar inhalation data in other species. These data will provide important information regarding the relationships between exposure levels, absorption amounts and toxic effects.

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ESTIMATING LETHAL AND SEVERE TOXIC EFFECTS IN MINIPIGS FOLLOWING 10, 60 AND 180-MINUTES OF WHOLE-BODY GB VAPOR EXPOSURE.

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Sexually mature male and female Gottingen minipigs were exposed to various concentrations of GB vapor via whole-body inhalation for 10, 60 or 180 minutes. Signs of nerve agent exposure were classified as either lethal, severe, moderate or mild. Maximum likelihood estimation was used to calculate the median effect levels (lethal (LCT<sub>50</sub>) and severe effects (ECT<sub>50</sub>)) for each gender-duration combination. Ordinal regression was used to model the concentration x time profile of the agent toxicity. Contrary to that predicted by Habers rule, LCT<sub>50</sub> and ECT<sub>50</sub> values increased as the duration of the exposures increased. LCT<sub>50</sub> values (with 95% confidence limits) for 10, 60 and 180-minute exposures in male minipigs were 72.5

(57.3-91.6), 105.7 (85.6-130.6) and 182.3 (145.2-228.9) mg·min/m<sup>3</sup> respectively. LCT<sub>50</sub> values (with 95% confidence limits) for 10, 60 and 180-minute exposures in female minipigs were 86.9 (69.2-109.2), 127.1 (100.7-160.4) and 174.3 (134.7-225.5) mg·min/m<sup>3</sup> respectively. The data were best fit using a probit slope of 15.7 and toxic load exponent of 1.38 (95% confidence limits of 1.25-1.51). Although, males were significantly (p =0.01) more sensitive than females to lethal effects of GB vapor, the ratios of lethal to severe concentrations were higher in female minipigs (99% ANOVA confidence) indicating that there is less difference between severely toxic and lethal dosages in the female as compared to male pigs.

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PRETREATMENT WITH CARBOXYESTERASE INHIBITOR CBDP SENSITIZES MICE TO SARIN: POTENTIAL MODEL FOR LOW-DOSE EXPOSURE ASSESSMENT.

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Low-level (sub-clinical) exposure to organophosphates (OPs), both insecticides and chemical warfare agents, continues to be a public health concern. To better understand the effects of such exposures, we have developed a mouse model that utilizes the selective carboxyesterase inhibitor, 2-(O-cresyl)-4H-1:3:2-benzodioxaphosphorin-2-oxide (CBDP), to render the mouse more sensitive to OPs. In this study we pretreated animals for 1 hr with either 1.5 mg/kg CBDP administered sc. or vehicle (propylene glycol/5% EtOH) and then administered sc. saline or sarin (0.05 or 0.1 x LD<sub>50</sub> - 0.4 ug/kg). The animals were sacrificed 24 hr later and cholinesterase activity was determined in blood and several brain areas (brain stem, prefrontal cortex, caudate, and amygdala). Analysis of the blood cholinesterase activity indicated that at the dose used, CBDP had little effect alone, but potentiated the inhibition produced by both doses of sarin. In all of the brain areas examined, neither CBDP nor sarin alone produced inhibition of brain CHE activity; however, CBDP pretreatment enabled sarin to inhibit CHE activity by 38 to 65%, depending on the dose and brain region. We also observed no behavioral effects of CBDP or sarin alone at the doses tested, but the combination produced classic signs of OP toxicity: hypothermia, lethargy, and muscle fasciculations. From these results, we conclude that by inhibiting carboxyesterase with CBDP pretreatment, the mouse becomes sufficiently sensitized to sarin for it to function as a model for human exposure. The advantages of using a mouse model include the ability to employ transgenic animals and microarray technologies to look for subtle changes induced by sub-lethal OP exposures. This research is funded by DoD contract DAMD17-00-C-0020.

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GENISTEIN PROTECTS MICE FROM RADIATION-INDUCED WEIGHT LOSS.

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Radioprotectors are chemical compounds that provide protection from ionizing radiation-induced injury. They have applications in clinical oncology, space travel, radiation site cleanup, radiological terrorism, and military scenarios. We demonstrated that the isoflavone genistein protects mice from radiation-induced lethality when administered 24 hr before gamma irradiation (Landauer et al., *J. Appl. Toxicol.*, 23, 379-385, 2003). In a clinical setting, radiation is administered at sub-lethal doses to destroy tumor cells. A frequent side effect of sublethal irradiation is a reduction of body weight. Moreover, weight loss has been demonstrated to be a prognostic indicator of reduced survival rates; therefore, the amelioration of radiation-induced weight loss is an important objective when using radiation therapy. In the present study, we characterized the effects of genistein on gamma radiation-induced weight loss in CD2F1 male mice. Mice were divided into four groups:(1) vehicle + sham irradiation, (2) genistein + sham irradiation, (3) vehicle + 7-Gy irradiation, and (4) genistein + 7-Gy irradiation. A single injection of genistein (200 mg/kg) or vehicle was subcutaneously administered 24 hr before either sham irradiation or a sublethal dose of cobalt-60 gamma irradiation (7 Gy at 0.6 Gy/min). Animals were weighed daily for 30 days after irradiation. We found that mice treated with vehicle or genistein that were sham-irradiated exhibited normal weight gain over the 30 days of the experiment. In contrast, a significant reduction in body weight for both groups of irradiated animals was observed. Beginning on day 3 after irradiation, the genistein/irradiation group exhibited decreased weight loss compared with the vehicle/irradiation group. By day 20 after irradiation, the body weight of the genistein/irradiation group had returned to control levels. However, the body weight of irradiated mice that did not receive genistein remained significantly below that of the other three treatment groups at 30 days after irradiation. These results demonstrate that genistein can mitigate weight loss in mice receiving a sublethal dose of gamma radiation.

## LOW DOSE SARIN PRODUCES LONG LASTING CHANGES IN CARDIOVASCULAR FUNCTION.

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Our previous studies showed that sarin, acetylcholinesterase (AChE) inhibitor and chemical warfare agent, has effects on brain gene expression at very low doses. To extend these studies to physiological function, we evaluated the cardiovascular effects of sarin in mice. Male C57BL mice (n=8) with radiotelemetric arterial catheters were injected with sarin subcutaneously (8 µg/kg, 0.05 x LD50) on two consecutive days. Arterial pressure (AP) was recorded (5Hz) under baseline conditions and 5, 12 and 30 days after treatment. We measured blood pressure and heart rate (HR) with analysis of variability in time and frequency domains. AP and pulse interval (PI) time series were submitted to spectral analysis by autoregressive method with variability measured in the low (LF, 0.1-1.0 Hz) and high (HF, 1-5 Hz) frequency ranges. The low dose of sarin did not affect blood cholinesterase activity, HR or AP. However, there was a more than 2 fold increase in PI variance which lasted for at least 30 days. PI variance after sarin was: 40±4, 97±17, 96±13 and 110±24 ms<sup>2</sup> before and 5, 12 and 30 days after treatment, respectively. Sarin also enhanced the LF and HF components of PI variability (19±3, 42±6, 44±8 and 63±12 ms<sup>2</sup>; 16±1, 45±11, 42±6 and 39±10 ms<sup>2</sup> before and days 5, 12 and 30 for LF and HF, respectively). Sarin treatment (12 days, n = 5) combined with muscarinic receptor blockade (atropine, 4mg/Kg, i.p.) produced an increase in HR from 478±14 to 623±34 bpm, while MAP did not change. Atropine markedly reduced PI variability from 97±10 to 9±2 ms<sup>2</sup> and also reduced LF from 41±10 to 1±1 ms<sup>2</sup> and HF from 43±7 to 6±2 ms<sup>2</sup> in sarin treated mice. Our results show that a dose of sarin which exerts no peripheral effects on cholinesterase activity produced marked and long lasting effects on cardiac function. These data raise concerns about possible public health effects of exposure to even sub-clinical levels of sarin. (This work was supported by US Army Medical Research and Materiel Command under contract DAMD17-00-C-0020).

## EVIDENCE OF CYSTEINE LOADING IN SKIN AND OTHER TISSUES IN SPRAGUE-DAWLEY RATS DOSED AT 4-HOUR INTERVALS WITH N-ACETYL-L-CYSTEINE (1, 200 MG/KG).

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Intracellular cysteine levels are a significant, rate-limiting factor for chemical detoxification via glutathione (GSH) conjugation. N-acetyl-L-cysteine (NAC) is an artificial source of cysteine and oral administration of NAC has shown to increase cellular GSH levels and protect against toxic injury from certain exposures. However, NAC has a relatively short half-life of approximately 4-6 hours and it is assumed that the beneficial effect of NAC also diminishes 2-4 hours after oral administration. In this study, we explored whether oral administration of a single bolus dose of 14C-NAC (1, 200 mg/kg in 2 mL d H<sub>2</sub>O) every 4 hours for up to 12 hours resulted in increasing levels of 14C dpm, GSH levels and glutathione-S-transferase (GST) activity in tissues with potential exposure to irritating chemicals. We found that the levels of 14C increased significantly with each successive NAC dose in the serum, skin, kidneys, thymus, lung, and liver at 4, 8, and 12 hours as compared with to tissues from negative control animals dosed with deionized water. 14C dpm for eyes, kidney, liver, serum, and thymus did not increase significantly following a third NAC dose at 8 hours as compared to levels at 4 hours suggesting that cysteine levels in these tissues had reached steady-state after two doses. 14C dpm for the skin and lungs were found to be elevated still further after 3 NAC doses indicating that cysteine levels had not reached steady-state. Studies are continuing to determine if repeat dosing increases GSH levels and GST activities in the tissues analyzed for 14C activity.

## LOW-LEVEL INHALATION EXPOSURE TO GB, GF AND VX INDUCES EXPRESSION OF NEURONAL APOPTOSIS AND REGENERATION GENES IN RAT.

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Since the description of Gulf War Syndrome and the terrorist events in Tokyo and Matsumoto in the 1990s, there has been increased interest in the toxic effects of low-levels of chemical warfare (CW) agents. A considerable body of literature suggests that exposures to organophosphorus (OP) compounds result in cognitive and neurological decrements. However, there is a paucity of literature regarding the mo-

lecular effects of low-level exposures to OP nerve agents. Although low-level exposures may not cause obvious pathology at the time of exposure, they may cause genetic alterations or changes in gene expression that may predispose personnel to injury or disease later in life. In the experiments described herein, male and female Sprague-Dawley rats were exposed to low-level levels of the aerosolized nerve agents Sarin (GB), Cyclosarin (GF), and VX. The ranges of exposure levels (0.0001- 0.033 mg/m<sup>3</sup> for 1 or 4 hrs.) used in these experiments are near levels inducing miosis. Control animals were exposed to air for the same time periods. Alteration of gene expression levels in the brain of the exposed animals was assessed using DNA microarray analysis, and was verified by RT-PCR. To date, our results indicate that low-level inhalation exposure to GB, GF, and VX results in the differential expression many genes that participate in neurological injury, cell death, and regeneration. These data reveal both agent specific and general organophosphate nerve agent toxicities. Furthermore, our analyses also reveal many differences in the extent and identities of the differentially expressed genes between the female and male animals. These data, along with parallel physiological measurements, strongly suggest that there are significant gender-associated differences in the toxicity of low-level exposures to organophosphate nerve agents.

## MECHANISM OF THE MIOTIC TOLERANCE OBSERVED FOLLOWING MULTIPLE EXPOSURES TO SARIN VAPOR.

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One of the first signs of exposure to sarin (GB) vapor is miosis. The present study was undertaken to investigate the ocular effects of repeated low-level exposure to GB vapor. Rats were exposed to GB vapor (3.93 ± 0.18 mg/m<sup>3</sup>) for 1 hour on each of 3 consecutive days. Exposed rats had >97% reduction in the ratio of pupil-to-iris radius following the first two exposures (n=15). However, following the third exposure, tolerance developed to the miotic effect of GB, with the ratio of pupil-to-iris radius only decreased by 40% (n=15). The light reflex in GB-exposed animals was also absent following the third exposure, but gradually returned over the next 7 days. AChE and BChE activity in the eye and blood were inhibited to a similar degree (50-70%) following each exposure, and the amount of GB recovered from the eye was similar following each exposure. Thus, the tolerance observed cannot be attributed to a reduction in the inhibitory effect of GB. Phentolamine (n=4) and propranolol (n=4) were administered to some animals before the third exposure to determine if enhanced sympathetic activity was responsible for the tolerance observed, but neither had an effect on the development of tolerance to GB vapor. Atropine (6 mg/kg i.m.; n=4) was administered to some animals before the first exposure to determine if excessive stimulation and subsequent desensitization of muscarinic receptors following inhibition of AChE may be responsible for the tolerance observed. Atropine pre-treatment prevented both the miosis associated with the first exposure, and the tolerance observed following the third exposure. The results of the present study demonstrate that multiple inhalation exposures to GB vapor result in tolerance to the miotic effect of GB that persists for about 7 days, and is likely the result of excessive stimulation of muscarinic receptors.

## THE URINE CONCENTRATIONS OF 2-AMINO-2-THIAZOLINE-4-CARBOXYLIC ACID AS A BIOMARKER FOR CYANIDE IN SMOKING AND NON-SMOKING MALE AND FEMALE VOLUNTEERS.

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It is known that 2-amino-2-thiazoline-4-carboxylic acid (ATCA) is a toxic metabolite (Nagasawa et al., OPPI 36, 178-182, 2004) of cyanide which causes tremors, convulsions and brain lesions (Bitner et al., NeuroToxicology 16, 115-122, 1995, NeuroToxicology 18, 191-200, 1997). ATCA inhibits cytochrome c oxidase approximately 200 times greater than cyanide (Kelly et al., 2005). ATCA appears to be a promising biomarker of cyanide as it is far more stable than cyanide (Logue et al., submitted to J. Chromatography B). Concentrations of ATCA were measured in urine of smoking or non-smoking volunteers using gas chromatography-mass spectrometry. Gender urine differences of ATCA were found in smokers indicating that women (319 + 284 ng/ml, N=11) had higher concentrations than men (115 + 36 ng/ml, N=8). In non-smokers, no differences in urine ATCA concentrations as a function of gender were observed in males (87 + 36 ng/ml, N=15) versus females (80 + 53 ng/ml, N=6). It is shown that the cyanide metabolite ATCA could be used to measure cyanide exposure if the factors of smoking and gender are considered.

INTERACTION OF 2-AMINO-2-THIOZOLINE-4-CARBOXYLIC ACID WITH CYTOCHROME C OXIDASE IMMOBILIZED IN AN ELECTRODE-SUPPORTED LIPID BILAYER MEMBRANE.

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Cyanide inhibition of cytochrome c oxidase (CCO) is thought to be responsible for cyanide toxicity. It has also been shown that the cyanide metabolite 2-amino-2-thiazoline-4-carboxylic acid (ATCA) (Nagasawa et al., OPPI, 36, 178-182, 2004) is formed chemically from cyanide. ATCA itself causes tremors, convulsions, and brain lesions in rodents (Bitner et al., NeuroToxicology, 16, 115-122, 1995, NeuroToxicology, 18, 191-200, 1997). In the current preliminary experiments, potential inhibition of CCO (isolated from bovine) by ATCA (Chem-Impex Interactions, Wood Dale, IL) was investigated using electrochemical sensor technology. Voltammograms were obtained during continuous exposure to CCO by 9.8  $\pm$  M ATCA in phosphate buffer (0.1 M, pH 7.4). The initial experiment found a 91% decrease in oxidative current (using cyclic voltammetry) of CCO after exposure to ATCA for 20 min. The inhibition of CCO by ATCA is generally similar to earlier results found with cyanide spectrophotometrically (Alexander et al., BBA, 912, 41-47, 1987) or electrochemically (Su et al., submitted to J. Electroanalytical Chem.), although the inhibition by ATCA is of greater magnitude than that of cyanide. It is suggested that the cyanide metabolite ATCA is a more potent inhibitor *in vitro* of CCO than cyanide.

VALIDATION OF REAL TIME QUANTITATIVE PCR AND SAMPLE PREPARATION PROCEDURE IN BIODISTRIBUTION STUDY OF ADENOVIRUS VECTORS.

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A reliable real time quantitative PCR (Q-PCR) assay and a standardized sample preparation procedure must be established prior to evaluation of the biodistribution of a gene therapy product. Demonstrated here are strategies implemented to achieve reliable and reproducible data in a biodistribution study of an adenovirus vector (Ad-vector) in mouse tissues. The well-defined primer-probe set and an optimized Q-PCR assay allowed for specific detection of the Ad-vector in mouse tissue samples with a reaction efficiency of greater than 96%. Applying Q-PCR limiting dilution assay, the copy number (1.0) of the Ad-vector DNA calculated from the size and concentration compared well with the experimentally determined value of 1.13 copy numbers. A standard curve ranging from  $10^1$  to  $10^7$  copies per reaction was shown to be reproducible over four-month study period with an average CV% of 2.0% for  $C_T$  ( $n=9$ ). Because a variety of tissue types and multiple steps were involved in the target detection, each step of the procedure was standardized. This included using identical amounts of various tissues for DNA isolation and equal volumes of each DNA sample for the PCR assay to ensure a consistent sample quality among individual samples. Applying these procedures, the concentration of DNA isolated with a Qiagen DNeasy kit was similar for each tissue type. Additionally, DNA samples from various control mouse tissues, each spiked with 10 copies of Ad-vector DNA resulted in an average of  $8.1 \pm 1.8$  (SD) copies being detected. Moreover, data generated from a group of animals treated with Ad-vector ( $n=5$ ), which covered a range from zero to several thousand copies of Ad-vector per  $\mu$ g DNA in eleven different tissues, have been reproduced with the same tissue lysate over a nine-month period. These data demonstrate the reliability and reproducibility of the complete procedure from sample preparation through PCR analysis. (Supported by NGVL, and NIH grant no. RR-01-001)

SINGLE-DOSE TOXICITY STUDY OF AN ONCOLYTIC ADENOVIRUS ADMINISTERED BY INTRAVENOUS INFUSION TO CYNOMOLGUS MONKEYS.

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Replication of recombinant adenovirus 01/PEME is attenuated in normal human cells by key pathways that control cell growth and apoptosis. However, these pathways, which are often dysregulated in tumor cells, allow 01/PEME to undergo vigorous replication, resulting in release of new 01/PEME to infect other tumor cells and produce oncolysis. The current study was designed to assess the safety of 01/PEME in cynomolgus monkeys. Female monkeys ( $n=2$ /group) were administered a single iv (saphenous) dose of either vehicle,  $1 \times 10^{11}$ ,  $5 \times 10^{11}$ ,  $1 \times 10^{12}$ , or  $5 \times 10^{12}$  viral particles/kg (p/kg). Animals, with the exception of the highest dose

group, were necropsied on study Day 25. Mortality and morbidity were observed at  $5 \times 10^{12}$  p/kg within 4 days of dosing and was associated with increased prothrombin and activated partial thromboplastin time values, mild thrombocytopenia, elevated transaminase values, and diffuse liver necrosis. Monkeys administered  $5 \times 10^{11}$  and  $1 \times 10^{12}$  p/kg exhibited dose-related transient increases in transaminase levels. 01/PEME was detectable in excreta by PCR but only a single incidence of active infectious virus was detected in a monkey administered  $5 \times 10^{12}$  p/kg. 01/PEME was measurable in blood in a dose-dependent manner, exhibiting multiphasic pharmacokinetic profile, and was detected in other necropsied tissues by PCR. Histological examination revealed intranuclear inclusion bodies in the lung and liver and inflammation. Livers from animals administered  $5 \times 10^{12}$  p/kg exhibited mRNA for hexon by rtPCR and transmission electron microscopy was negative for viral structures, thus collectively indicating abortive replication. These findings are consistent with other non-replicative adenoviral studies conducted in nonhuman primates.

FIFTEEN-MONTH REPEATED DOSE SUBCUTANEOUS ADMINISTRATION STUDY OF rhFGF-2 IN ATHYMIC NUDE MICE.

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[Background] Although the importance of the carcinogenicity property of human recombinant growth factors *in vivo* has been widely recognized, repeated dosing study has often been hesitated in concern with antibody production. In the present study, we successfully completed the chronic repeated dose study of recombinant human FGF-2 (rhFGF-2), with special attention to the tumor development, using athymic nude mice. rhFGF-2 was injected subcutaneously at the dose of 0.4, 4 and 40  $\mu$ g/head twice weekly in Crj:CD-1(ICR)-nu male and female athymic nude mice for 15 months and various analyses were performed. [Results] Survival rates attained to 25% at the end of 15-month treatment and it did not differ significantly between the rhFGF-2 groups and the control group treated with saline. As the neoplastic lesions, malignant lymphoma was observed in almost all the mice of all the groups died during the study and the incidence and onset were identical between the rhFGF-2 and the control groups. Additionally, a small number of other neoplastic lesions were observed with no relation to the treatment of rhFGF-2. As the non-neoplastic lesions, incidence of glomerulosclerosis in the kidney increased in number in the 40  $\mu$ g/head group, which might be related the mitogenic potency of FGF-2 on the glomerular epithelial cells. Increase in the subcutaneous fat tissues of dorsal skin in the 4 and 40  $\mu$ g/head groups, which resulted in significant increase in the body weights, was considered the outcome of rhFGF-2 that acted as the mitogen on the fat cells. Anti-rhFGF-2 neutralizing antibody was not detected in all the rhFGF-2 groups after the 15 months of repeated dosing even at the presence of rhFGF-2 in the serum. [Conclusion] The present study clearly indicated that rhFGF-2 did not develop the neoplastic lesions or unexpected toxicities in the systemic organs after the repeated subcutaneous treatment for 15 months in athymic nude mice.

PRECLINICAL SAFETY OF RECOMBINANT HUMAN THROMBIN.

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Recombinant human thrombin (rhThrombin) is being developed as an alternative to thrombin products purified from pooled human or bovine plasma and currently-marketed as topical hemostatic agents. Preclinical studies of rhThrombin were conducted prior to its evaluation as a topical adjunct to surgical hemostasis in clinical trials. Innate cynomolgus monkey inhibitors, including antithrombin III, bound  $^{125}$ I-rhThrombin both *in vitro* and after a single IV (3.5 U/kg) or SC (350 U/kg) dose demonstrating inactivation of rhThrombin following entry into the circulation (SE-HPLC detection). Mouse fibroblast cells incubated with 250 or 500 U/mL rhThrombin or bovine thrombin demonstrated viability after 24 and 48 hours with morphologic changes consistent with thrombin's action as a serine protease. Application of rhThrombin or bovine thrombin to rabbits at concentrations of 100, 1000 or 2000 U/mL resulted in no ocular or skin irritation for up to 72 hours. Repeated subcutaneous injection of rhThrombin or bovine thrombin (346 U/kg weekly for four weeks) in monkeys produced no treatment-related findings as assessed by clinical signs, clinical pathology and gross and microscopic pathology. Absorbable gelatin sponges soaked with rhThrombin or bovine thrombin (1000 U/mL), implanted for six weeks in monkeys following creation of a surgical liver wound, showed equivalent gross and microscopic wound healing characteristics with no systemic or local toxicities. Specific anti-rhThrombin antibodies and anti-prothrombin activator (PTA) antibodies were not detected after exposure to rhThrombin. Transient, low-titer antibodies specific to Chinese hamster ovary (CHO) host cell protein were detected in one of ten monkeys. rhThrombin was shown in preclinical studies to be safe when administered topically, subcutaneously or via an absorbable gelatin sponge.

## SAFETY ASSESSMENT OF MONOCLONAL ANTIBODY MAB216 + VINCRISTINE IN RABBITS.

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The monoclonal antibody MAb216 (VH4-34) is targeted against human B lymphocytes and is proposed for chemotherapy in combination with vincristine (VCR). We have evaluated MAb216 in NZ rabbits to determine a safe dose for human trials and to identify potential target organs of toxicity. Males and females were treated intravenously (iv) with a single dose of MAb216 at 0, 6.25, and 25 mg/kg (75 and 300 mg/m<sup>2</sup>) with or without iv vincristine (0.125 mg/kg, 1.5 mg/m<sup>2</sup>). All rabbits gained weight and had no significant clinical signs or mortality. On Day 3 all rabbits treated with 25 mg/kg MAb216 had reductions in white blood cells (WBC), neutrophils (ANS), and lymphocytes (ALY; females only). Reduced WBC persisted through Day 10. VCR treatment reduced reticulocytes (RET) in both genders. By Day 17, all hematology parameters returned to control values. There was a general trend towards lower platelet counts (PLC) in all MAb216 groups on Days 3 and 10; however, this may be due to increased clumping of samples caused by MAb216 rather than on a direct MAb216-induced thrombocytopenia. Flow cytometry analysis of bone marrow and mandibular lymph nodes showed modest, non-dose-related, but consistent decreases in the percentage of B cells in the bone marrow samples from MAb216-treated animals on Day 10, but not on Day 35. No histopathologic changes were attributed to treatment with MAb216. MAb216 was detected in rabbit sera by ELISA with mean values of 67 and 889 µg/ml at 6.25 and 25 mg/kg, with half-lives of 6 and 9 hrs, respectively. Rabbits produced an antibody response to the human protein, but antibody titer was far less at 10 days than at 35 days, suggesting minimal effect on circulating MAb216 following injection. In conclusion, iv treatment with MAb216 ± vincristine does not appear to induce sustained toxicity in rabbits. The no observable adverse effect level (NOAEL) is approximately 6.25 mg/kg (75 mg/m<sup>2</sup>). (Supported by NCI Contract N01-CM-87101, N01-CM-42203, and RAID Application No. 038).

## RISING DOSE TOLERABILITY STUDIES OF A CHIMERIC ANTI-PHOSPHATIDYL SERINE MONOCLONAL ANTIBODY (TARVACIN™) IN RATS AND CYNOMOLGUS MONKEYS.

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Tarvacin™ is a chimeric (human/murine) monoclonal antibody specific for phosphatidylserine that becomes exposed on the external surface of tumor (but not normal) vascular endothelium. Tarvacin™ is being evaluated for the treatment of human solid tumors. The objective of these studies was to determine the effects of Tarvacin™ administered as a single IV bolus to Sprague-Dawley rats and Cynomolgus monkeys. Four groups of rats (i.e., 3/sex/group) received 0, 2, 20, 60 or 200 mg/kg Tarvacin™ and were monitored for 7 days post-dose. Monkeys were divided into two groups (i.e., 1/sex/group). One group received 1 mg/kg (day 1) and 30 mg/kg (day 13) Tarvacin™. The other group received 10 mg/kg (day 1) and 100 mg/kg (Day 16) Tarvacin™. Monitoring was continued for 7 days post-dose except for monkeys receiving 100 mg/kg Tarvacin™ who were monitored for 14 days post-dose. Samples were collected at various time points for hematology and coagulation parameters. Findings in both species were limited to transient dose-dependent elevations in activated partial thromboplastin time (APTT) and prothrombin time (PT). To further evaluate the coagulation response, a study in rats showed that while APTT and PT were elevated 2.9- and 1.3-fold, respectively at 24 hours post-dose, bleeding time, fibrinogen, thrombin time (TT), and fibrin degradation products (FDP) were not significantly affected. Furthermore, an *in vitro* study with Tarvacin™-spiked rat plasma showed that Tarvacin™ interfered with the analytical determination of APTT. We conclude that Tarvacin™ administered as a single IV bolus is generally well tolerated in rats and monkeys with effects limited to prolonged APTT and PT.

## SAFETY EVALUATION OF A CHIMERIC ANTI-PHOSPHATIDYL SERINE MONOCLONAL ANTIBODY (TARVACIN™) IN MICE USING A FULL-THICKNESS EXCISIONAL DERMAL WOUND MODEL.

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Tarvacin™ is a chimeric (human/murine) monoclonal antibody specific for phosphatidylserine that becomes exposed on the external surface of tumor (but not normal) vascular endothelium. Tarvacin™ is being evaluated for the treatment of

human solid tumors. The objective of this study was to determine whether Tarvacin™ inhibits wound healing in a full thickness excisional dermal wound model in mice. Three groups of mice (i.e., 6/group/termination time point) received 0, 4, or 200 mg/kg/week Tarvacin™ intravenously. A fourth positive control group received 1 mg/kg/day dexamethasone subcutaneously (SC). All treatments began on day 0. On day 6, two full-thickness circular dermal wounds (6-mm diameter) were surgically created on the lower backs of female BALB/c mice. Twenty-four mice were sacrificed on days 14 and 19, respectively and wounds evaluated for healing. All animals survived until the scheduled time of termination. On day 14, percent wound closure (mean±SD) was 71.2±15.4, 81.8±17.1, and 86.3±4.7% in 0, 4, and 200 mg/kg Tarvacin™ groups, respectively. Wound closure was 57.3±19.3% in dexamethasone-treated controls. Histological evaluation (i.e., trichrome stains) of the wounds showed no qualitative difference in wound healing between the vehicle and Tarvacin™-treated groups. By day 19 all wounds from all groups had re-epithelialized with the exception of 3 out of 12 wounds in the dexamethasone control group. We conclude that repeat doses of Tarvacin™, up to 200 mg/kg/week, does not prolong time-to-healing in this murine model.

## REPRODUCTIVE AND DEVELOPMENTAL EFFECTS OF AVASTIN™, A MONOCLONAL ANTIBODY AGAINST VASCULAR ENDOTHELIAL GROWTH FACTOR.

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Avastin™ (bevacizumab) is a recombinant humanized monoclonal antibody directed against vascular endothelial growth factor (VEGF) that has been approved for use in colorectal cancer patients. It blocks the interaction of VEGF with its receptors, thereby inhibiting angiogenesis. Angiogenesis plays a role in pathologic processes such as cancer; it is also critical to several normal physiological functions, including corpus luteum development and fetal development. Avastin™ administration was thus expected to interfere with these normal functions. In cynomolgus monkey safety studies, female reproductive tract alterations were observed following administration of 10 or 50 mg/kg Avastin™ once or twice weekly for 13 or 26 weeks; the alterations included significant decreases in ovarian and/or uterine weight and number of corpora lutea, a reduction in endometrial proliferation, and an inhibition of follicular maturation. Importantly, evidence of reversibility was noted upon cessation of treatment. The doses that resulted in these effects were at least 2-fold greater than the human clinical dose. The teratogenic potential of Avastin™ was investigated in New Zealand White rabbits. Administration of 10, 30, or 100 mg/kg Avastin™ during the period of organogenesis induced adverse effects. Effects included decreases in maternal and fetal body weights, an increased number of fetal resorptions, and an increased incidence of specific gross and skeletal fetal alterations. The effects in fetuses were noted at doses below those which caused maternal toxicity, and that approximated the average serum concentration at the clinical dose. Although potentially tolerable in the oncology patient population, decreased reproductive fertility and adverse outcomes of pregnancy are risks associated with the administration of Avastin™.

## ACUTE TOXICITY STUDIES WITH A TRANSFORMING GROWTH FACTOR-BETA ANTAGONIST IN SPRAGUE-DAWLEY RATS AND CYNOMOLGUS MONKEYS.

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Transforming growth factor beta (TGF- $\beta$ ) is a multifunctional cytokine that plays a fundamental role in the regulation of the immune system as well as cell growth and differentiation. The importance of this growth factor is underscored by the role that aberrant TGF- $\beta$  expression plays in the initiation and/or progression of a number of diseases. Overexpression of TGF- $\beta$  has been implicated in the pathogenesis of fibroproliferative disorders, immunosuppression, as well as an increase in metastasis. Monoclonal antibodies have been demonstrated as an effective means by which inappropriate cytokine levels can be antagonized. In collaboration with Cambridge Antibody Technology, we have developed a human IgG4 monoclonal antibody, GC1008, that specifically neutralizes all three isoforms of TGF- $\beta$ . To assess the acute toxicological potential of GC1008, Sprague-Dawley rats and Cynomolgus monkeys were given a single, intravenous administration of vehicle, 0.5, 5 or 50 mg/kg of GC1008 and evaluated for 14 days. Evaluations included cageside observations, body weights, clinical pathology, toxicokinetics, necropsy, and organ weights. In addition, electrocardiograms, blood pressure, heart rate, body temperature, and respiration rate was monitored in monkeys. The half-life of GC1008 in rats and monkeys was dose-dependent and ranged from 6 to 12 days and 3 to 10 days, respectively. A single dose of 50 mg/kg of GC1008 was well tolerated in both

the rats and monkeys. Rats dosed with 50 mg/kg showed a significant, but not adverse increase in reticulocyte levels. In monkeys, there was a small, dose-independent reduction in total T-cytotoxic lymphocytes and CD8+ activated lymphocytes. None of the changes in either species were considered to be toxicologically relevant. Thus, under the conditions of these studies the no observed adverse effects level (NOAEL) for GC1008 in rats and monkeys was 50 mg/kg.

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#### SAFETY EVALUATION OF A HUMAN FUSION PROTEIN PRO97796 (BR3-FC).

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Abnormal signaling through B-cell activating factor (BAFF) has been implicated in the pathology of autoimmune disorders including rheumatoid arthritis and lupus. PRO97796 (BR3-Fc) is a human fusion protein designed to inhibit BAFF signaling by acting as a decoy receptor. To evaluate the safety and biologic activity of this candidate therapeutic, cynomolgus monkeys were administered 0, 2, or 20 mg/kg/week of PRO97796 by IV injection for 18 weeks. Clinical observations, hematology, clinical chemistry, urinalyses, gross pathology and histopathology were evaluated both during dosing and during a 23-week recovery period. Effects on B, T and NK cells were evaluated by FACS analyses of peripheral blood and lymphoid tissues. To evaluate effects on normal immune function, monkeys were given a primary vaccination with Pneumovax 23, a primary and booster vaccination with KLH, and a booster vaccination with tetanus toxoid. As anticipated, PRO97796 induced significant reductions (up to 50%) in peripheral blood B cells. Decreases were evident as early as week 4 post first dose and persisted throughout the dosing period. There were no consistent effects on T or NK cells. By IHC, there was an approximate 40% decrease in the size of lymphoid follicles and a significant decrease in splenic outer marginal zone B cells. All B cell effects were reversible following cessation of dosing. PRO97796 did not inhibit the ability to mount primary or secondary immune responses as measured by Pneumovax-, KLH- and tetanus-specific IgG and IgM responses. There were modest decreases in titers for KLH and tetanus IgG; however, decreases were statistically significant only for tetanus IgG and only at the low dose. Other than the anticipated effects on B cells, there were no drug-related clinical signs or changes in hematology, clinical chemistry, urinalyses, organ weights, gross pathology or histopathology. Overall, PRO97796 has been shown to have a good safety profile at doses up to 20 mg/kg in cynomolgus monkeys.

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#### CJC-1295, A LONG-ACTING GROWTH HORMONE RELEASE FACTOR ANALOGUE, IS WELL TOLERATED IN DOGS UP TO 14 DAYS.

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CJC-1295 is a synthetic modification of growth hormone releasing factor (GRF) which stimulates the secretion of insulin-like-growth factor (IGF-1) and the pulsatile release of growth hormone (GH). By applying the Drug Affinity Complex (DAC) technology to GRF, the peptide selectively and covalently binds to circulating albumin after subcutaneous (SC) administration, thus prolonging its  $t_{1/2}$ , as seen in dogs administered CJC-1295 ( $t_{1/2}=83$  h). CJC-1295 was evaluated SC in Beagle dogs following single doses of 2.5, 8, 24 and 40 mg/kg, 7-day repeat dosing at 40 mg/kg/day, and 14-day repeat dosing at 0, 2, 6 and 18 mg/kg/day. Treatment-related effects included transient decreased activity and emesis at single doses  $\geq 8$  mg/kg. Similar clinical signs were noted in the 7-day repeat-dose phase at 40 mg/kg/day and at  $\geq 6$  mg/kg/day in the 14-day study in Beagle dogs. In addition, the repeat dosing resulted in dose- and pH-dependent injection site irritation, seen at all CJC-1295 doses as scabs, signs of pain/discomfort, redness, swelling, thickening, or microscopically as inflammation, hemorrhage and minimal to mild necrosis. There were no treatment-related effects on survival, body weight, food intake, ophthalmology, ECG, blood pressure, urinalysis, or organ weight. Although decreases in hemoglobin and red-cell mass were noted at  $\geq 2$  mg/kg/day ( $\downarrow 16\%$ ), there was no clear dose-response pattern, making the toxicological significance of the finding unclear. Mild increases in cholesterol at  $\geq 26$  mg/kg/day ( $\uparrow 1.8$ -fold) were suggestive of possible altered lipid metabolism. There was partial or complete recovery of the findings after a 4-week treatment-free period. In conclusion, CJC-1295 was well tolerated in dogs following SC doses up to 18 mg/kg/day for up to 14 days.

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#### RODENT SUBCHRONIC TOXICITY STUDIES WITH THE PEPTIDE ANTIBIOTIC XMP.629.

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XMP.629 is a novel D-linked 9-amino acid peptide based on a natural antimicrobial protein, and has been tested in Phase I/II trials as a topical treatment for acne. Subchronic 30- and 90-day studies have been performed in the rat. Due to low oral

bioavailability, these studies used subcutaneous administration to maximize systemic exposure. In both studies, injection site lesions were noted at gross necropsy and histopathology. These findings included thickened skin, hemorrhage, necrosis and inflammation. Test article deposition at the site of injection was seen in the 30-day study. In both studies, systemic effects were seen that appeared to be indirect, secondary reactions to the injection site inflammation. These systemic effects in the 30-day study included  $\uparrow$  neutrophil and monocyte counts,  $\uparrow$  spleen weights,  $\downarrow$  Hgb, Hct and RBCs, and  $\uparrow$  reticulocyte counts. The 90-day study showed these alterations plus  $\uparrow$  liver weight,  $\uparrow$  lymphocyte, basophil and leukocyte counts, as well as histopathology findings of injection site fibrosis, granulation tissue, macrophage infiltration, Kupffer cell hypertrophy, mesenteric lymph node (LN) histiocytosis and splenic monocyte/macrophage hyperplasia. The  $\uparrow$  spleen weight and hematology alterations in both studies may indicate mild RBC hemolysis mediated by the strong inflammatory response. Some of the 90-day findings (such as injection site fibrosis and granulation tissue) illustrate a transition from acute to chronic inflammation. The 90-day findings of macrophage infiltration, Kupffer cell hypertrophy, and mesenteric LN histiocytosis likely indicate macrophage-mediated clearance of test article. PK data revealed that the mean high dose AUC0-24 seen in the 90-day study was 2110 hr\*ng/mL. By comparison, all patient serum samples from a Phase I trial were below the LOQ of 1 ng/mL. The results from these studies demonstrate a high safety margin and support the use of this novel compound in clinical trials.

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#### SAFETY AND EFFICACY OF GLYCOSYLATED UNDENATURED TYPE II COLLAGEN (UC-II) IN OBESE-ARTHRITIC DOGS.

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In large breed dogs, arthritis is very common because of obesity, injury, aging, or immune disorder. This study was therefore undertaken to evaluate clinical tolerability and safety of UC-II in obese-arthritic dogs. Fifteen dogs in three groups received either no UC-II (Group I) or UC-II with 1 mg/day (Group II) or 10 mg/day (Group III) for 90 days. Lameness and pain were measured on a weekly basis for 120 days (90 days treatment plus 30 days post-treatment). Blood serum samples were assayed for rheumatoid factor (marker of arthritis); creatinine and blood urea nitrogen (markers of renal function); and alanine aminotransferase- and aspartate aminotransferase (markers of liver function). Dogs receiving 1 mg and 10 mg UC-II/day for 90 days showed marked decline in pain and lameness after physical exertion, and the therapeutic efficacy was dose-dependent. UC-II at either dose did not produce any side effects or significant changes in serum chemistry, and was well tolerated. In addition, dogs receiving UC-II for 90 days showed increased activity level and lost a significant amount of body weight. Following UC-II withdrawal for a period of 30 days, all dogs experienced a relapse of overall pain, pain with limb manipulation, and exercise-associated lameness. These results suggest that daily treatment of arthritic dogs with UC-II ameliorates signs and symptoms of arthritis, and UC-II is well tolerated with no adverse effects (Supported by InterHealth Nutraceuticals Inc. and Beckman Coulter, Inc.).

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#### PRECLINICAL SAFETY ASSESSMENT OF RECOMBINANT PLAGUE VACCINE (rF1V) FOLLOWING REPEAT-DOSE ADMINISTRATION.

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A recombinant preventative vaccine conceived by the US Army Medical Research Institute of Infectious Diseases is being developed to provide protection against pneumonic plague. The vaccine comprises the F1 antigen (capsular protein of Y. pestis), genetically fused to the V antigen (virulence protein, secreted by Y. pestis) resulting in a single fusion protein (rF1V). Studies were conducted to evaluate the general toxicity and local reactogenicity of the rF1V vaccine. Toxicity was evaluated in CD-1 mice vaccinated by IM injection with two doses of rF1V in a volume of 100  $\mu$ L with or without Alhydrogel on Days 0, 28, 56 and 70. Total immunizing protein given in each dose was 20 or 60  $\mu$ g/animal. When normalized by the body weight, the amount of rF1V and Alhydrogel given to the mice were  $>1000$ -fold in excess of the anticipated human dose. Local reactogenicity was evaluated in New Zealand White rabbits using the same injection volume (0.5 mL) and formulations (40, 80, 160 and 320 mg/mL total antigen with 0.33% Alhydrogel) intended for use in the Phase 1 clinical trial. Reactogenicity at the vaccination sites was scored for edema, ulcer, and erythema using a scale from 0 for no symptoms to 4 for severe symptoms. Mice gained weight and had normal food consumption during the study. Clinical observations, clinical chemistry, clinical hematology, gross necropsy, organ weights and histopathology in mice indicated no overt toxicity related to rF1V administration. Dermal observation showed no adverse vaccination site reactogenicity in mice or rabbits. Localized inflammation, typical of foreign body host

response, was observed in mice and rabbits vaccinated with rF1V/Alhydrogel combinations. These results indicate a favorable safety profile for rF1V and support its use in a Phase 1 clinical trial. Funded by the Joint Vaccine Acquisition Program, Department of Defense (DoD) Contract DAMD 17-98-C-8024 and does not represent official DoD positions, policies, or decisions.

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PRECLINICAL SAFETY ASSESSMENT OF RECOMBINANT BOTULINUM VACCINE A/B (RBV A/B) FOLLOWING REPEAT-DOSE ADMINISTRATION.

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A recombinant preventative vaccine conceived by the US Army Medical Research Institute of Infectious Diseases is being developed to provide protection against the effects of botulism. The two recombinant antigens that comprise the rBV A/B vaccine, Antigen A and Antigen B, are derived from the 50 kDa C-terminal domains of the botulinum neurotoxin serotypes A and B. A comprehensive series of studies were conducted to evaluate the general toxicology, neurotoxicology and local reactogenicity of the rBV A/B vaccine. Toxicity was evaluated in CD-1 mice vaccinated with the three doses of rBV A/B with or without Alhydrogel by IM injection on Days 0, 28, 56 and 70 in a volume of 100  $\mu$ L. Total immunizing protein given in each dose was 0, 2, 4 and 8  $\mu$ g/animal. Local reactogenicity was evaluated in New Zealand white rabbits using the same injection volume (0.5 mL) and formulations (10, 20 and 40  $\mu$ g/mL total antigen with 0.2% (w/v) Alhydrogel) intended for use in the Phase 1 clinical trial. Mice gained weight and had normal food consumption during the study. Clinical observations, inoculation site reactogenicity, clinical chemistry, clinical hematology, gross necropsy, organ weights and histopathology indicated no overt toxicity related to rBV A/B administration. Qualitative and quantitative functional observational battery assessment indicated no adverse neurobehavior related to administration of rBV A/B. Dermal observations showed no adverse vaccination site reactogenicity in rabbits. A microscopic, mild inflammatory response was observed in the Day 28 injection site and was consistent with vaccine-Alhydrogel interaction. There was no indication of chronic inflammation in the Day 0 injection site. Together these results indicate a favorable safety profile for rBV A/B and support its use in a Phase 1 clinical trial. Funded by the Joint Vaccine Acquisition Program, Department of Defense (DoD) Contract DAMD 17-98-C-8024 and does not represent official DoD positions, policies, or decisions.

**812**

SPONTANEOUS HEMORRHAGE OF THE PANCREATIC ISLET IN SPRAGUE-DAWLEY RATS.

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In rats, although there is much information about age-related pancreatic islet changes such as  $\beta$ -cell hyperplasia characterized by enlarged islets, fibrous dissection, and hemosiderin deposition, little report is available on islet hemorrhage. The present study was designed to find the spontaneous hemorrhage in the pancreatic islet of non-treated Sprague-Dawley rats. Groups of 20 male and 20 female Crj:CD(SD)IGS rats each were allowed free access to a commercial diet containing high protein and fat, and were sequentially sacrificed at 8, 12, 18, and 26 weeks of age. During the study, clinical observations and measurements of body weight and food consumption were periodically performed, and hematology and serum glucose, insulin and estradiol levels were examined with histopathology of the pancreas. As results, no changes in blood and serum parameters tested were observed throughout the experimental period. Histopathologically, however, pancreatic islet hemorrhage in males was observed in one animal each at 12 and 18 weeks, and eleven animals at 26 weeks. In females, the same hemorrhage was observed only in one animal each at 18 and 26 weeks. This hemorrhage was accompanied by pigment deposition in the peripheral or central islets which were occasionally dissected by fibrous septa. A special staining revealed that the pigments contain iron, suggesting the previous occurrence of micro-hemorrhage. Other changes, except for hemorrhage, closely resembled the age-related changes reported previously. In conclusion, an obvious sex-difference exists in spontaneous hemorrhage of the rat pancreatic islets, and its incidence increased with aging.

**813**

NRF2 ACTIVATION INVOLVES AN OXIDATIVE-STRESS INDEPENDENT PATHWAY IN TETRAFLUOROETHYLCYSTEINE-INDUCED CYTOTOXICITY.

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Tetrafluoroethylcysteine (TFEC), a metabolite of the industrial gas tetrafluoroethylene has been known to cause both nephrotoxicity and limited hepatotoxicity that are associated with the covalent modification of specific intramitochondrial pro-

teins including HSP60, mtHSP70, AST, aconitase and  $\alpha$ -ketoglutarate dehydrogenase. Using the murine TAMH cell line as a useful *in vitro* model, we demonstrate a rapid and sustained induction of Nrf2, a cytosolic member of the "cap-and-collar" family of transcription factors, after a cytotoxic dose of TFEC (250  $\mu$ M). A functional correlation of this induction is established with the rapid translocation of cytosolic Nrf2 into the nucleus as assessed by both immunoblot and immunocytochemical methods. We have also detected a corresponding transcriptional and translational upregulation of Nrf2 regulated genes including GCL (both catalytic and modulatory subunits), heme oxygenase-1, and some GSTs. While Nrf2 activation is often linked to perturbation of cellular thiol status and/or oxidative stress, we were unable to detect any significant depletion of cellular glutathione nor oxidation of membrane cardiolipin. These data suggest that Nrf2 activation is likely independent of oxidative-stress, or at best, a result of a transient oxidative stress. Moreover, supporting evidence from DNA microarray and Western blot analysis indicates an early ER response after TFEC treatment, with a time-dependent upregulation of the ER responsive genes gadd34, gadd45, gadd153 and ndrl. These findings suggest an alternative avenue of exploration for Nrf2 activation, i.e. the phosphorylation of Nrf2 through ER-mediated protein kinases such as PERK. Overall, the results implicate a role for Nrf2 in the cellular response to TFEC toxicity, and suggest a novel ER role in a mitochondrially-initiated pathway of cytotoxicity. (Supported by NIEHS Center Grant P30ES07033, 5 R01 ES010849 and Pfizer Inc.)

**814**

NRF2-DEPENDENT ARE ACTIVATION IS A GAIN OF FUNCTION ALTERATION SPECIFIC TO DIFFERENTIATED AND FUNCTIONALLY ACTIVATED ASTROCYTES.

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The promoter region of several phase II detoxification enzymes contain a cis-acting enhancer known as the antioxidant response element (ARE). Increased expression of these enzymes contributes to the protection of neural cells from oxidative stress. Transgenic reporter mice were created to carry in their genome the core ARE sequence coupled to the human placental alkaline phosphatase (hPAP) reporter gene. Neural stem cells derived from these mice were treated with tert-butylhydroquinone (tBHQ), a potent ARE activator. *In vitro* cell migration assays demonstrated that only fully differentiated, functionally activated astrocytes responded to this ARE-dependent activation. Neither neural stem cells, differentiated neurons, oligodendrocytes, proliferating cells nor apoptotic cells showed this response. Adenovirus-mediated Nrf2 overexpression also revealed a massive increase in ARE activation in astrocytes but not progenitor cells. Transplantation of ARE-hPAP neural stem cells into mouse striatum demonstrated a similar pattern of ARE activation specific to astrocytes after malonate lesioning, suggesting that this gain of function was unique to the astrocytes in response to low-molecular-weight electrophilic compounds. Real-time RT-PCR analysis of Nrf2 dependent, ARE driven genes highlight the different extent of transcriptional upregulation between neural stem cells and their differentiated cultures after tBHQ treatment. This is the first developmental study to show that only differentiated, functionally activated astrocytes are responsible for ARE activation. Developmental expression of Nrf-dependent detoxification enzymes may have a strong impact on early life stage susceptibility to neurotoxicants. Support by: Grants ES08089 and ES10042 from the NIEHS

**815**

INDUCTION OF NAD(P)H:QUINONE OXIDOREDUCTASE 1 IN MOUSE LIVER DURING EXTRAHEPATIC CHOLESTASIS IS DEPENDENT UPON NRF2 ACTIVATION.

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Bile duct ligation (BDL), a rodent model of extrahepatic cholestasis, causes bile acid and conjugated bilirubin accumulation in liver and blood. Elevated levels of bile constituents in liver can cause oxidative stress and inflammation, which may cause hepatic injury if normal bile flow is not restored. Nuclear factor-E2-related factor 2 (Nrf2) induces gene expression in response to administration of antioxidants as well as during conditions of oxidative stress. Nrf2 regulates basal and inducible expression of numerous protective genes in liver, including NAD(P)H: quinone oxidoreductase 1 (Nqo1). We hypothesized that BDL increases mouse liver Nqo1 expression through activation of Nrf2. BDL or sham surgeries were performed on male C57BL/6 mice. Livers were collected at one, three, and seven days after surgery for analysis of Nqo1 mRNA, protein, and activity. BDL increased

Nqo1 mRNA expression in male C57BL/6 mouse livers by 3 fold at one and three days, and 7 fold at seven days, as compared to expression in livers from sham-operated controls. Nqo1 protein expression and activity in livers from male C57BL/6 mice were increased 2-3 fold and 4-5 fold at three and seven days after BDL, respectively. Parallel studies were performed with wild-type and Nrf2-null mice, with livers being collected three days after BDL or sham surgery. BDL increased Nqo1 mRNA and protein expression, as well as enzyme activity, in livers from wild-type mice, but not in livers from Nrf2-null mice. Together these data demonstrate that during BDL-induced extrahepatic cholestasis, Nqo1 expression and activity in mouse liver are induced via a Nrf2-dependent mechanism. (Supported by ES-09716, ES-09649, and ES-07079)

**816**

ALTERATIONS IN GENE EXPRESSION BY 2378-TETRACHLORO-P-DIBENZODIOXIN (TCDD) IN LIVERS OF NRF2 KNOCKOUT MICE.

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Nrf2 is an essential transcription factor for inducible and/or constitutive expression of a group of phase II detoxification and antioxidant enzymes. Previously we showed that the expression of glutathione S-transferase P1 gene was induced in cultured rat liver cells by dioxins and its expression was dependent on Nrf2/ARE (antioxidant responsive element) system. We aimed to globally identify TCDD-inducible genes that are dependent on Nrf2 exploiting Nrf2 knockout mice. Nrf2(-/-) mice and Nrf2(+/+) mice were produced by intercrossing Nrf2(+/+) mice. Forty micrograms of TCDD per kg body weight were administered orally to female Nrf2(-/-) and Nrf2(+/+) mice, and total RNA was extracted from the livers 24 hrs after the administration. The relative expression levels of genes were analyzed using Codelink DNA array. Identification of genes expressed/suppressed by TCDD and the relation analysis between altered genes were performed under support of KeyMolnet expert system (Institute of Medicinal Molecular Design Inc.). TCDD administration caused over 2-fold increase in the expression levels of 99 genes and 131 genes in Nrf2(-/-) and Nrf2(+/+) mice, respectively. On the other hand, the expression levels of 72 genes and 111 genes in TCDD-treated Nrf2(-/-) and Nrf2(+/+) mice, respectively, were decreased to less than half of untreated mice. Among the genes altered by TCDD, the expression of following genes was increased in both Nrf2(-/-) and Nrf2(+/+) mice, CYP1A1, CYP1A2, CYP1B1 and NQO1. In contrast, the expression of several metabolic enzyme genes (e.g., GSTP1, GSTM2 and UDP glucuronosyl transferase) and c-Jun were elevated only in Nrf2(+/+) mice but not in Nrf2(-/-) mice, suggesting that Nrf2 contributes to the inducible expression of these genes by TCDD. We surmise that the administration of TCDD might provoke the generation of endogenous inducers for the Nrf2/ARE system and activate the expression of phase II and antioxidant enzyme genes.

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PHOSPHORYLATION OF NRF2 BY CASEIN KINASE 2 (CK2) REGULATES ACTIVATION AND DEGRADATION.

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Phosphorylation of transcription factor Nrf2 is a potential mechanism of activation. In this study, we identified two phosphorylated types of Nrf2 and provide evidence that CK2-mediated Nrf2 phosphorylation plays a critical role in activation and proteasome-mediated degradation. Human Nrf2 has a predicted molecular mass (MM) of 66 kDa. However, western blot showed two bands at 96 and 116 kDa are increased in response to Nrf2 inducers, like arsenite (As), in several human cell lines. Over-expression of Nrf2 by transfection results in two similar bands. Nuclear fractions from As-exposed cells treated with  $\lambda$ -phosphatase or PP1 showed the loss of the two higher MM bands and the appearance of a lower MM band, suggesting both the higher MM forms of Nrf2 are phosphorylated. *In vitro* kinase assay using Nrf2 immunoprecipitation products as the substrate for purified CK2 indicated Nrf2 is a CK2 substrate and CK2 mediates two steps of Nrf2 phosphorylation. Although the mechanism of CK2 activation is unknown, our results support a role in which calmodulin (CaM) binding regulates CK2 activity. CaM and  $Ca^{2+}$  dramatically inhibits CK2 activity *in vitro*, suggesting  $Ca^{2+}$  mobilization may affect local CK2 activity. Accordingly, we found cold shock in  $Ca^{2+}$ -free media decrease cellular  $Ca^{2+}$  levels and caused Nrf2 hyper-phosphorylation, which in turn was prevented by CK2 specific inhibitors. Gel-shift assay showed the hyper-phosphorylated type Nrf2 (HP-Nrf2) can not bind to the  $\gamma$ -GCS<sub>h</sub> antioxidant response element, indicating phosphorylated-Nrf2, but not HP-Nrf2, has transcriptional activity. In contrast, HP-Nrf2 is more susceptible to proteasome-mediated degradation.

**818**

NRF3 NEGATIVELY REGULATES ARE-MEDIATED EXPRESSION AND ANTIOXIDANT INDUCTION OF DETOXIFYING ENZYME GENES.

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Antioxidant response element (ARE) and nuclear factors Nrf2 and Nrf1 are known to coordinately upregulate expression of a battery of detoxifying enzyme genes including NAD(P)H:quinone Oxidoreductase1 (NQO1) gene in response to antioxidants. This induction is critical in cellular protection against chemically induced oxidative stress and neoplasia. Nrf3 is a third member of Nrf family of proteins that was recently identified and cloned. In this study, we investigated the role of Nrf3 in ARE-mediated NQO1 gene expression and induction in response to antioxidants. Band and super shift assays with *in vitro* transcribed and translated proteins and nuclear extracts from Hep-G2 cells treated with DMSO and antioxidant tert-butyl hydroquinone (t-BHQ) and immunoprecipitation assays demonstrated that Nrf3 forms heterodimers with small Maf proteins that bound to NQO1 gene ARE. Overexpression of Nrf3 in Hep-G2 cells led to concentration dependent decrease in transfected and endogenous NQO1 gene expression and induction in response to t-BHQ. In addition, RNAi specific to Nrf3 reduced intracellular Nrf3 that led to increased expression and induction of transfected and endogenous NQO1 gene expression in response to t-BHQ. Deletion mutation analysis revealed that Nrf3 repression of NQO1 gene expression required heterodimerization and DNA binding domains but not transcriptional activation domain of Nrf3. The cotransfection of Nrf2 with Nrf3 rescued the repression effect of Nrf3. These results combined suggest that Nrf3 is a negative regulator of ARE-mediated gene expression. It presumably interferes with binding of Nrf2 and Nrf1 and/or titrates out small Maf proteins to repress ARE-mediated gene expression.

**819**

ANTIOXIDANT INDUCED NUCLEAR IMPORT AND EXPORT OF OXIDATIVE STRESS-RESPONSE FACTOR NRF2.

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Antioxidants protect cells against oxidative stress and neoplasia. This is achieved by antioxidant response element (ARE)-mediated coordinated induction of genes encoding chemopreventive proteins. These genes include detoxifying enzyme NAD(P)H:quinone oxidoreductase1 (NQO1). Nrf2 (NF-E2 related factor 2) is a transcription factor that binds to ARE and regulates activation of these genes. Under normal conditions, Nrf2 is retained in the cytoplasm by its inhibitor, INrf2. Exposure of cells to antioxidants leads to release of Nrf2 from INrf2. Nrf2 then accumulates in the nucleus leading to ARE-mediated gene expression. Nrf2 exits from the nucleus after it has turned on the gene expression, presumably to bind to INrf2 for its own degradation. In this report, we have characterized the nuclear import and export signals of Nrf2. We demonstrated that Nrf2 contains a bipartite nuclear localization signal (NLS) and a leucine rich nuclear export signal (NES) which regulate its shuttling in and out of nucleus. Immunofluorescence studies revealed that Nrf2 accumulates in the nucleus within 15 min of antioxidant treatment and exits around 6 h after the treatment. An Nrf2 mutant lacking NLS failed to enter the nucleus and displayed diminished expression and induction of downstream genes including NQO1. The Nrf2 NLS sequence fused to green fluorescence protein resulted in the nuclear accumulation of GFP indicating that this signal was sufficient to direct nuclear localization of heterologous protein. A NES was characterized in the C-terminus of Nrf2, deletion of which caused Nrf2 to display more nuclear localization as observed by immunofluorescence. This NES was sensitive to leptomycin B and could function as an independent export signal when fused to heterologous protein. These results led to the conclusion that Nrf2 localization is controlled by both nuclear import and export suggesting that overall distribution of Nrf2 is likely to result from a balance between these two processes. Antioxidants shift this balance in favor of nuclear accumulation of Nrf2 leading to activation of detoxifying genes.

**820**

COMPARTMENTAL OXIDATION OF THIOL/DISULFIDE REDOX COUPLES DURING EGF SIGNALING.

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Exogenously added reactive oxygen species (ROS) cause generalized oxidation of cellular components whereas endogenously generated ROS induced by physiological stimuli activate discrete signal transduction pathways. An increase in reactive

oxygen species (ROS) accompanies epidermal growth factor (EGF) binding to its receptor and potentiates the phosphorylation cascade that leads to MAP kinase activation and cell proliferation. Compartmentation is an important aspect of such signaling pathways but little is known about redox compartmentation during signal transduction. In the present study we examined the effects of EGF induced redox signaling on specific thiol/disulfide redox pools. We measured the redox states of cytosolic thioredoxin-1 (Trx1), nuclear Trx1 and mitochondrial thioredoxin (Trx2) using Redox Western blot methodologies during endogenous ROS production induced by EGF signaling. Glutathione redox state was measured by HPLC. Results showed that only cytosolic Trx1 undergoes significant oxidation during EGF signaling. Thus we demonstrate that EGF signaling involves subcellular compartmental oxidation of Trx1 in the absence of a generalized cellular oxidation.

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UPREGULATION OF MRP3 AND MRP4 BY SULFHYDRYL-MODIFYING COMPOUNDS IS MEDIATED BY THE TRANSCRIPTION FACTOR NRF2.

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The Multidrug Resistance-Associated Protein (Mrp) family is comprised of ATP-dependent transporters that mediate efflux transport of a variety of conjugated and unconjugated compounds from cells. In liver, Mrp3 and Mrp4 transport chemicals from hepatocytes into sinusoidal blood, and may serve to protect the liver from toxicity. Mrp3 and Mrp4 are upregulated by butylated hydroxyanisole (BHA) and oltipraz (OPZ), chemicals that have been shown previously to upregulate a number of genes involved in the protective response to oxidative stress, including NAD(P)H:quinone oxidoreductase 1 and heme oxygenase 1. Collectively this battery of antioxidant genes is transcriptionally regulated via Nuclear Factor E2 related factor 2 (Nrf2). During oxidative homeostasis, Nrf2 is sequestered in the cytosol by Kelch-like Ech-Associated Protein (Keap1); during oxidative challenge, modification of Keap1 sulfhydryl groups results in release and nuclear translocation of Nrf2. The purpose of this study was to determine whether Mrp3 and Mrp4 are part of the Nrf2-regulated antioxidant battery. OPZ and BHA upregulated Mrp3 and Mrp4 mRNA at least three fold in livers of wild-type mice. In Nrf2-null mice, basal expression of Mrp3 mRNA was diminished in liver, and the induction of Mrp3 and Mrp4 by OPZ and BHA was attenuated. In mouse hepatoma cells, known Nrf2 activators, such as diethyl maleate, oltipraz, ethoxyquin, tert-butylhydroquinone, catechol, and catechol dinitrobenzene, all increased expression of Mrp3 and Mrp4. These data suggest that a broad range of chemicals can transcriptionally upregulate Mrp3 and Mrp4, presumably through the actions of Nrf2. In conclusion, during cellular adaptation to an oxidative environment, Mrp3 and Mrp4 respond via Nrf2 to help excrete compounds or metabolites that are harmful to the cell, and therefore may play an important role in prevention of hepatic toxicity. (Supported by NIH grants ES-09716 and ES-07079)

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EPIGENETIC EFFECTS OF OXIDATIVE STRESS.

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For many years, research on oxidative stress focused primarily on determining how reactive oxygen species (ROS) damage cells by indiscriminate reactions with the macromolecular machinery of a cell, particularly lipids, proteins and DNA. However, many chronic diseases affiliated with oxidative stress, such as cancer, are not always a consequence of tissue necrosis, DNA mutations, or protein damage but rather to altered gene expression. Gene expression is highly regulated by the coordination of extra-, intra- and inter-cellular communication systems that will typically maintain tissue homeostasis by sustaining a balance between proliferation, differentiation and apoptosis. Therefore, much research has shifted to the understanding of how ROS can reversibly control the expression of genes at noncytotoxic doses through cell signaling mechanisms. Cell proliferation typically involves inhibition of gap junctional intercellular communication (GJIC) and the activation of mitogen activated protein kinase pathways (MAPK). We demonstrate that normal rat liver epithelial cells response to epidermal growth factor (EGF) will inhibit GJIC in addition to activating extracellular receptor kinase (ERK), a MAPK. However, inhibition of NADPH oxidase, which reduces oxygen to H<sub>2</sub>O<sub>2</sub>, with the very selective inhibitor, diphenyleneiodonium, prevented EGF from inhibiting GJIC suggesting that the generation of H<sub>2</sub>O<sub>2</sub> is an essential component of the intracellular pathway controlling GJIC. Furthermore, we previously demonstrated that reduced-glutathione (GSH) was also a necessary cofactor of H<sub>2</sub>O<sub>2</sub>-induced inhibition of GJIC. These results demonstrate that ROS and GSH play essential roles in controlling EGF-dependent control of GJIC. Therefore, the overly simplistic approach of either preventing the generation of ROS or accelerate the removal by antioxidants could deleteriously alter normal signaling functions. Funded by NIEHS (PA42ES04911).

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HISTONE H3.3 MODIFICATIONS DURING REACTIVE OXYGEN SPECIES INDUCED DNA DAMAGE.

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2, 3, 5-Tris-(glutathion-S-yl)hydroquinone (TGHQ), a reactive oxygen species (ROS)-generating toxicant, causes DNA single strand breaks (SSBs), premature chromatin condensation (PCC) and oncotic/necrotic cell death in renal proximal tubular epithelial (LLC-PK1) cells. Previous studies revealed an increase in the phosphorylation of histone H3, in response to TGHQ, concurrent with PCC, and only serine was phosphorylated in this event. However, western analysis revealed a decrease in phosphorylation of histone H3 at both S10 and S28, both of which are well-known sites of S phosphorylation during mitotic chromosomal condensation. Moreover, TGHQ-induced phosphorylation only occurred on the histone H3.3 isoform, and not on histone H3 isoforms H3.1 and H3.2. Comparing the amino acid sequence of all three histone H3 variants, histone H3.3 contains a unique S site at position 31 in the highly modified N-terminal tail region, suggesting that S31 might be an alternate site of histone H3 phosphorylation in response to TGHQ. To ascertain whether S31 is a novel site of histone H3.3 phosphorylation in response to TGHQ, wild-type and four histone H3.3 mutants, S10A, S28A, S31A, and S10/28A were constructed using alanine to substitute for serine residues at position 10, 28, 31, and 10 plus 28, respectively. Transient transfection of LLC-PK1 cells with either wild-type or mutant histone H3.3 constructs resulted in similar levels of his-tagged protein expression. Neutral red analysis revealed a decrease in TGHQ-induced toxicity in LLC-PK1 cells transfected with mutant S31A compared to the wild-type transfection (each with ~70% transfection efficiency). These studies suggest that modification of histone H3.3 at S31 is associated with TGHQ induced oncotic/necrotic cell death. To confirm that S31 is a novel site undergoing phosphorylation in response to TGHQ, current work is focusing on [32P]-orthophosphate incorporation into LLC-PK1 cells transiently expressing H3.3 wild-type or mutant proteins. (DK59491)

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EVALUATION OF PLASMA VON WILLEBRAND FACTOR (VWF) AND VWF PROPEPTIDE IN DRUG-INDUCED VASCULAR INJURY.

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Drug-induced vascular endothelial injury in toxicology studies is of concern to regulatory agencies and the pharmaceutical industry. The absence of a reliable marker of endothelial cell injury is an unmet need in experimental and clinical medicine. Plasma levels of von Willebrand factor (vWF) and vWF propeptide (vWFpp) were investigated as biomarkers of activated/injured endothelial cells following physiological and pathological stimuli in beagle dogs. In control dogs, repeat bleeding had no significant effects on vWF and vWFpp. Physiological stimulation of the vascular bed using deamino 8 D arginine vasopressin (5μug/kg DDVAP) caused a rapid but short-lived increase in vWF (30-100%) but no changes of vWFpp. Pathological stimulation of the vascular bed with an endotoxin (2mg/kg Lipopolysaccharide) produced an immediate and prolonged increase of vWF (40-220%) over 24-hours: additionally vWFpp increased markedly (1100-2300%), returning to near baseline by 24-hours. Plasma levels of vWF and vWFpp were measured in dogs following the administration of a novel Endothelin Receptor Antagonist (ETRA) and a novel potassium channel opener known to induce localized injury in the coronary vascular bed of some dogs. Both compounds caused vWF elevations similar to DDAVP even though they induced focal lesions in the coronary bed whereas DDVAP stimulates the entire vascular bed. In addition, both compounds induced a 30-300% increase in vWFpp. vWF and vWFpp were elevated following endothelial cell perturbation and drug-induced vascular injury in these dog studies, and warrant further investigation of these biomarkers for drug-induced vascular injury in preclinical toxicology studies.

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MORPHOLOGIC IMAGING OF EXTRACELLULAR MATRIX IN CHEMICAL-INDUCED DEVELOPMENTAL DISSECTING AORTIC ANEURYSM USING MULTIPHOTON FLORESCENCE AND SECOND HARMONIC GENERATION MICROSCOPY.

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To reveal the role of the extracellular matrix (ECM) in developmental dissecting aortic aneurysm (DAA), we studied the pathological progression of changes in elastin and collagen using two nonlinear imaging techniques, multiphoton flores-

cence microscopy (MPFM) and second harmonic generation microscopy (SHGM), in a recently-described model of thoracic DAA in newborn rats due to *in utero* exposure to relatively non-toxic doses of semicarbazide (SM). Sprague-Dawley rat dams were given SM (0.10 to 49.6 mg/kg i.p.) from days 14 to 20 of gestation. Fetuses were harvested on day 20 of gestation (E20), as newborns (P1) and at 7 (P7) and 28 days of age (P28); matched controls were from dams treated with saline only. Thoracic organs were removed for histopathology and MPFM and SHGM elastin study at an excitation wavelength of 800 nm. A narrow band pass filter centered at 400 nm with a 14 nm band width was used to collect the SHGM signal from collagen in standard sections. DAA occurred at birth, no E20 fetuses showed lesions in routine sections, although MPFM and SHGM demonstrated that collagen was significantly degraded at doses of 6.13 mg/kg SM. In P1, P7, and P28, DAA occurred in nearly 100% at all doses over 1.15 mg/kg. The massive dissections frequently extended to the carotids and other vessels of the aortic arch. Histologic study revealed healing and healed vascular lesions consisting of localized collections of blood in vascular media. Gradually disorganized or non-fibrillar structures and dose-dependent degradation of elastin and collagen were seen. Conclusion: MPFM and SHGM provide sensitive and high-resolution information on aortic elastin and collagen in developmental DAA. The extracellular matrix potentially plays a crucial role in developmental DAA. (Supported by NIH grants HL45616 and ES013038.)

## 826

### EFFECTS OF GONADAL STEROIDS ON CALCIUM CURRENTS AND ACTION POTENTIALS IN GUINEA PIG CARDIAC MUSCLES.

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It is known that there are gender differences in arrhythmia and it is possible that cardiac memory contributes to arrhythmia. Cardiac electrophysiological changes are thought to be important determinants of gender differences and cardiac memory. Previous research in arrhythmia has focused mainly on outward potassium currents that contribute largely to phase 3 of repolarization in action potential (AP). Clinical and basic studies suggest that other currents contributing to AP plateau also determine gender differences in repolarization and cardiac memory. However, in cardiac electrophysiology, it is not clear how gonadal steroids contribute to gender-related differences or how cardiac memory is induced. Accordingly, the purpose of the present study was to determine whether gonadal steroids influence  $\text{Ca}^{2+}$  currents ( $I_{\text{Ca}}$ ) and APs in male and female guinea pig cardiac muscles. [Methods] We evaluated  $I_{\text{Ca}}$  and APs in cardiac muscle using whole-cell patch-clamp and micro-electrode techniques. gonadal steroids used were 5 $\alpha$ -dihydroxytestosterone (DHT) and 17 $\beta$ -estradiol (EST). [Results] There was no difference in APs or L-type  $I_{\text{Ca}}$  ( $I_{\text{Ca},L}$ ) between male and female guinea pig ventricular myocytes. EST (0.1-10  $\mu\text{M}$ ) decreased  $I_{\text{Ca},L}$  in both males and females. In contrast, DHT (10  $\mu\text{M}$ ) had no effect on  $I_{\text{Ca},L}$ . EST (10  $\mu\text{M}$ ) decreased AP duration (APD) in both male and female guinea pig papillary muscle. DHT (10  $\mu\text{M}$ ) slightly increased APD in female guinea pig papillary muscle. DHT had no effect on APD in male guinea pig papillary muscle. Therefore, we evaluated other  $\text{Ca}^{2+}$  currents in cardiac muscles. [Conclusion] These results suggest that the gonadal steroids regulate  $I_{\text{Ca}}$  conditions that constitute AP, and contribute to gender-related differences in ventricular repolarization.

## 827

### NUCLEOSIDE ANALOG REVERSE TRANSCRIPTASE INHIBITOR (NRTI)-INDUCED $\rho^-$ RAT CARDIAC MYOCYTES IN CELL CULTURE.

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The advent of NRTI antiretroviral therapy has yielded substantial improvements in clinical outcomes for HIV-infected patients; unfortunately, the success of this therapy is constrained by the eventual development of a dose-limiting metabolic failure. Drug-induced interference with metabolic regulation is manifested as systemic-wide alterations in carbohydrate and lipid metabolism, which is believed to have mitochondrial origins of pathogenesis. The prevailing paradigm for NRTI-induced metabolic failure is that the phosphorylated analogs are competitive substrates for mitochondrial DNA Pol  $\gamma$ , thereby inhibiting mtDNA replication by truncating the elongating mtDNA copy. The inferred consequence is a decrease in mtDNA copy number ( $\rho^-$ ) leading to a depletion of mitochondrial-encoded proteins and a loss of aerobic metabolic capability leading to cell death. This paradigm was tested in h9c2 cardiac myocytes cultured in the presence of 0.1-50  $\mu\text{M}$  zidovudine (AZT) or didanosine (ddI); a separate set of cells cultured in 50 ng/ml ethidium bromide (EtBr) was included as a positive control. Cardiac myocytes survived in culture in the presence of up to 50  $\mu\text{M}$  AZT or ddI for up to 10 cell passages. Similar to what was observed with EtBr, both AZT and ddI caused a dramatic time- and dose-dependent depletion of up to 80% mtDNA, which was accompanied by a correspond-

ing and progressive dose and time-dependent decrease in cytochrome oxidase enzyme activity. The associated decrease in ATP concentration is suggestive of a progressive decoupling of mitochondrial oxidative phosphorylation. This bioenergetic phenotype of cardiac myocytes grown in the presence of NRTIs is consistent with the Pol  $\gamma$ -dependent mitochondrial depletion proposed to account for the metabolic failure associated with long-term therapy of HIV infected patients. (Supported by HL72715-02).

## 828

### EPHEDRINE AND CAFFEINE CAUSE SIGNIFICANT MORTALITY IN 14 WEEK OLD BUT NOT IN 7 WEEK OLD FISCHER RATS.

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Human consumption of a combination of ephedrine and caffeine has been associated with a number of adverse effects including changes in the electrocardiogram, myocardial infarction, hyperthermia, and in rare instances death. To study potential mechanisms associated with cardiotoxicity heart rate, body temperature, histological and ECG changes were assessed in 7 and 14 weeks old rats treated with a combination of ephedrine (25 mg/kg) and caffeine (30 mg/kg). No mortality was seen in ephedrine and caffeine-treated 7 week old rats, nor was any mortality observed in the 14 week old rats treated with ephedrine or caffeine alone. However, of the 14 week old rats treated with the combination of ephedrine and caffeine, 46% (6 of 13 animals) died within 5 hours of dosing. Treatment of both 7 and 14 week old rats with ephedrine and caffeine caused significant increases in QTc interval (from 116 to 190 msec and 118 to 190 msec, respectively) as well as significant increases in body temperature (from 37.3 to 38.5  $^{\circ}\text{C}$  and from 37.1 to 38.0  $^{\circ}\text{C}$ , respectively). Yet at this timepoint, prior to any signs of distress, the degree of hyperthermia in the 14 week old rats that died was significantly higher (40.6  $^{\circ}\text{C}$ ) than surviving 14 week old rats ( $p<0.0001$ ). Three hours post-treatment the percentage increase in the heart rate of 14 week old animals that died (154%) was significantly larger than the percentage increase in heart rate for both the 7 week old rats (121%,  $p<0.0001$ ) and the 14 week old rats that survived (129%,  $p<0.0001$ ). The combination of ephedrine and caffeine induced an increase in mitochondrial uncoupling proteins (UCP) 2 and 3 expression in 14 week old rats, and a decrease in expression of UCP2 and 1 adrenergic receptor in 7 week old rats. Histopathological data indicated interstitial hemorrhage, myofiber necrosis and vasospasm. These data are consistent with enhancement of catecholamine induced ischemic injury in 14 week old rats due to hyperthermia.

## 829

### ACUTE HEMORRHAGIC MYOCARDIAL NECROSIS AND SUDDEN DEATH OF RATS EXPOSED TO A COMBINATION OF EPHEDRINE AND CAFFEINE.

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Because of possible side effects of herbal medicines containing ephedrine and guarana-derived caffeine, including increased risk of stroke, myocardial infarction, and sudden death, the Food and Drug Administration recently banned the sale of ephedra-containing products, specifically over-the-counter dietary supplements. We report cardiac pathology in pilot studies in 7- and 14-week-old male F344 rats exposed by gavage to ephedrine (25 mg/kg) and caffeine (30 mg/kg) administered in combination for one or two days. The ephedrine-caffeine exposure was approximately 12- and 1.4-fold, respectively, above average human exposure, based on a mg/m<sup>2</sup> body surface-area comparison. Several of the exposed 14-week-old rats died or were sacrificed in extremis 4-5 h after the first dosing. In these hearts, changes were observed chiefly in the interventricular septum but also left and right ventricular walls. Massive interstitial hemorrhage, with degeneration of myofibers, occurred at the subendocardial myocardium of the left ventricle and interventricular septum. Immunostaining for cleaved caspase-3 and anti-phospho-H2AX, a histone variant that becomes phosphorylated during apoptosis, indicated multifocal generalized positive staining of degenerating myofibers and fragmenting nuclei, respectively. The Barbeito-Lopez trichrome stain revealed generalized patchy yellow myofibers, consistent with acute coagulative necrosis. In treated animals terminated after the second dosing, foci of myocardial degeneration and necrosis were already infiltrated by mixed inflammatory cells. The myocardial necrosis may occur secondarily to intense diffuse vasoconstriction of the coronary arterial system with decreased myocardial perfusion. Our work shows the direct relationship between combined ephedrine and caffeine exposure and cardiac pathology.

**830**

### EFFECTS OF HYPOKALIEMIA ON THE QT INTERVAL IN DOGS.

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Imbalance in plasma electrolytes, in particular potassium is known to affect ECG. Hypokalemia increases the duration of cardiac action potential; consequently it prolongs QT interval and is a risk factor for arrhythmia. The objective of this study was therefore to estimate the quantitative relationship between plasma potassium concentrations and the QT interval in dogs. Hypokalemia was induced by treatment with furosemide, a potent diuretic. One group of 3 dogs/sex received oral escalating doses of 5, 8, 12, 18, 30, 45 mg/kg of furosemide and one group of 2 dogs/sex received a single dose of 30 mg/kg followed by repeat daily doses of 60 mg/kg for 3 days. ECG were recorded before the start of the treatment and on each day of treatment, before dosing, then 1.5 and 3.5h after dosing. QT was measured from leads II and CV5RL. Blood was sampled immediately after each ECG recording, for measurement of the plasma concentrations of potassium. The slopes of the linear regression between QT and plasma potassium levels were calculated for each animal, with the corresponding coefficient of correlation ( $r$ ) and of determination ( $r^2$ ). Escalating doses of furosemide produced a progressive hypokalemia whereas an abrupt change occurred in animals treated with 60 mg/kg. The change in plasma potassium was paralleled by an increase in QT and QTc interval. There was a clear inverse relationship between  $K^+$  plasma levels and QT or QTc interval in all animals and overall, the correlation was better for QT and QTc recorded in CV5RL lead compared to DII. When  $K^+$  plasma levels were below approximately 3.5 mmol/L, QT was increased above normal values of our historical range. The mean coefficients of determination of the relation between QTc measured in CV5RL and  $K^+$  plasma levels were 0.42 for males and 0.44 for females whereas the mean slopes were -17.4 for males and -24.2 for females. In conclusion, hypokalemia is quantitatively associated with an increase in QT and QTc interval in dogs.

**831**

### DOES AUTONOMIC NERVOUS TONE DIRECTLY AFFECT THE QT INTERVAL?

T. Harada, J. Abe, M. Shiotani, Y. Hamada and I. Horii. *PGRD Nagoya Lab., Pfizer Inc., Taketoyo, Aichi, Japan.* Sponsor: M. Kurata.

Changes in the QT interval should be evaluated precisely in the early stages of drug development because QT prolongation can cause torsades de pointes, a life threatening polymorphic ventricular tachycardia. However, it is well known that the QT interval varies with changes in autonomic nervous tone irrespective of heart rate. In this study, we investigated the changes of the QT interval by shifting the RR interval from 400 to 700 msec, and the direct effect of autonomic nervous tone on the QT interval by measuring heart rate variability in dogs. Our result showed that the QT interval at the high HF (high vagal tone) or low LF/HF ratio (low sympathetic tone) was longer than that at the low HF or high LF/HF ratio, respectively, when the RR interval was constant at 400 or 700 msec. Besides, the effect of vagal tone on the QT interval might be somewhat stronger than that of sympathetic tone because HF, which reflects vagal tone, is much higher in dogs than in other animals and the significant differences in the QT interval between low and high LF/HF ratios were not detected in 2 animals. The present observations support the idea that sympathetic as well as parasympathetic tone regulate the QT interval and that the QT interval may be controlled physiologically by both sinus rhythm and myocardial characteristics, that is, autonomic nerves may regulate the QT interval directly as well as via the sinus node.

**832**

### SENSITIVITY OF CANINE AND RABBIT CARDIAC REPOLARIZATION ASSAYS FOR DETECTION OF POTENTIAL QT LIABILITY.

J. Kramer. *ChanTest, Inc., Cleveland, OH.* Sponsor: T. Narahashi.

The nonclinical evaluation of the potential for QT prolongation induced by non-antiarrhythmic drugs includes *in vitro* cardiac action potential repolarization assays. These evaluations can be critical for decision-making and their relative predictive value for identifying potential QT and torsade de pointes (TdP) liabilities is of particular importance. We compared the responsiveness of several widely-used test systems: canine and rabbit Purkinje fibers, *ex vivo* rabbit hearts, and hERG potassium channels in a panel of drugs that are known to be either positive or negative for TdP in humans. Torsadogenic compounds such as, cisapride, erythromycin, haloperidol, ketanserin, quinidine, dl-sotalol, and thioridazine gave positive signals in both canine and rabbit Purkinje fibers. However, other torsadogenic compounds such as bepridil, pimozide and terfenadine gave positive signals only in rabbit Purkinje fibers. Terfenadine- and cisapride-induced QT interval prolongation was confirmed in *ex vivo* Langendorff-perfusion rabbit hearts. Non-torsadogenic test compounds (e.g. amoxicillin, aspirin, captopril and loratadine) were negative in both canine

and rabbit Purkinje fibers. Our results indicate that *in vitro* measurements obtained in rabbit-derived test systems give fewer false negative responses and have greater predictive value than those obtained in canine Purkinje fibers.

**833**

### ACCURATE EVALUATION OF QT INTERVAL IN CONSCIOUS CYNOMOLGUS MONKEYS BY USE OF TELEMETRY ECG.

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The objective of this study was to determine the best method to evaluate the QT interval with respect to the heart rate in cynomolgus monkeys. Cynomolgus monkeys are widely used in cardiovascular research, but the characteristics of QT-RR formulas in cynomolgus monkeys have not been determined. We analyzed the physiological QT-RR relationship for practical evaluation of the QT interval in conscious cynomolgus monkeys by use of 24-hour telemetry ECG monitoring. As the QT interval varies dependent on the RR interval, 14 QT regression functions were evaluated by use of 12 pairs of QT-RR points per animal in 100 male cynomolgus monkeys. The accuracy of fit with the measured data was assessed according to the minimum Akaike information criterion. Among one-parameter linear regression function,  $QT = s \times (RR)^{1/2}$ , where  $s$  is the regression coefficient, gave the best fit, which was almost comparable to that of all multi-parameter regression functions. Our results also suggest that the QTc formula derived from the square root regression function is the most practical for evaluation of QT intervals in cynomolgus monkeys. Furthermore, ranges in diurnal variation and the patterns of blood pressure (BP), heart rate (HR), body temperature (BT), motor activity (MA) and ECG parameters (PR interval, QRS duration, RR interval, QT interval, JT interval, QTc and JTc) in 100 male cynomolgus monkeys were analysed using telemetry system. We believe that these results in cynomolgus monkeys are very important and quite useful for cardiovascular research of new drugs.

**834**

### NEW SAFETY ASSESSMENT ON LEFT VENTRICULAR VOLUME AND ITS FUNCTION IN MONKEYS USING THREE-DIMENSIONAL ECHOCARDIOGRAPHY.

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[Background and Purpose] Non-invasive determination of cardiac function over time is potentially important for safety pharmacology study and toxicity study. The objectives of this study are to evaluate the accuracy of three-dimensional echocardiography (3DE) measurement of left ventricular (LV) volume and LV function, and to examine effects of some drugs on LV function in monkeys. [Methods] 3DE (SONOS 7500, Philips Med. Sys.) was performed in isoflurane-inhalated male cynomolgus monkeys before and after intravenous infusion (5 or 10 min) of four drugs (propranolol, verapamil and dobutamine). End-diastolic volume (EDV), end-systolic volume (ESV), ejection fraction (EF), stroke volume (SV), cardiac output (CO) and heart rate (HR) were determined. LV volume was determined using 4D CardioView RT software (TomTec Imaging Sys) by average rotation method. [Results] By using cut sausage at various volumes, there was strong correlation between the actual volume by Archimedes method and volume determined by 3DE (ranging 1.5-7 mL, slope=0.99,  $R^2=0.999$ ), suggesting that 3DE is accurately measured the small-volume-materials. Propranolol (0.1 mg/kg/10 min,  $n=5$ ) caused an increase in ESV, not EDV, resulting in decreases in EF and SV. Verapamil (0.1 mg/kg/10 min,  $n=5$ ) produced increases in both EDV and ESV, and thereby SV maintained almost pre-value despite a slight decrease in EF. Dobutamine (0.01 mg/kg/5 min,  $n=5$ ) produced decreases in both EDV and ESV, and thereby the increased CO resulted from the increased SV and EF. [Conclusion] These results demonstrate that cardiac function can be estimated by LV volume and analytical parameters from 3DE, and that this method is possible to use for evaluation in safety pharmacology study and toxicity study.

**835**

### HERG LIABILITY: THINKING OUTSIDE THE BLOCK.

B. Wible. *ChanTest, Inc., Cleveland, OH.* Sponsor: T. Narahashi.

Direct block of the hERG potassium channel by non-antiarrhythmic drugs (NARDs) is a major cause of QT prolongation and torsade de pointes (TdP). Recently, we showed that certain NARDs may produce hERG liability not by direct block but by inhibition of channel trafficking. Trafficking defects are not detected with the usual patch clamp protocol or any of the surrogate assays such as rubidium flux, ligand displacement, or dye-based methods. We have developed a novel, comprehensive assay called HERG-Lite® that predicts hERG liability for both direct

blockers and trafficking inhibitors. The antibody-based, chemiluminescent assay uses two cell lines to monitor hERG surface expression: hERG-WT (wild-type) is a biosensor for trafficking inhibitors while hERG-SM (single mutant) detects channel blockers. We have screened an 880 compound diverse chemical library and found that ~35% of the compounds have a predicted hERG liability. These compounds fall into three classes. Class B compounds, such as cisapride and pimozide, are direct blockers of the channel and show increased surface expression of the SM. Class C compounds, such as thioridazine and trifluoperazine, are direct blockers but also show a predominant trafficking defect in the HERG-Lite® assay. Our studies show that ~40% of hERG blockers possess this dual liability. Class A compounds such as arsenic trioxide and pentamidine are non-blockers that produce hERG trafficking inhibition. The results of the HERG-Lite® screen indicate that inhibition of hERG trafficking is a prevalent mechanism for NARD-induced hERG liability.

**836**

#### VARIABILITY IN THE MEASUREMENT OF HERG POTASSIUM CHANNEL INHIBITION: EFFECTS OF TEMPERATURE AND STIMULUS PATTERN.

G. Kirsch. *ChanTest, Inc., Cleveland, OH.* Sponsor: T. Narahashi.

*In vitro* evaluation of drug effects on hERG K<sup>+</sup> channels is a valuable tool for identifying potential proarrhythmic side effects in drug safety testing. Patch clamp recording of hERG K<sup>+</sup> current in mammalian cells can accurately evaluate drug effects, but the methodology has not been standardized and results vary widely. Our objective was to evaluate two potential sources of variability: the temperature at which recordings are performed and the voltage pulse protocol used to activate hERG K<sup>+</sup> channels expressed in HEK293 cells. A panel of 15 drugs that spanned a broad range of potency for hERG inhibition and pharmacological class was evaluated at both room temperature and near-physiological temperature using several patch clamp voltage protocols. Concentration-response analysis was performed with three stimulus protocols: 0.5 s step-pulses, 2 s step-pulses or a step-ramp pattern. Block by two of the 15 drugs tested, d, l-sotalol (antiarrhythmic) and erythromycin (antibiotic), was markedly temperature-sensitive. HERG inhibition measured using a 2 s step-pulse protocol underestimated erythromycin potency compared with results obtained with a step-ramp protocol. Using conservative acceptance criteria and the step-ramp protocol, the IC50 values for hERG block differed by less than twofold for 15 drugs. Data obtained at near-physiological temperatures using a step-ramp pattern are highly repeatable and provide a conservative safety evaluation of hERG inhibition.

**837**

#### VALIDATION OF QT EVALUATION IN CONSCIOUS GUINEA PIGS WITH A TELEMETRY SYSTEM.

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Guinea pigs have constituted an attractive *in vivo* model to evaluate the effect of newly discovered drugs on the QT interval at the pre-clinical stage because of their small size, low cost, an action potential configuration similar to that of humans, and the availability of a lot of *in vitro* data on the animal. But no ECG measurement technique using a telemetry system has been fully established in guinea pigs as yet. The purpose of this study was to validate telemetry data in guinea pigs using drugs that are well known to prolong the QT interval in humans. Bepridil (10 mg/kg), terfenadine (4 mg/kg), cisapride (3 mg/kg), haloperidol (2 mg/kg), pimozide (5 mg/kg), quinidine (30 mg/kg), E-4031 (0.1 mg/kg) or thioridazine (6 mg/kg) were given to 6 animals each. The intravenous route was selected to save the amount of compound used. The pharmacokinetics of the drugs was also investigated using other animals. All the drugs clearly prolonged the QT interval 10-60 min post-dose, with 7 of the 8 drugs tested inducing a statistically significant prolongation. In the animals treated with haloperidol, flattening of the T-wave was also observed, which is liable to cause torsades de pointes. When QT interval changes were plotted against the serum concentrations of the drugs, the resulting curves indicated a delayed distribution of the compounds into the ventricle. In summary, the present study demonstrated that use of conscious guinea pigs with an implanted telemetry device is useful to detect drug-induced QT interval changes and evaluate their potential to cause arrhythmia in humans at an early stage of drug development using only a small amount of chemicals.

**838**

#### ASTEMIZOLE : SPECIES DIFFERENCES IN QT PROLONGATION AND ARRHYTHMOGENESIS.

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The current draft ICH S7B guideline requires that the potential of new drugs to provoke QT prolongation and cardiac arrhythmias should be investigated in conscious animal models. In order to compare the sensitivity of different non-rodent

species, 6 female beagle dogs and 6 female cynomolgus monkeys implanted with telemetric devices received a single oral administration of 30 mg/kg astemizole (an H2 receptor antagonist) or vehicle. ECG was recorded continuously for 12 hours, and checked 24 hours after administration. QTf (QT corrected with Fridericia's formula) and the occurrence of arrhythmias were monitored over the same period. QTf was prolonged in both species but primates were more sensitive than dogs (42±18% vs 14±4%, p<0.05). The maximal effect was observed after 10 hours in both species and QTf prolongation was still present at 24 hours (15±12% in primates; 7±3% in dogs). In primates, ECG shape was modified with the appearance of a U wave from 45 minutes after treatment in all animals. Isolated extrasystoles were observed in two animals, and more severe arrhythmias (bigeminism and polymorphic tachycardia including Torsades de pointe) were noted in one animal, leading to transient syncope. No arrhythmias were observed in dogs. Based on these results, primates would be considered as more sensitive to astemizole-induced QT prolongation. Nevertheless, the use of either species would have allowed identification of the QT prolongation hazard. The choice of dogs would have led to underestimation of the potential for arrhythmogenesis. Since the new guidelines emphasize both QT prolongation and the potential for arrhythmogenesis, the choice of species for *in vivo* investigation should ideally be discussed on a case-by-case basis, taking account of the response of the animal models to different chemical or pharmacological classes.

**839**

#### VALIDATION OF THE ISOLATED RABBIT HEART FOR CARDIOVASCULAR SAFETY ASSESSMENT.

E. Tanhehco, P. Senese and M. Gralinski. *CorDynamics, Inc., Chicago, IL.*

The potential to cause arrhythmias has become a major concern during lead optimization and assessment of all potential drug candidates, regardless of their therapeutic target. Unlike other experimental procedures such as those examining hERG channel binding and isolated Purkinje fiber assays, both hemodynamic and electrophysiologic effects of compounds can be observed simultaneously in our application of the isolated rabbit heart. In this screening study, we examined the hemodynamic and electrophysiologic effects of nine compounds with known cardiovascular actions. Hemodynamic function was assessed by measuring left ventricular pressure, coronary perfusion pressure, +dP/dt, and -dP/dt. Changes in electrophysiologic characteristics were determined by monitoring heart rate, monophasic action potential duration, and by measurements of PR, QT and QRS intervals. The compounds studied included flecainide, propranolol, dobutamine, amiodarone, E4031, terfenadine, sotalol, and verapamil. These compounds represent a wide range of mechanisms that affect cardiac electrophysiologic and/or hemodynamic function. Penicillin was used as a negative control. Each compound altered the function of isolated rabbit hearts in a similar manner to what is observed in humans, or in the case of E4031, consistent with what has been observed in other pre-clinical models. For example, terfenadine increased QT interval and verapamil decreased contractility. Our results demonstrate that our use of the isolated rabbit heart can serve as an effective cardiovascular toxicity screen, and as a physiologically relevant bridge between the typical ion channel assay, and *in-vivo* telemetry studies.

**840**

#### NON-SURGICAL TELEMETRY TECHNIQUES FOR ELECTROCARDIOGRAM (ECG) DETERMINATION IN DOGS AND MONKEYS DURING TOXICOLOGY STUDIES.

P. Shaver-Walker, M. Vezina, N. Leblond and C. Copeman. *IPN, CTBR Bio-Research Inc., Senneville, QC, Canada.*

Manual ECG recordings can be difficult to interpret due to movement artefact and excitement during restraint. The relatively large numbers of animals/study (avg 32-40) also creates logistical difficulties in obtaining the ECG at the appropriate time-points (around Cmax). Non-surgical telemetry was selected as an alternative to allow undisturbed ECG recording simultaneously from multiple animals. ECGs were obtained from 12 dogs (60 seconds) and 12 monkeys (30 seconds) during restraint in a sling (limb leads). The same animals were then fitted with specially designed jackets with an integrated pocket. A DSI telemetry transmitter with 2 biopotential leads was placed in the pocket and the 2 leads connected to stick-on gel electrodes in a cutaneous axial formation under the jacket. Jacketed animals were placed in their cages and the following day simultaneous ECGs were recorded from an anteroom at a time similar to the restrained recording. The ECGs were evaluated for quality (presence of artefact, readability of the ECG complexes) as well as heart rate (HR). Telemetry-derived ECGs exhibited little or no excitement-induced tachycardia, compared to most restrained ECGs, and improved readability due to reduced artefact. HR was reduced by ~ 80 BPM (45%) and ~ 120 BPM (40%) in dogs and monkeys, respectively. In dogs, the lower HR resulted in an expected increased incidence of sinus arrhythmia and other common dog-related ECG changes. It was concluded the non-surgical telemetry improved both the dog and

monkey ECG and reduced the time to record the ECGs. It allowed evaluation at more physiologically relevant HR in both species, but had a more profound improvement in readability for the monkey.

**841**

#### GENERATION OF BACKGROUND

#### ELECTROCARDIOGRAM (ECG) TELEMETRY DATA IN DOGS USING A POSITIVE CONTROL SUBSTANCE (DL-SOTALOL) FOR QT PROLONGATION.

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As part of the validation of a computerized telemetry data acquisition system (Ponemah P3 v. 3.23 and DSI Open Art v 2.2), data from the CTBR telemetry dog colony were obtained to evaluate the systems ability to analyze ECG waveforms from live animals. ECGs were continuously monitored using telemetry from six previously implanted beagle dogs for an approximate 24-hour period for baseline determination. The dogs then received oral doses of dl-sotalol at 5 and 10 mg/kg, with a one week washout between doses. On the day of dosing, the ECG was continuously recorded for 2 hours pre-dose and up to 24 hours post dose. Heart rate and the PR, QRS and QT intervals were measured and the QT was also corrected for heart rate using the Van de Water equation (QTcv). As expected, administration of dl-sotalol resulted in a dose-related prolongation of the QT and QTcv intervals as evidenced using values adjusted for baseline and also direct QT-HR plots. At 5 mg/kg, the QT interval was prolonged by an average of approximately 25 milliseconds (ms) and at 10 mg/kg by an average of approximately 90 ms. Most of the dogs also exhibited slight shortening of the QRS interval when plotted against the heart rate at the 10 mg/kg dose, but not the 5 mg/kg dose. The Ponemah software successfully detected the ECG waveform components when the heart rate was constant, but had some trouble when the heart rates were slower and sinus arrhythmia more prevalent. Therefore, the data generated by the system required verification and correction of the ECG data as needed. Although time consuming, it was considered to provide much more accurate data since every complex is measured compared to manual measurement techniques.

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#### THE ROLE OF CALPAIN-CALPASTATIN SYSTEM IN 2-BUTOXYETHANOL-INDUCED HEMOLYSIS.

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Administration of 500 mg/kg of 2-butoxyethanol (2-BE) to female Sprague-Dawley (SD) rats leads to hemolysis via its reactive metabolite, 2-butoxyacetic acid (2-BAA). This study was designed to test the hypothesis that calpain released from lysed red blood cells (RBCs) mediates the progression of hemolysis initiated by 2-BAA. Hematocrit was measured at 0, 3, 6, 9, 12, 24, 72, 120, 168 h after the oral intubation of 500 mg 2-BE/kg to female SD rats (200-250 g). Hemolysis evident as early as 3 h after 2-BE treatment, progresses until 24 h, well after the elimination of 2-BAA ( $t_{1/2} = 3$  h). Plasma calpain levels peaked at 3 h and remained high until 9 h after 2-BE intubation. Presence of high levels of calpain in the plasma during and after BAA elimination, suggests its role in the progression of injury. Incubation of RBCs (250  $\mu$ l buffer) with 5.6 U of calpain led to significant hemolysis by 2 h. It is known that after the low dose of 2-BE, rats and hematocrit values recover from the hemolytic episode by 168 h due to erythropoietic stimulation. Erythropoiesis leads to the production of new RBCs, known to be resilient to hemolysis, explaining much lower hemolysis on subsequently administered normally lethal dose of 2-BE given 7 days later. We hypothesized that overexpression of calpastatin, an endogenous inhibitor of calpain in the new RBCs will impart resistance to calpain-mediated degradation of RBCs. Calpastatin expression was assessed by Western blotting in whole RBC lysates from 0 to 120 h after 2-BE administration. Calpastatin increased significantly at 3 h and remained high till 72 h. Collectively, these results suggest that calpain released from lysed RBCs may mediate the progression of hemolysis initiated by 2-BAA while overexpression of calpastatin may explain the underlying mechanism of resiliency of young RBCs against calpain-mediated hemolysis. However, the calpastatin overexpression is not seen after 72 h, which suggests that other death proteins and their inhibitors may be players in mediation of progression/regression of hemolytic injury.

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#### ADHESION-MOLECULE CHANGES IN 2-BUTOXYETHANOL (BE) FISCHER F344 RAT MODEL FOR THROMBOTIC SEQUELAE OF HEMOLYTIC ANEMIAS.

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In hemolytic diseases, like sickle cell disease (SCD) and thalassemia, the mechanisms of thrombosis are poorly understood. Appropriate animal models would help increase our understanding of the pathophysiology of thrombosis. Gavage exposure

of female rats to BE leads to the development of hemolytic anemia and disseminated microthrombi. Using red blood cell (RBC) flow analysis, we demonstrated that formation of microthrombi was not secondary to RBC aggregation. Instead, we found that exposure to BE led to enhanced adherence of RBCs to the extracellular matrix, possibly secondarily to the recruitment of cellular adhesion molecules on the endothelial cell (EC)-RBC interface. We exposed female Fischer F344 rats to 250mg/kg of BE for up to 4 days, using 8 groups (5 animals/group) of rats 4 vehicle-treated control and 4 treated groups. One control and one treated group were terminated each day, after the 1st, 2nd, 3rd, and 4th daily dose. Blood was collected on each day for laboratory testing of coagulation markers. Platelet counts, prothrombin times, activated plasma thromboplastin times, fibrinogen, antithrombin-III levels, and D-dimers showed no differences between treated and control rats. The levels of endothelial intercellular adhesion molecule-1 (ICAM-1) in the BE-treated animals were approximately twice those of the control animals on days 2 and 3. On day 4, the levels in treated animals were 1.5 times those in controls. The thrombin-antithrombin levels in the BE-treated rats for all the time points were 3-7 times greater than those in the control rats. We suggest that RBC/EC interaction may be a potent catalyst for thrombosis. This hypothesis is consistent with the observations of increased RBC adherence, RBC/EC interaction, and increased ICAM-1 expression observed in humans during acute sickle-cell crises and provides an explanation for the relative failure of anticoagulant/antiplatelet strategies during acute thrombotic episodes in SCD.

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#### 2-BUTOXYETHANOL FEMALE RAT MODEL OF HEMOLYSIS AND DISSEMINATED THROMBOSIS: X-RAY CHARACTERIZATION OF OSTEONECROSIS AND GROWTH PLATE SUPPRESSION.

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We recently proposed a chemically induced rat model for human hemolytic disorders associated with thrombosis. The objective of the present investigation was to apply a non-invasive, high-magnification x-ray analysis (Faxitron radiography) to characterize the protracted bone damage associated with this model and to validate it by histopathology. Groups of female Fischer 344 rats were given 0, 250, or 300 mg of 2-butoxyethanol/kg body weight daily for 4 consecutive days. Groups were then sacrificed 2 hours or 26 days after the final treatment. The treated animals displayed a darkened purple-red discoloration on the distal tail. Histological examination of animals sacrificed 2 hours after the final treatment revealed disseminated thrombosis and infarction in multiple organs, including bones. Phosphotungstic acid-hematoxylin (PTAH) staining confirmed the presence of ante-mortem thrombosis. Faxitron radiography was used to image selected bones of rats sacrificed in 30 days (26 days after the final treatment). Premature thinning of the growth plate was seen in the calcaneus, lumbar and coccygeal vertebrae, femur, and ilium from the treated animals. Radiolucent areas were seen in the diaphysis of the femur of all treated animals. The bones were then examined histologically and showed a range of changes, including loss or damage to growth plates and necrosis of cortical bone. No thrombi were seen in the animals sacrificed at 30 days, but bone and growth plate changes consistent with prior ischemia were noted. The Faxitron proved to be an excellent non-invasive tool that can be used in future studies with this animal model to examine treatment modalities for the chronic effects of human thrombotic disorders.

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#### 2, 3, 7, 8-TETRACHLORODIBENZO-P-DIOXIN ALTERS FETAL MURINE CARDIAC GROWTH AND GENE EXPRESSION, AND LEADS TO CARDIAC HYPERTROPHY AND ALTERED CARDIAC FUNCTION AFTER BIRTH.

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2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD) and similar environmental contaminants have previously been demonstrated to be potent cardiovascular teratogens in developing piscine and avian species. In the current study, we investigate the effects of TCDD on murine cardiovascular development. Pregnant mice were dosed with 1.5, 3, 6, 12, and 24  $\mu$ g/kg TCDD on gestation day (GD) 14.5. No overt maternal or fetal toxicity was seen at this dose, and litter size and neonatal survival were normal in the offspring of TCDD-treated dams. At day GD17.5, fetal mice exhibited a dose-related decrease in heart-to-body weight ratio that was significantly reduced at a dose as low as 3  $\mu$ g/kg TCDD. In addition, cardiac proliferation, as measured by both proliferating cell nuclear antigen (PCNA) and 5-bromo-

2'-deoxyuridine (BrdU) staining, was reduced in GD17.5 hearts treated with 6 ug/kg TCDD. To determine if this reduction in cardiac weight was transient, or continued after birth, mice treated with 6 ug/kg TCDD were allowed to come to term and remain with the original dam. While heart weights had normalized by post-natal day 7, TCDD-treated mice had increased heart-to-body weight ratios and increased expression of the cardiac hypertrophy markers 21 days after birth. At post-natal day 21, mice from TCDD-treated dams demonstrated a pronounced ST-segment depression in response to isoproterenol, suggesting cardiac ischemia. Furthermore, basal and isoproterenol-responsive heart rate was lower in TCDD-treated mice. These results demonstrate that TCDD reduces heart weight during cardiac development, and results in a compensative cardiac hypertrophy and altered cardiac functionality after birth. Supported by NIEHS # ES012855-01 to E.A.T., and NIEHS # ES012335 to M.K.W.

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SUB-CHRONIC, LOW LEVEL 2, 3, 7, 8-  
TETRACHLORODIBENZO-P-DIOXIN (TCDD)  
EXPOSURE INCREASES BLOOD PRESSURE IN ADULT  
MICE AS MEASURED BY TELEMETRY.

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Hypertension is a prevalent disorder in industrialized regions. If left untreated, prolonged hypertension significantly increases the likelihood of coronary thrombosis, stroke, and renal failure. Previous studies have correlated adult TCDD exposure with increased incidence of cardiovascular disease in humans, including hypertension. The purpose of our study was to determine if sub-chronic, low level TCDD exposure increased arterial blood pressure, a risk factor for future cardiovascular disease-related mortality. An implanted telemeter system was used to monitor the blood pressure of the left carotid artery in 3-4 month old male C57Bl/6N mice. After telemeter implantation and a 7 day recovery period from surgery, baseline blood pressure was measured for 7 days. Following baseline measurement, mice were dosed with vehicle control (corn oil) or 150 ng/kg TCDD by oral gavage three times per week for 2 months, which will result in a steady state body burden of 1 ug/kg after 8 weeks. Within the first 36 hours after the first dose (t36) no change in mean arterial pressure (MAP) was observed. However, within the next 24 hours (t60) a trend for increased MAP was observed which continued up to 9 days (t216). After 9 days, a normalization of MAP occurred which continued through day 18 (t432). Telemeter output will continue to be monitored until the mice attain steady state TCDD body burdens at 8 weeks. These results suggest that low level TCDD exposure increases blood pressure, which is consistent with previous studies that used higher doses of TCDD in mice. However, the mechanism by which TCDD increases MAP followed by normalization remains to be elucidated. Hypertension is the primary risk factor for atherosclerosis. Others have shown that this sub-chronic dose of TCDD promotes atherosclerosis in ApoE null mice. The continuance of this telemetry study will reveal if increasing TCDD body burden continues to effect blood pressure regulation. Supported by ES012335 to MKW; P30ES12072 to UNM.

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A HARMONIZED PBPK MODEL FOR  
TRICHLOROETHYLENE RISK ASSESSMENT.

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The most recent EPA cancer risk estimates for trichloroethylene (TCE) were derived in part using PBPK models. In particular, risks of liver cancer based on tumors in mice were estimated using two different PBPK models, as well as with "calibrated" versions of these two models using re-estimated parameters obtained from Markov chain Monte Carlo analysis. In this effort, the developers of the two previous models have collaborated to develop a single harmonized PBPK model for TCE, and to characterize the reliability of the resulting model in providing dosimetry estimates in support of a risk assessment for TCE. The harmonized PBPK model provides a comprehensive description of all of the data used in the development of both of the previous models on the kinetics of trichloroethylene (TCE) and its metabolites, trichloroethanol (TCOH), and trichloroacetic acid (TCA), in the mouse, rat, and human, for both oral and inhalation exposure. Dose metrics that can be calculated with the model include the area under the concentration curve (AUC) for TCA in the plasma or liver, the peak concentration and AUC for chloral (CHL) produced in the tracheo-bronchial region of the lung, and the production of a thioacetyating intermediate from dichlorovinylcysteine (DCVC) in the kidney. It was concluded that there is currently not adequate data available with which to confidently parameterize a description for another metabolite of interest,

dichloroacetic acid (DCA). Model predictions of TCE, TCA, and TCOH concentrations in rodents and humans are consistent with variety of experimental data, suggesting that the model should provide a useful basis for evaluating cross-species and cross-route differences in pharmacokinetics for these chemicals. In the case of the lung and kidney target tissues, however, only limited data are available for establishing cross-species pharmacokinetics. As a result, PBPK model calculations for these dose metrics are highly uncertain.

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PHYSIOLOGICAL MODELING OF THE DERMAL  
ABSORPTION OF  
OCTAMETHYLCYCLOTETRASILOXANE (D4) AND  
DECAMETHYLCYCLOCLOPENTASILOXANE (D5).

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Studies have been conducted to assess the dermal absorption of D4 and D5 through human axilla skin *in vivo* and abdominal skin *in vitro*. A physiologically based pharmacokinetic (PBPK) model was previously developed to examine the dermal absorption of D4 in the rat and human. Here, we describe extension of the published PBPK model for inhalation of D5 in humans by adding a skin compartment for dermal application. The compartment model for D5 dermal absorption, based on the D4 model, included (1) volatilization of applied chemical from the skin surface, (2) a storage compartment in the skin tissue, (3) diffusion of absorbed chemical within the skin back to the skin surface, (4) evaporation of this chemical from the skin surface even though the applied dose was no longer present, and (5) uptake from the skin compartment into blood. Time course of blood and exhaled breath data from human volunteers were used to estimate model parameters. Based on model calculations, 0.05 % of the applied dose of D5 absorbed through the skin and into the body over a 24 hour period. Modeling results for the *in vivo* human dermal absorption study were generally consistent with the results of *in vitro* human dermal studies. A smaller proportion of applied D5 absorbed through axilla skin than absorbed with D4, although neither was very well absorbed by the dermal exposure route. For D4 and D5, more than 83% of the small amount of systemically absorbed chemical was eliminated by exhalation in the 24 hour period after application. With these compounds having low blood:air partitioning, extensive first-pass loss by exhalation reduces systemic exposures after dermal absorption. Sponsored by the Silicones Environmental, Health and Safety Council of North America.

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ALTERATION OF HEXACHLOROBENZENE  
DISPOSITION BY PCB126 COEXPOSURE AND  
APPLICATION OF PBPK MODELING IN A MEDIUM-  
TERM LIVER FOCI BIOASSAY.

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Hexachlorobenzene (HCB) is a probable human carcinogen which still exists in the environment despite the curtailment of its production and use. Human exposure to HCB is often accompanied by PCB126, a dioxin-like compound and the most potent congener of polychlorinated biphenyls. Earlier, we observed that PCB126 altered the kinetics of coexposed lipophilic chemicals. Here, we report our studies on HCB pharmacokinetics, with or without coexposure to PCB126. Our experiments included two separate kinetic studies (HCB alone and HCB+PCB126) integrated into a time-course medium-term liver foci bioassay. In both studies, fat had the highest concentrations of HCB, followed by the other tissues. The kinetics of tissue distribution of HCB are dramatically different between HCB alone group and the HCB+PCB126 combination group, suggesting complex interaction mechanisms. Using PBPK modeling as a hypothesis-testing tool, we carried out computer simulations based on the assumptions that PCB126 induced disruption at the levels of absorption, exsorption (i.e., the diffusion from blood to the lumen of GI tract), and fat metabolism. We utilized a previously constructed PBPK model for HCB to describe these altered disposition patterns. The model was composed of fat, liver, erythrocytes, blood plasma, GI lumen, and rapidly and slowly perfused tissues and incorporated the physiological changes related to partial hepatectomy in the time-course medium term liver foci bioassay protocol. The model adequately described the pharmacokinetics of HCB singly and in combination with PCB126. From our modeling exercises, we propose that the kinetic alterations were the results of a combination of the modification of HCB absorption and exsorption and the disruption of fat metabolism by PCB126. (This research was supported, in part, by a grant from CDC/NIOSH, 1 RO1 OH07556-01.)

PREDICTIVE PHYSIOLOGICALLY BASED  
PHARMACOKINETIC (PBPK) MODELING OF  
PYRETHRHOID PESTICIDES.

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Pyrethroids are a class of neurotoxic pesticides that have many different applications in agriculture, horticulture, and homes, and medicinal uses for animals and humans. Differences in the toxicity of pyrethroids are the result of their pharmacokinetic and/or pharmacodynamic properties. Rat LD<sub>50</sub> values for three type II pyrethroids, deltamethrin-105mg/kg, esfenvalerate-95mg/kg, and  $\beta$ -cyfluthrin-450mg/kg have been reported by their manufacturers. In an effort to develop human exposure-dose-response models for pyrethroid pesticides we have determined *in vitro* metabolism parameters in rat liver microsomes using a parent depletion approach. We are interested in whether intrinsic hepatic clearance (CLint) is a major determinant of target organ (brain) concentration and therefore a potential determinant of the relative toxicity of these three pyrethroids. We have determined Kmapp, Vmax and, CLint for deltamethrin (Km 3.09 $\mu$ M  $\pm$  0.82; Vmax 435.86 pmoles/min/mg MSP  $\pm$  27.79; CLint = 8.47E-3 L/hr/mg MSP), esfenvalerate (Km = 7.13 $\mu$ M  $\pm$  0.15; Vmax = 353.00 pmoles/min/mg MSP  $\pm$  28.15; CLint = 2.97E-3 L/hr/mg MSP), and  $\beta$ -cyfluthrin (Km = 16.13 $\mu$ M  $\pm$  2.43; Vmax = 810.40 pmoles/min/mg MSP  $\pm$  28.15; CLint = 3.02E-3 L/hr/mg microsomal protein). Deltamethrin is cleared nearly 3-fold more rapidly than esfenvalerate or  $\beta$ -cyfluthrin. These results do not indicate any correlation between the rate of hepatic clearance and the relative toxicity of these three pyrethroids. From literature derived data and these calculated CLint values; a preliminary PBPK model of exposure to deltamethrin was developed. Based on model predictions it appears that for these three pyrethroids in addition to CLint, critical determinants of pyrethroid target organ concentration include absorption/metabolism in the G.I. tract, and partitioning into and metabolism in the brain. (SJG was funded by NHEERL-DESE, USEPA CT826513. This abstract does not represent EPA policy)

ROUTE-TO-ROUTE EXTRAPOLATION OF METABOLIC  
INTERACTIONS IN MIXTURES OF ALKYLBENZENES  
USING A PBPK MODEL.

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PBPK models facilitate the conduct of route-to-route extrapolation of the extent of metabolism and tissue dose of chemicals. This aspect of the PBPK models has not been particularly well explored with chemical mixtures. The objective of this work was to evaluate the extent and consequence of competitive metabolic interactions in a ternary mixture of alkylbenzenes (toluene (T), m-xylene (X), and ethyl benzene (E)) following dermal, inhalation and oral routes of exposure considered individually or together. A previously validated human PBPK model for TEX mixture was used to simulate the area under the venous blood vs time curve (AUC) as well as the amount metabolized of each mixture component. The AUC associated with inhalation exposure (7 hr) increased from 1.1, 4.2 and 3.1 mg/L.hr to 1.3, 4.6 and 4.3 mg/L.hr, respectively for T (17 ppm), E (33 ppm) and X (33 ppm) due to interactions. Based on equivalent 24 hr AUCs, oral doses of 2.2, 4.8, and 4.6 mg/kg of T, E and X were calculated to be equivalent to the above inhalation exposures. At these oral doses, the AUCs of T, E and X were altered from 1.1, 4.2, and 3.1 mg/L.hr to 1.6, 5.3, and 4.9 mg/L.hr due to interactions during mixed exposures. Dermal exposure to TEX vapors at the same atmospheric concentrations as the inhalation study did not result in any significant metabolic interactions. In fact, further simulations indicated that the impact of interaction on AUC would be negligible following dermal exposures, at levels below the saturable vapor concentrations of TEX. The multiroute human PBPK model for TEX was then applied to simulate the pharmacokinetics of T, E and X for various exposure scenarios (inhalation and dermal exposure, inhalation and oral exposure, and all three routes of exposures together). This study demonstrates the unique usefulness of PBPK models in predicting tissue dose of mixture components during multimedia and multiroute exposures, on the basis interaction mechanism evaluated following a single route of exposure.

A PBPK MODEL FOR A MIXTURE OF DIOXIN-LIKE  
CHEMICALS.

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A physiologically based pharmacokinetic (PBPK) model for TCDD was used as the framework for models of TCDD, PCB126, PeCDF, and a mixture of these three chemicals. A goal was to assess how a model developed specifically for TCDD

would serve as a general Ah receptor model for individual dioxin-like chemicals as well as a mixture of dioxin-like chemicals. The model builds on previous TCDD models by linking P450s to measured activity (EROD, A4H). The model structure is the same for each chemical while parameters related to binding, metabolism, cytotoxicity, and absorption vary by chemical. The mixture model links the individual chemicals by competitive binding to the Ah receptor and CYP1A2. The National Toxicology Program (NTP) chronic bioassay data includes lung, liver, blood and fat concentrations as well as EROD and A4H activity. The individual chemical models were fit to the NTP data. The fit parameters were used in the mixture model to make predictions to compare with mixture data. The predictions for fat and liver concentration are very good, while the predictions of the other data are reasonable. The results suggest areas to further refine a general Ah receptor PBPK model.

A HUMAN PBTK MODEL DESCRIBING ACETONE  
KINETICS IN BREATH AND BLOOD AT DIFFERENT  
WORKLOADS.

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Typically, human PBTK models are developed by scaling animal PBTK models to man, based on animal and, at best, human blood data. We have over several years measured solvents in blood, breath and urine sampled from human volunteers. PBTK modeling of these data suggests difficulties to simultaneously describe the time courses of solvent in blood and breath. We attribute this to a washin-washout effect in the airways, an effect that may lead to erroneous exposure and risk estimates. The aim of this study was to develop a PBTK model that correctly describes blood, mixed-exhaled and end-exhaled breath acetone levels in humans experimentally exposed to acetone vapors at various workloads. A series of previously published 26 controlled exposures to acetone (Wigaeus et al 1983, Ernstgard et al 1999) in 18 male volunteers were used as a starting point. The exposures were carried out at rest and various levels of exercise (50, 100, 150 W) and pulmonary ventilation was measured. Arterial, venous and arterialized capillary blood and end- and mixed-exhaled air was analyzed for acetone. A PBPK model was developed in Berkeley Madonna based on previously published ones (Kumagai and Matsunaga 1995, Clewell et al 2001). These models include a mucous compartment to account for washin-washout in the airways. Both previous models were unable to simultaneously predict the experimental blood and end-exhaled and mixed-exhaled breath levels. No improvement was achieved by deleting the mucous compartment while freely adjusting alveolar ventilation. In contrast, a good fit to all experiments was achieved by setting the mucous clearance proportional to, rather than independent of, pulmonary ventilation. This exercise suggests that (1) washin-washout phenomena affects the kinetic behaviour of acetone considerably and (2) an adequately designed mucous compartment in the airways may be used to describe the blood and breath kinetics also at elevated workloads (supported by grants from the Swedish council for working life and social research).

EVALUATION OF ORAL AND INTRAVENOUS ROUTE  
PHARMACOKINETICS, PLASMA PROTEIN BINDING  
AND UTERINE TISSUE DOSE METRICS OF BISPHENOL  
A: A PHYSIOLOGICALLY BASED PHARMACOKINETIC  
APPROACH.

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Bisphenol A (BPA) is a weakly estrogenic monomer used in the production of polycarbonate plastics and epoxy resins. A physiologically based pharmacokinetic (PBPK) model of BPA pharmacokinetics in rats and humans was developed to provide a physiological context in which the processes controlling BPA pharmacokinetics (e.g. plasma protein binding, enterohepatic recirculation of the glucuronide (BPAG)) could be incorporated. A uterine tissue compartment was included to allow the correlation of simulated ER binding of BPA with increases in uterine wet weight (UWW) in rats. Intravenous and oral-route blood kinetics of BPA in rats and oral-route plasma and urinary elimination kinetics in humans were well described by the model. Simulations of rat oral-route BPAG pharmacokinetics were less exact, most likely the result of over simplification of the GI tract compartment. Comparison of metabolic clearance rates derived from fitting rat i.v. and oral-route data implied that intestinal glucuronidation of BPA is significant. In rats but not humans, terminal elimination rates were strongly influenced by enterohepatic recirculation. In the absence of BPA binding to plasma proteins, high ER occupancy was predicted at doses without uterine effects. Restricting free BPA to the measured unbound amount demonstrated the importance of including plasma binding in BPA kinetic models. The relationship between ER occupancy and UWW increases

fit expectations for a receptor mediated response with low ER occupancy at doses with no response and increasing occupancy with larger increases in UWW. (This abstract does not necessarily reflect USEPA policy.)

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#### LACK OF BIOACCUMULATION WITH REPEATED, PERIODIC EXPOSURES OF CYCLIC SILOXANES.

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Cyclic siloxanes, such as octamethylcyclotetrasiloxane (D4) and decamethylcyclotetrasiloxane (D5), have uses in a variety of consumer products. These siloxanes have unusual physical properties that make them attractive for their specific applications and give rise to novel pharmacokinetic properties. D4 and D5 have moderate volatility, high lipophilicity, and are cleared from the circulation by exhalation and methyl-group oxidation. High lipophilicity (fat:blood partition coefficients in the 1000 to 2000 range) typically leads to concern for bioaccumulation. Multi-dose route, multi-species physiologically based pharmacokinetic (PBPK) models for D4 and D5 have integrated physical chemical, metabolic and partitioning information to provide an understanding of the expected time course concentrations of D4 and D5 in tissues, including fat, during various usage scenarios. We simplified these models to evaluate time course plasma and fat concentrations during periodic daily exposures, likely to occur with low level exposures to consumers. The model was calibrated with blood and tissue concentrations in rats arising from 6 hr/day exposures of various durations of, 1 day, 14 day, and 6 months. The model had a central compartment with first-order metabolic clearance and either a single or two fat compartments that had variable diffusion-limitations for uptake. The fat concentrations at steady state were equal to the steady-state concentrations expected for a continuous exposure times the ratio of the daily exposure duration/24 hours. The approach to steady state and persistence after cessation for all exposure scenarios depended on the characteristic diffusional clearance from the deeper fat compartment. These siloxanes, despite high lipophilicity, do not bioaccumulate because they are rapidly cleared by exhalation and metabolism.

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#### IMPACT OF MODEL MISSPECIFICATION ON A PHYSIOLOGICALLY-BASED PHARMACOKINETIC MODEL FOR DMA.

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Inorganic arsenic in drinking water and organic arsenic used as herbicides both pose health risks. Using experimental data, a Physiologically Based Pharmacokinetic (PBPK) modeling has been applied to dimethylarsinic acid (DMA) in order to quantify dose metrics associated with health effects. A nine compartment intravenous PBPK model (venous, lung, lung blood, skin, kidney, urine, bladder, liver, and rest-of-body) was developed for mice that can be extrapolated to humans. This work describes part of the process of model development, and potential errors that can arise. To describe the pharmacokinetics, we initially assumed the simplest case, blood flow-limited distribution in every organ. However, this type of mathematical description was incomplete in that the amounts of DMA in the organs exceeded the dose. After examining the assumptions used for the lung, we decided that the blood flow-limited case was inappropriate for this organ. When we added membrane-limited terms to the lung blood equation, we could then account for lung tissue accumulation. Since the lung is a target organ, we wanted to ensure that we were describing accumulation correctly. The next organ to be described mathematically was the kidney. The fits obtained assuming zero order transport through the glomerular capsule were not satisfactory. An additional improvement included in the model is a saturable mechanism for the clearance of DMA in the kidney. In summary, this model describes the inclusion of more complex descriptions of the lung and kidney, resulting in a combination of different approaches used in PBPK modeling. (This abstract does not reflect EPA policy).

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#### HUMAN PBPK MODELING OF CLINICAL CASE REPORTS OF ETHYLENE GLYCOL OVERDOSING: INCORPORATION OF THERAPEUTIC INTERVENTIONS.

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The acute toxicity of ethylene glycol (EG) in humans and animals can proceed through three stages, each associated with a different metabolite: central nervous system depression (EG), cardiopulmonary effects associated with metabolic acidosis (glycolic acid; GA) and ultimately renal toxicity (oxalic acid; OX), depending upon

the total amount consumed and effectiveness of therapeutic interventions. A recently developed PBPK model for rats and humans was refined to include clinically relevant treatment regimens for EG poisoning such as hemodialysis and metabolic inhibition with ethanol or fomepizole. These modifications enabled the model to describe several human case reports which included analysis of EG and/or GA in blood or urine. Such data and model simulations provide important confirmation that the previously developed PBPK model can adequately describe the pharmacokinetics of EG in humans following low, occupational or environmentally relevant inhalation exposures, as well as after massive oral doses, even under conditions where treatments have been employed that markedly affect the disposition of ethylene glycol and glycolic acid. By integrating the case report data sets with controlled studies in this PBPK model, it was demonstrated that fomepizole, if administered early enough in a clinical situation, can be more effective than ethanol or hemodialysis in preventing the metabolism of ethylene glycol to more toxic metabolites. Hemodialysis remains an important option, however, if treatment is instituted after a significant amount of EG is metabolized or if renal toxicity has occurred. (Sponsored by the Ethylene Glycol Panel of the American Chemistry Council).

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#### DEVELOPMENT OF A PHYSIOLOGICALLY BASED PHARMACOKINETIC (PBPK) MODEL TO COMPARE DIFFERENCES IN DISPOSITION OF TRICHLOROETHYLENE(TCE), AND METABOLITES IN ADULT VERSUS ELDERLY RATS.

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Due to the increasing number of elderly in the American Population, the question as to whether the aged have different susceptibility to environmental contaminants needs to be addressed. Physiologically based pharmacokinetic (PBPK) models are used to extrapolate between rodents (toxicological database) and humans. We used an established PBPK model for TCE, a common environmental contaminant. The PBPK comparisons were made in two different F344 rat age groups: 6 months and 2 years old. A single inhalation exposure of 100 ppm for 1 hour was simulated as the basis for the comparison. The compartments evaluated were: brain, lung, adipose, kidney, liver, rapidly perfused and slowly perfused tissues. Both organ flow and organ size changes were examined. The metabolic scheme involved P450 oxidation of the parent TCE to both trichloroethanol (TCOH) and trichloroacetic acid (TCA). TCOH was further metabolized to TCA, and glucuronidated trichloroethanol(TCOG) for excretion by the kidneys. Acute neurotoxicity is related to the parent chemical concentration present in the brain, while chronic nephrotoxicity is related to TCOG in the kidneys. Simulation results show no differences in the concentration of TCE present in the brain for the two ages studied. There were obvious differences between ages in the time course of TCOG excretion, both at the early part of the time course and for the steady state component. In comparison to the young, the amount of TCOG excreted by the kidney in elderly rats was decreased by 12%, 24 hours after exposure. After repeating a second 1 hour exposure, the differences between the adult and elderly simulations were more obvious. In summary, the PBPK modeling results suggest that metabolite amount excreted may be different in the elderly, and that PBPK modeling should play a role in predicting potential experiments needed to confirm these predictions. (This abstract does not reflect EPA policy).

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#### A PBPK MODEL TO EVALUATE VARIABILITY IN RENAL CLEARANCE.

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A PBPK model was developed to better characterize human variability in renal clearance, and its contribution to overall variability in chemical disposition. Renal clearance is a function of filtration and secretion from the arteriolar circulation, minus reabsorption. Variability in renal clearance is relatively low in healthy adults due to the kidney's autoregulation of filtration and reabsorption, and its large functional reserve capacity. Based on recent data for clearance rates of various pharmaceuticals in healthy adults (Dorne et al. 2003), an adjustment factor of 1.8 times the mean accounted for 99 percent of the variability in renal clearance. This suggests that the standard default adjustment factor of 3.16, which is used in EPA's RfD/RfC process for intrahuman variability in pharmacokinetics, is adequate for renal clearance, although the default factor must also account for other kinetic processes including absorption and metabolism. The question arises as to how variability in renal clearance might increase when subpopulations are considered such as the aged, developmentally immature (neonates), or diseased individuals; or when a nephrotoxin compromises renal function. As a first step in evaluating variability

in renal clearance for subpopulations or nephrotoxicity, the renal compartment typical of most PBPK models was expanded to include terms for glomerular filtration and reabsorption, as well as simplified representations of kidney feedback controls based upon target levels of sodium and glucose in plasma and filtrate, and target levels for plasma and urine volume. Secretion is modeled as a first order elimination of free chemical (organic acids or bases) from plasma. Using this approach, the PBPK model provides a reasonably good simulation of normal adult renal clearance and response to perturbation. The next step is to apply the model to evaluate variability in chemical specific renal clearance due to reductions in filtration, reabsorption, or secretion, or reduced ability to maintain homeostatic control. [This abstract does not necessarily reflect EPA policy.]

## 860 DEVELOPMENT OF A PHYSIOLOGICALLY BASED PHARMACOKINETIC MODEL FOR SCH-D IN THE RAT.

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SCH-D, a CCR-5 receptor antagonist, is currently in development for the treatment of HIV and will be used in combination with several other anti-viral drugs. The possibility of pharmacokinetic and metabolic interactions exists as many of these compounds either inhibit or induce the enzymes responsible for the metabolism of SCH-D. In an effort to assess the other medications on the pharmacokinetic profile of SCH-D, a physiologically based pharmacokinetic model for SCH-D was developed in the rat. Compartments for this model included the brain, kidney, liver, fat, and rapidly perfused and slowly perfused. Pharmacokinetic data, including SCH-D plasma concentration-time profiles following both oral and iv administration, and SCH-D urinary excretion data, were used to estimate the oral absorption (KA), metabolic (KMET) and urinary excretion (KEX) rate constants. The data used for the pharmacokinetic analysis had been previously collected as part of the preclinical development program for SCH-D. The only data that was not already available were the SCH-D tissue:blood partition coefficients, which were determined using equilibrium dialysis. The model was validated against single and multiple dose toxicokinetic data that were also available. Application of the model indicated that following a single oral administration of 10 mg/kg peak SCH-D plasma concentrations increased by 12% when hepatic metabolism (Kmet) was reduced by half and decreased 19% when hepatic metabolism was doubled. In addition, the SCH-D plasma AUC(0-24 hr) increased 35% when hepatic metabolism was reduced by half and decreased by 43% when hepatic metabolism was doubled. This model represents the initial step in the development of a PBPK model for SCH-D in humans that will be used to more fully characterize potential pharmacokinetic and metabolic interactions between SCH-D and other anti-retroviral drugs.

## 861 PHYSIOLOGICALLY-BASED PHARMACOKINETIC MODEL FOR ALL-TRANS RETINOIC ACID IN PREGNANT CD-1 MICE.

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all-trans Retinoic acid (RA) is a well documented teratogen in laboratory animals as well as humans. When administered to mice on gestation day 11 (GD-11), RA causes severe alterations in fetal forelimbs and cleft palate. A nine compartment physiologically-based pharmacokinetic model (PBPK) for RA and a 4 compartment PBPK model for its metabolite, the 13-cis retinoic acid (13-cis RA) isomer, are under development to describe the uptake, fetal transfer, and systemic clearance of RA in the pregnant CD-1 mouse on GD 11. This model was adapted from a previously published RA model (Clewel, 1997) to include endogenous production of RA and induction of RA metabolism in the maternal liver. Maternal blood and fetal tissue kinetic data was collected from pregnant CD-1 mice on GD 11 after a single oral bolus dose (10, 30, 60, and 100 mg/kg) of RA suspended in soybean oil. Analysis of tissues and blood was by HPLC for RA and 13-cis retinoic acid (13-cis RA). The systemic clearance of RA by metabolism was described by two metabolic pathways; isomerization to 13-cis RA and a composite pathway representing both p450 and glucuronidation metabolic pathways. Placental transfer of RA and 13-cis RA to the fetal compartment were described as diffusion limited. Metabolism of 13-cis RA was described as a composite pathway including p450 and glucuronidation. To obtain adequate fits to the kinetic data the composite metabolic pathway for RA was assumed to be induced 5 fold by 4 hours after dosing. Diffusion limited transfer of RA and 13-cis RA to the embryo were also well described by the model for all doses tested. Dose-response analyses are ongoing to determine the relationship between fetal exposure and incidence or severity of malformation.

## 862 PREDICTION OF DI-N-BUTYLPHthalate (DBP) LEVELS IN PREGNANT RATS USING A PHYSIOLOGICALLY BASED PHARMACOKINETIC (PBPK) MODEL.

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DBP is a widely used phthalic acid ester and a reproductive toxicant. The monoester metabolite monobutyl phthalate (MBP) is the primary circulating form in rodents and is responsible for the adverse effects from DBP exposure. We previously developed a PBPK model to predict MBP levels in maternal and fetal rats during reproductive development (gestation days GD12-20). Physiological and chemical-specific parameters were obtained from peer-reviewed literature or fitted to data. Metabolism of DBP to MBP was modeled to occur in the gastrointestinal (GI) tract. Subsequent uptake of MBP, but not DBP, from GI was calculated using a first-order rate constant. Metabolism of MBP to MBP-glucuronide (MBP-G) occurs in liver and was modeled using Michaelis-Menten kinetics. Model predictions were compared to *in vivo* data from published studies that utilized oral dosing of DBP. This preliminary model predicted MBP levels qualitatively, but not quantitatively, primarily due to a lack of data on metabolism of MBP to MBP-G. To address this gap, we measured MBP and MBP-G levels in pregnant rats on GD19 following MBP intravenous (iv) administration. MBP to MBP-G metabolic parameters were fit to MBP plasma data following iv doses of 10, 30, and 60 mg/kg MBP. Our PBPK model was revised to incorporate these improved metabolic parameters and expanded to predict MBP-G levels after we observed that MBP-G accumulated in the fetus and amniotic fluid. The revised model effectively predicted initial MBP and MBP-G distribution and clearance but still underpredicted MBP and MBP-G levels at later time points. Sensitivity analysis indicated that maternal MBP levels were affected by the rate of uptake from GI; fetal plasma MBP and MBP-G levels were affected by placental diffusion and placenta-to-blood partition coefficient. This quantitative description of the critical determinants of MBP dosimetry and transport of MBP and MBP-G to the developing fetus will enable a better assessment of potential risks from DBP exposure.

## 863 PULMONARY FUNCTION IN RATS DURING PREGNANCY.

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Minute ventilation and tidal volume increase in humans during pregnancy. Currently little data exists on pulmonary function in rats during pregnancy, an animal model often used for developmental studies. Respiration will affect the pharmacokinetics of volatile compounds, and accurate estimates of minute ventilation will improve physiologically based pharmacokinetic models that predict target tissue concentrations for extrapolation to human exposure scenarios. We conducted a longitudinal study to evaluate pulmonary function in rats during pregnancy. Whole-body plethysmography was used to measure the breathing frequency, tidal volume, and minute ventilation approximately every other day from gestation day (GD) 1 to 21 in 8 timed pregnant and 8 nonpregnant female, Sprague-Dawley rats. Scaled minute ventilation was calculated from the minute ventilation and body weight of each rat. Multivariate analysis of variance methods for a repeated measures design were used to analyze the collected data. On GD 1, breathing frequency, tidal volume, minute ventilation, and scaled ventilation were  $97 \pm 2.7$  bpm,  $1.46 \pm 0.06$  ml,  $139 \pm 4.9$  ml/min, and  $34.1 \pm 1.2$  L/hr/kg for pregnant rats, and  $96 \pm 4.7$  bpm,  $1.42 \pm 0.04$  ml,  $134 \pm 5.5$  ml/min, and  $35.3 \pm 1.3$  L/h/kg for nonpregnant rats. On GD 21, breathing frequency, tidal volume, minute ventilation, and scaled ventilation were  $106 \pm 4.7$  bpm,  $1.95 \pm 0.06$  ml,  $204 \pm 12.4$  ml/min, and  $29.4 \pm 1.7$  L/hr/kg for pregnant rats, and  $92 \pm 4.7$  bpm,  $1.75 \pm 0.08$  ml,  $157 \pm 10.9$  ml/min, and  $36.3 \pm 2.2$  L/hr/kg for nonpregnant rats. Tidal volume was not significantly different between the two groups. Minute ventilation was significantly greater in pregnant rats compared to nonpregnant rats. The scaled ventilation was significantly lower in pregnant rats compared to nonpregnant rats due to the significantly greater body weight of the pregnant rats. This study provides important reference values to be used in pharmacokinetic models during pregnancy.

## 864 A PHYSIOLOGICALLY BASED PHARMACOKINETIC (PBPK) MODEL FOR THE PESTICIDE MONOMETHYLARSONIC ACID (MMA<sup>V</sup>).

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The monosodium salt of monomethylarsonic acid [MMA(V)] is a widely used organoarsenical herbicide. In lifetime feeding studies with MMA(V), the large intestine (focal mucosal ulceration) was the primary target organ in both male and female mice and rats and no treatment-related neoplastic effects were reported. We

have developed a PBPK model to describe the target tissue dosimetry of MMA in mice that can be extrapolated to humans for ultimate use in risk assessment. The model consists of two separate submodels (for mono- and di-methylated arsenic) linked by methylation in liver; tissue compartments include arterial and venous blood, gastrointestinal tissue, liver, lung, kidney, urinary bladder, skin and residual tissue. Chemical specific parameters for partition coefficients, gastrointestinal absorption, methylation in liver and urinary elimination were estimated using oral and i.v. kinetic data from our studies of MMA(V) and dimethylarsinic acid [DMA(V)] in mice. In general distribution into tissues was modeled assuming blood flow limited kinetics in plasma. Good visual fits were obtained for much of the data used to evaluate the model; however, kidney was often a notable exception, although fits to urinary clearance data were generally quite good. Sensitivity analysis suggests that parameters governing uptake into the kidney are highly influential for both kidney concentration and urinary clearance. This points to the need to further understand and evaluate mechanisms of renal uptake and elimination. (This abstract does not necessarily reflect EPA policy.)

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#### DEVELOPMENT OF A HYBRID REACTION NETWORK PHYSIOLOGICALLY BASED PHARMACOKINETIC MODEL OF BENZO(A)PYRENE AND ITS METABOLITES.

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PBPK modeling of mixtures is challenging in part because it requires information not only on the parent chemicals but also on any important metabolites. Such data are rarely available. To address this problem, Reaction Network (RN) modeling was used to describe the mammalian metabolism of benzo[a]pyrene (BaP), a priority environmental carcinogen. A RN model generates the reaction pathways automatically based on the chemical structures of the parent compounds and reaction rules. We have linked a RN model with a PBPK model to describe the distribution and disposition of BaP and seven of its metabolites. In the hybrid RN/PBPK model, BaP metabolism in the lung and liver was described by a RN model for BaP, in which the kinetics parameters were estimated using *in vitro* experiments with rat liver microsomes. In these *in vitro* studies, time-course profiles of BaP and 11 of its metabolites were measured using HPLC methods. The RN/PBPK model for BaP and its metabolites were calibrated using pharmacokinetics data from rats following intratracheal exposure to BaP (*Cancer Res*, 46:5655, 1986). Validation of the RN/PBPK model for BaP was performed using two different data sets in rats with intravenous (*Cancer Res*, 30:2893, 1970) and intraarterial (*J Pharmacol Exp Ther*, 226:661, 1983) exposure to BaP. The quality of the RN/PBPK model prediction demonstrates its ability to extrapolate from one dosing route to another and from low dose to high dose. The major advantages of applying RN/PBPK modeling to study toxicology are: (1) their capabilities of handling complex metabolic systems involving chemical mixtures; (2) their potential for predicting reaction networks of chemicals with limited knowledge on their metabolic pathways; and (3) their abilities to predict the reactive intermediates that are not readily measurable in experiments. (Supported by NIEHS Grant R01 ES09655).

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#### VALIDATION OF A HUMAN PHYSIOLOGICALLY-BASED PHARMACOKINETIC MODEL FOR VINYL ACETATE AGAINST HUMAN NASAL DOSIMETRY DATA.

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Vinyl acetate has been shown to induce nasal lesions in rodents in inhalation bioassays. A physiologically-based pharmacokinetic (PBPK) model has been used in human vinyl acetate risk assessment but has only previously been validated in rats. To provide validation data for the application of the model in humans, controlled human exposures to vinyl acetate were conducted. Air was sampled by a probe inserted into the nasopharyngeal region of five volunteers. Sampling was carried out during exposure to labeled <sup>13</sup>C<sub>1</sub>, <sup>13</sup>C<sub>2</sub> vinyl acetate during resting and light exercise. Both <sup>13</sup>C<sub>1</sub>, <sup>13</sup>C<sub>2</sub> vinyl acetate and the major metabolite <sup>13</sup>C<sub>1</sub>, <sup>13</sup>C<sub>2</sub> acetaldehyde from the nasopharyngeal region were analyzed in real time utilizing ion trap mass spectrometry (MS/MS). Experimentally determined concentrations of both vinyl acetate and acetaldehyde were then compared to predictions calculated by applying the previously published human model. Model predictions of vinyl acetate nasal extraction compared favorably with measured values of vinyl acetate as did predictions of nasopharyngeal acetaldehyde when compared to measured acetaldehyde. The results showed that the current PBPK model structure and parameterization is appropriate for vinyl acetate. These analyses were conducted from 1 to 10 ppm

vinyl acetate, a range relevant to workplace exposure standards but which would not be expected to saturate vinyl acetate metabolism at low concentrations. A risk assessment based on this model further concluded that 24 hour per day exposures up to 1 ppm do not present concern on cancer or non-cancer toxicity. Validation of the vinyl acetate human PBPK model provides support to these conclusions.

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#### DEVELOPMENT AND EVALUATION OF A PBPK MODEL FOR THE FATE OF 2-BUTOXYETHANOL IN HUMANS AFTER DERMAL AND INHALATION EXPOSURE.

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The objective of this study was the development of a PBPK model to describe the disposition of butoxyethanol (BE) in blood and its main metabolite 2-butoxyacetic acid in urine (BAA) after exposure to BE by inhalation and via the skin. An existing PBPK model for 2-butoxyethanol (Corley, 1994) was implemented and validated with published information and with experimental data obtained in human volunteers who were exposed by inhalation. In this PBPK model a detailed module for systemic uptake of substances via the skin (Kruse et al., 2004) was incorporated. The modeling of dermal permeation was achieved by the use of a multi-compartment model (vehicle, stratum corneum and viable epidermis) and systemic compartment. In the skin module the mass transport through the skin is determined by the thickness of the different layers in the skin and by substance related partition and diffusion coefficients. In a series of experiments the skin of human volunteers was exposed to vaporous BE and aqueous solutions of BE (50-90% w/w) and the resulting time courses of the BE concentrations in blood (during 8 hours) and the excretion rates of BAA in urine (up to 48 hours) were determined. These data were used for the fitting of the skin model in order to assign values to parameters of the model such as partition coefficient and diffusion coefficient. This was both done via a deconvolution method using the systemic response of a reference exposure via inhalation, and by direct fitting of the concentration time courses after dermal exposure using the PBPK model. The results of the two approaches were compared. In conclusion, a refined PBPK model for BE, including a mechanistic module for skin permeation was presented. The model is particularly well suited to simulate the time courses of BE in blood and BAA in urine following a variety of occupational scenarios including exposure via inhalation and/or dermal exposure to vapors or aqueous solution of BE.

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#### PHYSIOLOGICAL MODELING OF THE TOXICOKINETICS OF METHYL TERT-BUTYL ETHER (MTBE) AND ITS METABOLITE TERT-BUTANOL (TBA) IN HUMANS.

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MTBE, an oxygenated gasoline additive, has been investigated by several investigators for its pharmacokinetic behavior and toxicity. Animal and human PBPK models for MTBE have also been developed. The existing human PBPK models neither describe the kinetics of the oxidative metabolite (TBA) nor the kinetics of MTBE itself in exercising individuals, typical in work environments. The objective of this study was therefore to develop a physiologically-based (PB) model for simulating the toxicokinetics (TK) of MTBE and TBA in human volunteers performing workload (50 W). The PBTK model consists of five tissue compartments (liver, fat, richly perfused tissues, working muscle and resting muscle) interconnected by systemic circulation. The alveolar ventilation rate, cardiac output, and blood flows to tissues were calculated as a function of the workload exercised by exposed individuals. The human blood:air (17.7) and tissue:air (liver and richly perfused tissues 1.04, fat 5, and muscle 1.18) partition coefficients for the MTBE model were obtained from a previously published experimental study. The kinetics of TBA was simulated using a single compartment model, in which the volume of distribution was approximated to the body weight and the rate of elimination was described as a first order process. The integrated MTBE-TBA model, with its metabolism parameters estimated from the blood concentration vs time profile previously collected in human volunteers exposed for 2 hr to 5, 25 and 50 ppm (50 W workload), adequately described the whole dataset. Model simulations, consistent with the experimental data, indicate linearity in the range of these exposure concentrations and an intermediate hepatic metabolism. The toxicokinetic model developed in this study should be useful as a tool for interpreting the blood concentrations of MTBE and TBA in workers and general population. (Partially supported by Swedish council for working life and social research)

## A HUMAN DIETARY IODIDE PBPK MODEL TO EVALUATE THE EFFECTS OF PERCHLORATE ON THYROIDAL IODIDE CONTENT.

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Perchlorate is an environmental contaminant that is known to act on the thyroid by blocking radiolabeled iodide uptake into the thyroid gland in laboratory animals and humans. Thyroid function has been clearly altered in laboratory animals administered perchlorate, but not in humans. The objective of this research was to construct a dietary iodide PBPK model for the adult human to explore under what conditions perchlorate exposure would be predicted to deplete the thyroidal iodide stores (thyroid hormones) sufficiently to alter thyroid function. A human physiologically based pharmacokinetic (PBPK) model of thyroid hormone production and metabolism was constructed and adapted to an existing PBPK model for perchlorate and radiolabeled iodide (Merrill et al., 2004). The dietary iodide model accounts for a range of iodide diets and includes autoregulation of iodide uptake by the thyroid gland. Under brief perchlorate exposure conditions, such as those of Greer et al. (2002) (500, 100, 20 and 7 ug/kg/day oral administration for 14 days), perchlorate was not predicted to deplete thyroid iodide stores sufficiently to alter thyroid function. If a chronic exposure (years) to perchlorate occurs at these doses, the dietary iodide model predicts that the 100 and 500 ug/kg/day doses would cause depletion of thyroidal iodide stores sufficient to alter thyroid function (increase blood TSH levels in response to a decline in thyroid hormone production). For the two lower doses of perchlorate (7 and 20 ug/kg/day) the PBPK iodide model predicts that thyroid function would not be altered. This dietary iodide PBPK model can assist in policy decisions regarding public health issues with perchlorate in water supplies. The PBPK model for iodide and perchlorate forecasts that chronic exposure to 7 ug/kg day (245 ug/L for a 70 kg person) will not alter thyroid function in the adult euthyroid human.

## USE OF A PHYSIOLOGICALLY-BASED PHARMACOKINETIC MODEL TO ESTIMATE ABSORBED CARBARYL DOSE IN CHILDREN AFTER TURF APPLICATION.

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A physiologically based pharmacokinetic (PBPK) model was developed to investigate exposure scenarios of children to carbaryl following turf application. Physiological, pharmacokinetic and pharmacodynamic parameters describing the fate and effects of carbaryl in rats were scaled to establish the model structure for exposure to humans. Adjustments were made for differences in metabolism and physiology between children and adults. Michaelis-Menten kinetics were used to describe the metabolism of carbaryl to yield biomarkers of metabolism, including urinary 1-naphthol. Bimolecular rate constants,  $k_i$  (pM<sup>-1</sup> hr<sup>-1</sup>), were used to describe inhibition of acetylcholinesterase by the parent chemical. Rates for enzyme synthesis and reactivation were interposed within compartments to account for depletion of the enzymes. Exposure by hand-to-mouth activities for toddlers, and by dermal exposure for older children resulted in no observable cholinesterase inhibition (>99% of basal activity) in the brain and blood. Peak concentrations of carbaryl in the brain remained below the brain peaks observed in rats at the no observable adverse effect level (NOAEL) by an order-of-magnitude. Corresponding with the absorbed dose and subsequent distribution were the appearance of metabolite biomarkers in urine. Although this work was reviewed by EPA and approved for publication, it may not necessarily reflect official Agency policy.

## ASSESSING THE RISKS ASSOCIATED WITH CHILDREN INGESTING LEAD IN SCHOOL DRINKING WATER: PBPK MODELING AND RISK COMMUNICATION.

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Communities across the United States have become increasingly concerned about the many recent instances of lead being detected in school drinking water. Given lead's potential for developmental and behavioral toxicity at relatively low levels of exposure, concerns of parents, teachers, and administrators about the potential impact on children's health are understandable. Considering the uncertainties inherent in characterizing children's activity patterns as well as current regulatory debates about what levels of lead exposure are safe, effectively communicating the issues to various stakeholders can be a difficult process. We present the results of physiologically-based pharmacokinetic (PBPK) modeling (using the O'Flaherty model) conducted to predict blood lead levels and assess the risks to children exposed to lead in

school drinking water. This model was considered to be more appropriate for this exposure scenario than the traditional approach using the USEPA's Integrated Exposure and Uptake Biokinetic (IEUBK) Model for Lead in Children because of the episodic nature of the exposures, and allowed the calculation of incremental blood lead increases in any classroom with lead in drinking water sampling data. Our modeling illustrates that the for the school exposure scenarios evaluated, which included lead in drinking water concentrations of up to 1600 ppb (first-draw), virtually none would result in incremental elevations of blood lead greater than 3 micrograms per deciliter above blood lead levels associated with background exposures to lead. In addition to these data, we provide examples of effective risk communication materials and approaches used with various stakeholders, and an assessment of the scientific support for USEPA's action level of 20 ppb in school's drinking water.

## ENFUVIRTIDE DOES NOT IMPAIR HOST RESISTANCE TO INFECTIONS IN RATS.

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Enfuvirtide (ENF; T-20; Fuzeon) is a 36-amino acid peptide effective for the treatment of advanced HIV. In clinical trials, an increased rate of bacterial pneumonia was observed in patients treated with ENF. To assess whether ENF can suppress immunity and increase susceptibility of an organism to pathogens, two *in vivo* host resistance assays were conducted in Fischer-344 (CDF) rats challenged with infections of either *Streptococcus pneumoniae* or influenza virus. Test doses were 0, 25, 50, and 125 mg/kg ENF given twice daily as a subcutaneous injection. In the *Streptococcus pneumoniae* study, animals were treated with enfuvirtide for 28 days, infected on day 29, and sacrificed at 1, 4, and 24 hours post-infection. In the influenza virus study, animals were treated for seven days prior to infection, and groups were sacrificed at several time points for up to 21 days while continuing enfuvirtide treatment. Groups were composed of 10 females per dose per sacrifice time point. Each study contained a positive control group. The primary endpoint for each study was clearance of the infectious agent. Toxicokinetics were conducted in satellite animals. Cytokines and influenza-specific immunoglobulin G responses were determined in the influenza study. Neither study revealed an immunosuppressive effect of enfuvirtide treatment as clearance of the infectious organism was not impaired in the enfuvirtide-treated rats. Marked impairment of clearance of the infectious agent was observed in animals treated with the positive control agents. Cytokine responses in the influenza study were somewhat variable, but did not suggest any clear treatment-related effects of ENF. Similarly, the IgG levels were not consistent with a treatment-related effect, as levels were lowest in the low-dose treatment group, but similar to or greater than vehicle-controls in the middle and high dose groups. Exposure to ENF was confirmed by the toxicokinetic analysis. In total, these results suggest that enfuvirtide is not immunotoxic in rat host resistance models.

## EFFECTS OF METHYLPREDNISOLONE ON CELL PHENOTYPES IN A MURINE CANDIDA HOST DEFENSE MODEL.

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*Candida albicans* are dimorphic fungi that establish commensal relationships at mucosal surfaces of healthy individuals but have the potential to be pathogenic in immunocompromised hosts. The opportunistic feature of candidiasis and inclusive roles of different arms of the immune system in host defense makes this a compelling model for evaluating potential drug-dependent immunotoxicity. A murine host defense model, developed for evaluating innate and adaptive immunity by inoculating *C. albicans* intravenously (IV) or intramuscularly (IM), was used to measure changes in specific leukocyte subpopulations in the spleen and draining lymph nodes, respectively, by flow cytometry. Methylprednisolone was given as intraperitoneal injections at 25 mg/kg every other day to female CD1™ mice to determine the effects on the IV and IM candidiasis models. Treatment with methylprednisolone of mice inoculated with *C. albicans* IV resulted in decreased median survival time, body weight and lymphoid organ cellularity relative to control IV inoculated mice, indicating that overall host defense was impaired by this dosing regimen. Flow cytometric analysis of leukocyte subsets indicated changes in innate immune responses in mice given methylprednisolone as decreased absolute counts of MHC class II positive cells and decreased activation of neutrophils (decreased CD11b surface expression). Methylprednisolone-dependent changes in adaptive immune responses were observed in both IV and IM models as decreased lymphocyte (particularly CD4+ T cells and B cells) and dendritic cell counts in the draining lymphoid organs. Therefore, evaluation of the cellular signatures of normal immune responses to *C. albicans* infections can provide insight into the mechanisms of drug-induced impaired host defense.

AH RECEPTOR ACTIVATION ELEVATES INTERFERON GAMMA (IFN $\gamma$ ) AND INDUCIBLE NITRIC OXIDE SYNTHASE (iNOS) LEVELS IN THE LUNGS OF MICE INFECTED WITH INFLUENZA A VIRUS.

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We have previously shown that exposure of mice to the Ah receptor (AhR) ligand 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD) prior to infection with influenza A virus results in 4-fold higher levels of IFN $\gamma$  in the lung and decreases survival to an otherwise non-lethal infection. Using Ah receptor (AhR)-deficient mice, we show here that the AhR mediates the elevated IFN $\gamma$  levels in the lungs of infected mice. We also demonstrate that exposure to other AhR ligands, (polychlorinated biphenyl (PCB) 126, PCB 129 and tetrachloroazobenzene) enhances pulmonary IFN $\gamma$  levels in infected mice. These findings are significant because excessive IFN $\gamma$  production is associated with enhanced inflammation and severe tissue damage in many model systems. In addition to increasing pulmonary IFN $\gamma$  levels, exposure to TCDD alters the cellular source of this cytokine in the lung by elevating IFN $\gamma$  production by macrophages and neutrophils. In fact, we show here that the majority of the excess IFN $\gamma$  in the lungs of TCDD-treated mice comes from alveolar macrophages. Furthermore, elevated IFN $\gamma$  levels correlate with an increase in iNOS in the lungs of TCDD-treated mice. The de-regulated levels of IFN $\gamma$  and iNOS likely contribute to the pulmonary inflammation and decreased survival of TCDD-treated, virus-infected mice via a nitric oxide-dependent mechanism. Given that chronic inflammatory diseases of the lower respiratory tract are on the rise worldwide, and that high levels of both IFN $\gamma$  and iNOS have been associated with the pathology of these diseases, our data suggest that environmental exposure to AhR ligands may contribute to the development of these disorders. Thus, determining the mechanism that drives the elevated IFN $\gamma$  and iNOS levels in the lung will further our understanding of the causal relationship between activation of the AhR, dysregulated immune responses to viral infections, and these chronic inflammatory diseases.

INCREASED NUMBERS OF NEUTROPHILS IN TCDD-TREATED MICE INFECTED WITH INFLUENZA A VIRUS ARE CAUSED BY AN AH RECEPTOR-MEDIATED MECHANISM THAT IS LIKELY SPECIFIC TO THE LUNG.

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Increased numbers of neutrophils at the site of antigen challenge constitute one of the immunomodulatory effects of exposure to the aryl hydrocarbon receptor (AhR) ligand 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD). For example, exposure to TCDD doubles the number of neutrophils in the lungs of influenza virus-infected mice. Using AhR-deficient mice, we show here that the AhR mediates this increase in neutrophil number. Furthermore, *in vivo* depletion of neutrophils improves host resistance to influenza virus, indicating that the excessive pulmonary neutrophilia is detrimental to the host. We have previously shown that exposure to TCDD does not enhance neutrophil chemoattractants, up-regulate adhesion molecules on neutrophils, or delay neutrophil apoptosis in the lungs of infected mice. In this study we examined two alternative mechanisms by which AhR activation could result in excess neutrophils in the lung. First, we determined the number of neutrophils in the bone marrow and spleen, the primary sites of neutrophil production. Our results show that treatment with TCDD did not increase neutrophil numbers in these organs, suggesting that the elevated neutrophilia is specific to the lung. Second, we tested whether AhR activation increases lung injury and vascular permeability in infected mice. Lung lavage fluid lactate dehydrogenase, protein levels, and lung wet-to-dry weight ratios were not altered by exposure to TCDD, which indicates that excess neutrophilia is not due to increased lung injury and concomitant influx of neutrophils. In contrast to the lack of a direct effect on neutrophils or injury to the lung, we show that exposure to TCDD specifically enhances CYP1A1 levels in Clara cells, Type II pneumocytes and lung endothelial cells. Thus, our data suggest that lung epithelial/endothelial cells constitute potential targets for AhR-mediated deregulation of pulmonary inflammation.

$\Delta$ 9-TETRAHYDROCANNABINOL ALTERS HOST RESISTANCE TO INFLUENZA A/PR/8 INFECTION IN C57BL/6 MICE.

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The present study was designed to examine whether THC treatment decreases host resistance to influenza infection in the mouse. Mice were treated by oral gavage with 50 mg/kg THC or vehicle [corn oil (CO)] for 5 consecutive days and on Day

3 were infected by intranasal instillation with 50 plaque forming units (pfu) influenza virus A/PR/8 (PR8) or its respective vehicle, saline. Mice were sacrificed on Days 3, 7, 10, 15 and 21 post-infection (PI). Using real-time RT-PCR, changes in mRNA levels from apical and cardiac right lung lobes were determined for IL-4, IFN- $\gamma$ , and TNF- $\alpha$ . IL-4 mRNA was not detected in any of the treatment groups on any of the days. IFN- $\gamma$  and TNF- $\alpha$ , however, were significantly increased in CO/PR8 treated mice on Days 7 and 10 PI with a marked attenuation observed on Day 7 PI for IFN- $\gamma$  in the THC/PR8 treatment group, but not TNF- $\alpha$ . Cytometric bead array analysis revealed an increase in circulating serum IFN- $\gamma$  and TNF- $\alpha$  on Day 7 PI in mice treated with CO/PR8 that was attenuated by THC treatment. Treatment of mice with PR8 resulted in airway epithelial cell exfoliation on Day 3 PI with regeneration observed on Days 7 and 10 PI, followed by mucous cell metaplasia (MCM) on Days 15 and 21 PI. There is a trend toward increased alveolitis on Day 10 PI and MCM on Day 15 PI in lungs from THC/PR8 treated mice. Using image analysis and standard morphometric techniques, airway epithelial cell densities of intraepithelial mucosubstances (Vs) were determined on AB/PAS stained sections obtained from the left lung lobes. Sections from PR8 treated mice for Days 15 and 21 PI were characterized by viral-induced MCM with a trend toward increased Vs in sections from THC/PR8 treated mice. Collectively, these studies suggest that THC exacerbated the pathogenesis of influenza infection by altering host immunity. (Supported by NIH grant DA07908 and the MSU Foundation).

EXPOSURE TO THC, A MARIJUANA CANNABINOID, INDUCES ALTERATIONS IN TH1/TH2 CYTOKINE PROFILE RESULTING IN SUPPRESSION OF IMMUNITY AND INCREASED SUSCEPTIBILITY TO BREAST CANCER.

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In the current study, we tested the central hypothesis that exposure to THC can lead to enhanced growth of tumors that express low to undetectable levels of cannabinoid receptors by specifically suppressing the anti-tumor immune response. We demonstrated that the human breast cancer cell lines MCF-7 and MDA-MB-231 and the mouse mammary carcinoma, 4T1, express low to undetectable levels of CB1 and CB2 and that these cells are resistant to THC-induced cytotoxicity. Furthermore, exposure of mice to THC led to significantly elevated 4T1 tumor growth and metastasis due to inhibition of the specific anti-tumor immune response *in vivo*. The suppression of the anti-tumor immune response was mediated primarily through CB2 as opposed to CB1 expression on immune cells. Furthermore, exposure of mice to THC led to increased production of IL-4 and IL-10, suggesting that THC may specifically suppress the cell-mediated Th1 response by skewing the immune system to a Th2 response. This possibility was further supported by microarray data demonstrating the upregulation of a number of Th2-related genes and the downregulation of a number of Th1-related genes following exposure to THC. Finally, injection of anti-IL-4 and anti-IL-10 mAbs led to a partial reversal of the THC-induced suppression of the immune response to 4T1. Such findings suggest that marijuana exposure either recreationally or medicinally may increase the susceptibility to and/or incidence of breast cancer as well as other cancers that are resistant to THC-induced apoptosis. Supported by NIH grants (R01DA016545, R21DA014885, R01ES09098, R01AI053703, R01HL058641, R01AI058300, K12DA14041 and P50DA05274), The American Cancer Society (IRG-100036) and The Jeffress Memorial Trust Fund (J-741).

IMMUNE FUNCTIONAL AND HISTOPATHOLOGICAL ALTERATIONS FOLLOWING EXPOSURE IN FEMALE B6C3F1 MICE TO THE WIDELY USED VEHICLE ELMIRON®

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Elmiron is the trade name for  $\beta$ -cyclodextrin ( $\beta$ -CD), a cyclic polymer of 8- $\beta$ -1, 4-linked glucopyranosyl units, which is used in food and pharmaceutical preparations. Elmiron solutions have also been used as the vehicle for numerous toxicological studies, including studies evaluating various contaminants found in drinking water. Recent studies conducted by the National Toxicology Program have demonstrated that exposure of rats and mice to Elmiron induced infiltration of vacuolated histiocytes into multiple tissues, including the lymph nodes and spleen. Transmission electron microscopy identified these vacuoles as lysosomal structures that exhibited a variety of contents. Additional studies were conducted to determine if Elmiron altered immune function or pathology when administered to female B6C3F1 mice by gavage. Mice received doses of 0, 63, 125, 250, 500, and 1, 000 mg/kg for 28 days by gavage. An increase in liver weight was noted at the two

highest doses. With the exception of an increase in reticulocyte numbers, hematological parameters were not affected. The antibody forming cell response to sheep erythrocytes was not altered by Elmiron administration. Evaluation of the functional activity of the mononuclear phagocytic system demonstrated a slight decrease in vascular half-life of sheep erythrocytes and a significant increase in the percent uptake by the liver in the high dose animals. At histopathology, a slight to moderate presence of vacuolated histiocytes were noted in the spleen and mesenteric lymph nodes. These studies demonstrate that the extensively used vehicle Elmiron when administered at doses of 1000 mg/kg or greater may alter function and histopathology of macrophages in mice, consistent to effects reported in rat (Supported by the NIEHS Contract ES 05454).

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#### INTERACTIONS OF MERCURY WITH LIVER-STAGE RESPONSES TO MALARIA INFECTION.

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Malaria, a life-threatening parasitic disease transmitted by mosquitoes, has re-emerged in Amazonia over the past two decades. Among the many factors proposed for this re-emergence are changes in population distribution, failures of vector control and pharmacologic management, as well as local and global environmental changes including the widespread use of mercury (Hg) for gold extraction. Our previous studies with BALB/c mice have shown that pretreatment with Hg impairs host resistance to subsequent infection by sporozoites of the murine-specific *Plasmodium yoelii*. Hg also decreased production of nitric oxide by splenocytes, and down-regulated expression of both IFN-gamma and nitric oxide synthase. These effects appeared to involve hepatic mechanisms, since Hg did not affect parasitemia following infection with blood stage plasmodia. In addition, we reported that Hg pretreatment failed to prevent parasitemia in animals immunized twice with irradiated sporozoites. In these studies we explored early responses at the liver stage of infection in BALB/c mice pretreated with Hg by studying liver-stage infection and cytokine levels in animals pretreated with Hg and infected with *P. yoelii* sporozoites. At 40 hours following infection, Hg pretreatment unexpectedly decreased plasmodia in liver, detected by measurement of a plasmodial 18S rRNA sequence in the liver (specific for liver stages of *P. yoelii* parasites). However, at this time, in Hg treated mice, levels of IFN-gamma and IL-6 protein were reduced in spleen homogenates, and mRNA levels of these cytokines were reduced in liver. We also examined plasmodia in liver after a single immunization with irradiated sporozoites, followed one week later by infection with live sporozoites. In this model, we found no effect of Hg on early liver-stage immunity. These results indicate that Hg can affect early hepatic responses to malaria infection but that these effects may not predict the interactions of Hg with acquisition of immunity (defined by parasitemia).

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#### THE EFFECTS OF CIGARETTE SMOKE EXPOSURE AND PREGNANCY ON INNATE AND ADAPTIVE IMMUNE RESPONSES IN B6C3F1 MICE.

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It is well-known that cigarette smoke (CS) exposure *in utero* negatively affects the development and viability of the offspring, causing a host of defects including lower birth weight, nicotine-related neurological problems and alterations in immune function. Despite this, 20-30% of women in the United States still smoke. Although adult smokers experience some immunosuppression, the relationship between CS and the immune response has not been extensively examined for pregnant women. This study investigates the role of pregnancy on CS-induced immune modulation by examining innate and adaptive immunity in pregnant and non-pregnant B6C3F1 mice. Following inhalation of mainstream CS for 5d/wk (4hr/d from gestational d5 to parturition for the dams), inflammation was assessed via differential counts of bronchoalveolar lavage fluid and blood. Effects on tumor surveillance were evaluated *in vivo* by tumor challenge with cultured lymphoma cells and *ex vivo* by examination of cytotoxic T lymphocyte (CTL) activity. While no significant differences in lung inflammation were observed between the groups (i.e., air and CS or dams and virgins), pregnancy modulated the response to transplanted tumor cells in CS-exposed mice; tumor incidence in the smoke-exposed dams was 53.8%, while 87.5% of the exposed virgins demonstrated tumors. Furthermore, CTL activity measured in the spleens of both groups of females appeared higher in CS-exposed dams than in their virgin counterparts. These findings suggest that pregnancy may actually protect against the immunosuppressive effect of CS. Moreover, results begin to provide insight on the link between the immune response in smoking mothers and some of the adverse health outcomes affecting their offspring. Philip Morris Foundation Inc.

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#### SMOKING DURING PREGNANCY REDUCES IMMUNE TUMOR SURVEILLANCE MECHANISMS IN THE OFFSPRING: A TOXICOLOGICAL MODEL.

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Accumulating epidemiologic evidence suggests that prenatal exposure to intact cigarette smoke increases the incidence of cancer in the offspring. A study was proposed to examine the effects and underlying mechanisms of prenatal exposure to mainstream cigarette smoke (MCS) on offspring resistance to tumor challenge and surveillance mechanisms critical for the recognition and destruction of developing neoplasms. Pregnant B<sub>6</sub>C<sub>3</sub>F<sub>1</sub> mice were exposed by whole body inhalation to MCS for 5 d/wk (4 hr/d from gestational d5 to parturition) and smoke-induced effects on offspring host resistance to transplanted tumors, body/organ weight, natural killer cell and cytotoxic T-lymphocyte (CTL) activity, lymphoproliferation, cytokine levels, immune cell subpopulations and lymphoid organ histology were examined. At a concentration of smoke equivalent to smoking 2-3 packs of cigarettes, prenatally-exposed male offspring challenged at 5-wk of age with EL4 lymphoma cells demonstrated a >2-fold increase in tumor incidence (relative to age-/gender-matched air-exposed offspring). Furthermore, CTL activity in the smoke-exposed 5- and 10-wk-old male pups was significantly lower than the age-/gender-matched controls. Prenatal exposure to cigarette smoke also reduced (compared to age-/gender-matched controls) mitogen-stimulated T-lymphocyte proliferation in the 3-wk-old male offspring; circulating numbers of white blood cells and lymphocytes were increased in the smoke-exposed offspring. No effects were observed on body/organ weight, cytokine levels or immune cell subpopulations. Results demonstrate that exposure of pregnant mice to a relatively low dose of MCS decreases offspring resistance against nascent tumors and persistently reduces immune functions associated with tumor surveillance. This study suggests that children of mothers who smoke during pregnancy have a greater risk of developing tumors in later life. Supported by Philip Morris Foundation Inc.

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#### GESTATIONAL EXPOSURE TO PFOS SUPPRESSES IMMUNOLOGICAL FUNCTION IN F1 MICE.

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Perfluorinated hydrocarbons have been manufactured for over 40 years and have numerous applications in industry. This group of compounds has recently generated much interest as common metabolites (i.e., perfluorooctane sulfonate (PFOS); perfluorooctanoic acid (PFOA)) are found to be persistent in the environment, have been detected in blood samples of both wildlife and humans, and appear to have a common mechanism of action. Studies show that these perfluorinated compounds cause peroxisomal proliferation, hepatomegaly, altered steroidogenesis, and body weight decreases that are associated with a wasting syndrome; however, effects on immune function have not been extensively assessed. This study examined the immunotoxicological effects of PFOS on the developing immune system should exposure occur during pregnancy. Therefore, C57BL/6N pregnant dams (mated with C3H/HeJ males) were orally exposed with 0, 0.1, 1.0 or 5.0 mg/kg of PFOS during each day of gestation. F1 offspring were evaluated for immunological alterations at 4 and 8 weeks of age. In general, there were no treatment effects on body, spleen, or thymus mass, and limited effects on kidney and liver mass. Corresponding flow cytometric analysis of CD4/CD8 lymphocytic subpopulations in the thymus and spleen were not altered in 4- or 8-week F1 mice. However, assessment of functional immunological parameters at 8-weeks of age revealed functional deficits. Natural killer cell activity was dose-dependently suppressed in both male and female F1 adults, whereas IgM plaque forming cell (PFC) responses were only suppressed in male F1 adults. Our data indicate that prenatal exposure to PFOS can suppress functional immunological responses that are evident at adulthood, with increased vulnerability exhibited in the male F1 offspring.

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#### CHANGES IN THE OVINE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS DURING PREGNANCY AND LACTATION FOLLOWING CHALLENGE WITH ESCHERICHIA COLI LIPOPOLYSACCHARIDE.

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Lipopolysaccharide (LPS) is a gram-negative bacterial membrane component that is known to induce a systemic inflammatory response through the activation of blood monocytes and hepatic kupffer cells. These cells secrete the pro-inflamm-

tory cytokines IL-1 and 6, and TNF- $\alpha$ , which subsequently induce the hypothalamic-pituitary-adrenal axis (HPAA) to release cortisol, an anti-inflammatory hormone that assists in the regulation of the inflammatory response. We hypothesize that the ovine HPAA is attenuated in response to inflammatory stressors (LPS) during pregnancy and lactation and that this attenuation could potentially increase susceptibility to, and recovery from various bacterial induced inflammatory diseases such as mastitis and metritis. Initial studies were designed to determine various levels of HPAA activation at different LPS concentrations (0, 200, 400, 600 ng/kg) by measuring serum cortisol concentrations over time in ewes after i.v. challenge. This showed a maximal cortisol response in the 400ng/kg dose group, which was then used in the follow-up studies involving LPS challenge of ewes at different stages of pregnancy and lactation to assess whether or not the HPAA is attenuated in response to inflammatory stress. From these results we show that the HPAA is not attenuated in response to an inflammatory stressor, in fact there seems to be hyper-responsiveness of cortisol secretion over the duration of pregnancy. Unexpectedly, we also observed the thermogenic response to LPS was attenuated during early pregnancy.

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#### TETRACHLORODIBENZO-P-DIOXIN (TCDD) INHIBITS DIFFERENTIATION AND INCREASES APOPTOTIC CELL DEATH OF PRECURSOR T CELLS IN THE FETAL MOUSE THYMUS.

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TCDD causes thymic atrophy as well as alterations in thymocyte maturity in mice. Fetal mice appear to be highly sensitive to the immunotoxic effects of TCDD when it is administered during thymic development, between gestational days (gd) 10 to 18. Multiple mechanisms for thymic hypopcellularity have been suggested, including increased thymocyte apoptosis, a maturation arrest of thymocyte development, and decreased seeding of the thymus by hematopoietic progenitors. Treatment of pregnant C57BL/6 mice in these experiments with doses of 5 or 10  $\mu$ g/kg TCDD on gd 14 and 16 resulted in both decreased thymic cellularity as well as increased percentages of less mature CD4 $^+$ CD8 $^+$  thymocytes when examined on gd 18. The marker 7-AAD showed a decrease in viable thymocytes from TCDD-treated fetal mice, and a dose-related increase of thymocytes in early apoptosis. Relative to control, thymocytes from the 5 and 10  $\mu$ g/kg TCDD exposure groups displayed 1.92% and 5.32% respective increases in early apoptotic cells. Increases in apoptotic cells were primarily noted in the CD4 $^+$ CD8 $^+$  phenotype. As apoptotic cells are rapidly cleared from the thymus, these data support thymocyte apoptosis as contributing to fetal thymic atrophy after TCDD exposure. Histopathologic evaluation of the thymi demonstrated a decrease in organ size, an increase in pyknotic cells, disruption of organ architecture with a loss of distinction between cortical and medullary regions, and a decrease in cortical size in the TCDD treated feti. A decrease in thymocyte density was noted in all regions, but was marked in the cortical zones. A decrease in the frequency of mitoses in the epithelial, cortical, and medullary regions was also noted after TCDD treatment. These findings support TCDD's effects on the fetal thymus in causing thymic atrophy and increasing thymocyte apoptosis. The disruption in architecture, decreased cortical size, decreased thymocyte density, and decreased frequency of mitoses suggests a chemical effect on thymocyte maturation/proliferation as well.

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#### POST-NATAL EXPOSURE OF THE RAT TO CYCLOSPORINE DOES NOT RESULT IN PROLONGED IMPAIRMENT OF THE IMMUNE SYSTEM.

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Cyclosporine (CY) is an immunosuppressive drug widely used to prevent graft rejection in young patients. This study was performed to investigate the possible effects of post-natal exposure to CY on the development of the immune system in the Sprague-Dawley rat. 20 male and 20 female rat pups were given CY by the oral route at the dose level of 10 mg/kg/day from 4 days of age through to 28 days of age. An equal number of control pups received the vehicle (olive oil). Each dam raised 4 treated and 4 control pups. Immune tests, including blood and spleen lymphocyte subset determinations, a T-dependent antibody response to sheep red blood cells, a humoral immune response to KLH, and total IgG and IgM assays, were performed in various sub-groups of rats at intervals up to the age of 10 weeks. Lymphoid organs from different sub-groups of rats were weighed and submitted to histopathology at 2, 3, 4, 6 and 10 weeks of age. The 6-week post-treatment follow-up period was included to allow the detection of any persistent functional defects in the absence of the confounding influences of direct immune modulation. The period of exposure in the rat was intended to cover the developmental stages that occur in the human from late fetal development through to late infancy. The treated juvenile rats showed severe immune depression during exposure to CY, but recovered full immune function after cessation of treatment. These results did not suggest a developmental immunotoxic potential of CY following post-natal exposure in the rat.

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#### EFFECT OF TRIBUTYLTIN CHLORIDE ON NATURAL IMMUNODEFENCES IN THE F1 GENERATION IN MICE.

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Tributyltin (TBT) accumulated in edible fish or shellfish is suspected of having immunotoxic effects on animals. In terms of studies on the toxicity of TBT, little information is available about immunotoxicity in offspring exposed to low dose of TBT during lactation, especially about the effects on host resistance to bacteria. Thus, in this study, we investigated the effect of TBT on the natural immunodefense in offspring breast-fed by dams exposed to low doses of TBT using *in vivo* and *in vitro* experiments with *Escherichia coli* K-12 (*E. coli* K-12), a non-pathogenic bacterium. Pregnant C57BL/6 mice were given water containing 0, 15 or 50 ppm of TBT from parturition to weaning. For an *in vivo* infection experiment, offspring were i.p. inoculated with *E. coli* K-12 at weaning time, and the bacterial clearance from the peritoneal cavity and spleen was examined. For an *in vitro* experiment, to evaluate the effect of TBT on the natural immunity that plays an important role in the early stage of infection, the anti-bacterial functions of neutrophils and macrophages in peritoneal cavity, such as binding, phagocytic and killing activities, were examined. The infection experiment demonstrated that the bacterial clearance was significantly depressed in F1 breast-fed by dams exposed to 15 ppm (15 ppm F1) but not in F1 breast-fed by dams exposed to 50 ppm. The *in vitro* experiment revealed that the killing activity of neutrophils was significantly decreased in 15 ppm F1, supporting the result of the infection experiment. These results suggest that TBT impairs natural immunodefenses in the F1 generation through breast milk. We propose that it is necessary to accumulate additional information about the effect of low dose of TBT on host resistance to bacterial infection in offspring and that such maternal toxicity should be taken into account the risk assessment of TBT.

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#### ALTERED SPLENOCYTE FUNCTIONS IN FOURTEEN-MONTH OLD MICE PRENATALLY EXPOSED TO DIETHYLSТИLBESTROL.

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An excellent example of a fetal basis of adult disease is the linkage of *in utero* exposure to diethylstilbestrol (DES) and the manifestation of a variety of reproductive disorders and possibly immune-related illness in adults. While the long-term adverse consequences of prenatal DES-exposure on reproductive disorders are well known, there is a paucity of data with regards to immune outcome. We hypothesize that prenatal DES-exposure imprints on the immune system altering the response to subsequent exposure of DES in adult mice. To test this hypothesis, C57BL/6 mice were prenatally exposed to DES or vehicle only (oil) at one-year of age and alterations in the immune system examined. Female DES-exposed mice (DESprenatal/DESadult) had higher serum levels of interferon-gamma (IFNg) in response to administration of *Toxoplasma gondii* soluble antigens compared to female controls, which received oil during prenatal life (Oilprenatal/DESadult). Splenic lymphocytes from female DESprenatal/DESadult mice, when activated with Concanavalin-A (Con-A), also secreted higher levels of IFNg compared to female controls (Oilprenatal/DESadult) when examined at 14-months of age. This increase in IFNg in prenatal DES-exposed mice is not due to enhanced numbers of splenocytes or increased relative percentages of CD4 or CD8 cells. Con-A-activated T-cells from female DESprenatal/DESadult had increased expression of the co-stimulatory molecule, CD28. The above immune changes were not evident in the males prenatally exposed to DES. Further, prenatal DES exposure did not induce autoimmunity in non-autoimmune C57BL/6 mice. Overall, these results provide provocative evidence that prenatal DES exposure has long-term immune consequences as demonstrated by altered immune capabilities, which become evident following a secondary exposure to DES in adult life.

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#### T-CELL MEDIATED IMMUNITY IN ADULT SPRAGUE-DAWLEY RATS IMPLANTED WITH DEPLETED URANIUM.

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The density and tensile strength of depleted uranium (DU) make it ideal for projectiles used to penetrate armored vehicles and hardened bunkers. As exposure to heavy metals and radiation can impact reproductive, neurological, and immunological health, embedded DU shrapnel, which is radioactive and can dissolve and relocate to other tissues, may be cause for concern. This study, which is part of a multi-generational experiment, assessed the effects of embedded DU on thymic

development and T-cell function in adult Sprague-Dawley (SD) rats. Nine-week-old rats were implanted with 4, 8, or 12 DU pellets in the gastrocnemius muscle. Tantalum steel (Ta) pellets were used to bring each rat's pellet burden to 12. Only females were treated with 4 or 8 DU pellets. Rats implanted with 12 Ta pellets and untreated rats were used as negative controls. Thirty weeks post surgery rats were twice immunized (2 weeks apart) with  $2 \times 10^8$  sheep red blood cells (SRBC). Six days after the second immunization,  $1 \times 10^8$  SRBC was injected into the footpad to stimulate a delayed type hypersensitivity (DTH) response. Swelling was measured 24 h later. Upon necropsy, the thymus was evaluated for mass and thymocyte cellularity. No significant differences in the DTH response were found between control and DU groups. Thymic mass and total thymocyte numbers showed no differences between DU groups and controls for males. In females the non-surgery control had greater relative thymic mass and thymocyte counts than the DU treated groups and the Ta implanted control. Thymocyte viability did not differ among groups. In another part of this study, cyclophosphamide treatment significantly suppressed thymic mass, cellularity, and DTH response in younger adult rats, demonstrating the sensitivity of these assays for detecting immunosuppression. In conclusion, the doses of DU used in this study did not alter thymic mass, thymocyte cellularity, or T-cell mediated DTH response in 42-week-old SD rats following 33 weeks of exposure.

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IMMUNE FUNCTION IN ADULT RATS EXPOSED TO DBT IN DRINKING WATER.

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Organotins are used commercially as agricultural pesticides, antifouling agents and stabilizers for polyvinyl chloride (PVC) pipe. Mono- and di-substituted methyl and butyltins, used in PVC pipe production, are of concern to the USEPA as they leach from supply pipes into drinking water and have been reported to cause multisystem toxicity. As part of an ongoing study to evaluate immunotoxic effects of organotins, we assessed immune function after dibutyltin dichloride (DBT) exposure. Individually housed adult male and female Sprague-Dawley rats were given drinking water containing 0, 10 or 25 mg/L of DBT (final concentration) in 0.5% Alkamuls for 28 days. Water bottles were changed and water consumption was monitored twice weekly. Body weights (BW) were recorded weekly. Delayed-type hypersensitivity (DTH), primary and secondary antibody responses to sheep red blood cells, and natural killer (NK) cell activity were evaluated in separate groups on day 29. DBT consumption had no significant effect on BW although water consumption was significantly decreased in both sexes at 25 mg DBT/L, especially during the first two weeks. DTH response and NK cell activity were similar across all groups regardless of dose. Although IgM responses were similar in all groups, IgG responses were significantly elevated in males consuming 25 mg DBT/L compared to controls. Organotins are immunotoxic in adult and pre-weanling rats and although these data did not suppress the adult immune responses we examined, the elevated IgG responses we observed were similar to an enhanced antibody response observed by Smialowicz, et al. (1989, 1990) in F344 rats exposed to tributyltin oxide. This enhancement is not consistent with other studies that have demonstrated suppressed or no changes in antibody responses. Therefore, this study illustrates that the immunotoxic effects of organotins may be related to the age of the animal, exposure duration, timing of immunization, rat strain, and/or individual organotins. This abstract does not reflect EPA policy and was supported by UNC/EPA Cooperative Training Agreement CT829472.

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IN VIVO AND IN VITRO IMMUNOSUPPRESSIVE EFFECTS OF 1, 2:5, 6-DIBENZANTHACENE IN FEMALE BALB/C MICE.

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Although polycyclic aromatic hydrocarbons (PAHs) have been known to suppress immune responses, few studies have addressed the immunotoxicity of 1, 2:5, 6-dibenzanthracene (DA). In this study, we investigated the immunosuppression by DA, both *in vivo* and *in vitro*, in female BALB/c mice. To assess the effects of DA on humoral immunity as splenic antibody response to sheep red blood cells (SRBCs), DA was given a single dose or once daily for 7 consecutive days po with 30, 60 and 120  $\mu$ moles/kg. Acute oral administration of DA induced activities of hepatic ethoxresorufin *O*-deethylase and pentoxresorufin *O*-depentylase. In addition DA reduced the number of antibody forming cells (AFCs) in a dose-dependent manner. Subacute treatment with DA caused weight decreases in spleen and thymus. Interestingly, subacute DA inhibited the hepatic activity of methoxresorufin *O*-demethylase. The number of AFCs was dramatically decreased by DA in a dose-dependent manner. In a subsequent study, mice were subacutely exposed to same doses of DA without an immunization with SRBCs, followed by splenic and

thymic lymphocyte phenotypings using a flow cytometry and *ex vivo* mitogen-stimulated proliferation. DA exposed mice exhibited reduced splenic and thymic cellularity, decreased numbers of total T cells, CD4<sup>+</sup> cells and CD8<sup>+</sup> cells in spleen and immature CD4<sup>+</sup>CD8<sup>+</sup> cells, CD4<sup>+</sup>CD8<sup>-</sup> cells and CD8<sup>+</sup>CD4<sup>-</sup> cells in thymus. The number of CD4<sup>+</sup> IL-2<sup>+</sup> cells was reduced about 16%, 13% and 33%, following exposure of mice to 30, 60 and 120  $\mu$ moles/kg of DA, respectively. In *ex vivo* lymphocyte proliferation assay, DA inhibited splenocyte proliferation by LPS and Con A. These results demonstrated that DA-induced immunosuppression might be mediated, at least in part, through the IL-2 production, and caused by mechanisms associated with metabolic processes. (Supported by a grant from Korea Food and Drug Administration and a grant from KOSEF, Korea).

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EVALUATION OF THE IMMUNOMODULATORY EFFECTS OF DIBENZ(A, H)ANTHRACENE IN ADULT FEMALE B6C3F1MICE.

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Dibenz(a, h)anthracene (DBA) is an environmental contaminant classified on EPA's priority pollutant list. It is a polycyclic aromatic hydrocarbon formed by the incomplete combustion of carbon-containing compounds. For example, it has been identified in auto exhaust and cigarette smoke. The objective of this study was to evaluate the immunomodulatory effects of DBA in adult female B6C3F1 mice. Mice were exposed subcutaneously to DBA in corn oil (158, 500, 1580, and 5000  $\mu$ g/kg) daily for 28 days. Several quantitative measures and functional assays, e.g. the IgM antibody forming cell (AFC) response to the T-dependent antigen, sheep red blood cells, natural killer cell (NK) activity, anti-CD3 antibody-mediated proliferation, mixed leukocyte response (MLR), delayed type hypersensitivity (DTH) response and phenotypic analysis, were employed to evaluate humoral, innate and cell-mediated immunity. A dose-dependent decrease ranging from 45% to 100% in both total activity (AFC/Spleen) and specific activity (AFC/106 splenocytes) was observed with the three highest doses reaching the level of statistical significance. For NK activity, MLR and anti-CD3 antibody-mediated proliferation, a significant decrease of 36%, 27%, and 75% was observed at the highest dose level, respectively. The DTH response to the antigen, *Candida albicans*, was less sensitive to detect the immunosuppressive effect of DBA, and significant decreases were only observed when the dose reached the level of 40 mg/kg. The results of these studies indicate that DBA is a potent suppressor of a variety of immune functions at low exposure levels with the humoral immunity as the most sensitive. (Supported in part by the NIEHS Contract ES 05454).

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CYTOCHROME P450 1B1 (CYP1B1) IS REQUIRED FOR 7, 12-DIMETHYLBENZ(A)- ANTHRACENE (DMBA) INDUCED SPLEEN CELL IMMUNOTOXICITY IN C57BL/6N MICE.

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7, 12-Dimethylbenz(a)anthracene (DMBA) is a potent carcinogen that induces immunosuppression of both humoral and cell-mediated immunity in mice and other species. Previous studies have shown that CYP1B1 is required for bone marrow toxicity produced by DMBA in mice. Therefore, the purpose for this series of studies was to determine whether CYP1B1 was required for spleen cell immunotoxicity. Female C57BL/6N wild-type and CYP1B1 knockout (-/-) mice were treated with 0, 17, 50, or 150 mg/kg (cumulative dose) DMBA in corn oil by oral gavage once a day for 5 days. Several immunotoxicological assays were used to assess the effects of DMBA on systemic immunity. These included the *in vitro* T-dependent antibody response to sheep red blood cells (SRBC) measured using a direct plaque forming cell (PFC) assay, T and B cell mitogenesis induced by Con A and LPS, and nonspecific cell-mediated immunity was evaluated using an NK cytotoxicity assay. In addition, lymphocyte subpopulations were measured by flow cytometry using specific cell surface markers. Following 5 days of DMBA treatment, the body weights and spleen cell surface markers of the wild-type and CYP1B1 -/- mice showed no significant changes. A decrease in NK activity was found at the 50 mg/kg DMBA dose in wild-type mice, but not in the CYP1B1 -/- mice. The SRBC PFC response demonstrated that the IgM antibody response was suppressed by DMBA in wild-type mice in a dose-dependent manner (significant at 50 and 150 mg/kg). However, there were no changes in the SRBC PFC responses in any DMBA test group in the CYP1B1 -/- mice. Similarly, while DMBA suppressed B and T cell mitogenesis at the 50 and 150 mg/kg dose levels in C57BL/6N wild type mice, no effect was seen in CYP1B1 -/- mice. Thus, CYP1B1 appears to be critical for the immunosuppression of DMBA in mice, suggesting a role for bioreactive metabolites in the spleen cell immunotoxicity produced by DMBA. Supported by ROI ES05495 and P30 ES012072

NONCOPLANAR PCB-INDUCED IMMUNOTOXICITY  
IN A FISH MODEL IS NOT AHR-MEDIATED.

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Binding to, and activation of, the aryl hydrocarbon receptor (AhR) is well-defined and believed to be a pre-requisite for polychlorinated biphenyl (PCB)-induced toxicity, including immunotoxicity. In recent years studies have emerged in which noncoplanar PCB congeners have been shown to alter both innate and cell-mediated immune parameters in mammals. The objective of this study was to determine the impact of a noncoplanar (PCB 153) and coplanar (PCB 126) PCB congener on the immune response and to investigate an AhR-independent mechanism of PCB-induced immunotoxicity using a laboratory fish model. Bluegill sunfish, injected intraperitoneally (i.p.) with either 0.01 or 1.0  $\mu$ g PCB 126/g BW or 5.0 or 50.0  $\mu$ g PCB 153/g BW, were sacrificed after 3, 7, 14 or 21 d and hepatic CYP1A induction, phagocyte-mediated superoxide ( $O_2^-$ ) production and T- and B-lymphocyte proliferation were measured. Results demonstrated that while PCB 126 significantly increased hepatic CYP1A induction at all post-injection time points, immune parameters were unaffected by exposure. Conversely, in the absence of CYP1A induction, treatment of fish with 50.0  $\mu$ g PCB 153/g BW suppressed T- and B-lymphoproliferation for up to 7 d and increased phagocyte-mediated extracellular  $O_2^-$  production at 3 d post-injection. Based upon these results, studies were initiated to examine the role of the neuroimmune axis, specifically the serotonergic component, as a potential mechanism of PCB-induced immunotoxicity. Preliminary studies using parachlorophenylalanine (a specific inhibitor of the rate-limiting enzyme in serotonin [5-HT] synthesis, tryptophan hydroxylase) to determine if 5-HT is important for mounting an immune response have demonstrated that similar to that observed for other fish and mammals, 5-HT is important for regulating immune function in bluegill. Studies are ongoing to determine how exposure to PCB impacts this neuroimmune balance. Hudson River Foundation Graduate Fellowship and USACEHR No. DAMD 17-99-9011.

## METALLOTHIONEIN INFLUENCES ON IMMUNE CAPACITY IN METAL-STRESSED SYSTEMS.

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Metallothionein (MT) is a small molecular weight thiol rich protein. This protein associated thiols confer to MT its capacity to protect against heavy metal toxicity and oxidative stress. Moreover, MT also alters the immune response. Studies conducted in our laboratory have shown that MT plays a immunosuppressive role in an adaptive immune response. Antibody responses to T-dependent antigens are decreased when antigen is injected concurrently with MT. Similarly, challenging Mt-1/Mt-2 null mice with T-dependent antigen, or challenging in the presence of anti-MT antibody result in increased antibody levels. In this report, we examine the effects of MT gene dose on the inflammatory process. C57BL/6J (WT), B6-Mt-1 transgenic (B6-TGN) and B6-Mt-1/Mt-2 null (B6-KO) mice were injected with sterile thioglycollate. Inflammatory peritoneal cells were harvested at 4 and 24 hours and examined for GR-1 and CD11b expression by flow cytometry. Simultaneously, the livers were also harvested, and MT levels were measured by ELISA using a monoclonal anti-MT antibody (UC1MT). The total number of inflammatory cells responding to TG stimulus does not differ amongst the three strains at 4 and 24 hours after TG injection. However, the expression of CD11b did vary according to MT gene dose. Liver MT increased in proportion to MT gene dose at 4 hours, persisted at 24 hours in proportion to MT gene dose. *In vitro* treatment with H2O2 also illuminated differences in oxidant management and anti-oxidant enzymes (catalase and superoxide dismutase) between the three strains. Alteration in available MT may alter both intrinsic and inflammatory environments, which in turn would alter the expression of oxidant-sensitive molecules such as CD11b. These studies suggest that MT gene dose and MT protein levels play a complex but important role in management of immune and inflammatory processes. Supported by NIEHS ES07408 and NIEHS ES25490.

THE PARADOXICAL EFFECTS OF LEAD IN IFN $\gamma$ KNOCKOUT (KO) BALB/C MICE.

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Lead (Pb) exposure enhances IL-4 and inhibits IFN $\gamma$  production in wild type (WT) BALB/c mice. Here, we examined Pb effects on immunity in IFN $\gamma$ KO mice. WT and IFN $\gamma$ KO BALB/c mice (2-4 mon-old) were i.p. injected with PbCl<sub>2</sub> (50  $\mu$ g/100  $\mu$ L saline) 3x/wk for 3 wk beginning on day 1. Mice were immunized i.p. with KLH (100  $\mu$ g) at day 7 and day 14. Blood was collected at day 21; KLH delayed-type hypersensitivity (DTH) was performed at day 35. After DTH assessment, splenocytes (SPL) or popliteal lymph node cells (PLN) were assayed *in vitro* for cytokine production. Other mice were immunized with DNFB and assessed on

day 5 for contact hypersensitivity (CHS) after 24 hr. Pb significantly increased IgG1 anti-KLH levels in WT mice ( $30.3 \pm 13.2 \mu$ g/mL) compared to the controls ( $16.8 \pm 8.6 \mu$ g/mL); IgG2a anti-KLH levels also were increased by Pb. In addition, total IgE level was increased, but not IgE anti-KLH. In IFN KO mice, the IgG1 and IgG2a anti-KLH and total IgE levels were significantly lower than those of WT mice. Surprisingly, Pb significantly enhanced IgG1 and IgG2a anti-KLH and total IgE levels in the IFN KO mice. However, unlike with WT mice, Pb caused a greater percent increase of IgG2a than IgG1 anti-KLH, indicating enhanced Th1 immunity. Pb also enhanced the KLH DTH response in WT mice (control,  $0.22 \pm 0.1$  mm; Pb,  $0.41 \pm 0.09$  mm). Not surprisingly, minimal KLH DTH occurred in IFN KO mice ( $0.06 \pm 0.04$  mm); however, Pb induced a strong KLH DTH ( $0.32 \pm 0.1$  mm). In these mice, IL-4, IL-5 and IL-10, but not THF, IL-2 and IL-12, were increased in SPL and PLN supernatants. In contrast to KLH, DNFB CHS was detected in all groups, and Pb only slightly enhanced CHS. As with previous findings, Pb enhances Th2 responses in WT mice; however, Pb promoted type-1 immunity in the IFN KO mice, and enhanced KLH DTH > DNFB CHS, which is more CD8 T cell dependent. Supported by NIH grant ES11135.

## ROLE OF COPPER CHELATION, ALTERATION OF RED-OX BALANCE, AND STRESS MEDIATORS IN MODULATION OF CYTOKINE RESPONSES BY SODIUM METHYLDITHIOCARBAMATE.

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Sodium methylthiocarbamate (Metam Sodium, SMD) is the third most abundantly used conventional pesticide in the US. In this study, potential mechanisms by which SMD alters LPS-induced cytokine production in the peritoneal cavity of mice were examined. On the basis of the chemistry of SMD or preliminary data presented here, three mechanisms were selected for evaluation. They were evaluated by administration of 1) SMD or an equivalent amount of SMD-copper chelation complex to female B6C3F1 mice, 2) by administration of N-acetyl cysteine (NAC), which increases the amount of reduced glutathione (GSH) prior to SMD, and 3) by administration of a glucocorticoid antagonist (RU 486) and a beta-adrenergic antagonist (nadolol) prior to SMD to prevent the actions of important stress mediators. The combination of RU 486 and nadolol partly and significantly prevented the SMD-induced reduction of peritoneal IL-12 production in LPS-treated mice. In contrast, NAC exacerbated both the decreased production of IL-12 and the increase of IL-10 caused by SMD. The copper complex of SMD caused a greater increase in IL-10 than SMD, but it caused no suppression of IL-12, in contrast with SMD, which significantly suppressed IL-12. In addition, the effects of the major breakdown product of SMD, methyl isothiocyanate (MITC) on production of IL-12 and IL-10 were examined. MITC (at an equimolar dosage compared to SMD) caused a similar suppression of LPS-induced IL-12 and a greater increase in IL-10 in the peritoneal lavage fluid. These results indicate that all three of the hypothesized mechanisms of SMD action are probably involved in the dysregulation of TLR4-mediated cytokine production. However, MITC does not chelate copper and cannot act as a reducing agent (whereas SMD can), and it may act by an independent mechanism, such as formation of adducts with signaling proteins. This work was supported by NIH grant ES09158.

## SODIUM METHYLDITHIOCARBAMATE SUPPRESSES INNATE IMMUNITY: ROLE OF TLR SIGNALING AND CYTOKINE PRODUCTION.

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Sodium methylthiocarbamate (Metam Sodium, SMD) is the third most abundantly used conventional pesticide in the US. The study described here was done to determine if SMD acts to inhibit innate immunity induced through Toll-like receptors (TLR) and if inhibition of TLR signaling could be one of the mechanisms for this effect. Female B6C3F1 mice were treated with SMD (200 or 300 mg/kg, in water by oral gavage) 30 min before administration of lipopolysaccharide (LPS, 60 micro g/mouse, iv). Three hr later, cytokine concentrations in peritoneal lavage fluid, cellular lysates of peritoneal macrophages, and serum were determined using ELISA kits. In separate sets of mice treated in the same manner, signaling parameters in peritoneal macrophages were analyzed by Western blot or BioRad multiplex bead array 30 or 90 min after LPS. Cytokine mRNA in the same cells was analyzed by RNase protection assay. Finally, resistance to infection was assessed by administration of an LD40-LD100 dose of indigenous Escherichia coli. The results demonstrate that SMD decreased the induction of IL-12 in serum, peritoneal fluid, and macrophage lysates and increased the induction of IL-10 in the same samples. Changes in mRNA for these cytokines were similar to the changes in protein. This was associated with decreased activation of kinases late in the TLR4 signaling pathway (p38, JNK, and ERK), but not early in the pathway (IRAK-1). Activation of

AP-1 transcription factor was significantly inhibited, but the translocation of the p65 component of NF-kappaB to the nucleus was not inhibited. Resistance of mice to *E. coli* peritonitis was decreased significantly as indicated by lower survival times and decreased rates of survival. These results indicate that SMD is a potent modulator of TLR signaling and cytokine production, and it suppresses innate resistance to *E. coli*. An experiment with specific kinase inhibitors suggests that inhibition of TLR signaling may be the mechanism by which SMD modulates cytokine expression. This work was supported by NIH grant ES09158.

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IDENTIFICATION OF BIOMARKERS FOR DRUG-INDUCED STRESS: COMPARISON OF MOUSE AND RAT MODELS.

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Biomarkers for stress are needed to determine if signs of immunotoxicity that are detected in routine safety testing are caused by a non-specific stress response rather than direct immunotoxicity. The objective of this study was to compare the stress response in female (Sprague-Dawley) rats to that observed previously in female (B6C3F1) mice. Oral administration of ethanol at 5 g/kg in rats increased serum corticosterone levels from 1-12 hr after dosing and yielded an area under the corticosterone concentration vs. time curve (AUC) of 3328 ng/ml\*hr. Administration of ethanol at 5 g/kg in mice yields an AUC value of 1033 ng/ml\*hr, and dose of 6 g/kg yields an AUC value of 2239 ng/ml\*hr. In mice, ethanol at 6 g/kg significantly decreases the expression MHC class II proteins on cells in the spleen 24 hr after dosing. However, the larger stress response (corticosterone AUC) induced by ethanol in rats did not cause significant changes in MHC class II expression in the spleen or on B cells in the blood. The percentage of blood neutrophils was increased significantly in both species, but the increase was greater in mice than rats. The results for exogenous corticosterone administration were similar. The corticosterone AUC value for rats given one dose of corticosterone at 20 mg/kg was 1380 ng/ml\*hr and the value for rats given 30 mg/kg was 2852 ng/ml\*hr. The corticosterone AUC value for mice given corticosterone at 18 mg/kg was 3268 ng/ml\*hr. Neither dosage in rats affected MHC class II expression in the spleen or the blood, whereas significant decreases in MHC class II occurred in mice. At 24 hr, there was a small, significant decrease in thymus cellularity in mice, but not in rats. There was an increase in blood neutrophils in rats and a larger increase in mice. Thus, several immune parameters that are sensitive to corticosterone and ethanol-induced stress in mice are less sensitive in a rat strain commonly used in pre-clinical testing. This work was supported by a grant from Pfizer, Inc.

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ROLE OF CORTICOSTERONE IN STRESS-MEDIATED SUPPRESSION OF TUMOR RESISTANCE.

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Natural killer (NK) cells are involved in resistance to metastatic tumors. Many types of stress, including that induced by chemicals, reduce NK cell function. These experiments were performed to test the hypothesis that corticosterone (Cort), a hormone released during stress, exogenously administered to mimic an active stress response, is involved in NK cell-mediated resistance to metastatic tumors. Additionally, ethanol (EtOH), which increases Cort levels, was used as a model of chemically-induced stress. EtOH decreased ex-vivo NK cell killing of YAC-1 cells in Fischer-344 (F-344) rats, as measured by Cr-release assay. In one experiment, lytic units (LU) were  $29.0 \pm 1.13$ ,  $22.66 \pm 0.41$ , and  $12.97 \pm 0.44$  (mean  $\pm$  SEM) for naive, vehicle and oral EtOH groups (5g/kg), respectively (significance between naive and vehicle groups was  $p < 0.01$ , whereas, that between the EtOH and either naive or vehicle was  $p < 0.001$ ,  $n=3$ ). In the other experiment, LU were  $10.12 \pm 1.18$  and  $5.22 \pm 0.67$  for vehicle and EtOH groups ( $p < 0.01$ ,  $n=4$ ). *In vivo* NK cell function was evaluated by injecting MADB106 cells into F344 or Sprague-Dawley (SD) rats receiving Cort (20 mg/kg, or vehicle), or SD rats treated with EtOH (or vehicle). After two weeks, lung tumor nodules were counted. SD rats treated with Cort developed  $19.0 \pm 1.8$  lung tumors, and those treated with vehicle had  $8.6 \pm 1.1$  ( $p < 0.001$ ,  $n=4-5$ ). SD rats treated with EtOH had  $28.0 \pm 8.8$  tumors, and vehicle-treated animals had  $1.0 \pm 0.77$  ( $p < 0.01$ ,  $n=4-5$ ). F344 rats treated with Cort developed  $31 \pm 1.5$  lung tumors while vehicle animals had only  $20 \pm 1.5$  ( $p < 0.0001$ ,  $n=3-5$ ). These results indicate that both EtOH and exogenous Cort significantly reduce the ability of NK cells to kill target cells, and this has implications with regard to decreased resistance to metastatic tumors. They also suggest a role for Cort in this process. Next, we will further evaluate Cort involvement by blocking Cort activity or synthesis to confirm its involvement in this type of stress-induced decrease in NK cell mediated immunity. This work was supported by the Office of Multicultural Affairs, LSUHSC-S.

900

INVESTIGATION OF HABITUATION OR TOLERANCE IN THE STRESS RESPONSE AND IMMUNOMODULATORY ACTIONS OF ETHANOL.

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Acute administration of ethanol (EtOH) suppresses a number of immune parameters, and some of these effects are mediated by the vigorous stress response induced by EtOH. Some reports indicate that chronic EtOH administration in a liquid diet or in drinking water does not induce a stress response. However, there is uncertainty as to whether these models or a large dose of EtOH once a day would be more representative of the drinking patterns of alcohol-dependent persons. In the present study, the effects of daily administration of EtOH (4, 5, 6, and 7 g/kg by gavage) for 28 days were compared to the effects of a single dose of EtOH at the same dosages, as determined in previously published studies. In both cases, serum corticosterone concentrations were measured at 2, 4, 6, 8, and 12 hr after EtOH and immune parameters were measured 12 hr after EtOH. After the first dose of EtOH, there was a significant increase in corticosterone, which persisted for 8 hr. However, after the 28th daily dose, the increase in corticosterone was minimal and only persisted for 1 hr. As expected, immune parameters known to be suppressed by stress responses, such as the expression of MHC II on B cells and the lymphocyte to neutrophil ratio in the blood in the blood were affected less after 28 days of dosing than after a single dose. However, parameters for which suppression is not primarily mediated by the stress response, such as natural killer cell activity in the spleen, were affected to a greater extent after 28 days of dosing than after a single dose. These results demonstrate that mice become habituated (or tolerant) to the stress-inducing effects of EtOH but not to other immunotoxic mechanisms. This work was supported by NIH grants AA09505 and ES09158.

901

ACUTE AND CHRONIC EFFECTS OF ETHANOL ON MACROPHAGES.

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Alcohol consumption is a risk factor for infections. There is evidence for different effects of chronic alcohol drinking versus binge drinking on the outcome of patients with infections and/or cardiovascular diseases. Previous study results in our lab have demonstrated that acute ethanol (EtOH) interferes with the innate immune response. However, it is not clear whether chronic ethanol has the same effect. The purpose of the present study is to investigate the mechanisms by which chronic and acute ethanol affect LPS-induced cytokine production and TLR4 signaling. Mice or RAW264.7 cells were treated with lipopolysaccharide (LPS), which induces cytokine production through TLR4. Some groups of mice received acute EtOH by our binge drinking model and/or chronic EtOH at 20% (wt/vol) in the drinking water as the sole liquid source for 4 weeks. For cell culture, ethanol was directly added to the medium acutely or for 24/48 hours. The results demonstrate different effects of chronic ethanol versus acute ethanol on LPS-induced cytokine production *in vivo* as well as *in vitro*. Chronic EtOH induces over-production of cytokines in mice or RAW264.7 cells stimulated with LPS. Mice or RAW 264.7 cells previously exposed to chronic EtOH treatment became resistant to the acute suppressive effect of EtOH. EtOH, at least in part, has a direct effect on LPS-induced TNF-alpha production *in vivo*. These results indicate that acute and chronic EtOH act by different mechanisms to modify TNF-alpha expression. An initial experiment indicates that acute EtOH may act in the *in vitro* system by disrupting lipid rafts, which are known to be involved in TLR4 signaling. It is not known if this is also the case for chronic EtOH, but we speculate that chronic EtOH may induce membrane adaptations that prevent the effects of acute EtOH on lipid raft structure. Considering that septic shock is the number one cause of death in intensive care units and cardiovascular disease is a common cause of death in the US, these observations may have practical significance. This work was funded by NIH grants R01 AA009505 and R01 ES09158.

902

EVALUATION OF FLOW CYTOMETRIC ENDPOINTS FOR THE LYMPH NODE PROLIFERATION ASSAY (LNPA).

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The LNPA is being developed for use in detection of the potential for systemically administered drugs to cause clinical hypersensitivity reactions. This method, modified from the local lymph node assay, uses *in vivo* uptake of 3H-thymidine in the superficial cervical lymph node as the endpoint. The research reported here was performed to determine whether changes in lymphocyte subpopulation numbers or cell surface marker expression could be used as a non-radioactive endpoint in the LNPA. Groups of BALB/c mice were injected s.c. with saline or test drugs for three days, rested for two days, and sacrificed. The drugs tested were: assay control: strep-

tozotocin; negative - phenobarbital, metformin; positive - nevirapine, abacavir, lamotrigine, ofloxacin, zomepirac, clonidine, procainamide, and sulfamethoxazole. Leukocytes from draining lymph nodes and peripheral blood were analyzed for changes in cell surface proteins using flow cytometry. These markers were initially measured in lymph node cells: sIgE, IgA, CD45R/B220, CD4, CD8, CD25, TcR-beta, CD62L, CD44, CD71, CD54, CD86. In the lymph nodes, changes were seen in cell numbers only with streptozotocin, lamotrigine, abacavir and nevirapine. Changes in the percent of B220+ cells were seen with nevirapine, and lamotrigine. Alterations among T-cell populations were observed with nevirapine, lamotrigine, and abacavir with changes in proportions of CD4, CD8 and CD62L populations. Significant changes in absolute numbers among T-cell populations were seen only with streptozotocin and nevirapine, due to the larger variability in lymph node cell counts. No changes in CD25, CD44, CD71, CD54, or CD86 were seen with any drug. Changes in percentages of cells in peripheral blood were also seen following treatment with abacavir, lamotrigine, nevirapine, and ofloxacin. The expression of CD62L was increased only with nevirapine treatment. The presence of the alterations in expression of cell surface proteins and in the proportions of cells in both lymph nodes and peripheral blood are in general agreement with the results of the <sup>3</sup>H-thymidine-uptake results.

## 903

### ASSESSMENT OF EARLY IMMUNOLOGICAL PARAMETERS IN THE POPLITEAL LYMPH NODE ASSAY TO DETECT IMMUNOMODULATING DRUGS.

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Some drugs have the ability to induce immune-mediated drug hypersensitivity reactions in susceptible individuals. Validated predictive screening tools to evaluate these undesired side-effects are presently unavailable. The popliteal lymph node assay (PLNA) with reporter antigens (RA) is a promising candidate for this purpose. The present study was designed to provide valuable mechanistic information on the very early induction phase of drug-induced type 1 and type 2 immune reactions in the PLNA. In addition, we evaluated the use of potential predictive readouts in this assay. The anti-rheumatic drug D-Penicillamine (D-Pen) induced a type 2 response, characterized by an early influx of dendritic cells (DC) and B cells that expressed CD40, CD54, and CD86, but no CD80. First DC, but later B cells appeared to function as APC and plasma cells produced IgG1 in the presence of IL-4. Streptozotocin (STZ; chemotherapeutic drug) induced a type 1 response, showing an increase in activated CD8 cells (high expression of CD25 and CD69) and macrophages, accompanied by high concentrations of IFN- $\gamma$  and IL-12 from day 2 after exposure. CD40 expression was absent, but CD54, CD80, and CD86 were present predominantly on non-B APC, presumably macrophages. Apparently, these type 1- and type 2-inducing drugs produce drug-specific patterns of immune adjuvanting factors that stimulate very different, time-dependent profiles of cells, cytokine production, and expression of costimulatory molecules very shortly after the initial exposure to drugs. The parameters obtained with the PLNA provide information about the immunological mechanisms in the early induction phase of drug-induced hypersensitivity and may be useful in the development of an appropriate screening test to predict the allergenic potential of drugs in a preclinical phase.

## 904

### INVESTIGATING THE MECHANISM OF NEVIRAPINE-INDUCED SKIN LESIONS IN THE FEMALE BROWN NORWAY RAT.

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Nevirapine, a non-nucleoside reverse transcriptase inhibitor, often results in a severe idiosyncratic rash in patients. We previously found that nevirapine can cause a skin rash in Brown Norway (BN) female rats. The skin rash is immune-mediated because sensitivity can be transferred from a sensitized animal to a naïve animal with spleen cells. We set out to further characterize this novel animal model. In studies addressing the role of various leukocytes, we treated BN female rats for 5, 8 or 22 days. After primary exposure, followed by a month of a drug-free period, several rats were re-exposed to nevirapine for either 1 or 9 days. Upon completion of each treatment period, we used flow cytometry analysis to determine the change in cell populations present in the rat spleens, auricular and mesenteric lymph nodes (ALN, MLNs). Numbers of CD4/T cells decreased in the spleen, ALNs and MLNs, while CD8/T cell numbers increased in the ALNs and MLNs on primary and in the spleen on the secondary exposure. Activated T cells, B cells and macrophages were observed in the spleen, MLNs and ALNs. B cells and macrophages also expressed MHC II, CD80 and CD86 surface receptors. Low dose treatment for 2 weeks or co-treatment with tacrolimus led to tolerance to a dose that would otherwise lead to a rash. We suspected that this was immune tolerance, but both treatments also led to lower concentrations of nevirapine so it could be metabolic tolerance. However, when the tacrolimus co-treated rats were exposed

to aminobenzotriazole, a known P450 inhibitor, drug plasma levels were higher than previously observed in the sick animals, without making rats sick. This indicated the potential immune nature of tolerance, which when further investigated appeared to be long term, as final rechallenge of animals with nevirapine for 2 weeks, resulted in only 1 out of 6 animals developing the skin rash. These studies further characterize the mechanism of nevirapine-induced skin rash. (This research was supported by a grant from the Canadian Institutes of Health Research).

## 905

### ASSOCIATION OF ACETAMINOPHEN-INDUCED HEPATOTOXICITY WITH IMMUNOSUPPRESSION.

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Current evidence suggests that drug-induced liver injury is often caused by an allergic response (drug-induced allergic hepatitis, DIAH) induced by hepatic drug-protein adducts. The relatively low incidence of these reactions has led us to hypothesize that tolerogenic mechanisms prevent DIAH from occurring in most people. Here we present evidence for the existence of one of these regulatory pathways. When wild type and highly susceptible IL-4/-IL-10/- mice were treated with 300 mg/kg or 80 mg/kg of APAP, respectively, liver injury was concomitant with an influx of lymphocytes, macrophages, and granulocytes. Histological and flow cytometric analyses of the lymphoid organs (thymus, spleen, and lymph nodes) revealed that APAP treatment induced lymphocytolysis in both the T- and B-cell zone areas that was attributed in part to apoptosis. No signs of lymphocytolysis were apparent in the absence of significant hepatotoxicity. The decrease in cells in the lymphoid organs was associated with an increase of mononuclear cells infiltrating the liver. Following APAP treatment, the levels of IL-2, TGF-beta, IL-12, and IL-6 mRNA were decreased in liver mononuclear cells, suggesting that the infiltrating cells may not be capable of mounting an immune response. These findings suggest that the widespread lymphocytolysis following a hepatotoxic dose of APAP may inhibit an adaptive immune response to APAP adducts, thereby accounting for the lack of APAP-induced hypersensitivity reactions in most patients.

## 906

### ANTIBODIES AGAINST CYP2E1 AFTER EXPOSURE TO 1, 1, 1-TRIFLUOROETHANE AND 1, 1, 1, 2-TETRAFLUOROETHANE.

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Hydrofluorocarbons (HFC) are used as substitutes for the ozone depleting chlorofluorocarbons, e.g. for refrigeration. Associations between high exposure to refrigerants and pain in joints and influenza-like symptoms have been shown for refrigeration mechanics. These symptoms may be signs of autoimmune reactions. Exposure to similar substances (e.g. trichloroethylene, vinyl chloride and the anaesthetic halothane) has previously been associated with autoimmune diseases. The HFCs are metabolised by Cytochrome P450 2E1 (CYP2E1). Our hypothesis is that trifluoroacetaldehyde formed during metabolism binds to CYP2E1. If trifluoroacetyl-CYP2E1 is seen as non-self, antibodies against the adduct and/or against CYP2E1 may be produced. Antibodies against CYP2E1 have been found in humans accidentally exposed to hydrochlorofluorocarbons, a group of substances similar to the HFCs. The aim of this pilot study was to determine if experimental and/or occupational exposure to HFCs generates measurable increases in antiCYP2E1 titres. Healthy, male refrigeration mechanics were exposed to 500 ppm 1, 1, 1-trifluoroethane (n=9) and 1, 1, 1, 2-tetrafluoroethane (n=10) at two different occasions in an exposure chamber. Plasma sampled 22 hours post exposure was screened for antibodies against CYP2E1 with enzyme-linked immunosorbent assay. The titres were compared to titres from an unexposed group of controls (n=10). The study was approved by the Regional Ethical Committee at Karolinska Institutet. There was no difference in antibody level between the exposed subjects and the unexposed controls. However, since only healthy subjects were allowed to participate, sensitive individuals may have been excluded from the study. Also, the experimental and occupational exposure levels may have been too low to generate measurable autoimmune reactions. In conclusion, antibodies against CYP2E1 could not be detected in healthy refrigeration mechanics experimentally and occupationally exposed to HFCs.

## 907

### POTENTIAL AUTOIMMUNITY OF RECOMBINANT MOUSE IL-2 AS A CANCER THERAPEUTIC AGENT IN TUMOR-BEARING BALB/C MICE.

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Recombinant interleukin-2 (IL-2) has been developed to augment the immune response against cancer since IL-2 not only stimulates T lymphocytes but also enhances natural killer (NK) cell activity. In order to evaluate the immunological

safety of recombinant mouse IL-2 (rmIL-2) in cancer therapy, renal cell carcinoma was established in the flank by s.c. injection of renca cell line. Tumor-bearing BALB/c mice were treated i.p. injections with  $2 \times 10^5$  IU rmIL-2. Even though the tumor size was diminished, there were not significant recovery of body and relative lymphoid organ weights including thymic atrophy in rmIL-2 immunotherapy. On the one hand, emergence of autoantibodies (ANA, anti-dsDNA, and anti-histone) in blood was measured after rmIL-2 treatment. The results showed that the levels of ANA and anti-dsDNA did not significantly changed, but the level of anti-histone was increased significantly owing to rmIL-2 therapy. Distribution ratios of T cell subsets in thymus were analysed using flow cytometry. Without regard to dosage of rmIL-2, the ratio of CD3+CD4-CD8- T cells was increased in accordance with survival of solid tumor but that of CD4+CD8+ T cells was decreased dramatically. There was no effect in case of therapy with recombinant human IL-2 in tumor-bearing BALB/c mice model. These results indicate rmIL-2 immunotherapy is to induce the autoimmune potential, and the anti-histone measurement as a biomarker of autoimmunity is useful in cancer immunotherapy.

## 908

### SILICA FAILS TO INDUCE FIBROSIS IN SCAVENGER RECEPTOR DEFICIENT MICE.

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The role of the class A scavenger receptor (SR-A) has been well studied with regards to recognition and internalization of various environmental particles; however, its link to the downstream events of inflammation and fibrosis in the lung are not well understood. In the current study, SR-A-deficient mice were used to investigate the role of SR-A on the development of inflammation and fibrosis in the murine lung *in vivo*. Consistent with current models, histopathology from 129Sv wild-type mice exposed to silica revealed typical focal lesions, interstitial thickening with increased connective tissue matrix, and cell infiltrate into air space. In contrast, SR-A deficient mice exhibited little to no deposition of collagen, yet demonstrated enhanced accumulation of inflammatory cells. This increase in inflammatory cells in SR-A -/- lungs was determined to be largely composed of neutrophils. Results also demonstrated that wild-type and SR-A -/- AM possess the ability to recognize and uptake crystalline silica, although this response is attenuated in SR-A -/- AM. These results suggest that SR-A plays a role in the initiating events of silicosis, and is important to the resolution of inflammatory cells. Furthermore, because SR-A deficient mice fail to develop fibrosis following 6 months silica exposure, these results indicate that the function of SR-A is critical to the development of fibrosis.

## 909

### COMPARISON OF CHLORDECON AND ESTROGEN EFFECTS ON PHENOTYPIC MARKERS ON IMMUNOCYTES.

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Both chlordecone and estrogen accelerate the development of systemic lupus erythematosus (SLE) in female (NZBxNZW)F1 (NZB/WF1) mice. It has been hypothesized that chlordecone and estrogen influence autoimmunity in NZB/WF1 mice through a common mode of action; that is, that chlordecone acts as an estrogen mimic. Doses of chlordecone that shorten the time to onset of SLE are generally too low to produce significant estrogenic effects as assessed through classical uterine hypertrophy measurements. However, low doses of chlordecone might nonetheless produce estrogen-like effects on immunocytes. This was tested in experiments in which ovariectomized NZB/WF1 mice were treated with 17-beta estradiol (0.025mg/kg/day) or chlordecone (0.5 and 2.5mg/kg/day) using sustained-release pellets implanted subcutaneously. Control mice received pellets with matrix only. Flow cytometric analysis after five weeks of treatment found that estrogen treatment resulted in an altered distribution of B cell subsets, with a decrease in immature transitional CD21loCD24hi T1 and an increase in CD21hiCD24lo marginal zone B cells ( $p < 0.01$  by ANOVA). Chlordecone treatment had little effect on B cell subsets. Estrogen increased the expression of the germinal center marker CXCR5 ( $p < 0.05$ ) on B cells and the percent of mature splenic CD19-CD138+ plasma cells ( $p < 0.01$ ), a trend seen only weakly with chlordecone treatment. Also, estrogen treatment decreased CD4 and CD8 T-cell populations ( $p < 0.01$ ) and CD11b+ dendritic cells ( $p < 0.05$ ), while chlordecone treatment caused a moderate dose-dependent decrease of T cells, but had no effect on dendritic cells. On the other hand, chlordecone, but not estrogen, increased the level of MHC class II expression on splenic B cells ( $p < 0.05$ ). These results indicate that low doses of chlordecone, relevant to effects on autoimmunity, affect lymphocyte populations, but the pattern of changes is different from that produced by estrogen. Supported by ES07375 from NIH.

## 910

### ADAPTATION OF THE SYSTEMIC LUPUS ERYTHREMOTOSUS PRONE (NZB X NZW)F1 MOUSE STRAIN FOR AUTOIMMUNE TOXICOLOGY EVALUATION.

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Evaluation of unintended immunostimulation that can lead to autoimmune disease is very challenging to address in both preclinical and clinical studies. In absence of established methods for immunotoxicology evaluation, the systemic lupus erythematosus (SLE) model in (NZBxNZW)F1 mice was used for adaptation of a model of autoimmunity. (NZBxNZW)F1 mice are genetically prone to spontaneously develop autoimmune disease similar to SLE in humans and disease in this strain is characterized by development of anti-DNA antibodies, proteinuria and glomerulonephritis. We monitored female (NZBxNZW)F1 mice from 18 to 36 weeks of age for single stranded and double stranded DNA antibodies at bi-weekly intervals, proteinuria at weekly intervals and glomerulonephritis at 20, 28, 32 and 36 weeks of age. Detection of specific levels of anti-DNA antibodies and proteinuria indicated end stage development of disease in this study. The correlation of these two primary endpoints enabled us to adapt this model for earlier disease detection and enhanced ethical treatment of animals. In a separate study comparing SLE prone (NZBxNZW)F1 mice with the SLE resistant strains BALB/c and CD-1, female mice were immunized with keyhole limpet hemocyanin (KLH) in Complete Freund's Adjuvant at 8, 16 and 24 weeks of age. Ten days post immunization *ex vivo* splenocyte proliferation and cytokine production in response to KLH was evaluated. (NZBxNZW)F1 exhibited overall reduced antigen specific proliferation compared to BALB/c and CD-1. (NZBxNZW)F1 also produced less IFN- $\gamma$  and more IL-10 and KC at young age that did not increase over time like that observed for the other two strains tested. The adaptation of this SLE model and identification of a distinct profile of immune responses in (NZBxNZW) F1 mice can provide a new approach to autoimmune toxicology evaluation of immunomodulatory drugs in development.

## 911

### ASBESTOS-INDUCED AUTOIMMUNITY IN C57BL/6 MICE.

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Environmental impacts on autoimmunity have significant public health implications. Epidemiological studies have shown associations between exposure to airborne silicates, such as crystalline silica or asbestos, and autoimmunity, but the etiology remains unclear. A mouse model is needed to explore mechanisms of asbestos-associated autoimmunity. In order to determine whether asbestos could lead to autoimmune responses in mice, female C57Bl/6 mice were instilled intratracheally with tremolite asbestos, wollastonite, or saline. Serum samples were collected via saphenous vein bleeds and urine was checked for protein bi-weekly for 7 months, at which time tissue and serum samples were taken following sacrifice. Tremolite, but neither wollastonite nor saline, led to cellular infiltrations and fibrosis, as measured by histology and Lucifer yellow staining. By 26 weeks, the tremolite-instilled animals had a significantly higher frequency of positive anti-nuclear antibody (ANA) tests compared to wollastonite and saline groups. Of those positive ANAs, most showed homogeneous or combined homogeneous/specckled patterns on HEp-2 cells, and tested positive for antibodies to dsDNA by ELISA. Although no glomerulonephritis was apparent according to proteinuria tests, immune complex deposition was demonstrated in the kidneys of tremolite-instilled mice. There was an overall decrease in the mean IgG serum concentration in tremolite-instilled mice. Flow cytometry demonstrated a significant decrease in the percentage of CD25 T suppressor cells and an increase in the percentage of B1a B cells in the cervical lymph nodes of the tremolite-instilled mice, consistent with changes seen in autoimmune-prone mice. These data demonstrate that tremolite asbestos led to lung fibrosis as well as immunologic changes consistent with the development of autoimmunity. This study demonstrates a murine model of asbestos-induced autoimmunity, which can be used to elucidate the mechanisms leading to these responses. This work was supported by CDC Grant #CCR822091-01 and COBRE Grant #RR-017670.

## 912

### IMMUNOTOXICITY OF DICHLOROACETYL CHLORIDE AND DICHLOROACETIC ANHYDRIDE IN FEMALE MRL +/- MICE.

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Dichloroacetyl chloride (DCAC), one of the trichloroethene metabolites, is known to induce/accelerate autoimmune response in female autoimmune-prone MRL +/- mice. DCAC is proposed to acylate proteins resulting in formation of neoantigens that can lead to an autoimmune response. Similar acylation of proteins is expected

to occur by dichloroacetic anhydride (DCAA). Therefore, to evaluate the mechanism of neoantigen formation, we compared the immunotoxicity of DCAC and DCAA. Female MRL  $+/+$  mice (5 weeks old) were intraperitoneally treated (twice/week) with 0.2 mmol/kg of DCAC or DCAA in corn oil for 6 weeks and overall immune response was evaluated in serum samples. Total IgG, its isotypes and IgM were quantified by using radial immunodiffusion, IgE was determined by ESLIA. Total IgG and IgG1 were increased in DCAC (IgG 177% and IgG1 272%) and DCAA-treated (IgG 124% and IgG1 142%) mice as compared to controls. No changes were observed in IgG2a, IgG2b, and IgG3 levels in all groups. The IgM in DCAC and DCAA groups were decreased as compared to controls. IgE was significantly increased in DCAC-treated (200%) group as compared to controls and DCAA group. Our study shows that DCAC and DCAA elicit similar immune responses, DCAC being more pronounced apparently due to its greater acylating potential.

**913**

#### IMMUNOTOXICITY OF OLEIC ACID ANILIDE AND ITS HYDROLYSIS PRODUCTS IN FEMALE MRL $+/+$ MICE.

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An epidemic of a multi-systemic disease, known as the toxic oil syndrome (TOS), was caused by edible oil denatured with 2% aniline. Oleic acid anilide (OAA) has been suggested as one of the most likely etiologic agents responsible for TOS based upon their presence in TOS-related oils. The aim of this study was to evaluate the immune response of OAA and its hydrolysis products (aniline and oleic acid). We treated female autoimmune-prone MRL  $+/+$  mice with equimolar doses of OAA, aniline or oleic acid (0.8mmol/kg), i.p., twice a week for 6 weeks. The immunoglobulins IgE, IgG and its isotypes IgG1, IgG2a, IgG2b, and IgG3 were analyzed in the serum. Also, serum levels of cytokines including G-CSF, MIP-1a, IL-4, IL-6, IL-12 P40/P70, IL-13 were analyzed by cytokine arrays and cytokine protein multiplex immunoassay. Exposure to OAA and oleic acid caused significant increases in IgG, IgG1, IgG2a and IgG2b levels as compared to aniline and control groups, whereas IgG3 value increased only in OAA-treated mice. The IgE levels in OAA (637 ng/ml), aniline (568 ng/ml) and oleic acid (473 ng/ml) groups were significantly higher than the controls (219 ng/ml). Among cytokines, only G-CSF increased in aniline (110 pg/ml) and OAA (84 pg/ml) treated groups vs. control (57 pg/ml), while decreased in oleic acid (24 pg/ml) treated group. These studies provide a complex picture, which supports the contention that OAA and/or its hydrolysis products cause perturbation in the immune response and this could be one of the potential mechanisms leading to autoimmune-type of symptoms observed in TOS-related cases.

**914**

#### A RESPIRATORY SAFETY PHARMACOLOGY ASSESSMENT OF HYDROCODONE BITARTRATE AND NALTREXONE HYDROCHLORIDE.

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The effects of hydrocodone bitartrate and hydrocodone bitartrate + naltrexone hydrochloride on the respiratory function of the albino rat were investigated in this study. Male Cr:CD®(SD)IGS BR VAF/Plus® rats were administered a single oral gavage dose of hydrocodone bitartrate at 0, 125, 250, 500, 600 or 1200 mg/kg body weight, or a combination of hydrocodone bitartrate/naltrexone hydrochloride at 125/1.5625, 250/3.125, 500/6.25, 600/7.5 or 1200/15 mg/kg body weight, at a fixed ratio for each component of 80:1. Following dose administration, surviving animals were placed in 'head out' plethysmographs to for assessment of respiratory parameters. Administration of hydrocodone bitartrate alone resulted in 11 deaths at doses ranging from 125 to 1200 mg/kg. When hydrocodone bitartrate was administered in combination with naltrexone hydrochloride, there were only 2 compound-related deaths at the high dose of 1200/15 mg/kg, indicating that naltrexone hydrochloride had an antagonistic effect on the action of hydrocodone bitartrate. Oral administration of hydrocodone bitartrate alone also resulted in a dose-related decrease in respiratory rate in these rats at  $\geq 125$  mg/kg beginning at 15 minutes post-dose. This change was similar to the respiratory depressant effect seen following administration of morphine in an earlier safety pharmacology study. When hydrocodone bitartrate was administered concurrently with naltrexone hydrochloride, the effect on respiratory rate was less pronounced and of a shorter duration than that seen in the hydrocodone-treated animals. These results show that naltrexone hydrochloride has a protective effect on the action of hydrocodone bitartrate. The presence of naltrexone hydrochloride increased the viability and alleviated the respiratory depression effect of hydrocodone bitartrate in the hydrocodone-treated animals, indicating that the combination of hydrocodone bitartrate and naltrexone hydrochloride ameliorate the toxic effects of hydrocodone bitartrate.

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#### PARTICLE OVERLOAD IN RAT LUNG FOLLOWING INHALATION TO A POORLY SOLUBLE COMPOUND.

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Inhalation of poorly soluble particles induces a range of toxicological responses in the lung as a result of particle overload, which is reported as a rodent specific event. Cmpd AVE, an interleukin-4 expression inhibitor, is a highly insoluble compound being evaluated for the treatment of asthma. As part of a preclinical safety program, a 13-week inhalation toxicity study was conducted in rats using a dry powder formulation of micronized crystalline Cmpd AVE (monohydrate) blended in lactose. Rats were exposed to ambient air (air control), lactose monohydrate powder (vehicle control) or Cmpd AVE in lactose at 0.5, 5 or 50 mg/kg/day by aerosol via nose-only inhalation exposure for 4 hours/day for 13 weeks (high dose equates to a 70 mg/g lung burden). Indices of toxicity, including histopathology endpoints, were evaluated at the end of the 13-week exposure period. Treatment-related microscopic changes were observed in the lungs and tracheobronchial and mediastinal lymph nodes at 5 and 50 mg/kg/day, and were consistent with particle overload. Microscopic lung changes consisted of increased numbers of hypertrophied alveolar macrophages containing birefringent crystalline material (interpreted as Cmpd AVE) and hyperplasia/hypertrophy of alveolar type II epithelial cells; the amount of accumulated material and associated tissue reaction was dose-related. Macrophage aggregates containing crystalline material were also present in the regional lymph nodes at 50 mg/kg/day. Raman microprobe imaging confirmed the macrophage material as Cmpd AVE. In a 14-day rat inhalation study conducted at similar doses, crystalline material in the respiratory tract was not observed. Results of the 13-week study indicate that prolonged inhalation exposure to a poorly soluble compound can induce particle overload in the rat. The human safety relevance of this rat lung change is not known.

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#### ALTERED SUSCEPTIBILITY TO 1-NITRONAPHTHALENE OF RAT NASAL MUCOSA AFTER CHRONIC OZONE EXPOSURE.

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1-Nitronaphthalene (1-NN) and ozone are ubiquitous, cytotoxic air pollutants. Increased rates of photochemical formation of nitroarenes from naphthalene have been observed in irradiated air as the concentration of ozone increases. Previous studies have shown that 1-NN toxicity in the lung is considerably higher in the ozone-treated rats compared to corresponding filtered air-treated rats. The present study was designed 1) to evaluate acute injury from 1-NN in the nasal mucosa of rats, and 2) to determine whether previous long-term exposure to ozone would alter the susceptibility of the nasal mucosa to 1-NN. Adult male Sprague-Dawley rats were exposed to filtered air or 0.8 ppm ozone for 8 hour per day for 90 days (night time exposure). On the 91st day, rats were treated with 0, 12.5, or 50 mg/kg 1-NN by intraperitoneal injection. Following filtered air, 1-NN treatment resulted in injury in the nasal mucosa of rats at the 50 mg/kg dose. In general, 1-NN injury was not cell-type specific. The nasal transitional epithelium (NTE) sustained the most inflammation and injury. Goblet cells in respiratory epithelium were unaltered. In ozone-exposed, corn oil-treated rats had prominent, wide-spread mucous cell metaplasia and an occasional hyperplasia occurred only in the NTE. In ozone-exposed rats, the NTE showed less injury from 1-NN. Chronic ozone exposure did not appear to alter susceptibility to 1-NN-induced injury in the nasal regions other than the NTE. We conclude that the nasal mucosa of rats is a target of systemic 1-NN, and previous ozone exposure markedly lessens the severity of injury in the NTE where the histologic changes induced by ozone-exposure preexist. This study suggests that mucous cell metaplasia may be an adaptive/protective cellular response which is capable of protecting the nasal mucosa from toxic insults. Supported by ES00628, ES04311, ES04699, and ES05707.

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#### QUANTITATION OF AIRWAY SPECIFIC GENE EXPRESSION OF ENDOTHELIN-1 AND IT'S RECEPTORS DURING NAPHTHALENE INJURY AND REPAIR IN THE ADULT MOUSE LUNG.

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The potential for airway repair following acute injury may be a key factor in the initiation or exacerbation of a number of xenobiotic induced lung diseases. Endothelins have important regulatory roles in normal tissues and diseases as potent vasoconstrictors / bronchoconstrictors and are mitogens for epithelial cells and other lung cells. Previous work by our laboratory has demonstrated that ET-1 protein is elevated during the proliferative phase of bronchiolar injury and repair and

that addition of ET-1 to mouse bronchioles *in vitro* stimulates epithelial cell proliferation. In this study we used a well-defined model of bronchiolar injury and repair in which acute Clara cell injury is produced in mice by the bioactivated toxicant, naphthalene. Measurements of airway specific gene expression of endothelin-1 and the Type A or B endothelin receptors (ET-1, ETR-A and ETR-B) were determined using real time RT-PCR at 6 hrs, 1, 2, 4, 7, 14, and 21 days following naphthalene injury. We found airway RNA expression for ET-1, ETR-A and ETR-B remained unchanged one day following treatment. Two days following treatment airway RNA expression decreased two to five fold and did not return to control levels by 21 days after treatment. Previous studies with this model have demonstrated that two days following treatment is when maximal airway epithelial cell proliferation occurs. Therefore, it is likely that these expression changes represent part of the mechanism involved in attenuating the cell proliferation that occurs following airway injury. It is particularly interesting is that expression levels of ET-1, ETR-A and ETR-B do not return to control levels 21 days following injury, as airway epithelial repair is complete 14 and 21 days following injury. How these sustained expression decreases will impact future injury and repair is unknown. Supported by NIEHS 04311, 05707 and by California TRDRP grants 6KT-0306, 11RT-0258.

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PROTEOMIC CHARACTERIZATION OF BRONCHOALVEOLAR LAVAGE FLUID (BALF), LUNG, AND SERUM IN A MOUSE EMPHYSEMA MODEL INDUCED BY ELASTASE.

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The purpose of this study was to characterize gene expression changes in the lung associated with elastase-induced emphysema in mice. Male C57BL/6 mice were administered porcine pancreatic elastase (0 or 37.5 U/Kg BW) by intra-tracheal instillation. Groups of control or treated mice (n = 6) were sacrificed at one or four weeks after dosing. Anatomical pathology and lung morphometry were used to evaluate the induction of emphysema. BALF, lung tissue, and serum were digested with trypsin. BALF was analyzed by cLC-FTICR-MS using the Accurate Mass and Time tag strategy; lung tissue and serum were digested and analyzed by cLC-MS/MS. The group's mean ion current for each BALF protein was used as a measure of abundance for each of the 491 proteins identified. 106 proteins were up-regulated by elastase treatment after 4 wk; 28 of these were up- and 5 down-regulated at 1 wk. 28 proteins were down regulated after 4 wk; 1 of these was up- and 6 down-regulated at 1 wk. The content of albumin in BALF was not altered, suggesting that the blood-air barrier was not significantly damaged by the elastase treatment. Proteins consistently up regulated include meltrin alpha (ADAM 12), which interacts with growth factors; surfactant pulmonary associate protein; glutathione S-transferase mu; glutathione peroxidase 1; and apolipoprotein A. Proteins down-regulated included glutathione S-transferase alpha 3; peroxiredoxin 5; and several members of the alcohol and aldehyde dehydrogenase families. 17 proteins identified as hypothetical from RIKEN cDNA were differentially abundant at 1 wk, but not 4 wk. These proteins represent a knowledge gap for understanding pulmonary function. Further analysis of this dataset, and integration with pulmonary gene profiles and metabolomic datasets from the same study, will lead to a better understanding of the elastase-induced emphysema model in mice. Sponsored by BMI IR&D.

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CHARACTERIZATION OF ELASTASE-INDUCED EMPHYSEMA IN MOUSE LUNG PER HISTOPATHOLOGY, MORPHOMETRY, AND BRONCHOALVEOLAR LAVAGE (BAL) MEASUREMENTS.

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An intra-tracheal instillation of elastase induces specific lung damage, mimicking pulmonary emphysema. However the induction regimen and characterization criteria vary among investigators, making common usage of the model difficult. The objective of this project was to establish definitive pulmonary emphysema in mice as a prototype animal model. Young C57BL/6 mice were subjected to intra-tracheal instillation of porcine pancreatic elastase (37.5 U/kg BW) or saline at ~0.07mL (6 mice/dose/timepoint). A group of mice was sacrificed at 1 or 4 wk postdosing and BAL was performed on isolated lung with PBS. The supernatant from the first two washes (BALF) were analyzed for LDH, NAG, protein, and cytokines. Cell pellets from all 6 washes were used for cytological evaluation. Another group was necropsied at 1 and 4 wk postdosing and lungs were preserved and processed for light microscopy. Lung morphometry was performed using Image Pro® Plus. There were no mortality or body weight effects throughout 4 weeks. Mild or moderate alveolar emphysema was present in all dosed mice by 1 week. Multifocal lesions were pre-

dominantly subpleural and most frequently involved the diaphragmatic lobe. This was accompanied by significant increase in mean linear intercept and lung volume. A mixed inflammatory cell infiltrate (macrophages, granulocytes, and lymphocytes) was present surrounding or adjacent to areas of emphysema. Alveolar emphysema was present at 4 weeks but less severe than at 1 week postdosing. BAL macrophage counts were significantly elevated in the dosed mice at both times. BALF NAG concentration increased significantly in the dosed group at 1 week, while BALF IL1 $\beta$  and IFN- $\gamma$  concentrations were significantly decreased at 4 weeks. In summary, the current elastase regimen produced consistent, reliable and quantifiable lung damage in mice that was sustained up to 4 weeks postdosing without notable in-life toxicity. (Funded by Battelle BSTI IR&D)

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THE USE OF LUNG MECHANICS FOR THE PHENOTYPING OF LUNG RESPONSES TO CIGARETTE SMOKE EXPOSURE IN MICE.

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Cigarette smoke exposure in mice produces airspace enlargement resembling emphysema, and the mouse has become an acceptable model for the study of the human disease. Genetic predisposition is an important determinant for the development of emphysema in humans, thus we reasoned that the availability of resistant and susceptible mice strains for the development of emphysema would be important for the study of genetic determinants in mice which could then apply to the human disease. We exposed 7 strains of mice, 6 animals and 6 controls per strain, to 2 cigarettes a day utilizing a nose only exposure apparatus during 6 months. At the end of the exposure all animals underwent lung mechanics measurements (elastance and P-V curves) while alive and anesthetized utilizing the flexiVent (SCIREQ Inc., Montreal, Canada). Animals were then sacrificed, the lungs dissected and prepared for histology. Based in the results of the mean linear intercept (Lm) and the lung mechanics we identified 3 groups of susceptibility for the development of emphysema: NZW- resistant, no Lm increase and no changes in elastance; C57BL6, A/J, SJL- mildly susceptible, Lm increase but no changes in elastance; Pallid and AKR-susceptible, increased Lm and decrease in elastance. BAL/c strain developed marked increase in elastance and decreases in Lm found to be secondary to diffuse lung fibrosis. In conclusion lung mechanics measurements in mice are essential for a proper phenotyping of emphysema. Based in these findings we have identified a resistant strain, NZW and a susceptible AKR for the development of emphysema. Furthermore using lung mechanics we have identified a strain which could become a model for lung fibrosis.

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EVALUATION OF RELATIVE CONTRIBUTIONS OF VAPOR PHASE AND PARTICULAR MATTER TO THE TOTAL CYTOTOXICITY OF CIGARETTE SMOKE.

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The purpose of this study was to evaluate the relative contribution of vapor phase and particular matter to the total cytotoxicity of cigarette smoke. Smoke was generated from research cigarettes 2R4F, US Burley, and Brazilian Flue cured cigarettes using an AMESA smoke generation system, diluted with clean air to achieve target wet total particulate matter (WTPM) concentration, and then directed to cell media containers placed within an incubator maintained at 37°C. Cytotoxicity of whole cigarette smoke or vapor phase was measured using the neutral red cytotoxicity assay in BALB/c 3T3 cells. Two experiments were performed for each cigarette and four doses (three cultures/dose) were used in each experiment. The exposure duration was one hour and the neutral red cytotoxicity assay was performed 24 hours after exposure. Dose-response curve fitting and LC<sub>50</sub> calculation for relative cell survival data were performed using the CalcuSyn program. Dose-related decrease in cell survival was observed in all three cigarettes for both whole smoke and vapor phase. The mean LC<sub>50</sub> for vapor phase were 39.3, 72.2, and 94.1 µg/L (WTPM), for 2R4F, Brazilian flue cured, and US Burley cigarettes, respectively. The mean LC<sub>50</sub> for whole smoke were 30.9, 58.0, and 90.7 µg/L (WTPM), for 2R4F, Brazilian flue cured, and US Burley cigarettes, respectively. Vapor phase contributed from 65 to 99% of total toxicity of cigarette smoke in the doses studied in these three cigarettes. The ratios of mean LC<sub>50</sub> for whole smoke versus vapor were 78.6, 80.3, and 96.4%, for 2R4F, Brazilian flue cured, and US Burley cigarettes, respectively. These results indicate that the neutral red cytotoxicity assay protocol used in current study is suitable to evaluate the cytotoxic effects of vapor phase or whole cigarette smoke, and vapor phase is the dominant factor in determining the cytotoxicity of cigarette smoke. (Funded by Battelle BSTI IR&D fund)

## LUNG INFLAMMATION IN RATS AFTER ACUTE EXPOSURE TO CIGARETTE SMOKE.

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We have reported (SOT, 2004) that acute exposure of rats to cigarette mainstream smoke induces changes in inflammatory mediators in bronchoalveolar lavage fluid (BALF). Here, we investigate how the changes in inflammatory mediators are reflected in changes in BALF neutrophils after acute and subchronic exposure to cigarette mainstream whole smoke (WS), gas-phase depleted particulate phase (PP), and gas phase (GP). Sprague-Dawley rats (8/group) were exposed to smoke from University of Kentucky filtered reference cigarettes. Acute exposure was 2 x 1 hour to WS and PP at concentrations of 0 (sham) to 1200  $\mu$ g total particulate matter (TPM)/l, or to equivalent concentrations for GP; subchronic exposure was 2 x 1 h/day for 35 days to WS at 500 and 750  $\mu$ g TPM/l, PP at 750  $\mu$ g TPM/l and an equivalent concentration for GP. Inflammatory mediators (IL-1 $\beta$ , CINC-1, CINC-3, MCP-1, fractalkine) and free lung cells (FLC) were quantified in BALF (flow cytometry) and respiratory parameters were measured (plethysmography). Acute exposure to WS and PP resulted in a concentration-related increase of inflammatory mediators. GP had no effect. Effects of PP were more pronounced than WS. At 1200  $\mu$ g TPM/l, FLC consisted of 16% neutrophils in the WS-group, 25% in PP and 2% in GP (sham: 2%). After subchronic exposure, neutrophils were concentration-dependently increased. At 750  $\mu$ g TPM/l, neutrophils were 20% higher after PP than after WS; GP was equal to sham. A reduction of 40% in respiratory minute volume was found during exposure to WS and GP; for PP, no reduction was seen. This indicates that PP-exposed rats took up more TPM, which causes the inflammatory changes, than rats exposed to WS. Effects after acute exposure were qualitatively the same as those observed after subchronic exposure. Acute inhalation exposure of rats may be a useful short-term *in vivo* assay for the evaluation of the inflammatory effects of cigarette smoke.

## FOCAL PROLIFERATIVE LESIONS IN A/J MOUSE LUNG FOLLOWING 5-MONTH EXPOSURE TO CIGARETTE MAINSTREAM SMOKE.

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Incidence and multiplicity of grossly-observed and microscopic alterations of the respiratory tract of nose-only, mainstream smoke-exposed A/J mice (50, 200 or 400 mg total particulate matter/m<sup>3</sup> from 2R4F cigarettes; 3 hr/d, 5 d/wk for 5 months) was compared with filtered air controls. Animals were necropsied at the end of exposure (5 months) or allowed to recover for an additional 4 or 7 months (9 or 12 months from initiation of exposure). Lungs were visually inspected for tumors at all necropsies and examined histopathologically at 9 and 12 months. At 5 months no tumors were recorded. At 9 months the incidence of visual tumors was similar for all groups whereas multiplicity was significantly increased in the mid-dose smoke group (0.90 versus 0.55 for controls). At 12 months, multiplicity was increased over the 9-month necropsy, but there were no dose-related trends in multiplicity or incidence. Histopathological examination of the lung revealed increased numbers of proliferative lesions and identified the presence of focal hyperplasia. At 9 months, the multiplicity of focal lung lesions was 1.4 in controls but was decreased by smoke exposure and averaged 1.0 for all smoke-exposed groups. At 9 months, the percentage of focal hyperplasia decreased with increasing smoke concentration from 62.5% to 36.4% to 16.7% for the 50, 200 and 400 mg/m<sup>3</sup> exposures respectively, compared to controls (71.4%). At 12 months the high-dose smoke exposure group had increased multiplicity of 2.3 lesions compared with 1.6 among controls and the percentage of hyperplastic lesions was similar between groups. Microscopic examination of the lung increased the number of lesions recorded and identified focal hyperplastic lesions. In addition, smoke exposure was associated with a shift from predominately focal hyperplasia in control animals to adenomas in smoke-exposed animals at the 9-month time point.

## INFLUENCE OF SUB-CHRONIC CIGARETTE SMOKE EXPOSURE ON THE PROGRESSION OF MYOCARDIAL HYPERTROPHY IN SPONTANEOUSLY HYPERTENSIVE RATS (SHR).

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In search of an experimental model for cardiac risk by smoking we exposed 8-week-old male SHR to cigarette mainstream smoke (MS) from the Kentucky Reference Cigarette 2R4F (450  $\mu$ g total particulate matter/l) or to fresh air (sham) for 30, 60,

and 90 days (2 hours/day, 5 days/week) to investigate the development of myocardial hypertrophy (heart/body weight ratio, mRNA expression of hypertrophy markers via real-time RT-PCR, and protein expression via immunoblots). Basal heart function was determined *in vitro* by Langendorff-perfusion. Blood pressure and heart rate were monitored *in vivo* via telemetric analysis. Due to a reduced increase in body weight, the MS groups had an approximately 20% larger increase in heart/body weight ratio after just 30 days ( $5.83 \pm 0.29$  versus  $4.94 \pm 0.35$  mg/g;  $p < 0.01$ ) accompanied by elevated basal heart function (left ventricular developed pressure:  $103.9 \pm 5.9$  vs.  $83.3 \pm 6.5$  mm Hg,  $p < 0.01$ ). The mRNA expression of myocardial hypertrophy markers, i.e., atrial natriuretic factor, ornithine decarboxylase, and TGF- $\beta$ , increased in both ventricles (more pronounced at 90 days than at 30 days). In the sham groups these markers were less increased in the left ventricle and did not change over time in the right ventricle. Increases in ANF and TGF- $\beta$  were confirmed on the protein level in the 90-day MS group. There were no measurable differences for blood pressure or heart rate between MS and sham groups. The mRNA expression for calcium-handling proteins (SERCA2A and NCX) did not change. At the same time, there was less  $\beta$ -adrenergic response in heart function to isoproterenol (100 nM). SHR exposed to MS showed a faster progression of myocardial hypertrophy with a reduction in  $\beta$ -adrenergic response. This effect is independent of hypertension and occurs in both ventricles. The data suggest the acceleration of cardiac dysfunction development by sub-chronic MS exposure in rats.

## CIGARETTE SMOKE ENHANCES ENDOTOXIN-INDUCED PULMONARY INFLAMMATION.

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Long-term cigarette smoke exposure induces chronic pulmonary inflammation, leading to asthma, bronchitis, and emphysema. However, the short-term effects of smoke inhalation are less clear, since they are not generally associated with significant changes in lung morphology. Indeed, acute smoke exposure may adversely affect the lung only if underlying disease is present. To test this hypothesis, Syrian hamsters pretreated with 0.2 mg E. coli endotoxin were passively exposed to cigarette smoke for 2 hrs per day over a period of 3-5 days. Controls were also given endotoxin, but continuously exposed to room air instead. All the animals were euthanized 24 hr after the last smoking session. Bronchoalveolar lavage (BAL) was then performed to measure the pulmonary inflammatory response. Compared to controls, animals receiving both endotoxin and cigarette smoke showed a 155% increase in lavaged neutrophils at 3 days (19.6% vs 7.70%;  $p < 0.05$ ) and a 203% increase at 5 days (16.47% vs 6.33%;  $p < 0.05$ ). Elevations in lymphocyte counts were also seen in the smoke-exposed group at both time points (67% and 57%, respectively). Such results indicate that short-term inhalation of cigarette smoke can enhance pre-existing lung inflammation. More importantly, these findings provide further evidence that persons with underlying lung disease may be adversely affected by even limited amounts of second-hand smoke.

TETRAHYDROCANNABINOL CAUSES PULMONARY CELL MITOCHONDRIAL INJURY *IN VITRO* AND *IN VIVO*.

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Habitual marijuana smoking is associated with chronic bronchitis and inflammation of the upper bronchial airways. We have demonstrated previously that *in vitro* exposure of lung epithelial cells to whole marijuana smoke or to tetrahydrocannabinol (THC), the primary psychoactive component of marijuana, results in decreased cellular ATP levels. Examination of mitochondria in these cells using the fluorescent potentiometric probe, JC-1, revealed THC-induced loss of mitochondrial membrane potential. This loss was only partially reversible following THC removal and was attenuated by cyclosporin A, suggesting a role for the permeability transition pore. Particulate smoke extracts from marijuana cigarettes also impaired mitochondrial membrane potential whereas tobacco or placebo (0% THC) marijuana smoke extracts produced minimal effects. We have established an *in vivo* smoke inhalation model using the rat. Using a cross-flow nose-only inhalation model system, 20 min exposure to marijuana smoke produced blood THC levels ranging from 2-15 ng/ml and lung levels of 46-76 ng/g wet wt tissue. Values varied as a function of total smoke consumed. Following *in situ* JC-1 staining of lungs by intratracheal infusion, red JC-1 fluorescence, characteristic of mitochondrial staining, was greatly diminished throughout the lungs of rats exposed to marijuana smoke. These results suggest that inhalation of marijuana smoke may have deleterious effects on lung cell energetics and may contribute to adverse health effects observed in humans. (Supported by NIH Grant R37 DA03018).

SPONTANEOUSLY HYPERTENSIVE RATS ARE SUSCEPTIBLE TO AIRWAY DISEASE INDUCED BY SULFUR DIOXIDE.

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Rodent models of chronic pulmonary diseases induced by sulfur dioxide (SO<sub>2</sub>), elastase or tobacco smoke have limited utility because of their lack of chronicity of inflammation, and they demonstrate limited sensitivity to a given experimental manipulation. We hypothesized that disease susceptibility from experimental exposures require unique genetic predisposition. Spontaneously hypertensive (SH) rats, with genetic susceptibility to cardiovascular disease, and Sprague Dawley (SD) rats were exposed: 1) nose-only to SO<sub>2</sub> at 0, 250 or 350 ppmx5h/dx4d; 2) to porcine pancreatic elastase (PPE), intratracheal (IT) at 0, 400, 800 or 1200 U/kg; or 3) to SO<sub>2</sub> (only SH rats pre-exposed to PPE at 0 or 800 U/Kg, IT, 1 week prior) at 0 or 350 ppm, 5h/dx4d. Pulmonary functional and biological impairments were evaluated. PPE caused severe pulmonary injury and inflammation in SD rats. The severity of acute injury was less in SH rats but inflammation persisted for a longer time following exposure. In contrast to PPE, SO<sub>2</sub>-induced neutrophilic inflammation was 30-40 fold higher in SH than in SD rats, and was associated with an increase in alveolar macrophages. These changes in SH rats persisted for up to 7 days. Although the inflammatory response was higher in SH rats, SO<sub>2</sub>-induced changes in breathing parameters were not significantly different between SH and SD rats. We then hypothesized that SO<sub>2</sub>-induced inflammation would be more severe in PPE-pretreated SH rats. Neither pulmonary injury nor inflammation was greater in PPE-pretreated SH rats, except for a slightly greater increase in alveolar macrophages. Similarly, the plethysmographic measurement of breathing parameters indicated marginally greater PenH values following the PPE+SO<sub>2</sub> exposure. In conclusion, SH rats demonstrated several fold greater inflammatory response to SO<sub>2</sub> than SD rats, but the strain differences were not marked in regards to effects on breathing parameters. Also, PPE-induced injury is not exacerbated by subsequent SO<sub>2</sub> exposure. (Does not reflect USEPA policy).

HOST-DEPENDENT INCREASE IN LUNG EPITHELIAL MEMBRANE PERMEABILITY AFTER O<sub>3</sub>.

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Ozone (O<sub>3</sub>), an ambient air pollutant and strong oxidant, when inhaled can lead to an inflammatory response characterized by polymorphonuclear (PMN) cell infiltration of lung tissue and PMN recruitment into the airspaces. To better understand the role of host defense in O<sub>3</sub>-induced inflammation we investigated the lung response of 9 genetically diverse inbred strains of mice (129/Svlm, A/J, BALB/cJ, BTBR, C3H/Hej, C57Bl/6J, DBA/2J, FVB/NJ, and CAST/EI) to a 3 h exposure to 2 ppm O<sub>3</sub>. Age-matched male controls were exposed to filtered air (FA). Based upon the number of PMNs in bronchoalveolar lavage (BAL) fluid collected at sacrifice, 6 or 24 h post-exposure, we selected resistant, C3H/Hej (CHJ), and a susceptible, 129/Svlm (129S), strains for further evaluation to determine if epithelial membrane integrity, was a co-determinant of host vulnerability to O<sub>3</sub>. Additional CHJ and 129S mice were exposed to FA or O<sub>3</sub> and evaluated 24 h post-exposure. BAL results replicated the differential inflammatory cell responses observed previously. Mice, not undergoing BAL, were anesthetized and were studied *in vivo* with a non-invasive radio-isotopic marker (Tc99m-DTPA) to assess epithelial membrane integrity. Transference of the permeable marker (MW = 490 dalt, molec radius = 0.57 nm) from the lung to the vasculature was monitored by scintigraphy and recorded dynamically. In O<sub>3</sub>-susceptible 129S mice, Tc99m transference half-times were decreased on average by 43% (n=6, epithelium more permeable) as compared to half-times of FA controls (n=6); whereas for O<sub>3</sub>-resistant CHJ mice, transference half-times were equivalent between FA and O<sub>3</sub> exposed mice (n=6 each). If O<sub>3</sub>-treated 129S mice were permitted to recover, Tc99m transference half-times returned to normal values within 7 days. These data suggest that O<sub>3</sub>-induced inflammatory cell responses are strain dependent, and that airway recruitment of PMNs following an acute exposure to O<sub>3</sub> may co-depend upon the integrity of the epithelial membrane. Research sponsored by NIH awards: HL62641, ES012496, ES011961.

EVALUATION OF THE SENSITIVITY TO INHALED OXIDANTS IN CSB MICE.

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To study oxidative stress as a mechanism for the health effects caused by air pollution, Cockayne syndrome B (CSB) mice were exposed to the model oxidant, ozone. CSB-/- mice are deficient in their transcription coupled repair. CSB-/- and +/- mice

were either exposed for 8 hours to 0.8 ppm ozone or clean air. Clear pathological changes in the lung due to ozone, as well an increase of neutrophils were observed in both CSB-/- and CSB+/- mice. The ozone exposed CSB-/- mice show a stronger increase in antioxidant levels (total glutathione and uric acid) and a significant increase in TNFa in broncho alveolar lavage fluid compared to their +/- wild types. These results are compared with the gene expression profile in whole lung tissue using the micro-array technique. We concluded that ozone, as a model oxidant, affects the CSB-/- mice more than its wild type counterpart. The data provide support to use this model in future studies to investigate the role of oxidative stress in health effects due to air pollution.

ZINC DEFICIENCY ENHANCES PRO-INFLAMMATORY RESPONSES AFTER OZONE EXPOSURE.

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Previous studies have demonstrated that humans are differentially susceptible to adverse health effects induced by exposure to ozone. Serum analysis have shown that the elderly (people >65 years) are deficient in several key vitamins and trace elements, including zinc (Zn). The trace element Zn is essential for the function of several key signaling molecules and antioxidant enzymes, meaning insufficient dietary Zn intake and consequently low intracellular Zn levels could potentially alter the susceptibility to air pollution. We have established an *in vitro* model of human respiratory epithelial cells (A549 cells) grown under Zn-deficient (Zn-DF) or Zn-inadequate (Zn-AD) conditions. Culturing these cells under Zn-DF conditions significantly reduces their intracellular Zn levels and the activity of key Zn-dependent enzymes, such as Cu, Zn-SOD, while the activity Zn-independent antioxidant enzymes, such as catalase and glutathione peroxidase, remain unchanged. Zn-AD and Zn-DF cells grown on membranes were exposed to 0.2 ppm ozone for 5 hours and subsequently analyzed for LDH release and the release of pro-inflammatory cytokines, such as IL-8, MCP-1, and IL-6. Ozone exposure caused similar LDH release in both Zn-AD and Zn-DF cells. Interestingly, ozone induced greater release of IL-8, MCP-1, and IL-6 into the apical compartment of Zn-DF cells as compared to Zn-AD cells, while ozone-induced cytokine release into the basolateral compartment was similar in Zn-DF and Zn-AD cells. These data suggest that Zn-DF respiratory epithelial cells are more susceptible to adverse inflammatory effects induced by ozone exposure and that ozone-induced cytokine release is greater towards the apical side than the basolateral side. Overall, these results suggest that the nutritional status of individuals could influence their susceptibility to air pollutant-induced health effects.

MECHANISMS REGULATING ACTIVATION OF MACROPHAGES IN THE LUNG FOLLOWING OZONE INHALATION.

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Studies from our laboratory have demonstrated that the toxicity of inhaled ozone is due, in part, to cytotoxic inflammatory mediators released from activated alveolar macrophages. Thus following ozone inhalation, alveolar macrophage production of nitric oxide, generated from inducible nitric oxide synthase (NOSII), tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), and prostaglandin E2 (PGE2) produced via cyclooxygenase-2 (COX-2) are increased. Moreover, blocking macrophages or inflammatory mediators abrogates ozone-induced toxicity. In the present studies we analyzed mechanisms regulating macrophage activation in the lung following ozone inhalation. Triggering receptor on myeloid cells (TREM-1) is a newly identified receptor thought to be involved in amplifying the inflammatory response by stimulating the release of proinflammatory mediators from phagocytic cells. Treatment of C3H/OuJ mice with ozone (0.8 ppm, 3 hr) caused a time-dependent induction of expression of TREM-1 mRNA in alveolar macrophages which was evident 12-24 hr after exposure. Similar activity was observed in C3H/HeJ mice which possess a mutated non-functional Toll-like receptor 4 (TLR-4), making them insensitive to bacterially derived endotoxin. These mutant mice were found to be resistant to ozone-induced toxicity, as measured by protein accumulation in bronchoalveolar lavage fluid. This was correlated with reduced expression of NOSII and COX-2 protein in the lung and delayed expression of the anti-inflammatory cytokine, IL-10, demonstrating the importance of TLR-4 in the pathogenesis of inflammation and tissue injury induced by ozone. Our findings that mutations in TLR-4 do not affect ozone-induced expression of TREM-1 suggest that there are multiple independent pathways mediating macrophage activation in the lung following ozone inhalation. Supported by NIH grants ES04738, GM34310, ES05022 and ES007148.

3 $\beta$ -HYDROXY-5-OXO-5, 6-SECOCHOLESTAN-6-AL, A MAJOR OZONATION PRODUCT OF CHOLESTEROL, INDUCES APOPTOSIS IN CARDIOMYOCYTES.

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Ozone toxicity induces pulmonary dysfunction in humans and animals. Since ozone does not penetrate far ( $<2\text{ }\mu\text{m}$ ) into the lung parenchyma, the adverse effects of ozone are thought to be mediated via the ozonation products formed at the air/lung interface. Thus, phospholipids, cholesterol, and cholesterol esters present in the epithelial lining fluids and in the alveolar cell membranes are among the primary targets for the reaction of ozone. The interaction of ozone with these lipids results in the formation of a number of toxic metabolites including 3 $\beta$ -hydroxy-5-oxo-5, 6-secocholestan-6-al (Chol-Seco), the major ozonation product of cholesterol. Most studies to date have concentrated on the effects of ozone and the ozonation products on the lung parenchyma. The present study was undertaken with the purpose of determining the extra-pulmonary effects of ozone mediated via cholesterol ozonation using cardiomyocytes derived from the embryonic rat myocardium (H9C2 cells). The H9C2 cells were treated with varying low concentrations (0-10  $\mu\text{M}$ ) of Chol-Seco for 12-24 h at 37 $\pm$ 0.5 °C. After incubation, the cells were fixed and analyzed using flow cytometry and immunocytochemistry. It was observed that Chol-Seco induces a time and dose dependent increases in cardiomyocyte apoptosis. The lowest dose of 2  $\mu\text{M}$  Chol-Seco for instance, was found to cause 14 $\pm$ 1.1% apoptosis against DMSO controls that only showed 3 $\pm$ 0.1% apoptosis. At 5 and 10  $\mu\text{M}$  Chol-Seco, the percent apoptotic cells at 24 h were 45 $\pm$ 3.8 and 52 $\pm$ 2.0, respectively. Apoptosis was also seen at 12 h and 18 h, with the highest dose inducing greater cell death. Anexin staining showed a similar pattern. These results suggest that cholesterol ozonation product(s) is/are potent inducers of apoptosis in cardiomyocytes and might play a role in ozone induced myocardial injury. (Funding support from NIEHS ES10018 and LBRN is acknowledged.

INCREASED SUSCEPTIBILITY OF HYPERTHYROID RATS TO OZONE: EARLY EVENTS AND MECHANISMS.

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Previous studies have determined that ozone-induced lung damage and inflammation are much greater in hyperthyroid vs. normal rats at 18 hours following exposure. The purpose of the present investigation was to study early events and mechanisms underlying the increased sensitivity to ozone in a hyperthyroid state. Specifically, the degree of lung epithelial cell barrier disruption, extracellular lining fluid antioxidant status, and the release of inflammatory mediators were examined. To create a hyperthyroid condition, mature male Sprague-Dawley rats were implanted with time-release pellets containing thyroxine; control rats received placebo pellets. After 7 days, the animals were exposed to air or ozone (2 ppm, 3 h). Immediately following the end of the exposure, bronchoalveolar lavage (BAL) fluid and cells were harvested. BAL fluid albumin levels and total antioxidant status were examined. In addition, levels of PGE2, MIP-2, MCP-1, and TNF- $\alpha$  were determined in BAL fluid and following *ex vivo* culture of BAL cells. The results of this study are consistent with the following hypotheses: 1) a marked increase in the permeability of the alveolar-capillary barrier is an early event underlying the increased susceptibility of hyperthyroid rats to ozone, however this does not appear to be due to overall changes in BAL fluid antioxidant potential; 2) early increases in MIP-2, but not PGE2, are involved in the enhanced lung response to ozone in a hyperthyroid state; 3) inflammatory mediator production (i.e., PGE2, MIP-2, MCP-1, and TNF- $\alpha$ ) by alveolar macrophages plays a minimal role in the initial responses to ozone in a hyperthyroid state.

INHIBITION OF TRANSFORMING GROWTH FACTOR BETA MNRA TRANSCRIPTION BY ANTISENSE OLIGONUCLEOTIDES IN AMIODARONE TREATED HAMSTERS.

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Pulmonary fibrosis is an often fatal disease characterized by cellular hyperplasia and excessive accumulation of extracellular matrix components with remodeling of the lung. The precise series of events leading to such scarring are not well understood. Amiodarone is an antiarrhythmic drug that is known to cause pulmonary fibrosis. Amiodarone induces an increase in transforming growth factor beta (TGF $\beta$ ) and such increase plays a significant role in the fibrotic process. The hypothesis of the current study is that blocking TGF $\beta$  mRNA transcription will modulate amiodarone induced pulmonary fibrosis in hamsters. Golden Syrian hamsters were

treated with TGF $\beta$  antisense oligonucleotides by aerosolization. Following such treatment animals were exposed to amiodarone by intratracheal insufflation. The hamsters were euthanized 24 hours after treatment, bronchoalveolar lavage (BAL) samples obtained and the lung tissue either frozen in liquid nitrogen or fixed in formalin and embedded in paraffin. TGF $\beta$  levels were measured in BAL samples by Western blot analysis. TGF $\beta$  levels were significantly decreased in animals treated with the antisense oligonucleotides as compared to control. BAL samples from treated and control animals were analyzed by light microscopy to determine the level of inflammatory cells present in the lung. A significant decrease was observed in the number of inflammatory cells present in the BAL fluid from antisense treated animals as compared to controls. Immunohistochemical analysis of frozen tissue sections from animals treated with antisense oligonucleotides prior to toxicant exposure exhibited a decrease in TGF $\beta$  levels as compared to controls. Morphologic study revealed reduction in areas of interstitial thickening in animals treated with TGF $\beta$  antisense oligonucleotides as compared to the controls. Antisense oligonucleotides to TGF $\beta$  mRNA inhibited, but did not completely prevent cytokine production in animals exposed to amiodarone and reduced alterations in lung architecture.

INVESTIGATION OF PREVENTION OF AMIODARONE- AND DESETHYLMIODARONE-INDUCED TOXICITY IN HUMAN PERIPHERAL LUNG EPITHELIAL CELLS HPL1A.

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Amiodarone (AM) is a highly effective antidysrhythmic used in the treatment of serious ventricular and supraventricular dysrhythmias. The mechanisms underlying AM-induced pulmonary toxicity (AIPT) have not been elucidated. Evidence suggests an important role for N-desethylamiodarone (DEA), a major AM metabolite, in AIPT. The immortalized, non-transformed human peripheral lung epithelial cell line (HPL1A) is morphologically and biochemically very similar to normal human peripheral lung epithelial cells. HPL1A cells have many characteristics that make them excellent candidates for assessing the mechanisms of toxicant action in human peripheral lung epithelial cells. In this study, HPL1A cells were grown to approximately 80% monolayer confluence, transferred to 96-well plates and incubated with AM or DEA concentrations varying from 0 to 1 mM. At 2, 4, 8, 12, 24, and 36 hours, viability was assessed using the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay. Time- and concentration-dependent losses of cell viability were observed, with DEA being more cytotoxic than AM at shorter incubation times. AM toxicity, expressed as the concentration that produces a 50% loss of cell viability, drastically increased with the incubation time. In contrast, DEA toxicity showed little variation over the incubation time period. Prior incubation with the spin trap a-phenyl-N-t-butylnitron (PBN, 10  $\mu\text{M}$ ) for 30 minutes provided only slight protection against AM/DEA-induced toxicity. These results support that long-term rather than acute exposure of HPL1A cells to AM is associated with an increased loss in cell viability. The observed cytoprotective effect of the spin trap suggest that AIPT may be in part mediated by a free-radical process. HPL1A cells represent a potentially competent model for elucidating the mechanisms of the AM-induced adverse pulmonary effects in humans. (Supported by Canadian Institutes of Health Research grant number MOP-13257).

GENE MICROARRAY ANALYSIS IN A RAT MODEL OF SMOKE INHALATION-INDUCED ACUTE LUNG INJURY.

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In a rat model of acute lung injury (ALI)/acute respiratory distress syndrome (ARDS)-like injury, we examined 28, 757 genes cDNA microarrays for early transcriptome alterations in the homologized lung tissue at 24 hours post-smoke insult. Data indicated that genes responsible for membrane enzyme, transporter, hemoglobin, signal transducer, binding activity, and transcription regulation were dysregulated. Smoke inhalation-mediated alterations in the identified genes were largely sorted into many signaling transduction pathways, including chemokine/cytokine, hormone, DNA binding, transcription factor, and G-protein receptor. Moreover, genes encoding different oxidoreductase, transferase, and isomerase were affected. Among them, the rapid inductions of sulfotransferase (Sult-n) and inositol/phosphatidylinositol kinase (Pik3cb) suggest that the metabolism of smoke toxins were prominent in the acute phase of smoke inhalation-induced ALI/ARDS. These findings reveal that there are complex molecular cascades involving disturbances in different subcellular compartments, influencing thereafter the normal cellular functions, leading to ALI/ARDS (Supported by ALA).

## EXPRESSION AND CHARACTERIZATION OF TRPM8 RECEPTORS IN THE LUNG EPITHELIUM.

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Several members of the Transient Receptor Potential (TRP) family of ion channels have been identified as physiological temperature sensors. These receptors respond to changes in temperatures, ranging from hot to cold, as well as chemical agonists such as menthol, capsaicin etc. Transient Receptor Potential Cation Channel, Melastatin subfamily, member 8 (TRPM8) has been shown to function as a cold sensing channel within the mammalian somatosensory system and acts by converting thermal stimuli into bio-chemical and electrical signals. Although much emphasis has been placed on the expression of TRPM8 in cold-sensitive sensory neurons, it is currently unknown whether functional TRPM8 receptors are expressed in the human respiratory tract. Preliminary studies in our lab have demonstrated the expression of TRPM8 mRNA in the bronchiolar epithelial (BEAS-2B), alveolar epithelial (A549), and normal human bronchiolar epithelial (NHBE) cells using RT-PCR. Additional studies aimed to assess the expression of functional TRPM8 channels in these cells demonstrated that menthol, the prototypical agonist of TRPM8, produced significant increases in the intracellular calcium concentrations that ranged from 1.4-fold for A549 cells to 5.6- and 5.8-fold for BEAS-2B and NHBE cells, respectively. The EC<sub>50</sub> values for induction of calcium flux were 2 ± 1 mM, 0.02 ± 0.009 mM and 1.5 ± 0.8 mM in BEAS2B, A549 and NHBE cells, respectively. Furthermore, the influx of calcium was inhibited by capsazepine, an antagonist of TRPM8 receptors. These data were consistent with the expression of functional TRPM8 channels in respiratory epithelial cells. Expression of these receptors in the respiratory tract may represent a significant finding with respect to adverse respiratory responses to cold air (e.g. asthma, cough). Support was provided by Grant # HL069813.

## AEROSOLIZED HYALURONAN MODIFIES ENDOTOXIN-INDUCED LUNG INJURY IN A TIME-DEPENDENT MANNER.

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Inhalation of an aerosolized preparation of low-molecular weight (150 kDa) hyaluronan (HA) was previously shown by this laboratory to prevent experimentally induced pulmonary emphysema without causing additional lung inflammation. As a result, aerosolized HA has been proposed as a potential treatment for human emphysema. However, other investigators have found that low-molecular weight HA may be proinflammatory, prompting us to determine whether aerosolized HA could possibly enhance inflammation in a model of pulmonary disease other than emphysema. Using endotoxin-induced lung injury, we tested the effects of aerosolized HA on the acute inflammatory response. Syrian hamsters were exposed via nebulization to a 0.1% solution of 150 kDa HA in water for 2 hrs, either immediately before or after intratracheal instillation of 0.1 mg E.coli endotoxin (controls received endotoxin alone). All animals revealed a rapid influx of inflammatory cells into the lung which peaked within the first 24 hrs. While microscopic examination did not show any significant differences between HA-treated and untreated lungs with regard to the magnitude of the inflammatory response, a marked elevation in the percentage of lavaged neutrophils (69% vs 5.4%; p<0.05) was seen at 4 hrs post-endotoxin in animals receiving HA after endotoxin administration. In contrast, administration of HA prior to endotoxin resulted in a decline in the proportion of lavaged neutrophils at 4 hrs (2.1% vs 5.0%; p<0.05). These results indicate that HA modifies endotoxin-induced lung injury in a time-dependent manner. The fact that HA stimulates a neutrophil influx only when given after induction of pulmonary inflammation suggests that it may specifically interact with activated inflammatory cells (e.g. macrophages) that recruit neutrophils to the lung. How these findings might affect the therapeutic potential of HA remains to be determined.

## IMMUNE RESPONSE TO ZYMOSEN-INDUCED PULMONARY INFLAMMATION IN RATS.

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1→3-β-Glucans have been associated with increased pulmonary inflammation in mold-related indoor air problems. The objective of the present investigation was to determine the immune response to zymosan-induced pulmonary inflammation in SD rats. Rats received a single dose of zymosan A (2.5 mg/kg body weight) via intratracheal instillation (IT) and were euthanized on days 1, 4, 6, and 8 post IT. Inflammation and lung injury were assessed by measuring (1) neutrophil (PMN) infiltration into bronchoalveolar lavage fluid (BALF) and (2) albumin, total protein and lactate dehydrogenase levels in BALF. Alveolar macrophage activation was determined by chemiluminescence (CL). Immune response was investigated via im-

munophenotyping of lymphocytes and lymphokine production. Immunophenotyping was performed on BAL cells and lung-associated lymph node cells. Lymphokine production was measured from lymph node cells with or without concanavalin A stimulation by an enzyme-linked immunosorbent assay. Upon challenge with zymosan, rats exhibited increased inflammation and injury at the early time points post-IT exposure. Although elevations in PMN infiltration and CL had returned to control levels on day 4, lung-associated lymphocytes continued to proliferate and reached a maximum on day 6. The ratio of CD4 to CD8 T cells in the lymph node and BAL was lower in zymosan-treated rats than in control rats, indicating a greater increase in CD8 T cells as compare to CD4 T cells. Zymosan also increased the number of infiltrating NK cells, B cells, and T cells in BAL at all time points. B cells in BALF were found to be highest in number on day 1 for zymosan-treated rats. The ratio of T/B cells in BALF increased significantly on day 6 and 8. Zymosan treatment increased IL-2, IL-10 and IFNγ but not IL-4 production in lymphocytes. These data along with immunophenotyping of lymphocytes suggests that helper CD4 T and cytotoxic CD8 T cells are involved in the immune activation caused by zymosan treatment. In summary, rats exposed to zymosan had increased inflammation and altered lymphocyte profile, indicating an activation of innate and/or adaptive immune response in rats.

## HYDROGEN SULFIDE EXPOSURE CAUSES INTRACELLULAR ACIDIFICATION OF RAT NASAL RESPIRATORY EPITHELIAL CELLS.

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Hydrogen sulfide (H<sub>2</sub>S) is a naturally occurring gas that is also generated by several industries. The potential for widespread human inhalation exposure to this toxic gas is recognized as a public health concern. Human epidemiological investigations and experimental laboratory animal studies have confirmed that the nasal epithelium is a sensitive site for H<sub>2</sub>S-induced pathology. Cytochrome oxidase (Co) inhibition has been postulated as one mechanism of H<sub>2</sub>S toxicity. Inhibition of Co disrupts the electron transport chain and impairs oxidative metabolism, leading to decreased ATP production and the build-up of lactic acid. While Co is a sensitive marker of H<sub>2</sub>S exposure, there is an incomplete correlation between H<sub>2</sub>S-induced nasal lesions and Co inhibition. Another mechanism by which H<sub>2</sub>S (a weak organic acid) could cause nasal injury is intracellular acidification and cytotoxicity. Literature reports show that changes in intracellular pH due to exposure to organic acids such as vinyl acetate can lead to cell death. To further understand the mechanism by which H<sub>2</sub>S damages the nasal epithelium, nasal respiratory epithelial cells were isolated from naive rats; loaded with the pH-sensitive dye, SNARF-1; and exposed to air or 400 ppm H<sub>2</sub>S for 90 min. Vinyl acetate (1000 μM) was used as a positive control. Intracellular pH was measured by flow cytometry and cell lysates were used to quantify total protein and Co activity. Flow cytometric analysis showed that nasal respiratory epithelial cells exposed to air maintained a pH of 7.6 ± 0.05. A significant decrease in intracellular pH occurred following exposure to either vinyl acetate (15% decrease from control) or H<sub>2</sub>S (5% decrease from control). Co activity in air-exposed cells was 1.39 ± 0.01 Δabs/min/mg protein, while Co activity in cells exposed to H<sub>2</sub>S was not detectable. The intracellular acidification of nasal respiratory epithelial cells by H<sub>2</sub>S demonstrates a novel mechanism of H<sub>2</sub>S-induced nasal respiratory cellular toxicity.

## PERTURBATION OF COPPER HOMEOSTASIS AND EXPRESSION OF COPPER-BINDING PROTEINS IN CADMIUM-RESISTANT LUNG FIBROBLASTS.

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To probe molecular mechanisms of cadmium (Cd) damage to the lung extracellular matrix (ECM) we developed Cd-resistant (CdR) cells derived from rat fetal lung fibroblasts (RFL6) by incubation with graded doses of Cd. CdR cells have been shown to display upregulation of metallothionein (MT) and glutathione (GSH), two metal scavenging agents, coupled with downregulation of lysyl oxidase (LO), a copper (Cu)-dependent enzyme necessary for crosslinking collagen and elastin in the ECM. Here we examined cellular uptake and distribution of Cu and compared expression of LO with other Cu-binding proteins in CdR cells. A <sup>64</sup>Cu 1-h pulse assay revealed that the amount of radioactivity associated with CdR cells was 2.1-fold of the parental Cd-sensitive (CdS) control. Thus, LO deficiency in CdR cells is not due to a reduced cell uptake of Cu, a cofactor of LO. Antibody affinity chromatography assays indicated a low level of <sup>64</sup>Cu bound to the LO fraction amounting to 9% of the CdS control as compared to 1, 400% of the control of <sup>64</sup>Cu associated with the MT fraction in CdR cells pulsed with isotope for 4 h. This suggests that the high level of cellular Cu in CdR cells at least in part resulted from elevated levels of cellular MT. Steady-state protein levels as determined by Western blot

showed that there were > 8-fold increases in MT, 1.0-1.6-fold in Cu/Zn-superoxide dismutase (SOD), 1.5-2.5-fold in CCS which delivers Cu to SOD, 1.0-1.5- fold in Mn-SOD and 1.0-1.5-fold in  $\gamma$ -glutamylcysteine synthetase, a GSH synthetic enzyme, in CdR cells resistant to 10-80  $\mu$ M Cd compared to the CdS control. In contrast, all LO species, i.e., the 46 kDa preproenzyme, the 50 kDa proenzyme and the 32 kDa mature enzyme were reduced by 84%, 70% and 98%, respectively. These results point out that downregulation of LO is linked with upregulation of other Cu-binding proteins and alteration in Cu homeostasis in CdR cells (Supported by NIH grant R01-ES11340 & Philip Morris ERP).

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PHOTOCHEMICAL REACTIONS ENHANCE INFLAMMATORY RESPONSES IN HUMAN LUNG CELLS.

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The chemistry of hazardous air pollutants has been studied for many years, yet little is known about how these chemicals, once interacted with urban atmospheres, affect healthy and susceptible individuals. During this study, environmental irradiation chambers (also called smog chambers) use natural sunlight to induce the natural photochemically stimulated transformations of environmental pollutants. These smog chambers have been used for more than 30 years to investigate and develop chemical mechanisms of species found in the atmosphere and used in regulatory air quality models. Smog chambers, coupled with an *in vitro* system, using A549 cells, were used to investigate methanol, toluene, and 1, 3-butadiene in combination with a synthetic urban smog mixture. Once released into the atmosphere, these air pollutants interact with hydroxyl radicals and ozone, which are created by photochemical processes, to produce many identified and unidentified products. Once these chemical reactions occur, the toxic potential of these atmospheric pollutants is currently unclear. In this study, A549 cells were exposed simultaneously to irradiated and non-irradiated chamber mixtures for five hours. Post exposure, adverse health effects were determined by measures of increased cellular stress (cytokine release) and cytotoxicity. Exposure to the photochemically generated products of 1, 3-butadiene, toluene, and methanol induced increases in both cytotoxicity and IL-8 gene expression compared to 1, 3-butadiene, toluene, or methanol alone. The smog chamber/*in vitro* exposure design was used to investigate the toxicity of chemicals after photochemical reactions and interactions with the urban atmosphere on healthy and susceptible individuals using representative *in vitro* samples. This research helps connect the gaps in the literature to explain the toxicity found from exposures to multiple environmental pollutants found in an urban atmospheric setting, and provides a way to obtain a more truthful estimate of toxicity of ambient releases of pollutants.

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INHALATION EXPOSURE TO TERTIARY AMYL METHYL ETHER (TAME) ALTERS THE ACTIVITIES OF CYTOCHROME P450 ISOZYMES IN HEPATIC AND RESPIRATORY TISSUES OF SPRAGUE DAWLEY RATS.

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Fuel oxygenates, as mandated by amendments to the Clean Air Act of 1990, are blended with gasoline to help reduce harmful vehicle exhaust emissions. To date, methyl-tert butyl ether (MTBE) is the most common oxygenate employed to produce cleaner burning fuels. TAME, as well as ethyl-tert butyl ether and diisopropyl ether, are less commonly used oxygenates; but with the recent launch of the MTBE phase-out program due to health concerns, the demand for TAME may increase. The full impact of TAME on human health, however, is yet to be determined. The aim of this study was to examine the alterations in CYP450 activities in rat nasal mucosa, lung and liver immediately following, and 24 h after, an acute 6 h inhalation exposure to TAME. CYP450 activity was measured in microsomes using specific enzyme probes. TAME (100ppm, 0 h or 24 h) exposure resulted in inhibition of CYP1A1 (69% & 53%), CYP1A2 (96% & 65%), CYP2A3 (89% & 41%), CYP2B1 (44% & 79%), CYP3A (28% & 21%) and CYP4B1 (36% & 23%) in nasal mucosa. Exposure to 300ppm (0 h or 24 h) inhibited nasal mucosal CYP1A1 (79% & 45%), CYP1A2 (80% & 61%), CYP2A3 (84% & 49%), and CYP2B1 (83% & 70%). CYP4B1 was inhibited 27% at 24 h only. In lung, TAME (100ppm or 300ppm) inhibited CYP2A3 (31% & 88%) and CYP2B1 (36% & 22%) at 0 h with recovery to control levels at 24 h. Lung CYP4B1 activity increased 23% immediately following the 300ppm exposure but was subsequently inhibited by 26% at 24 h post-exposure. Hepatic CYP2B1 activity increased at 0 h (87%) and 24 h (207%) following the 300ppm exposure. CYP1A1 activity increased (44%) 24 h following 300ppm only. TAME (100ppm) inhibited CYP3A (16%) at 0 h whereas inhibition of CYP2A3 (27%) and an increase in CYP2E1 activity (19%) occurred 24 h post-exposure in liver. These alterations in CYP450 activity following TAME exposure may lead to adverse human health outcomes upon co-exposure with other agents, such as other solvents in gasoline, by increasing bioactivation and/or delaying detoxication via this system.

**944**

BIOPERSISTENCE OF ROCK WOOL (RW) IN THE RAT LUNG AFTER SHORT-TERM INHALATION VIA NOSE.

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Rock wool (RW), a man-made vitreous fiber, has been used as residential and commercial thermal and acoustic insulation. IARC classified RW as a Group III carcinogen. For the evaluation of the safety of RW, data on the biopersistence of RW in the lung are required. We evaluated the biopersistence of RW in the rat lung after short-term inhalation via the nose, by determination of the length and diameter of each fiber and the total number of fibers. Male Fischer 344 rats (n=24) were exposed to 30 mg/m<sup>3</sup> RW (Nittobou) for 3 hours/day for 5 consecutive days in nose-only inhalation chambers. The rats were divided into four groups, and six rats each were euthanized at 1 hour, and 7, 14, and 28 days after the inhalation exposure. The lungs were removed, and prepared using a freeze-drying method. The dry tissue was ashed by low-temperature ashing. RW was collected after filtration. The size of each fiber and the total numbers of fibers were determined by phase contrast microscopy and WINROOF software. The geometric mean values of the length of the fibers were 8.49 mm at 1 hour, 9.57 mm at 7 days, 7.68 mm at 14 days, and 8.36 mm at 24 days. The geometric mean values of the diameter of fibers were 1.48 mm at 1 hour, 1.51 mm at 7 days, 1.31 mm at 14 days, and 1.41 mm at 24 days. The total numbers of fibers in the lung expressed as percentages of that at 1 hour were 94.1% at 7 days, 76.9% at 14 days and 59.3% at 28 days. The time in which the number of total fibers was halved was 38 days. The time in which the number of long fibers (>20 mm) was halved was 29 days. Although the length and diameter of the fibers in the lung did not change dramatically during the experiment, the total number of fibers decreased. The decrease in the total number of fibers may have been due to excretion by mucociliary movement, or phagocytosis of macrophages at bronchus and trachea. For the further evaluation of the safety of RW, the effects of long-term exposure should be evaluated for a longer observation time.

**945**

TWO-WEEK INHALATION STUDIES WITH DISK-SHAPED PARTICLES OF POTASSIUM TITANATE COMPOUNDS: TERRACESS PS AND TERRACESS LS.

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Inhaled fibrous particles are known to have potential fibrogenic and carcinogenic activities that are related to fiber dimensions and physicochemical characteristics. Terracess PS (MgK<sub>2</sub>TiO) and Terracess LS (LiK<sub>2</sub>TiO) are manufactured as disk-shaped particles to avoid potential fiber toxicity. Groups of 10 male rats were exposed whole-body to Terracess PS (TPS) or Terracess LS (TLS) aerosols at concentrations of 0, 5, 25, or 100 mg/m<sup>3</sup> for 6 hrs/day for 9 days over a two-week period. The mass median aerodynamic equivalent diameter of the aerosols was 3.6 to 4.3  $\mu$ m for TPS and 2.9 to 3.3  $\mu$ m for TLS. No adverse effects were observed in exposed rats in clinical observations, body weights, and clinical pathology including urine, hematology, and blood chemistry parameters. Lung and lung/brain weight ratio were not affected. No adverse histopathological effects were observed in the respiratory tract including nose, larynx/pharynx, trachea, and lungs. Inhaled Terracess particles were mostly phagocytized by free alveolar macrophages (AMs) in the alveolar airspaces. The adjacent alveolar walls enclosing dust-laden AMs showed normal structure. Some inhaled test particles were detected in thoracic lymph nodes via lung clearance processes. The absence of adverse effects in the alveolar walls was likely due to the test material's low reactivity and disk shape. Based on the lack of adverse effects in the respiratory tract, the no-observed-adverse-effect-level for Terracess PS and Terracess LS was 100 mg/m<sup>3</sup> and the lung responses were considered similar to "nuisance" type dusts.

**946**

CHRONIC LUNG TOXICITY IN RATS DUE TO EXPOSURE TO DIFFERENT CONCENTRATIONS OF THE MINERAL OIL.

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This work was done to study the histopathological effect of chronic exposure to mineral oil (BS148) on the lung of rats. The study was done on 80 rats divided into 4 groups (20 rats each). The first group (used as control) was exposed to atmospheric air. The second group to mineral oil at 100 mg/m<sup>3</sup> concentration, the third group was exposed to 200 mg/m<sup>3</sup> and the fourth one to 400 mg/m<sup>3</sup> concentration (the inhalation done for 3.5 h/d, 4 d/w for 12 weeks). By the end of 12th week the

animals were sacrificed and their lungs were taken for histopathological study. The histopathological results revealed that all lungs of the control group were normal. Of the 2nd group, lungs of 8 rats(40%) showed mild affection in the form of mild alveolar macrophage infiltration. 75% of rats of the 3rd group showed mild to moderate alveolar macrophage infiltration while, 100% of the lungs of the 4th group were affected, 40 % of which showed severe alveolar macrophage infiltration. From this work, it can be concluded that inhalation exposure of rats to mineral oil (BS148) resulted in concentration dependent lung affection.

**947**

**SENSORY IRRITATION RESPONSE TO ATP,  $\alpha$ ,  $\beta$ -METHYLENE-ATP AND ADENOSINE AEROSOLS.**

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In many organs, including the lungs, sensory nerves may be activated via purinergic signaling pathways. The current experiments were aimed at examining the potential role of purinergic activation of the nasal sensory irritation response. This response is mediated by trigeminal sensory nerves and is characterized by a decreased breathing frequency due to a pause at the start of each expiration. Female B6C3F1 mice were exposed for 15 minutes to aerosolized ATP,  $\alpha$ ,  $\beta$ -methyleneATP (a P2X receptor agonist) or adenosine (an A receptor agonist) in a double plethysmograph and expiratory pause duration was measured. Aerosolized ATP (10 mg/ml) acted a sensory irritant, inducing an expiratory pause of 150-200 msec duration within 2 minute of the start of exposure. The response diminished to approximately 20 msec within 5 minutes of the onset of exposure. The response to ATP aerosol was significantly lower in capsaicin-pretreated mice, providing evidence for the participation of TRPV1 (capsaicin)-receptor expressing C fibers. Airways resistance (sRaw) was not increased over control (saline) aerosol levels by ATP aerosol suggesting that nasal sensory nerve C fiber activation does not necessarily induce an obstructive response in the mouse. The P2X agonist  $\alpha$ ,  $\beta$ -methyleneATP also produced a rapid response (within 2 minute of the onset of exposure) that diminished as the exposure progressed. In contrast, adenosine aerosol produced a biphasic response with an early response (pause duration 150-200 msec, within 2 minute of the onset of exposure) followed by a decline and then a delayed response (pause duration ~80 msec) from 10-15 minutes of exposure. This response pattern is similar to that caused by exposure to acetic acid or ethyl acetate vapor. These results suggest purinergic signaling pathways may play a role in initiating the sensory irritation response and both A and P2X receptor families may be involved.

**948**

**EFFECTS OF GROUP HOUSING ON REPRODUCTIVE PARAMETERS IN THE SEXUALLY MATURE CYNOMOLGUS MONKEY.**

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Cynomolgus monkeys represent the most commonly used nonhuman primate species in toxicology. During reproductive and developmental toxicity studies, animals are housed singly in order to avoid interference with reproductive/developmental parameters imposed by social rank and associated behaviour. Current recommendations and anticipated European guidelines will enforce group housing for primates in order to provide improved social interactions and more enriched environments. Whilst potential effects on reproductive functions are not relevant for group-housed juvenile and immature primates, this aspect becomes critical for group housing of mature animals. The current project evaluates the effects on reproductive parameters of single housing into isosexual group housing of female monkeys (maturity proven by vaginal bleedings) and male monkeys (maturity proven by sperm in the ejaculate). A group of seven males was studied for a period of 26 weeks and a group of eight females was studied for a period of 9-10 months. Semen samples and testicular volumes were assessed in 3-4 week intervals. Vaginal smears were collected daily throughout the study period. Social rank was determined using a food challenge test. Among group-housed males, testicular volumes declined by 30-40 % within 6 weeks in three animals with lowest social rank and remained lower until week 13. Unexpectedly, thereafter testis size increased in these animals. Sperm motility and sperm numbers were not consistently altered in these subordinate animals. For females that had regular menstrual cycles when housed singly, menstrual cycles became more irregular during group housing. The social rank of females was difficult to ascertain. In conclusion, group housing of sexually mature animals is currently not recommended for reproductive toxicity studies. Further research (different group sizes, duration of group formation, etc.) is needed to clarify whether circumstances permitting group housing for reproductive toxicity studies can be identified.

**949**

**28-DAY INHALATION AND REPRODUCTIVE/DEVELOPMENTAL SCREENING TOXICITY STUDIES OF TETRAMETHYLSILANE IN SPRAGUE-DAWLEY RATS.**

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Tetramethylsilane (TMS) is a volatile, highly flammable liquid used as an industrial solvent. The potential toxicity of TMS, a High Production Volume chemical (HPV), was evaluated in a combined repeated exposure toxicity study with a reproductive/developmental toxicity screening test. The study design utilized an enhanced version of the protocol in accordance with the OECD and EPA test guidelines Nos. 422 and 870.3650, respectively. Developmental neurotoxicity endpoints including Functional Observational Battery (FOB) and Motor Activity were performed on male and female rats. The test substance was administered six hours a day, seven days a week by inhalation to 10 rats/sex/group at 0, 200, 1000, and 5000 ppm. All animals survived to the scheduled necropsy. There were no clinical signs or effects on body weights and food consumption among the groups. No treatment-related changes were observed in any FOB or motor activity parameters. There were no alterations in hematology, serum chemistry, organ weights, organ to body weight ratios or macroscopic examination of organs/tissues among the groups. Histopathologic examination of tissues and organs for control and high exposure animals demonstrated no significant microscopic findings. No effects were observed in any of the reproductive parameters evaluated. One female in 200 ppm group was found non-gravid but the remaining females produced litters that were similar in all respects to control litters. Based on the results of this study, the NOAEL for tetramethylsilane in rats via inhalation route was considered to be 5000 ppm. (Sponsored by the Silicones Environmental, Health and Safety Council).

**950**

**GLYCIDEOXYPROPYLTRIMETHOXYSILANE (TMSPGE): ONE-GENERATION REPRODUCTION STUDY IN THE RAT.**

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The potential effect of TMSPGE, an HPV chemical, was evaluated in a one-generation reproductive toxicity study. The study investigated the effects of TMSPGE to the rat on reproductive performance. The test substance was administered by gavage, once daily to males for a 70-day pre-pairing period, during the pairing period and until the last litter reached day 7 post partum. Females received the test substance during a 14-day pre-pairing period, and during pairing, gestation and lactation periods. Doses were 0, 250, 500 or 1000 mg/kg bw/day. Signs of discomfort after dosing were noted in all treated female animals; clinical signs were not observed in any male animal. Mean food consumption was not affected by treatment with TMSPGE. At 1000 mg/kg bw/d, mean body weight gain of males during pre-pairing was slightly decreased, resulting in a slightly lower mean body weight at the end of pre-pairing. This reduction was considered to be test substance related. During and after pairing, lower absolute body weights at 1000 mg/kg bw/d persisted, while body weight gain was similar to that of the control. Body weights of females was not affected by treatment. There were no test substance related macroscopic or microscopic findings. At 1000 mg/kg bw/d, statistically significant increased mean relative liver and kidney weights were noted for males and females. No effects were observed in any of the reproductive parameters evaluated. In the liver of high dose males, the severity of glycogen deposition was slightly increased; this finding was not considered to be test substance related. A slightly increased severity of tubular hyaline change occurred in the kidneys of high dose males; this change reflects an increased accumulation of alpha-2-microglobulin which is a male rat specific phenomenon. No test substance related findings, clinical signs or macroscopic findings were noted for any litter. The NOAEL (parental) is 500 mg/kg bw/d; the NOAEL (reproductive effects) is 1000 mg/kg bw/d. (Sponsored by the Silicones Environmental, Health and Safety Council).

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**DEVELOPMENTAL TOXICITY AND COMBINED MALE AND FEMALE FERTILITY STUDIES IN RATS EXPOSED TO 2, 3-DICHLORO-1, 3-BUTADIENE (DCBD) BY INHALATION.**

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In a fertility study, Crl:CD(SD)IGS BR rats (24/sex/group) were exposed by inhalation to 0, 1, 5, or 50 ppm DCBD (6 hr/day) during prematting (8 wks; 5 days/wk), cohabitation of mating pairs (up to 2 wks, 7 days/wk), postcohabitation for males (-7 days) and from conception to implantation (days 0-7 of gestation [G]). Estrous cyclicity was evaluated during prematting (last 3 wks) and cohabita-

tion. Reproductive and potential target organs, sperm parameters, and day 21G fetuses (viability, weight, external alterations) were evaluated. In a developmental study, pregnant Crl:CD(SD)IGS BR rats (22/group) were exposed to 0, 1, 10, or 50 ppm DCBD (6 hr/day) on days 6-20G; dams were necropsied on day 21G and fetuses were evaluated (viability, weight, and external, visceral and skeletal exams). During the in-life portion of the studies, body weight, food consumption, and clinical observation data were collected. Gasping and labored breathing occurred during the first exposure in both studies at 50 ppm. Fetal weight was decreased at 50 ppm in the developmental toxicity study. In the fertility study, decreased parental body weight, weight gain, food consumption and food efficiency and degeneration of the nasal olfactory epithelium were observed at 50 ppm. There were no effects on reproductive function, fetal viability or malformations in either study. The NOEL for reproductive toxicity was 50 ppm. The NOEL for effects in parental rats was 5 ppm based on adverse effects on body weight and food consumption parameters, and nasal olfactory epithelial toxicity at 50 ppm. The NOEL for maternal and developmental toxicity was 10 ppm based on reduced maternal weight gain and food consumption and fetal weight at 50 ppm in the developmental toxicity study.

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LACK OF FERTILITY OR ESTROUS CYCLE EFFECTS OF A FLUORINATED ORGANIC ETHOXYLATE SURFACTANT IN RATS.

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The purpose of this study was to determine the repeatability of fertility and estrous cycle effects observed at doses of 25 mg/kg/day and above, with no dose-response, in a previously conducted reproduction study and to establish a NOEL for reproductive parameters. Crl:CD(SD)IGS BR rats were dosed once daily by gavage at 0, 1, 10, or 25 mg/kg/day. Following 72 days of dosing (pre mating), the P1 males and females were cohoused within their respective treatment groups to produce F1 litters. Clinical observations, body weight, and food consumption in P1 rats were recorded weekly throughout the study; estrous cyclicity was evaluated during pre mating (last 3 wks) and during cohabitation. F1 litter viability, weights, and clinical observations were determined at birth and on day 4. After litter production, all P1 rats were given a gross pathological examination, the liver (target organ) was weighed, the liver and reproductive organs saved, and uterine implantation sites and ovarian corpora lutea counted. Liver and gross lesions from randomly selected P1 rats in the control and 25 mg/kg/day groups were examined microscopically. Treatment related effects were limited to increased liver weight in P1 rats at 25 mg/kg/day, which was not accompanied by gross or histological changes. This effect was considered a non-adverse physiological adaptive response. The NOEL for P1 rats, F1 litters, and reproductive parameters, including fertility, was 25 mg/kg/day, the highest dose tested. These results support the conclusion that the apparent increase in estrous cycle length and decreased fertility reported in the first study are unlikely to be test substance-related. Thus, the overall LOEL for reproductive parameters is 100 mg/kg/day, based on reduced pup survival and weight during lactation observed in the first study. The overall NOEL for reproductive parameters is 25 mg/kg/day, which is the same as the NOEL for subchronic toxicity observed in both studies.

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DEVELOPMENTAL IMMUNOTOXICITY OF DEXAMETHASONE AND CYCLOSPORIN A.

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Introduction. As pre and postnatal exposure can result in various types of immunotoxic effects in the offspring, incorporation of immunotoxicological determinations is considered in reproductive toxicology studies. The impact of maternal treatment and direct dosing of pre-weaning rats with dexamethasone and cyclosporin A on development of the immune system was investigated. Materials and Methods. Two developmental toxicity studies were performed in Wistar rats. In the first study rats were treated subcutaneously with 20 or 100 µg dexamethasone/kg from gestation day (GD) 0 to postnatal day (PND) 21. In the second study dams were treated orally with 5 or 15 mg cyclosporin A/kg from GD 6 to PND 21 and a selection of pups was dosed directly with 12.5 mg cyclosporin A /kg from PND 14-21. Enhanced immunopathology was performed. Immune function was studied by flow cytometric lymphocyte subset analysis in blood and by T cell-dependent antibody response to Keyhole Limpet Hemocyanine. Results. Dexamethasone induced maternal toxicity, decreased pup weights and immunopathological effects on thymus, spleen, bone marrow and mesenteric lymph nodes. The KLH-specific IgM and IgG antibody titers were not affected. Cyclosporin A induced a dose related increased post-implantation loss. The KLH-specific IgM and IgG antibody titers were not affected. The KLH-specific IgM antibody titer measured in pups dosed directly with cyclosporin A was increased (as compared to non-treated pups). The increase was similar in dosed pups derived from cyclosporin-treated dams and control dams. Conclusion. These data suggest that maternal dexamethasone and cy-

closporin A treatment up to dose levels of 100µg/kg and 15 mg/kg, respectively, influences postnatal immune system-related parameters in the offspring to a low extent. The pre-weaning rat (directly dosed) seems to be a more appropriate model for investigating possible effects on the development of the immune system than the adult rat.

**954**

BEHAVIORAL ABNORMALITY ASSOCIATED WITH ATTENTION DEFICIT HYPERACTIVE DISORDER IN RAT OFFSPRING PRENATALLY EXPOSED TO 5-BROMO-2'-DEOXYURIDINE.

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Our previous study revealed that rat offspring prenatally exposed to 5-bromo-2'-deoxyuridine (BrdU) exhibited hyperactivity. In the present study, the behavioral abnormalities induced by prenatal exposure to BrdU were further examined and a possible association with problematic behaviors of attention deficit hyperactive disorder (ADHD) was evaluated. Sprague-Dawley rats were treated with BrdU 50 mg/kg, IP or carboxymethylcellulose (CMC), its vehicle, on gestational days nine through 15, and their offspring (BrdU rats and CMC rats, respectively) were subjected to behavioral tests at five to six weeks of age. A continuous locomotor activity monitoring system revealed that locomotor activity increased when lights were out and gradually decreased during the night cycle in CMC rats. In BrdU rats, locomotor activity was kept increased throughout the night cycle. In elevated plus maze task, the number of open arm entries and time spent per one open arm entry, both of which are indices of impulsivity, were significantly increased in BrdU rats when compared with those of CMC rats. In the Y-maze test, spontaneous alternation behavior was significantly lower in BrdU rats. Long-term potentiation, a cellular mechanism of memory, was not different between BrdU and CMC rats. Thus, lowered spontaneous alternation behavior in BrdU rats was not caused by a deficiency of short-term memory but by attention deficit. These data suggest that BrdU rats showed ADHD-like behavior abnormalities including hyperactivity, impulsivity, and attention deficit.

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MATERNAL DETOXICATION VIA CYP1A1 PREVENTS METABOLIC ACTIVATION AND EMBRYO TOXICITY FOLLOWING ORAL EXPOSURE TO BENZO(A)PYRENE.

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Maternal smoking can produce numerous adverse outcomes in pregnancy, including lower birthweights. Recently, CYP1A1 polymorphisms were associated with an increased risk of low birthweight in infants born to mothers who smoked during pregnancy. Benzo[a]pyrene (BaP), a known carcinogen and mutagen in cigarette smoke, also causes fetal toxicity. BaP must be metabolically activated to produce its toxic effects. CYP1A1 plays a paradoxical role in the metabolism of BaP. It oxidizes BaP to DNA-reactive carcinogenic species, but this oxidation is required for conjugation and elimination. Thus, the role of CYP1A1 in eliminating or potentiating BaP toxicity must be evaluated in the context of the animal. To explore the role of CYP1A1 on BaP toxicity during gestation, we exposed *Cyp1a1(+/-)* and *Cyp1a1(-/-)* dams to 12.5 mg/kg/day oral BaP from gestational day(GD)4 to 10. There was nearly 100 percent *in utero* lethality and fewer implantation sites in *Cyp1a1(-/-)* dams. In contrast, there was little BaP-mediated toxicity to fetuses of *Cyp1a1(+/-)* dams, even at 125 mg/kg/day. To determine whether the lethality was due to a maternal or fetal effect of CYP1A1, we mated *Cyp1a1(+/-)* females with *Cyp1a1(-/-)* males and *Cyp1a1(-/-)* females with *Cyp1a1(+/-)* males. Dams were treated with 12.5 mg/kg/day BaP from GD4-10. Weight gain during gestation was significantly less in *Cyp1a1(-/-)* dams compared with *Cyp1a1(+/-)* dams ( $0.8 \pm 0.4$ g v.  $14.6 \pm 0.5$ g). DNA adducts in maternal liver, placenta and fetal tissues were dramatically higher in *Cyp1a1(-/-)* dams compared with *Cyp1a1(+/-)* dams. The genotype of the fetuses made no difference in terms of lethality or DNA adduct formation. We conclude that the presence of CYP1A1 in the mother is an important determinant in protection against oral BaP-induced teratogenesis and embryo toxicity. Supported in part by NIH grants P30 ES06096 and R01 ES08147.

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MATURATION OF HEPATIC CYTOCHROME P450 ISOZYMES IN DEVELOPING SPRAGUE-DAWLEY RATS.

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Cytochrome P450s (CYP450s) play a key role in the metabolic activation and inactivation of a wide range of xenobiotics. The ontogeny of a number of CYP450 isozymes has recently been delineated in human liver. There is relatively little such

information about immature rats, a primary animal model for infants and children. Our study was designed to characterize the ontogeny of several toxicologically-relevant CYP450 isozymes (CYP1A1/2, CYP2B1/2, and CYP2E1) as well as total CYP450 in Sprague-Dawley rats. Liver microsomes were prepared from unsexed naive rats at days 5, 10, 15, 21, 30, 40, 50 and 60 post-birth. On days 5, 10, and 15, samples were pooled and analyzed as a single sample. CYP1A1/2, CYP2B1/2 and CYP2E1 activities were measured using the substrates 7-Ethoxresorufin, 7-Pentoxyresorufin and para-Nitrophenol, respectively. CYP1A1/2 and CYP2B1/2 activities were measured using a spectrofluorimeter; CYP2E1 and total CYP450 were measured with a spectrophotometer. CYP1A1/2 activity was very low on day 5 and 10, but near-maximal by day 30. CYP2B1/2 displayed a similar pattern, with low activity on day 5, a 2-fold increase on days 10 and 15, peak activity days 30 and 40, followed by somewhat lower values on days 50 and 60. In contrast, CYP2E1 activity was relatively high by day 5 and exhibited moderately higher, but stable values the remainder of the developmental period. Total CYP450 levels were quite low on days 5, 10 and 15, with an abrupt increase to maximal levels by day 30. The differential expression of CYP450 isozymes during development may be an important factor in age-dependent susceptibility to pesticides, solvents and other toxicants metabolized by CYP450s.

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RESISTANCE TO PCB INDUCTION OF P450 1A (CYP1A) ACTIVITY AND REACTIVE OXYGEN SPECIES (ROS) PRODUCTION IN A POLLUTED KILLIFISH (*FUNDULUS HETEROCLITUS*) POPULATIONS.

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New Bedford Harbor MA and Newark Bay NJ are highly contaminated with polychlorinated biphenyls (PCBs). During early fish development, exposure to PCBs results in activation of the aryl hydrocarbon receptor (AHR), induction of cytochrome P450 1A (CYP1A) and increased production of Reactive Oxygen Species (ROS). Previous studies have demonstrated that first generation killifish (*Fundulus heteroclitus*) embryos from Newark Bay and New Bedford Harbor are resistant to PCB induction of CYP1A expression and mortality. We hypothesized that PCB induced P450 activity is associated with increased ROS production and that contaminated site killifish are resistant to PCB induction of ROS production. Killifish embryos from contaminated sites and a reference site were exposed to vehicle (acetone) or the potent CYP1A inducer, PCB126 (0.3nM), from day two to day seven post fertilization (pf). Superoxide production (detected with dihydroethidium) was measured on day 7 pf and *in ovo* CYP1A activity (ethoxresorufin-o-deethylase, EROD) was measured on day 9 pf. Treatment with PCB126 induced CYP1A activity and reactive oxygen species (ROS) production in reference site, but not polluted site embryos. To determine if PCB induced ROS production is dependent on AHR activation and induction of responsive P450s, reference site embryos were treated with the AHR/CYP1A inhibitor a-naphthoflavone (ANF) alone (367 nM), or the P450 inhibitor piperonyl butoxide (PBO) alone or in combination with PCB126. ANF blocked PCB126 induction of CYP1A activity, but did not affect ROS production. PBO treatment inhibited PCB induction of CYP1A activity and ROS production. Our results suggest that chronic contaminated killifish populations are resistant to PCB induction of CYP1A activity and ROS production and that PCB induced P450 activity plays an important role in ROS production.

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SUB-CHRONIC SODIUM ARSENATE EXPOSURE AFFECTS FERTILITY AND EARLY DEVELOPMENT IN HOMING PIGEONS: A MODEL FOR MIGRATORY BIRDS.

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Arsenic is found in high levels in surface waters near mine sites and in organoarsenic pesticide manufacturing plants where migratory birds are exposed and could result in fertility or developmental toxicities as shown for some mammalian species. These studies investigate the effect of repeated low doses of sodium arsenate on fertility, embryonic and neonatal development in homing pigeons as a model for migratory birds. Homing pigeons received doses of 0.5, 1.0, or 2.0 mg/kg of sodium arsenate a total of four times. Dosed birds were allowed to randomly mate and breed. Fertility and neonatal growth rates were recorded over four breeding cycles. Eggs were collected for embryo extraction at 2 days, 6 days, 8 days, and 12 days. Bone development was observed utilizing the Von Kossa staining method on sagittal sections of 8-day embryos. RT-PCR was performed on 2-day, 6-day and 12-day embryos for several developmental markers. Significantly lower fertility rates and 24-hour birth weights were observed for the treated birds as compared to the control birds. Morphological studies indicated no difference in bone development between the two groups. Skeletal Alpha-Actin was expressed consistently in 2-day, 6-day, and 12-day embryos and Cardiac Alpha Actin was expressed

consistently in 6-day and 12 day embryos with no difference between treated and control groups. Embryonic Heart Myomesin was expressed in all 12-day embryos and a single 6-day treated embryo. Sub chronic sodium arsenate exposure in pigeons corresponded with significantly lower fertility and birth weights and may cause the disruption of developmental gene expression, particularly embryonic heart myomesin. These data indicate that exposure to arsenate through mining contaminants and/or pesticide production may cause population-level effects in migratory bird species through decreased reproductive capacity. This study was funded in part by USDA Hatch Grant NEV 00727 and the Nevada Agricultural Experiment Station.

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TRUE HERMAPHRODITISM IN A CD-1 MOUSE.

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True hermaphroditism was diagnosed in a 20 week old female CD-1 mouse. Although no macroscopic abnormalities were recorded in the gonads, the uterus was dilated and the cervix thickened. Microscopically, bilateral ovaries were present. The right ovary was composed of several large central corpora lutea and variable amounts of ovarian stroma. Surrounding this were small numbers of seminiferous tubules, lined by Sertoli cells and lacking spermatogonia. In the adjacent connective tissue, a well defined section of epididymis was present. The left ovary was composed of variably atrophic seminiferous tubules lined by frequently vacuolated Sertoli cells. Small amounts of ovarian stromal tissue was also identified. Immunohistochemical analysis using Testosterone, 3 beta-hydroxy steroid dehydrogenase, p450c17, Vimentin and alpha-Smooth muscle actin was performed. In addition, chromosomal analysis was carried out to determine the presence of numeric or structural defects of the sex chromosomes

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CIGARETTE SMOKE INHALATION IN JUVENILE RATS: A POTENTIAL ANIMAL MODEL OF ADOLESCENT NICOTINE ADDICTION.

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Chronic administration of nicotine increases the density of nicotinic receptors in human and rodent brain. An increased susceptibility of juvenile rats to this receptor upregulation has been reported using continuous nicotine exposure with osmotic minipumps. The purpose of this study was to validate a model of nicotine addiction in juvenile rats inhaling cigarette smoke for 7 days. Rats were exposed in whole-body inhalation chambers to mainstream cigarette smoke generated from 2R4F cigarettes or filtered air (controls). Exposures were to 100 mg total particulate material/m<sup>3</sup> for 4 hours/day for 7 days. Subgroups were sacrificed immediately after the seventh exposure and after a 3-day hold period. Endpoints included evaluations of behavioral changes, body weight, and concentrations of nicotine and cotinine in blood and brain. Acetylcholine nicotinic receptor (nAChR) upregulation in the brain was determined by quantifying the extent of [<sup>125</sup>I]epibatidine binding in brain sections by receptor autoradiography. Concentrations (mean, n = 4) of nicotine in brains of juvenile (733 ng/g) and adult brains (985 ng/g) were not significantly different immediately following the seventh exposure. Nicotinic receptor upregulation was observed in brains of both juvenile and adult rats; however, the responses in the n. accumbens, cerebral cortex, thalamus and hippocampus were more profound in the juvenile as compared to adult rats. Exposure resulted in significant depression of heart rate and heart rate variability. These data confirm that nicotine inhaled as a component of cigarette smoke for only 7 days during adolescence produces robust increases in nAChR density, a hallmark of nicotine addiction. The effects in specific brain regions were more pronounced in juveniles than in adults despite similar nicotine and cotinine concentrations in whole brain. This animal model may be useful for studies investigating the mechanisms underlying nicotine addiction in juveniles.

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"SENSORIMOTOR PERFORMANCE DEFICITS, NEURONAL CELL LOSS, AND ELEVATED GLIAL ACIDIC PROTEIN EXPRESSION IN PURKINJE CELLS AND CA1 OF THE HIPPOCAMPUS IN ADULT OFFSPRING FOLLOWING MATERNAL EXPOSURE TO NICOTINE".

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The present studies were carried out in the offspring at adulthood following maternal exposure to nicotine via osmotic pump. Timed pregnant female Sprague-Dawley rats (300-350 gm) were treated with nicotine (3.3mg/kg, in bacteriostatic

water via s.c. implantation of the pump) from gestational days (GD) 4-20. Control animals were treated with saline via s.c. implantation of the pump. Male and female offspring on postnatal day (PND 90) were evaluated for sensorimotor functions, changes in the ligand binding for various types of nicotinic acetylcholine receptors in the cortex, and pathological alterations in the cerebellum and hippocampus. Neurobehavioral evaluations included, beam-walk-score, beam-walk time, incline plane and grip time response. Only male offspring from nicotine treated mothers showed significant deficits in beam-walk time and incline plane whereas both male and female offspring from nicotine treated mothers showed significant impairments in forepaw grip time. Ligand binding densities for [<sup>3</sup>H]epibatidine, [<sup>3</sup>H]cytisine and [<sup>3</sup>H] $\alpha$ -bungarotoxin did not show any significant changes in the cortex. Histopathological evaluation using cresyl violet staining showed a significant decrease in Purkinje cells in the cerebellum, and an increase in glial fibrillary acidic protein (GFAP) immunostaining in the white matter in both male and female offspring from nicotine treated mothers. CA1 subfield of the hippocampus from the female offspring of nicotine treated mothers showed a decrease in surviving neurons and an increase of GFAP immunostaining. These data suggest that maternal exposure to nicotine produces significant neurobehavioral deficits and pathological alterations in the cerebellum and CA1 subfield of the hippocampus in adult offspring. Supported by a grant from the External Research Program of Philip Morris, USA.

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**"INCREASED EXPRESSION OF GLIAL FIBRILLARY ACIDIC PROTEIN IN THE MOTOR CORTEX AND HIPPOCAMPUS, AND NEUROBEHAVIORAL DEFICITS IN THE OFFSPRING FOLLOWING GESTATIONAL EXPOSURE TO IMIDACLOPRID".**

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Imidacloprid belongs to a new generation of insecticides known as neonicotinoids. It has one of the fastest growing sales of any insecticide worldwide due to its selectivity for insects and its safety to human population. However, its neurotoxicity due to developmental exposure is not known. In the present studies, timed pregnant Sprague-Dawley rats (300-350 gm) were treated with a single intraperitoneal injection of imidacloprid (337 mg/kg, 0.75XLD<sub>50</sub> in corn oil) on gestational day 9. Control rats were treated with corn oil. On postnatal day 30, the offspring were evaluated for changes in the ligand binding for nicotinic acetylcholine receptors (nAChR) and muscarinic acetylcholine receptors (m2 mAChR), neurobehavioral performance and pathological alteration in brain regions. Beam-walk time, incline plane and forepaw grip time showed significant impairments in the offspring from imidacloprid treated mothers. Ligand binding densities for [<sup>3</sup>H]cytisine for  $\alpha 4\beta 2$  type nAChR did not show any significant change, whereas [<sup>3</sup>H]AFDX 384, a ligand for m2mAChR showed significant increase in the cortex of the offspring from imidacloprid treated mothers. Histopathological evaluation using cresyl violet staining did not show any alterations in surviving neurons in different brain regions. A significant increase in glial fibrillary acidic protein (GFAP) immunostaining in the motor cortex and hippocampus CA1 and dentate gyrus of the offspring from imidacloprid treated mothers was observed. These data suggest that gestational exposure to a large toxic dose of imidacloprid produces significant neurobehavioral deficits, and an increased expression of GFAP in different brain regions of the offspring on PND 30. These changes may have long-term adverse health effects later in life.

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**NONCHOLINESTERASE MECHANISM(S) OF CHLORPYRIFOS NEUROTOXICITY INDICATED BY GENE EXPRESSION PROFILES OF RAT BRAIN TISSUE.**

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Chlorpyrifos (CPF) is a widely used organophosphate insecticide that affects the nervous system by reversibly inhibiting the activity of cholinesterase (ChE). Recent studies have suggested that low doses of CPF disrupt brain development and cognitive function by mechanisms that do not involve the inhibition of acetylcholinesterase (AChE). Few reports in the literature have addressed the effect of CPF on the nervous system using transcriptomic profiling. In this study brain tissues isolated from rats 4 days after treatment with 1.0mg/kg, 10 mg/kg and 50mg/kg of CPF were processed for Affymetrix RAE230A Genechip analysis. Statistical analysis showed that the expression level of 151 genes changed in the brain isolated from CPF-treated animals. Genes playing a role in the G-protein pathways and sodium, potassium and cation transport showed down-regulation. Also down-regulated were responders to hypoxia and oxidative stress, as were the

genes involved in the regulation of synapse proteins and membranes. Transcripts for the genes involved in membrane synapse transmission and brain development showed up-regulation as well as those genes connected to transcription factors, protein phosphorylation and intracellular protein transport. Interestingly, the gene for the precursor of beta amyloid and the putative rat homolog of the human gamma synuclein gene, which have been implicated in neurodegenerative pathogenesis including Alzheimer's disease, were up-regulated after CPF treatment. This finding is consistent with the reports that CPF neurotoxicity involves pathways other than cholinesterase. Further analysis of the molecular and biological functions of these genes will likely provide a better understanding as to the mechanism(s) of CPF neurotoxicity.

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**OXIDATIVE MECHANISMS CONTRIBUTING TO THE DEVELOPMENTAL NEUROTOXICITY OF NICOTINE AND CHLORPYRIFOS.**

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Nicotine and chlorpyrifos are developmental neurotoxicants that, despite their differences in structure and mechanism of action, share many aspects for damage to the developing brain. Both are thought to generate oxidative radicals; in the current study, we evaluated their ability to produce lipid peroxidation in two *in vitro* models of neural cell development (PC12 and SH-SY5Y cells) and for nicotine, with treatment of adolescent rats *in vivo*. Nicotine and chlorpyrifos, in concentrations relevant to human exposures, elicited an increase in thiobarbituric acid-reactive species (TBARS) in undifferentiated cells, an effect that was prevented by addition of the antioxidant, Vitamin E. Initiating differentiation with nerve growth factor, which enhances nicotinic acetylcholine receptor expression, increased the TBARS response to nicotine but not chlorpyrifos, suggesting that the two agents act by different originating mechanisms to converge on the endpoint of oxidative damage. Furthermore, nicotine protected the cells from oxidative damage evoked by chlorpyrifos and similarly blocked the antimitotic effect of chlorpyrifos. Treatment of adolescent rats with nicotine elicited increases in TBARS in multiple brain regions when given in doses that simulate plasma nicotine concentrations found in smokers or at one-tenth the dose. Our results indicate that nicotine and chlorpyrifos elicit oxidative damage to developing neural cells both *in vitro* and *in vivo*, a mechanism that explains some of the neurodevelopmental endpoints that are common to the two agents. The balance between neuroprotectant and neurotoxicant actions of nicotine may be particularly important in situations where exposure to tobacco smoke is combined with other prooxidant insults. Support: NIH ES10387 and ES10356, and Philip Morris USA.

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**DEVELOPMENTAL CHLORPYRIFOS EXPOSURE TARGETS SEROTONIN FUNCTION AND RELATED BEHAVIORS.**

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Developmental exposure to chlorpyrifos (CPF) causes persistent changes in serotonergic (5HT) systems. We administered 1 mg/kg/day of CPF to rats on postnatal days 1-4, a regimen below the threshold for systemic toxicity. When tested in adulthood, CPF-exposed animals showed abnormalities in 5HT synaptic transmission and in behavioral tests that depend on 5HT. Both 5HT1A and 5HT2 receptor subtypes were upregulated in multiple brain regions, suggestive of reduced 5HT synaptic activity. In agreement, the ability of 5HT to stimulate adenylyl cyclase showed a distinct switch from stimulatory to inhibitory actions in females. For behavioral testing, we adopted two tests from standard procedures utilized in evaluations of 5HT systems in anxiety and depression. In the elevated plus maze, animals treated with CPF spent more time in the open arms, indicative of increased risk-taking behavior. In a test for anhedonia, the CPF-exposed group showed a decreased preference for chocolate milk versus water, indicative of decreased importance of rewarding stimuli. Both these findings are characteristic of a deficit in 5HT synaptic activity. Developmental CPF exposure also has lasting effects on cognitive function. We replicated our earlier finding, that developmental CPF exposure impairs the normal sex differences in 16-arm radial maze spatial learning and memory. During acquisition training control male rats performed more accurately than control females. CPF treatment eliminated this sex difference. Females exposed to CPF showed a reduction in working and reference memory errors, reducing their error rate to that equivalent of control males. Conversely, CPF-exposed males exhibited an increase in working and reference memory errors. To measure the CPF-induced functional impairment of 5HT function a dose-effect function of the 5HT2 antagonist ketanserin is used. Our results indicate that otherwise subtoxic neonatal CPF exposure produces lasting changes in 5HT synaptic activity and related behavioral performance. NIH ES10387, ES10356, ES07031.

YOUNG ANIMALS ARE DEFICIENT IN BOTH HEPATIC P450 AND CARBOXYLESTERASE DETOXIFICATION OF DELTAMETHRIN, A PYRETHROID PESTICIDE.

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It is known that young rats are markedly more sensitive to the acute toxicity of deltamethrin, a pyrethroid pesticide. To determine the underlying mechanism for that increased sensitivity, the deltamethrin detoxification capability of liver from postnatal day 21 (PND21) rats was compared to the detoxification capability of liver from adult (PND90) rats. All animals were male, Long-Evans rats; n=4 per age. Two primary detoxification routes were examined: carboxylesterase (CaE) activity in liver homogenates and P450 detoxification in liver microsomes. Deltamethrin detoxification was assessed by measuring the disappearance of deltamethrin over time using an HPLC method. It was found that hepatic P450s metabolized deltamethrin much more efficiently than CaEs, and that adult liver (either enzyme) metabolized deltamethrin more rapidly than PND21 liver (adult P450 =  $197.8 \pm 18.8$ ; PND21 P450 =  $97.3 \pm 6.8$ ; adult CaE =  $15.4 \pm 1.2$ ; PND21 CaE =  $4.8 \pm 1.4$ ) (ng deltamethrin metabolized/min/mg protein). Adult liver P450s metabolized deltamethrin twice as fast as the PND21 liver P450s, and adult liver CaEs metabolized deltamethrin 3.2 times faster than PND 21 hepatic CaEs. In a related investigation to determine which P450s are responsible for deltamethrin detoxification, various rat Supersomes® were assessed for deltamethrin detoxification potential. It was found that both 1A1 and 2B1 metabolized deltamethrin. In sum, young animals are deficient in enzymes which detoxify deltamethrin; this lack of detoxification capability may explain the marked age-related sensitivity to deltamethrin. *This is an abstract of a proposed presentation and does not reflect Agency policy.*

EFFECT OF ORGANOPHOSPHORUS INSECTICIDES AND THEIR METABOLITES ON DNA SYNTHESIS IN ASTROGLIAL CELLS.

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Though little attention has been given to the possibility that glial cells may represent a target for the developmental neurotoxicity of organophosphorus (OP) insecticides, recent evidence, obtained in particular with chlorpyrifos (CP), suggests that developmental exposure to this compound may indeed target astrocytes. To substantiate and expand these observations, we carried out a series of *in vitro* studies utilizing fetal rat astrocytes and a human astrocytoma cell line, 1321N1 cells, to investigate the effect of the OPs CP, diazinon (DZ) and parathion (P), their oxygen analogs chlorpyrifos oxon (CPO), diaxonon (DZO) and paraxon (PO), and their metabolites 3, 5, 6-trichloro-2-pyridinol (TCP), 2-isopropyl-6-methyl-4-pyrimidol (IMP) and para-nitrophenol (PNP), on cell proliferation. In fetal rat astrocytes and astrocytoma cells maintained in serum CP, DZ, P, CPO, DZO, and PO induced a concentration-dependent inhibition in [<sup>3</sup>H]thymidine incorporation with a very similar potency (IC<sub>50</sub>s between 45 and 57  $\mu$ M). Among the other metabolites, PNP was the most potent (IC<sub>50</sub>s = 81 and 70  $\mu$ M for rat astrocytes and astrocytoma cells, respectively), while TCP and IMP were much less effective (IC<sub>50</sub>s > 100  $\mu$ M). Cytotoxicity appears to account only for a small part of the effect on DNA synthesis. OP insecticides and their oxons were 3-6 fold more potent in inhibiting [<sup>3</sup>H]thymidine incorporation when cells were synchronized in the G0 phase of the cell cycle and re-stimulated by carbachol or EGF. These results suggest that OP insecticides and their oxons affect astroglial cell proliferation and that the transition from the G0 to the G1/S phase of the cell cycle may be particularly sensitive to the action of these compounds. (Supported in part by ES09601/EPA-R826886, ES07033).

PON1 MODULATES OP TOXICITY DURING DEVELOPMENT.

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Paraoxonase (PON1) is an HDL-associated enzyme that hydrolyzes multiple substrates, including organophosphorous compounds such as the nerve agents sarin and soman, and the highly toxic oxon forms of diazinon and chlorpyrifos. PON1 activity is determined in part by a Q192R coding region polymorphism that affects its catalytic efficiency and in part by differences in PON1 expression levels, which vary widely (>13 fold). Young children represent a particularly susceptible population for OP exposure due to low abundance of PON1. To address the involvement of PON1 in protecting against OP-related neurotoxicity during development,

PON1 knockout (PON1<sup>-/-</sup>) mice, or mice expressing PON1 (human or mouse), were exposed chronically (PN4 to PN21) to low levels of chlorpyrifos oxon (CPO). Endpoints included cholinesterase activity, histopathology, behavior, and gene expression. As early as PN4, PON1<sup>-/-</sup> mice were more sensitive than wild-type mice to inhibition of brain cholinesterase by CPO. At PN22 and persisting as long as 4 months, chronic developmental exposure of PON1<sup>-/-</sup> mice to 0.18 or 0.25 mg/kg/d CPO resulted consistently in perinuclear vacuolization of cells and abnormal distribution of neurons in the neocortex, with an increase in the number of affected cells at 0.25 mg/kg/d. In mice expressing PON1, this pathology was less severe and was only observed at 0.25 mg/kg/d. The neurobehavioral test battery did not reveal any effects on the specific behaviors chosen for assessment. However, from PN16-19, PON1<sup>-/-</sup> mice exposed chronically to 0.18 or 0.25 mg/kg/d exhibited striking hyperactivity immediately following CPO administration. These studies used multiple endpoints to reveal toxicity associated with low level OP exposure, and indicate that PON1 modulates OP toxicity during development. Supported by T32 AG0057, ES09601/EPA-R826886, ES09883, ES04696, ES07033, ES11387.

REPEATED EARLY POSTNATAL EXPOSURE TO CHLORPYRIFOS AFFECTS WORKING MEMORY PERFORMANCE IN THE 12-ARM RADIAL MAZE.

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Developmental exposure to chlorpyrifos (CPS) is thought to be associated with cognitive deficits in children. The current study investigated the effects of repeated early postnatal CPS exposure on spatial memory. Using an incremental dosing regimen, Sprague-Dawley rats were orally gavaged daily with either corn oil or a low CPS dosage of 1mg/kg from postnatal day (PND) 1-21; a medium dosage of 1 mg/kg from PND1-7, 2 mg/kg from PND8-14, and 4 mg/kg from PND15-21; or a high dosage of 1.5 mg/kg from PND1-7, 3 mg/kg from PND7-14, and 6 mg/kg from PND15-21. Rats were then tested beginning on PND36 for 4 weeks on a working memory task using a 12-arm radial maze paradigm. Developmental CPS exposure elicited significant reduction in cholinesterase activity that persisted up to 19 days after the cessation of exposure. Behavioral testing indicated significant treatment effects as demonstrated by decreased arm accuracy within and between training blocks. The number of total accurate choices made during the first eight arm visits was significantly decreased by CPS in both sexes. CPS treated rats also made significantly fewer correct choices prior to making their first error. However, as the animals became more familiar with the radial arm maze, performances in both control and CPS treated rats improved with respect to both total arm accuracy within first eight visits and accurate choices before first arm error. In the fourth week of testing, control males were significantly more accurate than control females, committing fewer errors and visited more arms before first error. Repeated exposure to CPS reversed this effect. These data suggest that repeated early postnatal exposure to CPS can alter spatial working memory in a gender specific manner that may persist beyond the period of exposure. (Supported by NIH R01 ES 10386).

DISRUPTION OF PERFORMANCE IN THE 12-ARM RADIAL MAZE IN RATS EXPOSED TO METHYL PARATHION DURING THE EARLY POSTNATAL PERIOD.

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Developmental exposure to methyl parathion (MPS) is suspected to contribute to cognitive deficits in children. The current study investigated the effects of repeated early postnatal exposure to MPS on spatial memory. Using an incremental dosing regimen, Sprague-Dawley rats were orally gavaged daily with either corn oil or methyl parathion at a low dose of 0.2 mg/kg from postnatal day (PND) 1-21; a medium dose of 0.2 mg/kg from PND1-7, 0.4 mg/kg from PND 8-14, and 0.6 mg/kg from PND15-21; or a high dose of 0.3 mg/kg from PND1-7, 0.6 mg/kg from PND8-14, and 0.9 mg/kg from PND15-21. These dosages are known to produce no overt signs of toxicity. Rats were then tested beginning on PND36 for 4 weeks (4 sessions per week for 16 total sessions) on a spatial working/reference memory task using a 12-arm radial maze paradigm. MPS exposure elicited significant ( $p < 0.05$ ) reduction in cholinesterase (ChE) activity that persisted up to 19 days after the cessation of exposure. Assessment of spatial working memory was made using two criteria; total number of correct choices in the first eight arm visits and the number of accurate arm choices before making the first error. The total number of correct arm choices in first eight arm visits was significantly reduced ( $p < 0.05$ ) in both MPS treated males and females as compared to controls. However, there were no treatment effects on the number of correct arm choices before making the first error. As the animals gained more experience became more fa-

miliar with the maze, the total number of accurate arm choices increased with decreasing latency over successive four-day training blocks in all groups. These data suggest that early postnatal exposure to MPS alters spatial working memory but to a lesser degree and is less persistent than CPS. (Supported by NIH R01 ES 10386).

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#### NGF GENE EXPRESSION AND PROTEIN LEVELS IN THE JUVENILE HIPPOCAMPUS AFTER REPEATED EXPOSURE TO CHLORPYRIFOS AND METHYL PARATHION.

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Phosphorothionates are the largest subclass of the organophosphate (OP) insecticides and are considered to be more toxic than the other subclasses. Two of the most commonly used phosphorothionates are the diethyl OP chlorpyrifos (CPS), and the dimethyl OP methyl parathion (MPS). The toxic effects of these compounds are attributed to the ability of their reactive metabolites to inhibit acetylcholinesterase (AChE) but there are differences in the patterns of AChE inhibition between the two insecticides. In the developing rat, the most dynamic aspects of cholinergic innervation take place during the first postnatal weeks. Neurotrophins, such as nerve growth factor (NGF), play an important role in this development and are regulated, in part, by neuronal activity. Given the excitatory signaling associated with OP exposure, it is possible that exposure to these two insecticides during that period could differentially disrupt the regulation of NGF levels and alter normal cholinergic development. The purpose of this project was to investigate the effects of CPS and MPS on gene expression and protein levels of the neurotrophin NGF in the hippocampus after repeated exposure (postnatal day 10-20) to CPS and MPS in rats. Using quantitative real-time RT-PCR, decreased NGF mRNA levels were observed in the hippocampus of both male and female rats exposed to the high dosage of CPS (6 mg/kg) but exposure to the high dosage of MPS (0.9 mg/ml) decreased hippocampal NGF mRNA levels in females only. However, there were no effects on NGF protein levels after either CPS or MPS treatment. The effect observed in NGF mRNA but not protein levels could be due to several factors such as the presence of multiple NGF transcripts in the hippocampus with only a select few controlling the level of NGF protein in the hippocampus or possible stabilization of the NGF protein as a result of OP exposure (Supported by NIH 1 P20 RR17661-01).

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#### EFFECTS OF POSTNATAL TREATMENT WITH CHLORPYRIFOS AND METHYL PARATHION ON CHOLINERGIC PARAMETERS IN RAT BRAIN REGIONS.

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The effects of early postnatal exposure to the commonly used organophosphorus insecticides chlorpyrifos (CPS) and methyl parathion (MPS) were investigated. Cholinesterase (ChE) activity, choline acetyltransferase (ChAT) activity, and muscarinic acetylcholine receptor (mAChR) densities were examined in several brain regions, which include cerebral cortex, corpus striatum, hippocampus, medulla/pons, and cerebellum. Using an incremental dosing regimen, pups were orally gavaged daily with one of seven treatments. 1) corn oil (control) 2) CPS low dose of 1 mg/kg from postnatal day (PND) 1 to 21; 3) CPS medium dose of 1 mg/kg from PND 1-5, 2 mg/kg from PND 6-13, and 4 mg/kg from PND 14-21; 4) CPS high dose of 1.5 mg/kg from PND 1-5, 3 mg/kg from PND 6-13, and 6 mg/kg from PND 14-21; 5) MPS low dose of 0.2 mg/kg from PND 1-21; 6) MPS medium dose of 0.2 mg/kg from PND 1-5, 0.4 mg/kg from PND 6-13, and 0.6 mg/kg from PND 14-21; 7) MPS high dose of 0.3 mg/kg from PND 1-5, 0.6 mg/kg from PND 6-13, and 0.9 mg/kg from PND 14-21. ChE activity, assayed spectrophotometrically, was inhibited in a dose related manner on PND 20 (4-62%), 25 (11-26%) and 30 (1-26%). PND 40 showed no inhibition. ChAT activity was determined by radioenzymatic microdetermination on PNDs 20, 25, 30 and 40. Males exposed to MPS exhibited statistical decreases in ChAT activity in the cerebral cortex and hippocampus on PND 20 and 25. The only change in female ChAT activity was an increase seen in the medulla/pons on PND 20 and 30. No statistical differences were seen at PND 40 suggesting recovery has occurred. Total and sub-types of mAChR densities were investigated on PNDs 12 and 20 by radioligand receptor binding assay. Several changes were noted. For example, the levels of total mAChR in male and female hippocampus were found to decrease by 17-31% at PND 20 in the high dose of CPS and MPS treated pups. These results indicate that pups exposed postnatally to CPS and MPS exhibited alterations in cholinergic biochemistry. (Supported by NIH R01 ES 10386).

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#### DEVELOPMENTAL PYRETHRIN EXPOSURE ALTERS DOPAMINERGIC NEUROCHEMISTRY RESULTING IN HYPERACTIVITY AND ENHANCED TOXICITY OF MPTP.

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The dopamine transporter (DAT) plays an integral role in control of locomotor activity and serves as the gateway for the parkinsonism-inducing toxin MPTP. Previously, we have observed that perinatal exposure of mice to 3 mg/kg of the pyrethroid pesticide deltamethrin increases DAT levels in their offspring at 28 days. In this study, we sought to determine the dose-related effects of low level deltamethrin exposure (0.3, 1, or 3 mg/kg every 3 days throughout gestation and lactation) on dopaminergic neurochemistry and locomotor activity in developing mice. We also determined whether deltamethrin exposure affects the dopaminergic toxicity of MPTP. At 5 weeks of age, striatal DAT levels were significantly increased by 21, 35, and 70% in offspring of the 0.3, 1, and 3 mg/kg groups, respectively. At these same doses, total locomotor activity was significantly increased by 98, 231, and 285%. The increased locomotor activity in deltamethrin treated offspring was attenuated by the dopamine receptor antagonist haloperidol, suggesting a primary role of the dopamine system in the locomotor promoting effects of deltamethrin. Finally, MPTP exposure (2 x 10 mg/kg s.c.) at 12 weeks of age resulted in a greater reduction of striatal dopamine in deltamethrin mice (77% in the 3 mg/kg group) compared to controls (66%). These data suggest that the developing dopaminergic system is particularly sensitive to deltamethrin exposure during the perinatal period even though there is no direct treatment of the offspring. The effects observed here are particularly notable since the dosage levels employed are 4-40-fold below the developmental NOAEL of 12 mg/kg and suggest that low-level deltamethrin exposure results in hyperactivity and increased risk of dopaminergic damage. (Supported by NIH R21ES012315 and F32ES013457).

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#### NEONATAL EXPOSURE TO NICOTINE INDUCES INCREASED SUSCEPTIBILITY TO PARAOXON EXPOSURE AT ADULT AGE.

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Low dose exposure of neonatal mice to nicotine has earlier been shown to induce an altered behavioral response to nicotine in adulthood. Organophosphorus insecticides are known to affect the cholinergic system by inhibition of acetylcholinesterase. This study was undertaken to investigate whether neonatal exposure to nicotine makes mice more susceptible to a known cholinergic agent, paraoxon. Neonatal, 10-day-old, male mice were exposed to nicotine-base (33 mg/kg b.wt.) or saline s.c. twice daily on 5 consecutive days. At five months of age the animals were exposed to paraoxon (0.17 or 0.25 mg/kg b.wt. (29% and 37% inhibition of cholinesterase, respectively)) or saline s.c. every second day for 7 days (total of 4 injections/mouse). Before the first paraoxon injection, the animals were observed for spontaneous motor behavior. The spontaneous behavior test did not reveal any differences in behavior between the treatment groups. Immediately after the spontaneous behavior test the animals received the first injection of paraoxon and were observed for acute effects of paraoxon on spontaneous behavior. The acute response to paraoxon in the spontaneous behavior test was a decreased level of activity in mice neonatally exposed to nicotine. Control animals showed no change in activity in response to the paraoxon injection. Two months after termination of the paraoxon treatment, the animals were again tested for spontaneous motor behavior. Animals neonatally exposed to nicotine and exposed to paraoxon as adults showed a deranged spontaneous behavior, including hyperactivity and lack of habituation. In conclusion, neonatal nicotine exposure makes adult mice more susceptible to a low-dose paraoxon exposure, resulting in a persistent change in spontaneous motor behavior.

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#### NEONATAL EXPOSURE TO A SINGLE LOW DOSE OF A TYPE I PYRETHRIN (BIOALLETHRIN) AFFECTS SPONTANEOUS BEHAVIOR AND LEARNING IN ADULT MICE OF DIFFERENT STRAINS.

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In earlier studies we have reported on developmental neurotoxic effects after neonatal exposure to pyrethrins. Neonatal NMRI mice exposed to a single oral dose of bioallethrin once daily during postnatal day 10 through 16 displayed deranged spontaneous behavior, changes in muscarinic cholinergic receptors in the cerebral cortex in adult mice. These effects were dose-response related. The task of this study was to determine whether neonatally exposed mice were affected by just a single low dose of bioallethrin. Two mice strains were used, NMRI and C57bl. NMRI

mice were orally given bioallethrin at two doses, 0.01mg/kg b.wt. and 0.7mg/kg b.wt. on postnatal day 10. C57bl mice were orally given bioallethrin, 0.07mg/kg b.wt. and 0.7mg/kg b.wt. Control mice for both strains orally received the 20% fat emulsion vehicle. Spontaneous behavior was observed in two-month-old mice and then again at four-months of age (C57bl only). Swim maze test was conducted at three months of age for NMRI and four months of age for C57bl mice. The results of the spontaneous behavior testing showed persistent and a significantly higher activity level in locomotion, rearing and total activity for the mice exposed to a single oral dose of bioallethrin (0.7mg/kg b.wt.) for both mouse strains. In C57bl mice the spontaneous behavior was even altered after the low dose of bioallethrin, 0.07mg/kg b.wt. The swim maze test revealed that C57bl mice exposed to bioallethrin at a dose of 0.7mg/kg b.wt. performed significantly worse than the control groups and the mice exposed to bioallethrin 0.07mg/kg b.wt., this was seen as their ability to relearn was impaired. The major findings of the present study show that just a single low dose of bioallethrin can cause the same developmental neurotoxic effect on spontaneous behavior as repetitive doses over one week of the neonatal period. This indicates the presence of a greater defined critical window during development when persistent effects can be induced by the pyrethroid bioallethrin.

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#### STRUCTURAL IMPACT OF DIAZINON AND MOLINATE ON NEURITE OUTGROWTH IN N1E-115 CELLS.

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Sponsor: J. Casida.

Cholinesterases are classically known for their role in hydrolyzing neurotransmitters. Recent studies also suggest that they may have a broader role, particularly in the development of nervous system. Sequence similarities to lipases, esterases, and cell adhesion molecules appear to reflect their structural role in neurite outgrowth. Deviations in normal levels of acetylcholinesterase at specific times in nervous system development appear to contribute to observed neuroanatomic abnormalities such as altered neurite outgrowth. In this study, we explored the influence of diazinon and molinate on the normal outgrowth of neurites of the neuroblastoma cell line N1E-115. Diazinon, an organophosphorous insecticide and acetylcholinesterase inhibitor, and molinate, a thiocarbamate herbicide, were both evaluated for their ability to alter neurite outgrowth and inhibit acetylcholinesterase activity in N1E-115 cells. Three concentrations of each pesticide (1, 10, and 100 $\mu$ M) were used to assess cell viability, acetylcholinesterase activity, and neurite outgrowth. Based on the concentrations tested significant inhibition of acetylcholinesterase was not observed for both diazinon and molinate; however, we observed changes in neurite outgrowth. Using a probe line method to produce unbiased parameter estimates, we examined the number and size of cells and neurites following exposure to both pesticides. Preliminary results indicate a decrease in neurite outgrowth in N1E-115 cells following treatments with diazinon and molinate, suggesting broader modes of action for both pesticides.

**977**

#### NEUROPATHOLOGICAL FINDINGS IN DEVELOPMENTAL NEUROTOXICITY TESTING: COMPARISON OF QUALITATIVE AND QUANTITATIVE EVALUATIONS.

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The USEPA Developmental Neurotoxicity Test (DNT) Guideline (OPPTS 870.6300) includes both qualitative histopathological evaluation and quantitative (morphometric) analysis. Quantitative assessments involve brain weight and linear measurements of several brain regions (minimally the neocortex, hippocampus, and cerebellum) in immature and adult offspring. OECD has released draft guidelines for DNT studies in which quantitative assessments are not required, but are recommended to assist in interpretation of brain weight or qualitative changes. The ability of qualitative and quantitative assessments to detect changes in the brain following developmental exposure was compared by examining the results of 31 guideline DNT studies. Qualitative neuropathological assessments were performed in pups on post-natal day 11 or 21 in all 31 studies; assessments in adults were performed in 28 studies. Morphometric evaluations were performed for pups in 21 studies and for adults in 19 studies. Qualitative neuropathologic effects were seen in only 2 studies (once in pups, once in adults); neither finding involved effects in the brain. Morphometric changes were seen in 12/21 evaluations for pups, 10/19 evaluations for adults. In 18 studies evaluating both ages, 11 found changes at both ages, 2 in adults only, and 1 in pups only. Changes in brain weight were not predictive of morphometric changes. Brain weight changes were seen in 12/30 studies in pups and 6/28 studies in adults; in 7 cases each for pups and adults, morphometric effects were found in the absence of brain weight changes. These results indicate that

morphometric evaluations detect changes not detected by qualitative histopathological evaluations or changes in brain weight, and vice versa. These data suggest all three procedures may uniquely contribute to neuropathological assessment in the DNT study. This abstract does not necessarily reflect the policy of the USEPA.

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#### A SIMPLE METHOD TO ESTIMATE RAT PUP EXPOSURE IN DIETARY DEVELOPMENTAL NEUROTOXICITY STUDIES.

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Verification of pup exposure during lactation is a major concern in developmental neurotoxicity studies, especially via dietary exposure. An accurate, sensitive method was developed to estimate rat pup compound consumption (CC) during lactation using levels of compound assayed in milk. Milk concentrations were determined and pup CC was estimated for two compounds in 3 range-finding studies for lactation days (LD) 4/5, 11 and 17/18 over a maternal dietary range of 50-350 ppm. Dams were fed treated diets from gestation day 6 to LD 21 (LD 17 in one study). Milk from at least 5 dams/group was collected, following SC injection with oxytocin (20 U/ml, 0.05 ml), manually by Pasteur pipettes on LD 4/5 (dams were lightly anesthetized for this sample) and by pump on LDs 11 and 17/18. Plasma was collected on the same dams on LDs 4/5 & 21 and for culled pups on postnatal day (PND) 4/5 and for weanlings on PND 21 in two studies. Milk and plasma were analyzed for levels of test compound by solvent extraction, liquid-liquid partition and Gas Chromatographic quantitation (LOD/LOQ = 0.01/0.05 ppm). Pup body weights (BW) on PNDs 4, 11 and 17, and pup BW gain for PNDs 0-4, 7-11 and 14-17 for the litters of dams sampled were used to estimate milk consumption (MC) by the method of Sampson and Jansen (J.Pediatric Gastroenterology and Nutrition, 3: 613; 1984). MC (g) was determined for each time interval by the formula: MC = 0.0332 + 0.0677 (BW) + 0.8775 (BW gain/24 hr). Pup CC was calculated for each time interval as CC (mg/kg BW) = (MC) (% compound in milk)(1000 mg/g) / BW in kg. Estimated pup doses positively correlated with plasma levels of each compound, determined for pups and dams from the same litters

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#### THALIDOMIDE-INDUCED OXIDATIVE STRESS IN RAT DORSAL ROOT GANGLIA (DRG) CELL CULTURES.

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Thalidomide has beneficial effects in treating inflammatory, malignant and infectious diseases. Its potential therapeutic use is often limited by the onset of peripheral sensory neuropathies, the mechanisms for which are unknown. Studies suggest that thalidomide-induced free radicals may have a mechanistic role in its activity and lead to present efforts to determine whether thalidomide is causing oxidative stress and redox misregulation in the peripheral nervous system. DRGs, isolated as mixed neuronal and glial cell populations from GD 17 rat embryos, were grown under conditions that favor neuronal proliferation. HPLC analysis showed that glutathione (GSH) concentrations were not significantly altered following 50 and 100 mM thalidomide treatment for 2, 6, 24 and 48 hr. In contrast, glutathione disulfide (GSSG) concentrations increased significantly by 2.7 fold after 48 hr of 100 mM thalidomide exposure. After 24 hr and 48 hr, respectively, thalidomide shifted the intracellular redox potential towards the positive by +32.7 mV and +30.4 mV for 100 mM and +26.0 mV and +22.5 mV for 50 mM thalidomide treatments. These results show that thalidomide is causing oxidative stress sufficient to alter the redox potential of cultured cells and could be affecting essential cell activities that depend on a reduced cell environment. Further analysis attempted to determine whether differential responses to the drug occurred in neuronal and glial cells of the mixed culture. Qualitative analysis using confocal microscopy and CMFDA fluorescence showed a prolonged decrease in neuronal fluorescence after thalidomide treatment while glial cell fluorescence decreased only marginally and recovered soon after treatment, possibly, compensating for neuronal losses. These results show that thalidomide causes oxidative stress in DRG mixed neuronal and glial cell cultures and that differences exist in the response of associated cell types as they cope with changes related to oxidative stress.

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#### EFFECTS OF DEVELOPMENTAL HYPOTHYROIDISM INDUCED BY PTU ON BRAIN FUNCTION IN RATS.

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It has been known that lack of thyroid hormone during development leads to structural, mental and behavioral changes in human and animals. In recent years, social concerns have increased about developmental neurotoxicity induced by environ-

mental chemicals, especially substances that affect the level of thyroid hormone. Hypothyroid rat experimentally induced by propylthiouracil (PTU) is considered to be a useful animal model for the developmental neurotoxicity. In the present study, we examined the effects on brain function in PTU-induced hypothyroidism rats in accordance with EPA DNT guideline. PTU was administered to dams orally at dosages of 0, 0.4, 1.0 and 2.5 mg/kg from Gestation Day 18 until postnatal day (PND) 21, and the effects on their offspring were evaluated. Although reduced concentration of triiodothyronine (T3) in 1.0 and 2.5 mg/kg groups, and thyroxin (T4) in all treated groups were detected on PND 22, these changes did not persist into adulthood. Reduced body and brain weight, and delay of eye opening were observed in 1.0 and 2.5 mg/kg groups. In the measurement of motor activity, tendency of reduction on PND 16, and increase on PND 21 and after, were detected. In the E-maze learning test, decreased ability was observed in infant and adults. In addition, decreased response in infant and increased response in adults were observed in acoustic startle response test. As a result, the changes in motor activity, learning ability and startle response were detected in PTU-induced hypothyroidism rat, and some of their responses varied by measurement time points that DNT guideline requires.

**981** EFFECT OF HYPOTHYROIDISM INDUCED BY PERINATAL EXPOSURE TO PTU ON CEREBELLAR GENE EXPRESSION.

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Certain kinds of developmental neurotoxicants are considered to work by affecting the level of thyroid hormone, which is essential for the brain development of both human and experimental animals. Hypothyroid rat experimentally induced by propylthiouracil (PTU) is now a useful animal model for the developmental neurotoxicity. The purpose of this study is to clarify the developmental alterations in gene expression caused by the decrease of thyroid hormones using this model. We quantified a series of genes in the cerebellum of rat exposed perinatally to various doses of PTU by quantitative RT-PCR on postnatal day (PND) 22 and after maturation. We especially focused on the genes implicated in the formation of neuronal network or synaptic function, including growth associated protein-43, brain-derived neurotrophic factor (BDNF), L1, neuronal cell adhesion molecule, synaptophysin, post synaptic density-95 and NMDA receptor 2A/2B (NR2A/NR2B), and also examined the control developmental profile of these genes mRNA. PTU-induced hypothyroidism in rats resulted in up-regulation of NR2B mRNA and down-regulation of NR2A and BDNF mRNAs in the cerebellum on PND 22, but no PTU-treatment-related alteration of these gene expressions were observed after maturation. Our additional examinations of NR2A and NR2B gene expression in cerebellum during earlier period demonstrated that PTU-treatment-related changes of NR2A and NR2B gene expression were evident on PND14, but not on PND4. There were no changes in other genes on PND22 and after maturation. These results suggested that PTU-induced hypothyroidism could delay switching from NR2B to NR2A subunits in the maturation of NMDA receptor. Developmental alteration of NMDA receptor, as changes in proportion of NR2A and NR2B subunits may provide a novel mechanism of developmental neurotoxicity underlying hypothyroidism.

**982** GABERIC FUNCTION IS ALTERED FOLLOWING DEVELOPMENTAL HYPOTHYROIDISM: NEUROANATOMICAL AND NEUROPHYSIOLOGICAL EVIDENCE.

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Thyroid hormone deficiency during development produces changes in the structure of neurons and glial cells and alters synaptic function in the hippocampus. GABAergic interneurons comprise the bulk of local inhibitory neuronal circuitry and a subpopulation of these interneurons contain the calcium binding protein, parvalbumin (PV). A previous report indicated that severe hypothyroidism reduced PV staining in the neocortex (Berbel et al., 1996). The present study examined GABA-mediated inhibition and immunocytochemistry of PV-containing interneurons in the dentate gyrus of the hippocampal formation. Animals were deprived of thyroid hormone *in utero* and throughout lactation by exposing pregnant dams to propylthiouracil (PTU) via the drinking water (0, 3, 10 ppm) from GD 6 to weaning of the offspring. This regimen reduced maternal serum T4, increased TSH and had no effect on T3 at the low dose. Both T3 and T4 were reduced in the offspring at weaning but returned to control levels in adulthood. Synaptic inhibition of the perforant path-dentate gyrus synapse was evaluated using *in vivo* field potentials and paired pulse techniques in adult offspring. PTU-induced reduction in paired pulse depression and augmentation in facilitation were observed, indicating a suppression of GABA-mediated inhibition. Immunocytochemical staining for PV was reduced in the dentate gyrus and the neocortex. A cross-fostering study revealed

that postnatal hormone deficiency was required for an alteration in PV staining. Altered staining persisted to adulthood despite return of thyroid hormones to control levels. These data indicate that thyroid hormone insufficiency during the early postnatal period alters interneuron expression of PV and compromises GABA-mediated synaptic transmission. (Funded by USEPA, does not reflect USEPA policy).

**983** VITAMIN E PROTECTS AGAINST THE BEHAVIORAL DEFICITS PRODUCED BY NEONATAL SODIUM VALPROATE EXPOSURE IN MICE.

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We have previously reported that pre- and/or postnatal exposure to sodium valproate (VPA) produces delays and regressions in neurobehavioral development which resembles behavioral features of autism. Recently, we observed an elevation of a marker of lipid peroxidation, urinary 8-isoprostane, in children with autism. As generation of oxidative metabolites contributes to toxicity induced by VPA, the current studies examined whether antioxidant pretreatment would protect mice against VPA-induced behavioral disruptions. Male BALB/c mice were treated with alpha-tocopherol (250 mg/kg s.c.) diluted in corn oil from PND 11-15 followed by VPA (400 mg/kg s.c.) on PND 14. Mice treated with VPA and pretreated with the corn oil vehicle showed a reproducible delay in the development of the negative geotactic response on PND 15-17, loss of the established mid-air righting response on PND 15, as well as hyperactivity on PND 16-19. These behavioral disruptions were eliminated or partially ameliorated by pretreatment with vitamin E. Administration of VPA produced an elevation in hippocampal, but not cerebellar GFAP levels. The increase in hippocampal GFAP was not ameliorated by vitamin E pretreatment. Together, these results suggest that oxidative stress may play a role in the functional deficits produced by VPA, and that GFAP does not serve as a sentinel biomarker for the behavioral effects of VPA. (Supported by NIH ES05022, NIH/EPA ES011256 and NIH ES11279)

**984** CAMPTOTHECIN-INDUCED APOPTOSIS IN NEURAL STEM CELLS AND DIFFERENTIATED CULTURES THROUGH TWO DIFFERENT MECHANISMS.

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We use both human and mouse neural stem cells (NSCs) as a model to evaluate the mechanism(s) involved in camptothecin (CA) induced cytotoxicity. Results from ultrastructural damage, TUNEL staining and Caspase 3 activation indicated that CA, a topoisomerase I inhibitor, induced apoptotic cell death in both stem cells and their differentiated cultures. CA (2.5 - 5  $\mu$ M) induced cleavable complex formation *in vitro* in pBR322 plasmid DNA. Cell cycle analysis showed that CA induced 50% and 95% cell loss in S and G2/M phases, respectively, and a 17% increase in G0/G1 phase during the undifferentiated stage. After CA treatment, there was a significant decrease in the rate of BrdU pre-incorporation (25% to 0.5%), suggesting that these S-phase stem cells are more susceptible to CA-induced cell death. In differentiated (post-mitotic) cultures, neurons were more vulnerable to CA-induced cytotoxicity than astrocytes. The same dose of CA induced a significant increase in ROS production, an extensive mitochondrial membrane potential change and calcium influx. CA-induced apoptosis could be suppressed by the G1/S phase blocker desferoxamine and adenovirus-mediated p53 siRNA only in stem cells but not the differentiated cultures. However, the differentiated cells showed increased susceptibility to glutamate-induced cytotoxicity compared to their progenitors. NMDA receptor antagonism, MK801, could significantly attenuate CA-induced apoptosis in differentiated cultures but not their progenitors. Since previous studies already showed a significant increase in the expression of certain NMDA receptors after stem cell differentiation, glutamate-induced toxicity may be involved in CA-induced apoptosis in differentiated cultures. Therefore, CA induces apoptosis in neural stem cells and their differentiated cells through two different mechanisms. (Supported by ES08089 (JAJ) and ES10042 (JAJ) from the NIEHS)

**985** THE ROLE OF AMYLOID- $\beta$  AND  $\alpha$ -SECRETASE CLEAVED AMYLOID PRECURSOR PROTEIN ON NEUROGENESIS IN ADULT HUMAN NEOCORTICAL SLICE CULTURES.

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Neurogenesis in the adult brain is increased in certain pathological states and may provide a basis for neuronal replacement therapy in neurodegenerative diseases like Alzheimer's disease. Alzheimer's disease may be caused by the abnormal processing

of the amyloid precursor protein (APP) and the accumulation of amyloid- $\beta$ . The amyloid precursor protein can be proteolytically cleaved into multiple fragments, many of which have distinct biological actions. For instance, a high level of amyloid- $\beta$  is toxic and the  $\alpha$ -secretase cleaved APP (sAPP $\alpha$ ) is neuroprotective. While studies have shown evidence of enhanced neurogenesis in Alzheimer's disease, the fragments derived from amyloid precursor protein that may be responsible for this and the possible mechanisms for neurogenesis are unknown. A novel *ex vivo* model of Alzheimer's disease was established by culturing fresh adult human neocortical tissue. Histologically normal tissue removed from the temporal cortices of patients undergoing surgery for temporal lobe epilepsy was used for all experiments. The tissue was rapidly processed to generate 400-micron slices that were cultured in defined medium for up to three weeks. Slices were treated with aggregated and non-aggregated forms of amyloid- $\beta_{1-42}$  or sAPP $\alpha$ . Amyloid- $\beta$  increased levels of overall cell death as well as neuron cell death. We tested the neuroprotective potential of sAPP $\alpha$  and a peptide derived from it in tissue exposed to amyloid- $\beta$ . We examined the incorporation of BrdU and expression of immature neuronal markers in order to elucidate the role of amyloid- $\beta$  and sAPP $\alpha$  in neurogenesis. Support Contributed By: Grants ES08089 and ES10042 from the NIEHS.

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BISPHENOL-A, AN ENVIRONMENTAL CONTAMINANT THAT ACTS AS A THYROID HORMONE RECEPTOR ANTAGONIST *IN VITRO*, INCREASES SERUM THYROXINE AND ALTERS RC3/NEUROGRANIN EXPRESSION IN THE DEVELOPING RAT BRAIN.

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Sponsor: R. Zoeller.

Bisphenol A (BPA, 4, 4 isopropylidenediphenol) is an industrial chemical used as a monomer in the production of polycarbonate plastics and epoxy resins. It is found in a number of products including food can linings, plastic water and milk bottles, adhesives and dental fillings and sealants, indicating potentially high human exposure. BPA has been found in human urine as well as in amniotic fluid and fetal serum, showing that it can pass through the placenta. BPA can competitively bind to the thyroid hormone receptor (TR) and acts as an antagonist of TR *in vitro*. To determine the *in vivo* effects of BPA exposure, we treated pregnant Sprague-Dawley rats with either 0, 1, 10 or 50 mg/kg/body weight BPA dosed onto wafers daily, from gestational day 6 through weaning and 1 male and 1 female pup were sacrificed on postnatal days 4, 8, 15, and 35. Serum total T4 was elevated by maternal exposure to 10 and 50mg/kg and there was no difference in male and female pups. Serum TSH was unaffected. RC3/Neurogranin, a known TH-responsive gene, was up-regulated by BPA exposure in the dentate gyrus of P15 males. These results indicate that BPA may act as an antagonist on TR beta regulation of TSH, but not on TR alpha regulation of RC3. To determine if BPA binds with the same affinity to both receptors, competitive binding assays with isolated recombinant TR alpha and TR beta were performed. BPA bound to both receptors with the same affinity, a Ki of  $1.44 \times 10^{-5}$  M. These studies show that BPA exerts an effect on thyroid hormone action during development and that these effects may be TR isoform specific.

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EXPOSURE OF RHESUS MONKEYS TO COCAINE THROUGHOUT GESTATION RESULTS IN DECREASED SENSITIVITY TO COCAINE IN ADULTHOOD: EFFECTS OF COCAINE ON TIMING BEHAVIOR.

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These studies examined whether animals exposed to cocaine prenatally would respond differently than control animals when challenged with several doses of cocaine as adults. Treatment groups consisted of untreated controls (n = 7) and animals that received total gestational exposure (TGE) to cocaine (n = 5). For the TGE group, nonpregnant females were treated IM with cocaine for up to several months prior to mating with doses of 1.0 to 3.0 mg/kg/injection, 3 times per day, 5 days per week. After pregnancies were detected (ca. gestational day 30-40) doses were escalated until birth as animals became tolerant to cocaine-induced appetite suppression: the dose was escalated by 0.5 to 1.0 mg/kg/injection up to maximums ranging from 3.5 to 7.5 mg/kg/injection at term (ca. 3/4 to 1 1/2 gram per day, human equivalent). At birth, TGE offspring weighed significantly less and had smaller head circumferences than controls, a finding also reported in human studies. At 6 years of age, cocaine dose-response curves were obtained: 15 min after the iv injection of 0.3, 1.0 or 1.75 mg/kg, subjects performed a timing task for food treats. The task required that subjects hold a response lever in the depressed position for at least 10 sec but no more than 14 sec. Endpoints included total lever holds and percent

correct holds. 2 way ANOVA (dose, treatment group) showed significant dose effects and that while there was no group effect on percent correct holds, there was a significant effect of group on total holds (p = .017), with the controls being significantly more sensitive to the disruptive effects of cocaine. These data demonstrate that gestational exposure to cocaine can cause long-lasting (permanent?) alterations in sensitivity to subsequent exposure to cocaine as evidenced by disruption of complex brain functions in primates.

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OBSERVATION OF CATECHOLAMINERGIC NEURONS IN THE FETAL RAT BRAIN EXPOSED TO THE GENOTOXIC COMPOUND, 5-BROMO-2'-DEOXYURIDINE (BrdU).

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5-Bromo-2'-deoxyuridine (BrdU), a thymidine analogue, is incorporated into DNA as 5-bromouracil during the synthesis phase of the cell cycle, and has been used extensively as a tool for labeling proliferating cells in cancer research and developmental neuroscience. Doses ranging from 10 to 100 mg/kg are commonly injected into animals. However, BrdU has genotoxicity. It was reported that mutagenesis occurred when BrdU triphosphate was incorporated into DNA, and when the BrdU-containing DNA replicated. We earlier reported that prenatal exposure to BrdU at a dose generally administered to adult rodents (50 mg/kg) induces locomotor hyperactivity in rats, suggesting that fetal brain is very sensitive to BrdU-induced genotoxicity. We also demonstrated that BrdU induced cortical dysgenesis (lower cellularity and disrupted arrangement of immature cells in the cortical plate) in the fetal rat brain. Since the dopaminergic nervous system is often implicated in hyperactivity (e.g. neonatal 6-hydroxydopamine lesioning rat and dopamine transporter knockout mouse models), we evaluated catecholaminergic neurons in the fetal rat brain after BrdU treatment. Pregnant rats were administered 50 mg/kg of BrdU on gestation days 9.5-15.5. The fetal brains were collected at gestation day 16.5, serially sectioned by vibratome and processed for immunohistochemistry using anti tyrosine hydroxylase (TH) antibody. The TH immunoreactivities were observed in a wide variety of areas including frontal neocortex, caudate putamen, substantia nigra, ventral tegmental area, and locus caeruleus. There were no dramatic differences between the control and the BrdU-treated brains. As noted above, the BrdU-induced abnormality involved the cortical plate. Our data support the view that while the neurons differentiating in cortical plate are principally involved in the BrdU-induced developmental neurotoxicity, catecholaminergic cells are not among them.

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HETEROGENEOUS SENSITIVITY OF THE EMBRYONIC NERVOUS SYSTEM TO THE GENOTOXIC AGENT BrdU IN MICE.

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In embryogenesis, the development of the neural system starts at an early stage and continues to develop after the postnatal period. Therefore, the fetal nervous system is a potential target for exogenous chemical insults. However, few research efforts focus on the examination of fetal brain relative to the adult. 5-Bromo-2'-deoxyuridine (BrdU), a thymidine analogue, is incorporated into any mitotic cell, and has been used as a marker of proliferating cells. BrdU is also considered a genotoxic agent. Previously, our studies demonstrated that prenatal BrdU exposure (50 mg/kg, intraperitoneally) on gestational day (GD) 9 to 15 induced abnormal behaviors and disrupted the monoaminergic nervous system in rat offspring. The fetal brain, after BrdU exposure, showed the induction of cell death in specific areas and abnormal structure of cortical plate. In this study, to examine more detailed effects of BrdU exposure on the fetal brain, C57BL/6 mice were exposed to 100 mg/kg BrdU intraperitoneally on GD9 or 11. Fetal brains were obtained 24 hours after the treatment, and examined in histopathological observations. BrdU did not induce cell death in fetal brains treated on GD9. However, treatment on GD11 increased cell death in specific areas such as the neocortex and striatum. No induction of cell death was observed in the substantia nigra, raphe and pons, which are nuclei of monoaminergic systems, even though BrdU was incorporated into these areas. The results suggest that the 1) sensitivity of the fetal brain to genotoxicity is different among fetal brain areas and developmental stages, 2) substantia nigra, raphe and pons seem to be tolerant to the genotoxic agent, indicating that monoaminergic neurons are more likely to carry mutations after prenatal exposure to genotoxic agents.



## 990 THE AHR IN CELL GROWTH AND DEATH.

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Since its first description as a dioxin-binding protein in the 1980s, the aryl hydrocarbon receptor (AhR) has been studied primarily for its control of biologic responses to environmental agonists. However, in the last few years it has become apparent that the AhR, which clearly did not evolve to recognize environmental pollutants, likely plays an important physiologic function. Indeed, the ability of the AhR to directly regulate important cellular genes and factors such as Bax, the estrogen receptor, Rb, and NF- $\kappa$ B hints at a critical role for the AhR in cell growth and death. The presence of constitutively active AhR in cells that exhibit aberrant growth and apoptosis regulation, i.e. neoplastic cells, further supports this hypothesis. In this symposium we will present several examples of AhR-mediated control of cell growth and death and will begin to detail the molecular mechanisms through which this control is manifest. In some studies presented herein, AhR function is revealed with exogenous agonists such as TCDD or PAH. In other cases, constitutive AhR function is demonstrated by modulation of AhR expression and activity in the absence of exogenous ligands. In all cases, the roles that the AhR may play in normal cellular physiology and the consequences of disrupting these functions with environmental agonists are discussed.



## 991 LIGATION OF AHR LEADS TO UP-REGULATION OF APOPTOTIC GENES THROUGH DRE-DEPENDENT AND -INDEPENDENT PATHWAYS INVOLVING NF- $\kappa$ B AND CONSEQUENT INDUCTION OF APOPTOSIS IN THYMOCYTES.

P. S. Nagarkatti. *Pharmacology and Toxicology, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, VA*.

Previous studies from our laboratory have shown that TCDD triggers apoptosis in thymocytes mediated through Fas-Fas ligand (FasL) interactions. However, the precise role of AhR in regulating apoptosis or the expression of Fas and FasL in thymocytes is not clear. In the current study, we noted that TCDD induced/enhanced the expression of several apoptotic genes in the thymus, including Fas and Fas ligand (FasL) in AhR wild-type but not AhR KO mice. TCDD-mediated upregulation of FasL expression was seen only in thymic stromal but not T cells. Fas but not FasL gene promoter was found to contain a functional DRE. In contrast, in FasL gene promoter region, potential NF- $\kappa$ B binding sites were detected. When activation of NF- $\kappa$ B was further investigated, it was noted that TCDD treatment led to AhR-dependent nuclear translocation and co-localization of p50 and p65 subunits in thymic stromal cells. When TCDD-exposed thymic stromal cells were mixed with untreated thymic T cells, increased apoptosis was detected in T cells that involved Fas-FasL interactions. TCDD, at higher doses, also triggered the mitochondrial pathway of apoptosis. However, cross-talk between death receptor and mitochondrial pathways was not necessary for apoptosis inasmuch as, TCDD-treated Bid knock-out mice showed thymic atrophy and increased apoptosis, similar to the wild-type mice. Together, these findings demonstrate that activation through AhR leads to DRE-dependent induction of several apoptotic genes including Fas in thymocytes, and furthermore, also leads to activation of NF- $\kappa$ B that regulates FasL expression on thymic stromal cells. Thus, TCDD disrupts normal T cell-stromal cell interactions that play a critical role in T cell differentiation, leading to induction of apoptosis in T cells.



## 992 AHR CONTROL OF B LYMPHOCYTE DEATH AND GROWTH.

S. H. David, H. Ryu, J. K. Emberley, L. L. Allan and J. J. Schlezinger. *Environmental Health, Boston University School of Public Health, Boston, MA*.

Environmental agonists of the aryl hydrocarbon receptor (AhR) compromise a variety of immunologic functions including antigen presentation and B and T cell effector responses. Here, we discuss how the AhR mediates both direct and indirect effects on immature and mature B lymphocytes. With regard to indirect effects, we will present studies demonstrating that AhR agonists, such as the prototypic PAH, DMBA, induce AhR bearing bone marrow stromal cells to deliver an apoptosis signal to neighboring AhR- B cells. The intricate signaling pathway activated within the B cells that results in their demise involves caspases 2, 3, 8, and 9, Bid, Bax and other elements of the mitochondrial signaling pathway. Stress activated kinases (e.g. JNK) are also involved. Regarding direct effects on the B cell compartment, we demonstrate that stimulation of human peripheral B cells with signals mimicking activation of the innate and adaptive immune responses (i.e. signaling through toll-

like receptor 9 and CD40 respectively) induces high levels of constitutively active AhR. Possible roles that this constitutively active AhR may play in human lymphocytes will be discussed.



## 993 REGULATION OF MAMMARY TUMOR GROWTH THROUGH THE ARYL HYDROCARBON RECEPTOR.

S. H. Safe. *Veterinary Physiology, Texas A & M University, College Station, TX*.

The aryl hydrocarbon receptor (AhR) is widely expressed in cancer cell lines and tumors, and AhR ligands inhibit growth of some cancers. Research in this laboratory has focused on development of selective AhR modulators (SAhRMs) for cancer chemotherapy, and on understanding the growth inhibitory mechanisms of AhR action in breast and other cancer cell lines. The antiestrogenic activity of AhR agonists in estrogen receptor (ER)-positive breast cancer cells is complex and involves multiple pathways that are dependent, in part, on individual genes/gene promoters. Inhibitory AhR-ER crosstalk is associated with AhR ligand-induced proteasome-dependent degradation of ER, increased metabolism of 17 $\beta$ -estradiol (E2), AhR interaction with cis-acting dioxin responsive elements, AhR displacement of ER from some promoters, and induction of transcription factors. These multiple inhibitory pathways in ER-positive cells primarily target E2-responsive genes that are involved in cell cycle progression and DNA synthesis. However, results of ongoing studies also show that AhR agonists inhibit growth of ER-negative breast cancer cells and other ER-independent cell lines, and the mechanisms of these responses will also be discussed. (Supported by NIH ES09106 and ES04176)



## 994 CURRENT INSIGHTS INTO AHR-MEDIATED LIVER CELL CYCLE CONTROL.

C. J. Elferink. *Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, TX*. Sponsor: D. Sherr.

Liver homeostasis in response to pathogen infection or drug toxicity is achieved by the removal of damaged hepatocytes and their replacement to maintain a constant liver cell mass. The AhR is a ligand-activated transcription factor known to regulate both apoptotic and proliferative processes. Our goal is to understand mechanistically how the AhR contributes to tissue homeostasis by regulating cell growth and cell death. The AhR ligand 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) is the prototype for a class of compounds responsible for a range of toxic or adaptive endpoints. A notable response to TCDD is the AhR-mediated G1 cell cycle arrest detected in certain liver-derived cells in culture, and in the regenerating liver *in vivo*. Studies exploring the molecular basis for the AhR-dependent growth arrest have identified multiple mechanisms contributing to the arrest response, including direct and indirect transcriptional regulation, and processes influencing growth factor signaling.



## 995 AHR CONTROL OF APOPTOSIS INDUCED BY AGENTS CAUSING LYSOSOMAL DAMAGE.

J. J. Reiners. *Institute of Environmental Health Sciences, Wayne State University, Detroit, MI*. Sponsor: D. Sherr.

Agents which damage lysosomes can activate the intrinsic apoptotic pathway. This occurs as a consequence of lysosomal protease cleavage of Bid, and tBid-mediated cytochrome c release. Photodynamic therapy with the lysosomal sensitizer NP6, ceramides, and TNF all induce the release of lysosomal proteases into the cytosol of murine hepatoma 1c1c7 cells. Apoptosis induced by these agents is Bid-dependent, and can be suppressed by a sphingomyelin derivative that stabilizes lysosomes. In contrast, variants of the parental line deficient in the aryl hydrocarbon receptor (AhR) are resistant to the pro-apoptotic effects of the three agents. However, the variants are comparable to the parental line in terms of their susceptibilities to pro-apoptotic agents that do not damage lysosomes. Surprisingly, the relative sensitivities of AhR-deficient cells to different classes of apoptotic inducers are similar to that seen in several human disorders collectively referred to as lysosomal storage diseases. Analyses of lysosomes from AhR-deficient cells indicated that they were markedly more resistant to breakage induced by reactive oxygen species, and deficient in proteases, including those involved in Bid cleavage. The latter property appears to reflect a global defect in the processing/trafficking of proteases to the lysosomes. Introduction of the AhR into deficient cells corrected the defect and restored sensitivity to lysosomal disrupters. These studies suggest that the AhR, in the absence of exogenous ligands, influences the stabilities and contents of lysosomes.

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## ORGANOPHOSPHATES & CARBAMATES: CHOLINERGIC & NONCHOLINERGIC MECHANISMS.

R. C. Gupta. *Toxicology, Murray State University, Hopkinsville, KY.*

Organophosphates (OPs) and carbamates (CMs) are commonly used as pesticides in agriculture, industry, and around the home/garden. Some OPs have also been used in chemical warfare and terrorism, while some CMs have been used to treat diseases such as myasthenia gravis and dementia. Interestingly, the CM pyridostigmine is used prophylactically to protect soldiers from possible OP nerve agent exposure. Many compounds of both classes are extremely toxic and lack selectivity, thus their inadvertent/accidental use continues to pose a threat to human and animal health, aquatic systems and wildlife. While these compounds have a wide variety of toxic effects, neurotoxicity elicited by inhibition of acetylcholinesterase (AChE) is generally of primary concern. In recent years, a variety of novel mechanisms for OPs and CMs have been proposed which may modulate cholinergic neurotoxicity or lead to non-cholinergic effects. Molecular studies of AChE and its interaction with inhibitors has allowed a more thorough characterization of the structure, function, and regulation of this enzyme. *In vitro* studies indicate that NGF-differentiated SY5Y cells may distinguish between OPs that produce OPIDN and those that do not, and may therefore be a suitable mechanistic model for exploring the effects of different classes of OPs. Evidence suggests that while cholinergic mechanisms play a critical role in the initial stage of cholinergic toxicity, neuronal damage/death may occur through non-cholinergic mechanisms including oxidative stress. The Food Quality Protection Act of 1996 requires EPA to consider the cumulative risk to pesticides with a common mechanism of action. In the case of OPs and CMs, this common mechanism is initiated by inhibition of AChE (via phosphorylation or carbamylation, respectively). However, noncholinergic mechanisms are not currently considered in risk assessment. This symposium will review the latest developments in understanding of molecular mechanisms (cholinergic and non-cholinergic) for these toxicants, as well as discuss current strategies of cumulative risk assessment for pesticides acting through a common mechanism.

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## CONTEMPORARY PROTEOMICS IN ACETYLCHOLINESTERASE AND RELATED $\alpha$ , $\beta$ HYDROLASE-FOLD PROTEINS AS TARGETS OF TOXICOLOGICAL MANIFESTATIONS AND GENETIC DISORDERS.

P. Taylor, Z. Radic, L. Jennings and S. Camp. *Pharmacology, University of California, San Diego, La Jolla, CA.* Sponsor: R. Gupta.

Knowledge of sequence and structures of acetylcholinesterase (AChE) as a target of nerve agents enables one to design sensitive fluorescence sensors for remote detection of OP modified enzymes, develop means by which the nerve agent may be scavenged by derivatives of the target and analyze the target for structural differences that may be determinants of susceptibility among individuals. We describe how we might employ a fluorescent derivative of AChE itself for remote or field sensing of nerve agents or insecticides. Since the sensor is a conjugate of AChE itself, false positives are minimized. Perturbations of the active center gorge give rise to distinctive spectra, this allows one to distinguish types of inhibitor. In turn, the conjugates that give rise to the fluorescence changes can be analyzed by digestion with proteases, the active center peptide isolated and the offending OP identified by mass spectrometry. Analysis is sufficiently sensitive that it can be conducted with enzyme from brains of OP exposed mice. Moreover, precise kinetics of alkylphosphorylation, aging and reactivation can be followed by the respective masses of the active center peptide. Application of genomics and proteomics comes through the analysis of naturally occurring mutations in the AChE gene to ascertain whether any of these mutations confer an unusual susceptibility to alkylphosphates. Several synonymous and non-synonymous single nucleotide polymorphisms have been found in the open reading frame and others in potential regulatory regions in the human gene. Whether they play a role in affecting enzyme expression, catalytic activity or stability of the enzyme has yet to be determined. Mutations in related  $\alpha$ ,  $\beta$  hydrolase-fold proteins, such as neuroligin, govern expression and processing in their biosynthesis. In selected cases, disorders such as autism appear to be related to altered expression of proteins forming heterophilic adhesion interactions in synapses. Supported by R37GM8360 and DAMD170220025.

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## ORGANOPHOSPHORUS AND CARBAMATE CHOLINESTERASE INHIBITORS: USES AND MISUSES OF A COMMON MECHANISM.

C. N. Pope. *Physiological Sciences, Oklahoma State University, Stillwater, OK.*

Cholinesterase inhibitors (anticholinesterases) have been used in the treatment of human and animal disease, to control insect vectors and parasites, and as weapons in chemical warfare and terrorism. Their uses and misuses are based on a common

mechanism of action initiated by inhibiting acetylcholinesterase (AChE). AChE is pivotally important in the dynamic regulation of cholinergic neurotransmission in the central and peripheral nervous systems where it rapidly and efficiently degrades the neurotransmitter, acetylcholine. Marked inhibition of AChE leads to accumulation of acetylcholine, with consequent over-stimulation of cholinergic receptors and resulting alteration of cellular functions. The wide distribution of cholinergic terminals within the central and peripheral nervous systems is the basis for an array of functional alterations possible following AChE inhibition. Although the molecular modification (e.g., carbamylation vs. phosphorylation) of AChE with different inhibitors can be an important determinant of effect (e.g., duration of action) leading to differential actions, a number of reports suggest that additional sites of action may be important in their pharmacologic or toxicologic effects. A number of enzymes (esterase and non-esterase), neurotransmitter receptors (cholinergic and non-cholinergic) and cell signaling components are sensitive to direct interaction with some anticholinesterases. In many cases, the toxicologic relevance of these additional sites is unclear. Recent studies of the interactive toxicity of binary mixtures of the organophosphorus insecticides chlorpyrifos, parathion and methylparathion indicate, however, that non-cholinesterase targets may be important in cumulative toxicity under some conditions. Interaction between anticholinesterases and other macromolecules may contribute to differences in acute toxicity among different inhibitors, to neurodevelopmental toxicity, and to more subtle, long-term sequelae sometimes reported in patients following anticholinesterase poisoning. (Supported by STAR grant 825811 from USEPA and R01 ES009119-05 from NIEHS).

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## IN VITRO MODELS FOR TESTING NEUROTOXICITY OF ORGANOPHOSPHORUS COMPOUNDS AND PROTECTIVE EFFECTS OF OP HYDROLASE.

E. Tiffany-Castiglioni. *Veterinary Anatomy and Public Health, Texas A&M University, College Station, TX.*

OPs produce potent neurotoxic effects in humans, including OP-induced delayed neuropathy (OPIDN). The human neuroblastoma cell line SY5Y at various stages of differentiation can distinguish between OPs having different toxic effects on the nervous system. We have examined the potential for the 200 kD neurofilament protein (NF200) and other neuronal proteins to serve as indicators for neurite damage in a differentiated SY5Y cells. Mipafox, which induces OPIDN, increases NF200 protein expression in SY5Y cells differentiated with human beta-nerve growth factor (NGF, 20 ng/ml) when SY5Y cells are exposed to 0.3 or 30 microM mipafox during the last 5 days of neurite extension. However, mipafox produces little change in NF200 protein levels in SY5Y cells exposed continuously throughout neurite elongation. In contrast, paraoxon (up to 30 microM), which does not produce OPIDN, does not produce any change in NF200 levels. The upregulation of NF200 by mipafox may represent a compensatory response to neurite degeneration. Two other neuronal proteins, growth associated protein 43 (GAP43) and microtubule associated protein 2ab (MAP2ab) show no changes in response to OP treatment in NGF-treated cells. Protein expression of NF200 may therefore be an indicator by which the sensitivities of SY5Y cells to mipafox and paraoxon are distinguishable at the molecular level. This cell culture system has been further used to test the ability of an OP hydrolase enzyme from genetically engineered *E. coli* to degrade OP compounds and eliminate or reduce their neurotoxicity. We found that anti-AChE activity of paraoxon (maximum 3 microM) and anti-NTB activity of mipafox (250 microM) in SY5Y cells were completely prevented by degradation with OPH. Furthermore, biodegradation of mipafox with OPH significantly reduced modulation of these neuroskeletal protein biomarkers by OPs. These results indicate an alternative approach and test system for investigating structure-activity relationships of OPs and their bioremediation by OP hydrolase.

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## OXIDATIVE STRESS INVOLVEMENT IN NEUROTOXICITY OF ORGANOPHOSPHATES AND CARBAMATES.

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Acute administration of organophosphates or carbamates results in a rapid increase of acetylcholine producing a rapid onset of seizures, convulsions, and subsequent neuropathology. The mechanisms involved in the pathogenesis of neuronal damage appear to be linked to free radical-mediated injury. This hypothesis-based investigation was undertaken to determine whether a causal relationship exists between oxidative stress and neurotoxicity induced by an organophosphate diisopropylfluorophosphate (DFP) or the carbamate carbofuran. Male rats exposed to an acute dose of DFP (1.5 mg/kg, sc) or carbofuran (1.5 mg/kg, sc) developed convulsions, seizure, and muscle fasciculations within 15-20 min, with maximal severity at 1 h, lasting for >2 h. At the time of peak severity, when AChE was markedly depressed

(> 75%), maximal increases in F2-isoprostanes and F4-neuroprostanes (markers of lipid peroxidation and ROS, 2-6-fold increase), citrulline (marker of NO/NOS and RNS, 4-8-fold increase), as well as depletion of high-energy phosphates (ATP, 36-60% and PCr, 28-52%) occurred in selected brain regions (cortex, amygdala, and hippocampus). Total adenine nucleotides and total creatine compounds were significantly reduced (36-58% and 28-48%, respectively). Pretreatment of rats with an antioxidant vitamin E (100 mg/kg, ip/day for 3 days) or the spin trapping agent PBN (200 mg/kg, ip) prevented significantly the increases in citrulline and the declines in high-energy phosphates. Detailed analysis of pyramidal neurons of CA1 region of hippocampus revealed that DFP or carbofuran caused marked decreases in total dendritic length (60-70%) and spine density (40%). These neuropathological and biochemical changes were completely prevented when rats were pretreated with a NMDA receptor antagonist memantine (18 mg/kg, sc). From these data, it is concluded that a causal relationship exists between AChE inhibitor- induced oxidative stress and neuropathology.

 **1001** EPA PERSPECTIVE: SCIENTIFIC APPROACHES FOR ASSESSING THE CUMULATIVE RISK OF ORGANOPHOSPHORUS AND N-METHYL CARBAMATE CHOLINESTERASE INHIBITING PESTICIDES.

A. Lowit. *Office of Pesticide Programs, USEPA, Washington, DC.* Sponsor: R. Gupta.

The Food Quality Protection Act of 1996 requires EPA to consider the cumulative risk to pesticides with a common mechanism of action. Under FQPA, cumulative risk is estimated for the common mechanism toxic effect used to establish that common mechanism group. In the case of the organophosphate (OP) and N-methyl carbamate pesticides, this effect is inhibition of acetylcholinesterase either through phosphorylation or carbamylation of the active site. The OP and the N-methyl carbamates were established as common mechanism groups by EPA in 1999 and 2001, respectively. The revised cumulative risk assessment (CRA) for the OPs was released to the public in June 2002; the preliminary CRA for the N-methyl carbamate is expected to be released to the public in the summer of 2005. This talk will discuss relative potency factor and pharmacokinetic approaches to estimating cumulative risk in addition to a discussion of key challenges in developing cumulative risk assessments. The development of physiologically-based pharmacokinetic/dynamic (PBPK/PD) models has increased rapidly in the last few years. However, the typical database of toxicology studies submitted for purposes of pesticide registration do not contain the appropriate types of pharmacokinetic and metabolism data needed to develop complex PBPK/PD models. This talk will also discuss a case study where targeted PK and metabolism experiments were conducted on carbaryl specifically for developing PBPK/PD models.

 **1002** PROTEOMICS AND ANTIBODY MICROARRAYS: APPLICATIONS IN TOXICOLOGY.

R. C. Zangar. *Pacific Northwest National Laboratory, Richland, WA.*

It is now possible to sequence a whole genome and predict the complete proteome of an organism. This information, when used in combination with sophisticated techniques for protein analysis such as tandem mass spectrometry (MS), allows for the rapid analysis of hundreds or thousands of proteins in a single biological sample. These proteomic technologies offer the ability to rapidly determine which of the identified proteins are altered in response to toxicity, xenobiotic exposure or various disease states. One specialized application of proteomics technology is the analysis of protein modifications. Since many of the toxic effects of xenobiotic exposure and oxidative stress are associated with covalent protein modifications, this area of research is expected to result in significant advances in our understanding of the molecular basis for toxicity. Another rapidly developing technology is protein arrays, which can be used for broad comparisons of protein-protein interactions or quantitative analyses. In comparison to MS-based analyses, protein microarrays generally have greater throughput but are limited to a select group of proteins. Overall, these developing technologies are expected to provide a broader insight into the mechanisms of toxicity and identify new markers of disease and toxicity.

 **1003** ANALYSIS OF MARKERS OF CANCER AND XENOBIOTIC EXPOSURE USING ANTIBODY MICROARRAYS.

R. C. Zangar<sup>1</sup>, S. M. Varnum<sup>1</sup>, D. S. Daly<sup>1</sup>, A. M. White<sup>1</sup>, C. Y. Covington<sup>2</sup>, S. Wiley<sup>1</sup>, B. D. Thrall<sup>1</sup> and R. D. Stenner<sup>1</sup>. <sup>1</sup>*Pacific Northwest National Laboratory, Richland, WA* and <sup>2</sup>*University of California School of Nursing, Los Angeles, CA.*

Proteomic and DNA microarray analyses are identifying large numbers of potential markers for toxicity and disease. Once this set of proteins is determined, additional studies are needed to define the utility of these biomarkers under various clinical or

experimental conditions. We have developed a sandwich enzyme-linked immunosorbent assay (ELISA) system in a high-density microarray format that can simultaneously assay 20 or more proteins. This system has sensitivities, dynamic ranges and quantitative characteristics comparable to commercial 96-well ELISAs, but the microarray system can perform these analyses using a few ul of sample. We are also developing software for the rapid evaluation of ELISA microarray data, including a variety of quality control measures. We have used this system to measure protein levels in nipple aspirate fluid, serum and cell-culture medium. These latter studies have provided insight into the molecular mechanisms that regulate the extracellular release of potential cancer biomarkers and have helped elucidate a mechanism of cell-signaling-pathway crosstalk involving regulated shedding of receptor ligands. Overall, the exceptional sensitivity, throughput and quantitative characteristics make the ELISA microarray system an attractive method for quantifying the levels of multiple proteins in large numbers of small samples.

 **1004** FINDING NEW SIGNAL TRANSDUCTION PARADIGMS USING PROTEIN-DOMAIN MICROARRAY.

M. T. Bedford, A. Espejo and J. Daniel. *MD Anderson Cancer Center, Bastrop, TX.* Sponsor: R. Zanger.

Protein domains mediate protein-protein interaction by binding to short peptide motifs in their corresponding ligands. These peptide recognition modules are critical for the assembly of multiprotein complexes. We have arrayed GST fusion proteins, with a focus on protein interaction domains, onto nitrocellulose coated glass slides to generate a protein-domain chip. Arrayed protein-interacting modules included WW, SH3, SH2, 14.3.3, FHA, PDZ, PH and FF domains - that focus on signal transduction pathways; and Chromo, Bromo, Tudor, SWIRM, CW and MBT domains - that are found in chromatin-accessioned proteins and will focus on "reading" the "histone code". Using peptides, we demonstrate that the arrayed domains retain their binding integrity. With this approach we recently identified tudor domains as a protein module that can recognize lysine methylate proteins. This protein-domain chip not only identifies potential binding partners for proteins, but also promises to recognize qualitative differences in protein ligands (caused by post-translational modification) from different sources. In addition, the effects of combinatorial posttranslational modifications on protein-protein interactions, at the level of transmembrane domain receptors, signalling molecules and histone tails can be investigated.

 **1005** PROTEOMIC ANALYSIS OF SERUM PROTEINS DURING ACUTE ACETAMINOPHEN TOXICITY IN RATS REVEALS ACUTE PHASE AND ANTIOXIDANT RESPONSE.

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The serum proteome is an accessible biofluid that can be monitored for changes reflecting organ toxicity and host response. In this study, proteomic analysis of serum from acetaminophen (APAP) intoxicated rats was performed to identify altered serum proteins that might provide insights into acute liver toxicity. Fasted male, Fisher 344 rats, at five/group, were given an oral suspension of 0 (control), 150 and 1500 mg/kg of APAP. Sacrifice times of 6, 24 and 48 hr after APAP represented pre-toxic, toxic and recovery periods, respectively, for the high dose as verified by serum enzymes and histopathology. The control and low dose APAP groups showed few changes in liver pathology and serum proteins over the 48 hr period. Serum profiling was performed by two dimensional (2D) gel electrophoresis and mass spectrometry (MS). Prior to separation, serum was prepared by immunosubtraction of abundant proteins that were removed by affinity chromatography. Proteomic analysis showed the serum proteome was most greatly perturbed at the peak of toxicity. The numbers of significant serum protein changes different from controls were 1, 68 and 17 proteins at 6, 24 and 48 hr, respectively, after a toxic dose of 1500 mg/kg APAP. Increased or newly appearing serum proteins identified by MS at the 24 hr time after APAP included known structural, enzymatic and acute phase proteins, and some previously unidentified proteins including antioxidant enzymes. Followup studies at 24, 72 and 120 hr with 1500 mg/kg APAP showed maximal liver injury at 24 hr which had subsided by 72 hr and recovery by 120 hr. There was a correspondence found between the intensity of liver damage and serum cytokines and antioxidant enzyme activity. Results of proteomic analysis suggest an increase in serum antioxidant enzyme activity triggered by acute phase response to APAP toxicity may reflect a protective host response to acute liver injury.



## 1006 IDENTIFYING PROTEIN MODIFICATIONS RESULTING FROM OXIDATIVE STRESS USING LC/ESI/MS.

I. A. Blair, S. Lee and T. Oe. *Pharmacology, University of Pennsylvania, Philadelphia, PA.* Sponsor: R. Zanger.

Oxidative stress results in the formation of reactive oxygen species (ROS), which can damage cellular macromolecules such as DNA and proteins. DNA damage results directly from ROS, or from ROS-derived lipid-hydroperoxides that break down to the alpha, beta-unsaturated aldehyde genotoxins, 4-hydroperoxy-2-nonenal, 4-oxo-2-nonenal, 4-hydroxy-2-nonenal, and 4, 5-epoxy-2(E)-decenal. Lipid hydroperoxides are also formed enzymatically during oxidative stress from 5-lipoxygenase (5-LOX), 15-LOX, cyclooxygenase-1 (COX-1), and COX-2. There are numerous reports that 4-hydroxy-2-nonenal can covalently modify proteins. However, in many cases 4-oxo-2-nonenal shows increased reactivity when compared with 4-hydroxy-2-nonenal. Therefore, we have used LC/ESI/MS to systematically characterize the lesions that can occur in proteins. Functional studies have focused on histone proteins because of the possibility that lipid hydroperoxide-mediated epigenetic effects may be induced during oxidative stress. Using LC/MS/MS, 87 % amino acid coverage of the tryptic fragments derived from histone H4 was identified. Treatment of histone H4 with 4-oxo-2-nonenal resulted in decreased intensity of peptide D66-K77 by more than an order of magnitude (as determined by LC/ESI/MS) when compared with the unmodified protein. However, the Staph. Aureus V8 fragment N64-E74 was present in almost the same amount as in the unmodified H4. This indicated that modification had occurred on H(75)A(76)K(77). Using a combination of deuterium isotope labeling and LC/MS/MS, it was demonstrated that a cyclic peptide had been formed on the HAK motif. Current experiments are directed at demonstrating whether this modification can occur on histone proteins during oxidative stress *in vivo*. Modification of the HAK motif by lipid hydroperoxides is also being examined in other functionally important proteins. Supported by NIH RO-1 CA95586 and RO-1 CA91016.



## 1007 COMPARATIVE PROTEOMIC ANALYSIS OF CONTROL AND TUMOR-BEARING MOUSE PLASMA BY GEL-LC-MS/MS.

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In SJL mice, growth of RcsX lymphoma cells induces an inflammatory response by stimulating V $\beta$ 16+ T cells. During inflammation, given serum protein levels can increase (e.g. acute phase reactants) or decrease (e.g. albumin), and most of these altered proteins are thus potential biomarkers. Although blood plasma is a valuable sample for biomarker discovery for diseases or for novel drug targets, it is a highly complex proteome. We have focused on a comprehensive comparison of the plasma proteomes from control and RcsX tumor bearing SJL mice, and have developed a simple immunoaffinity spin filter method to remove albumin and IgG from 2  $\mu$ l of plasma. Depleted samples were resolved by 1D-SDS-PAGE, and the entire gel lane was cut into 30 slices and in-gel digested with trypsin. The extracted peptides were analyzed by using an Applied Biosystems nano-LC/MS/MS system, and the MS/MS spectra were analyzed on the Agilent's Spectrum Mill MS Proteomics Workbench. This analysis resulted in the identification of a total of 1113 non-redundant mouse plasma proteins; more than 498 in control and 816 in RcsX tumor bearing SJL mouse plasma. The molecular weights ranged from 2-876 kDa, covering the PI values between 4.22-12.09, and included proteins with predicted transmembrane domains. By comparing the plasma proteomic profile of control and RcsX-tumor-bearing SJL mice, we found significant changes in the levels of many proteins in RcsX-tumor-bearing mouse plasma. Most of the up-regulated proteins were identified as acute-phase reactants. Also, several unique proteins i.e., Proteasome subunits, L-selectin, Fetuin-B, 14-3-3 zeta, MAGE-B4 antigen, etc, were found only in RcsX tumor bearing SJL mouse plasma- either secreted, shed by membrane vesicles, or externalized due to cell death. These results affirm the effectiveness of this approach for protein identification from small samples, and for comparative proteomics in animal models of human disorders.



## 1008 UPDATE ON MECHANISMS FOR ENVIRONMENTAL TOBACCO SMOKE-INDUCED HEALTH EFFECTS.

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Epidemiological evidence has demonstrated an association between exposure to environmental tobacco smoke (ETS) and disease. Diseases associated with ETS exposure include a variety of cancers, cardiovascular disease, COPD, asthma and a number of perinatal manifestations, to name a few. While many of these exposure/disease associations are strong, the mechanism(s) by which ETS exposure

influences the etiology of these diseases remains unclear. There are a great number of confounding factors which make ascribing any ETS-induced mechanism for disease difficult. Included amongst these factors is the immense complexity of the constituents which make up ETS. ETS is comprised of both a vapor phase and particulate phase with over 4000 chemicals identified to date. One must not only consider the effect of individual compounds but must also consider potential synergism between compounds. Another factor which needs to be considered is the fact that exposure to tobacco smoke compounds is substantially less than for smokers, due to dilution of the smoke. Chemical reactivity or "aging" of the ETS components can also modify their chemical nature. Also, an individual's susceptibility to the harmful effects of ETS exposure can be greatly influenced by their genetic makeup. While quite complex, certain mechanistic themes are beginning to emerge as characteristic of ETS's association with these diseases. The speakers will provide recent advances in our understanding of the mechanisms by which ETS can influence the etiology of these diseases and provide a linkage between the epidemiological and biochemical/pathophysiological bases of disease. The symposium will have two presentations each dealing with: ETS and cardiovascular disease, ETS and cancer and ETS and respiratory diseases.



## 1009 IMPACT OF WORKPLACE ETS EXPOSURE ON RISK FACTORS FOR CORONARY HEART DISEASE.

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Environmental Tobacco Smoke (ETS) remains an important source of workplace exposure to environmental contaminants in many bars, restaurants and casinos throughout the US. This study was designed to investigate the impact of workplace ETS exposure on risk factors associated with coronary heart disease and biomarkers of oxidative stress, and the potential moderation of these effects by antioxidant supplementation. Non-smoking bartenders, cocktail servers and casino workers in the Las Vegas and Reno, NV areas, exposed to ETS in the workplace but not in the home were recruited for the study. Following initial baseline analyses of risk factors for coronary heart disease and biomarkers of oxidative stress, subjects were randomized into one of three antioxidant supplement groups. Group 1 received a placebo. Group 2 received a low daily dose of an antioxidant cocktail approximating the recommended dietary allowances (RDA) (75 mg Vitamin C, 15 mg Vitamin E and 55 ug selenium). Group 3 received a higher daily dose approximating one-half the established upper limit for daily intake of these three antioxidants (1000 mg Vitamin C, 500 mg Vitamin E and 200 ug selenium). Subjects returned at 6 month intervals for up to two years to re-evaluate their risk factors and biomarkers. 8-hydroxy-2'-deoxyguanosine, homocysteine, C-reactive protein, total antioxidant capacity, oxidized LDL, cholesterol, LDL cholesterol, HDL cholesterol, superoxide dismutase, glutathione peroxidase and cotinine were determined in these subjects. Baseline values for this ETS exposed population were compared to national databases and each arm of the study was compared to assess the impact of the different levels of antioxidant supplementation. Preliminary results of this study suggest that antioxidant supplementation moderates some of the oxidative stress markers tested. This study was funded in part by NIEHS grant ESO9520.



## 1010 IMPACT OF PRENATAL CARDIOVASCULAR DISEASE RISK FACTOR EXPOSURE ON MITOCHONDRIAL DAMAGE AND ADULT HEART DISEASE DEVELOPMENT.

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Although cardiovascular disease (CVD) has typically been considered an adult disease, it is a slow, complex inflammatory disease that can start in childhood and progress with age. Numerous factors can increase the risk of CVD development, and many have been shown to cause vascular mitochondrial damage and dysfunction; however, whether gestational and/or neonatal exposure to CVD risk factors influence the development of adult heart disease and mitochondrial dysfunction has not been fully investigated. Because the events that lead to atherosclerosis probably begin decades before the clinical manifestations of the disease become evident, the impact of prenatal exposure to environmental tobacco smoke (ETS) on adult CVD development was investigated in a mouse model of atherosclerosis to test the hypothesis that *in utero* ETS exposure promotes adult atherosclerotic lesion forma-

tion and mitochondrial damage. Atherosclerotic lesion formation, mitochondrial damage, antioxidant activity and oxidant load were determined in cardiovascular tissues from adult apoE  $-/-$  mice that were exposed to either filtered air or ETS *in utero*, and fed a standard chow diet from weaning until sacrifice. All measured parameters were significantly altered in male mice exposed to ETS *in utero*. These data support the hypothesis that prenatal ETS exposure is sufficient to promote adult cardiovascular disease development.

## 1011 IMMUNOLOGIC DETECTION OF CARCINOGEN-DNA AND PROTEIN ADDUCTS.

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Immunologic methods using polyclonal and monoclonal antibodies have been developed for measurement of carcinogens bound to DNA or protein. These antibodies can be used in competitive ELISA to measure adducts in DNA isolated from blood or tissue samples or for immunohistochemical detection of adducts in single cells or paraffin or frozen tissue sections. Protein adduct antibodies can be used for quantitation of albumin adducts in blood. These methods have been used to monitor human exposure to environmental carcinogens including those in cigarette smoke. Polycyclic aromatic hydrocarbon (PAH)-DNA adducts are elevated in mononuclear cells of women who smoke compared to nonsmokers but there was no significant effect of passive smoke exposure. Since PAH are ubiquitous environmental carcinogens, exposure from diet may limit ability to detect passive exposure. 4-Aminobiphenyl (4-ABP)-DNA adducts have been measured in paraffin sections of breast tumor and adjacent non tumor tissue of women with breast cancer. Exposure to 4-ABP is a less ubiquitous than to PAH. Adducts were significantly higher in non tumor tissue of women who smoke compared to those not exposed to active or passive cigarette smoke. While adducts were slightly higher in tissues of those with passive smoke exposure, these differences were not statistically significant. In some situations where it is not possible to get sufficient DNA for adduct analysis, protein adducts have been measured as a surrogate. Children of mothers who smoke had significantly higher levels of cotinine and PAH-albumin adducts than did children of nonsmoking mothers. These studies suggest that measurement of DNA or protein adducts using immunologic methods can provide useful information of individual exposure to environmental carcinogens. However, detection of DNA adducts in those with passive smoking exposure has not found significant increases compared to nonsmokers.

## 1012 ETS IMPACT ON ALLERGIC AIRWAYS RESPONSES TO ANTIGEN.

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Epidemiologic evidence supports a role for maternal smoking and for ETS exposure in subsequent development of allergic airway responses to antigens. We have been investigating ETS-antigen interactions in mice exposed to aerosolized, nose-only (N/O) ovalbumin (OVA) as young adults after being exposed *in utero* to ETS. We tested the hypothesis that *in utero* ETS exposure will subvert establishment of aerosol-induced tolerance to inhaled antigens, resulting in induction of adult-onset asthma. Pregnant mice were exposed to ETS or filtered air throughout pregnancy (10mg/m<sup>3</sup> TSP; 5hr/day). From 6-8 wks of age, offspring were exposed N/O to OVA or saline, as above. At 9 wks, ETS/ OVA-exposed mice exhibited small but significant increase in OVA-specific IgE compared to air/OVA-exposed mice. Subsequently, mice were injected i.p. with 100 $\mu$ g OVA in alum, followed by aerosol sensitization with 5% OVA (priming) and sacrifice at 16 wks. Air exposure *in utero* followed by inhaled OVA at 6 wks resulted in minimal levels of OVA-specific IgE after priming, consistent with establishment of aerosol tolerance. In contrast, ETS *in utero* followed by inhaled OVA at 6 wks produced elevated levels of OVA-specific IgE after priming, consistent with subversion of aerosol tolerance by ETS. Lungs of the ETS- and OVA-exposed (N/O; then primed) mice exhibited significantly increased inflammatory changes compared to lungs from air-exposed mice with the same OVA exposures. Again, this is consistent with subversion of aerosol tolerance by ETS. Plethysmograph measurements revealed expected Penh increases in air/saline/primed mice compared to air/OVA/primed mice. This is consistent with establishment of aerosol tolerance in OVA/primed mice. Surprisingly, ETS exposures resulted in sharply decreased Penh values in saline/primed mice. Their Penh profiles were not different from those of OVA/primed mice. These results are not consistent with ETS subverting aerosol tolerance. This apparent discrepancy between pulmonary function results and pathology/immunology results is being investigated.

## 1013 GESTATIONAL STAGE-SPECIFIC ETS-INDUCED OXIDATIVE DNA DAMAGE.

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Sidestream tobacco smoke contains carcinogens and long-lived prooxidant radicals. Both humans and animals exposed to sidestream or environmental tobacco smoke (ETS) show cellular increases in 8-hydroxy-2'-deoxyguanosine (8-oxo-dG), a pro-mutagenic lesion. An important issue is whether transplacental exposure of fetuses to the carcinogens or oxidants received by mothers in ETS may result in DNA damage, with possible carcinogenic consequences. Tobacco smoking by fathers could in this way contribute to risk of childhood cancer. We investigated whether exposure of pregnant rats to ETS would result in an increase in 8-oxo-dG in the tissues of their fetuses. Timed pregnant SD rats were exposed to ETS from Kentucky 1R4F research cigarettes in inhalation chambers. Levels of 8-oxo-dG were analyzed by HPLC/EC. Tissues analyzed were liver, lung, kidney, heart, and brain. Increases in 8-oxo-dG occurred selectively in certain tissues. Changes were relatively small and somewhat variable, but were of statistical significance. Results varied with the stage of gestation. For the mothers, there were increases in 8-oxo-dG with ETS exposure for gestation days 4-16, in liver and kidney, but not in heart or brain. However, when exposure was continued until the end of gestation, days 4-21, no differences were seen in any tissue. For the fetuses, exposure for days 4-16 resulted in increased 8-oxo-dG in kidneys only. Fetal exposure for days 4-21 led to increased 8-oxo-dG in livers and in female brains, but not in other tissues. The effects in the livers measured on day 16 were significantly different compared with day 19, for both mothers and fetuses. These results imply that (1) ETS can indeed act transplacentally to cause potentially mutagenic changes in fetuses; (2) sensitivity is tissue-specific and varies during ontogeny, probably due in part to degree of differentiation and maturation of relevant enzymes; and (3) pregnant females may have special sensitivity to reactive oxygen damage from ETS.

## 1014 EFFECTS OF ENVIRONMENTAL TOBACCO SMOKE ON CYTOKINES AND NEUROTROPHINS IN THE NEONATAL LUNG.

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Epidemiological studies have associated ETS with asthma in early childhood. The role of Th2 cytokines in allergic response has been well demonstrated. Recent work suggests that neurotrophins are critical mediators between allergic inflammation and airway hyperresponsiveness. To determine the alteration of cytokine response and neurotrophin profile in newborn rhesus monkeys caused by ETS during perinatal period, exposure was started at 50 days gestational age at a total suspended particulate concentration of 1 mg per cubic meter, 6 hours per day, 5 days per week, and continued to 2.5 months postnatal age. Bronchoalveolar lavage (BAL), measurement of protein levels of interleukin (IL)-2, interferon-gamma, IL-4, IL-5, BDNF, and nerve growth factor (NGF) were done one day after the exposure. Compared with control animals, significant increase was found in the differential percentage of monocytes, lymphocytes, and eosinophils in BAL recovered cells (287%, 347%, 478% of control, p<0.05, respectively), total protein level in the BAL fluid (228% of control, p<0.05), levels of IL-4 and BDNF in the cell culture supernatant of peripheral blood mononuclear cells (PBMCs) (170% and 440% of control, p<0.05, respectively), plasma level of BDNF (335% of control, p<0.01), or IL-5 level in the lung tissue extract (159% of control, p<0.05), respectively. Statistically decreased levels of IL-2 in PBMC culture supernatant (81% of control, p<0.05) and NGF in BAL fluid (24% of control, p<0.01) were also determined. According to the results obtained from newborn monkeys, we conclude that perinatal exposure to ETS causes chronic pulmonary inflammation and BDNF-associated Th2 weighted imbalance of cytokine profile.

## 1015 IN VITRO TOXICITY TESTING OF AIR POLLUTANTS: PROS AND CONS.

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The increasing evidence for adverse health effects resulting from acute and chronic exposure to air pollution has driven efforts to identify which components cause these effects and by what mechanisms. This, in turn, has created need for rapid, cost-effective methods for *in vitro* screening and mechanistic studies. The respiratory tract is the first target of aerosols, but *in vitro* studies of this system are complicated by species differences, varied regional anatomy and physiology, as well as the fact that aerosols interact at the gas/air inter-phase of the lung surface. In addition to containing over forty different cell types that may respond quite differently to a

given stimulus, host susceptibility factors that may be driven by genetics, and previous exposures may impact on observed effects. The purpose of this session is to review issues regarding the selection of models, exposure methods, doses, and endpoints and to determine how these systems may reflect inhalation exposures in the exposed population.

## 1016 CELL POPULATIONS WITHIN THE RESPIRATORY TRACT.

C. G. Plopper. *Anatomy, Physiology and Cell Biology, University of California, Davis, CA.*

The respiratory system serves as one of the principal interfaces between the organism and its environment. The nature of the effects induced by inhaled materials, such as particulates, reactive gases, and pathogenic microbes, on the respiratory system depends on the aerodynamic properties of the three-dimensional architecture of the conducting air passages and interactions occurring between inhaled toxicants, the airway surface lining and the epithelial lining cells. The respiratory system is highly complex architecturally and the cells organized to form the unique compartments resulting from this organization are very heterogeneous. Each set of cells organizes its own unique microenvironment. The epithelial cell populations change in phenotypic expression in different locations within the tract. Nasal cavity, larynx, trachea, bronchi, bronchioles and alveoli each have their own populations. Even where the populations are structurally similar, they often have different metabolic and functional potential. The extracellular matrix and the mesenchymal cells of the interstitium also vary greatly by position within the tract. Meaningful *in vitro* studies require well-defined cell populations.

## 1017 SYSTEMS FOR EXPOSING LUNG CELLS TO AEROSOLS.

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*In vitro* systems of cultured lung cells offer considerable advantages in assessing the mechanisms by which aerosol toxicants may affect an organism. However, there are significant challenges in assuring that the materials to which the cells are exposed accurately represent the appropriate physical/chemical form of these potential toxicants. In conventional culture, cells are cultured on plastic substrata and collected materials from the aerosol of interest are mixed with the culture medium. Although this method is relatively simple and the concentrations of the materials in the medium can be known, changes in the physical/chemical composition during the collection and resuspension of the aerosol components are unavoidable, and responses to gas-phase components cannot easily be measured. Recently, several methods have been developed to expose lung cells to aerosols in a more physiological manner. These methods include exposing cultured cells or precision-cut lung slices intermittently to aerosols in roller bottles or rockers. Alternatively, direct air-liquid interface exposure systems have been developed. In these systems, cells are grown on a semi-permeable membrane, with culture medium present below the membrane. The apical surfaces are then directly exposed to the atmosphere. Advantages include more physiological exposure and the ability to quantify both the exposure concentrations and in many cases, the actual doses received at the cell surface. Disadvantages include the limitations of only short-term exposures, and the difficulty of performing simultaneous parallel doses or atmospheres. Critical features for appropriate use of these models include adequate pH regulation and humidity, confirmation of uniformity of exposures both among wells and across the surface of individual wells, a means of assessing the dose to the cells, and appropriate controls for exposure to clean air. Careful attention should also be paid to the reproducibility of the exposures and the cellular responses. Supported by FreedomCAR and Vehicle Technology Program of the US Department of Energy, Office of Energy Efficiency and Renewable Energy.

## 1018 ANALYTICAL TOOLS FOR ASSESSING AIR POLLUTION TOXICITY USING DIFFERENT *IN VITRO* CELL CULTURE MODELS.

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The primary targets for inhaled air pollutants are cells lining the respiratory tract and resident monocytes/macrophages. Several cell culture models are available to assess air pollution toxicity *in vitro*. These cell culture models are based on either immortalized cell lines, representing certain cell types of the respiratory tract, or primary cells obtained from either laboratory animals or humans. Immortalized cell lines and primary cells can be cultured either under submerged conditions or on membranous support at air-liquid interface (ALI) conditions. Furthermore, pri-

mary respiratory epithelial cells cultured under ALI conditions undergo mucociliary differentiation into a pseudotrified epithelium, which mimics many of the features seen *in vivo*, including large beds of beating cilia. These *in vitro* models of respiratory epithelial cells or macrophages are a valuable tool to determine cellular mechanisms of air pollution toxicity. For example, detailed analysis of signal transduction cascades induced upon exposure to air pollutants can be examined by applying commercially available pharmacological agents. In addition, manipulation of the expression of defined genes using techniques such as siRNA-mediated knock-down and plasmid-mediated overexpression are helpful in determining cellular mechanisms of air pollution toxicity. Furthermore, activities and interactions of signaling components can easily be examined using *in vitro* approaches. Some of these techniques can only be applied to a limited extent in primary cells or cells grown on membranous support. However, cell culture models using respiratory cells grown on membranous support can examine whether there is any polarity with regards to mediator release. In addition, these cell culture models facilitate co-culture with different cell types, which are useful to determine cell-cell interactions. In the context of emerging scientific questions regarding the mechanisms by which air pollutants exert their toxicity on the respiratory system, the advantages and disadvantages of using these various *in vitro* models will be discussed.

## 1019 *IN VITRO* CORRELATION WITH *IN VIVO* EXPOSURES.

R. Devlin and L. Dailey. *USEPA, Research Triangle Park, NC.*

In principle, *in vitro* toxicology studies offer several advantages over *in vivo* animal or human studies, for example by providing a way to rapidly screen toxicants in lieu of *in vivo* exposures, or by characterizing mechanisms in ways not possible with *in vivo* studies. However, to be of optimal use, there must be confidence that the information obtained from such studies can be applicable to *in vivo* exposure studies. This presentation will describe information obtained from exposure of human primary lung epithelial cells and alveolar macrophages to air pollutants. Studies will be presented which show that the response of cultured epithelial cells to different PM size fractions or components is comparable to lung inflammation observed in rats or humans exposed to the same particles. *In vitro* mechanistic studies will also be described which use cutting edge molecular biology approaches to better understand the pathophysiological effects observed following *in vivo* exposure of animals and humans to air pollutants. This abstract does not necessarily reflect EPA policy.

## 1020 TOXICOLOGY INFORMATION AND DATA UPDATE.

P. Wexler. *Toxicology and Environmental Health Information Program, National Library of Medicine, Bethesda, MD.*

As the Web and its online resources continue to expand and change rapidly, there is an increasing plethora of databases, documents, and other digital tools available to the toxicology community. Toxicologists with even formidable research skills are often knowledgeable about only a small fraction of what is available, and they are often not kept apprised of the newest developments. This symposium highlights major Web-based resources from all sectors - government, academia, industry, nonprofits - which can benefit toxicologists in their daily research and other work. This program takes a look at a few of these resources. The National Library of Medicine's Toxicology and Environmental Health Information Program is a major purveyor of toxicological information and data through its TOXNET system and other databases, geared to both the professional and the public. The Center for Research on Environmental and Occupational Toxicology (CROET) focuses on health and safety in the workforce and supports a number of information activities, including its Toxicology Information Center. The National Institute of Environmental Health Sciences (NIEHS) and the National Toxicology Program offer a number of unique online databases and make available online numerous test reports and the Report on Carcinogens. EPA has recently unveiled its Distributed Structure Searchable Toxicity (DSS-TOX) database, another powerful tool of potential interest.

## 1021 THE NATIONAL LIBRARY OF MEDICINE'S (NLM) WORLD LIBRARY OF TOXICOLOGY, CHEMICAL SAFETY AND ENVIRONMENTAL HEALTH.

P. Wexler. *Toxicology and Environmental Health Information Program, National Library of Medicine, Bethesda, MD.*

NLM's Toxicology and Environmental Health Information Program (TEHIP) provides an extensive suite of databases and other information resources for the toxicology community, ancillary professional groups, and the public. In addition to collaborative arrangements with other government agencies and non-profit organizations, NLM also offers links to other relevant sites. With increasing inter-

dependence among countries and the expansion of information and telecommunication technologies, it has become increasingly obvious that a coordinated global approach to toxicology information is a resource waiting to happen. NLM's World Library of Toxicology, Chemical Safety and Environmental Health is a Web-based tool that attempts to fill the void. With information supplied, maintained and updated by country national representatives, the World Library serves as a forum to stimulate collaborative projects between nations and to minimize duplication of effort, providing a special benefit to the developing world. Among the many information sources included are those provided by governmental organizations and non-government groups, and universities and other educational programs. Also presented is legislation, country gateway information, databases, TOXLINE references, news, publications and meeting announcements.

 **1022** WEB-BASED RESOURCES SPONSORED BY THE NATIONAL INSTITUTE OF ENVIRONMENTAL HEALTH SCIENCES (NIEHS) AND THE NATIONAL TOXICOLOGY PROGRAM (NTP).

L. L. Wright. *US Department of Health and Human Services, National Institute of Environmental Health Sciences, Research Triangle Park, NC.* Sponsor: P. Wexler.

The presentation begins with a brief overview of NIEHS and NTP web-based resources with information on major programs, research portfolios, announcements, extramural funding opportunities through grants, contracts as well as postings of in-house vacancies including pre-doctoral, post-doctoral, staff-fellow and full-staff employment opportunities. Thereafter discussions focus on the resources developed by the NTP: its mission, participating agencies, announcements (Federal Register, press releases, meetings and post-meeting updates), annual plans, official reports, toxicity testing programs and summaries in databases searchable by substance, organ site, lesion morphology, models and alternative models, historical controls and more. Database queries follow through to links providing full-text of technical reports and publications relevant to the search

 **1023** THE CENTER FOR RESEARCH ON OCCUPATIONAL AND ENVIRONMENTAL TOXICOLOGY (CROET).

F. Berman. *Oregon Health and Science University, Portland, OR.* Sponsor: P. Wexler.

The Center for Research on Occupational and Environmental Toxicology (CROET) Description: CROET comprises more than 100 scientists and research staff exploring a range of questions relating to the prevention of injury and disease - and the promotion of health - in the workforce of Oregon and beyond. In addition to the conduct of basic and applied research on a variety of occupational safety and health issues, CROET is charged with providing outreach and education to business, labor and professionals through conferences, direct consultation and, most importantly, via the web. CROET's website, [www.CROETweb.com](http://www.CROETweb.com), is an occupational safety and health resource directory that contains links to 1000+ occupational safety and health (and toxicology) resources that focus on day-to-day workplace issues. Links are hand selected and organized topically under a variety of headings, including Occupations and Industries, Chemical and Biological Hazards, Ergonomics Issues and other Workplace Safety Issues. The website also provides links to a variety of toxicology and other databases, including the full suite of National Library of Medicine databases. Link selection criteria and guidelines for the evaluation of health-based websites will be discussed. Center for Research in Occupational and Environmental Toxicology Oregon Health and Science University Portland, Oregon, USA

 **1024** DSSTOX STRUCTURE-SEARCHABLE PUBLIC TOXICITY DATABASE NETWORK: CURRENT PROGRESS AND NEW INITIATIVES TO IMPROVE CHEMO-BIOINFORMATICS CAPABILITIES.

A. M. Richard<sup>1</sup> and B. A. Rogers<sup>1</sup>. <sup>1</sup>USEPA, Research Triangle Park, NC and <sup>2</sup>NCCU Student COOP, USEPA, Research Triangle Park, NC. Sponsor: L. King.

The EPA DSSTox website (<http://www.epa.gov/nheerl/dssto>) publishes standardized, structure-annotated toxicity databases, covering a broad range of toxicity disciplines. Each DSSTox database features documentation written in collaboration with the source authors and toxicity experts, standardized chemical structure annotation, inclusion of data fields designed to enhance the utility of these files for structure-activity exploration and discovery, and full and open public access. In addition, the DSSTox website offers a range of information resources and tools to encourage public participation in the project and to facilitate use of these structure-searchable databases in relational database applications and structure-activity relationship (SAR) model development. Long-term goals of the DSSTox project are to:

expand database offerings into a wider range of toxicology study areas; coordinate development and adoption of standardized toxicity data fields that will expand structure-activity exploration capabilities across wide-ranging areas of toxicology; incorporate XML chemical structure (NIST-ICHI) identifiers; and encourage and facilitate expanded chemo-bioinformatics capabilities in toxicogenomics. In collaboration with the NIEHS/National Center for Toxicogenomics, and the Chemical Effects in Biological System Knowledge Base (CEBS), DSSTox standard chemical fields are being incorporated into the annotation of toxicogenomics datasets resulting from chemical exposure. This will create linkages from CEBS to DSSTox historical toxicity databases, and enable sophisticated searches across multiple domains of information (chemical structure analogs, toxicity endpoint, gene, pathway, etc.). Chemical structure, and the chemistry underlying toxicity in biological systems, provide a natural common metric for exploration across diverse types of biological data and will be an essential information component to the next generation of predictive toxicology and chemo-bioinformatics capabilities. This abstract does not represent EPA policy.

 **1025** CURRENT AND FUTURE SCIENCE-BASED APPROACHES TO DRUG SAFETY EVALUATION: AN ASSESSMENT OF POTENTIAL CANCER RISK.

M. Moore. *DGRT, NCTR, Jefferson, AR.*

Currently the preclinical safety evaluation of pharmaceuticals for potential cancer risk includes the application of genetic toxicology assays and the rodent cancer bioassay. While data from rodent carcinogenicity studies are generally available prior to marketing approval, data from short-term studies is used to assess cancer risk during the drug development phases. Although this has generally been a successful approach there is wide discussion as to the appropriate role of the genetic toxicology assays and the possibility that other assays such as the Syrian hamster embryo (SHE) test and the shorter term transgenic cancer bioassays (Trp53 and rasH2) might be useful. The emerging "omics" technologies provide for possible new and improved approaches to understanding whether new drugs might cause an increased cancer risk. In addition, both present and future technologies have the potential to be applied within the context of the clinical trials. This session will provide a forum to discuss these issues. The first speaker will provide an overview of the current approaches and issues relevant to preclinical drug safety assessment. Additional speakers will discuss the current thinking on the use of genetic toxicology assays, the SHE assay and the transgenic cancer bioassays. The last two speakers will consider both the present and potential future applications of "omic" technologies and our ability to utilize individuals in clinical trials to provide a better assessment of the potential human cancer risk from pharmaceutical drugs.

 **1026** OVERVIEW OF THE CANCER SAFETY ASSESSMENT IN CDER/FDA.

A. Jacobs. *USFDA, Rockville, MD.*

It is difficult to ascertain the carcinogenic potential of drugs from epidemiologic data. Thus, the Center for Drug Evaluation and Research (CDER) of the US Food and Drug Administration relies on short-term genetic toxicology studies to assess risk to patients involved in clinical trials and on rodent carcinogenicity studies to assess cancer risk for drug approval. Carcinogenicity studies are conducted according to ICH guidelines, generally for drugs for chronic conditions. Protocols for carcinogenicity studies are submitted by drug sponsors to the CDER executive carcinogenicity assessment committee (exec-CAC) for concurrence. The exec-CAC may suggest protocol revisions, such as elimination of unnecessary measurements and even lowering of the top dose. After the studies are completed, the exec-CAC also gives concurrence for what findings are considered to be drug related. The evaluation of cancer risk is the result of an integrated assessment of what is known about the drug, and risk is considered in the context of the clinical benefit

 **1027** THE ROLE OF GENETIC TOXICOLOGY ASSAYS IN A WEIGHT-OF-EVIDENCE CANCER ASSESSMENT FOR NEW PHARMACEUTICALS.

D. Jacobson-Kram<sup>1</sup>. <sup>1</sup>FDA, Rockville, MD and <sup>2</sup>Office of New Drugs, Center for Drug Evaluation and Research, USFDA, Rockville, MD.

The Center for Drug Evaluation and Research (CDER) relies on two-species rodent bioassays to determine potential carcinogenicity of new drugs. However, the results of these studies are typically not available until the time of drug approval. As a result, CDER relies on surrogate markers of carcinogenicity during the course of drug development. Clinical trials often involve exposure of healthy subjects and

large numbers of patients to pharmacologically active doses of the drug for protracted time periods. Typically, CDER review divisions rely on genetic toxicology studies to assess cancer hazard during this phase of drug development. Positive results in *in vivo* genetic toxicology studies are rare; however, positive *in vitro* chromosomal aberration assays and positive mouse lymphoma assays are not infrequent. Review divisions must decide if it is ethical for sponsors to proceed with a clinical trial, often in healthy subjects, in the face of positive genotoxicity findings. Some review divisions have requested that sponsors perform Syrian hamster cell transformation assays or p53 carcinogenicity studies before proceeding to repeated dose clinical trials. Current CDER policy gives sponsors a broader range of options in coping with positive genetic toxicology results. These include a weight-of-evidence assessment as to whether the results present a hazard to humans under the conditions of the clinical trial, use of mechanistic studies to understand and explain positive findings and the use of additional *in vitro* and *in vivo* studies to address the relevance of positive findings.

**1028** TRANSGENIC CANCER BIOASSAYS: A USEFUL ADDITION TO CANCER SAFETY ASSESSMENT OF NEW PHARMACEUTICALS?

R. D. Storer, *Safety Assessment, Merck Research Laboratories, West Point, PA.*

International guidelines allow for use of a short-term cancer bioassay (26 weeks) in transgenic mice as a substitute for one of the two required long-term rodent bioassays in the preclinical safety evaluation of pharmaceuticals. The substitution may be allowed, or encouraged, when preclinical safety and/or pharmacology data suggest that an alternative assay could provide additional information not likely to be obtained from a second long-term bioassay. Utilization of these models in preclinical safety evaluation can significantly reduce animal use, time and manpower. The two models which have gained general acceptance by both sponsors and regulators are the CB6F1-RasH2 mouse hemizygous for the human H-ras transgene and the B6.129N5-Trp53 mouse heterozygous for a p53 null allele. These models have shown potential to provide, in addition to a short-term tumorigenicity endpoint, insight into mechanisms involved in tumor induction. Utilization of the p53+/- model is of particular value for compounds with residual concern that genotoxic activity may contribute to tumorigenesis; the RasH2 model however may be accepted as an alternative without regard to evidence of genotoxic potential. In considering the utility of the p53 model, many genetic toxicologists view the ICH test battery and available adjunct assays as sufficient for evaluating the potential risk of genotoxic carcinogenesis and for making decisions with respect to initiation of clinical trials. However, results from a p53 assay can make an important contribution to the weight of evidence assessment of mode of action (genotoxic vs non-genotoxic) from any tumor findings in the long-term bioassay. In addition, since results from a short-term bioassay can be obtained relatively early in drug development, there is the potential for more timely assessment of cancer risk for individuals in clinical trials. For the RasH2 model, responses to non-genotoxic carcinogens and the rationale for use of this model will be discussed in the context of the debate as to which classes of compounds in this category are most important to detect.

**1029** INTEGRATION OF OMIC DATA INTO CANCER RISK ASSESSMENT.

Y. Dragan, *Hepatic Toxicology, NCTR, Jefferson, AR.*

In order to minimize the risk of cancer development, two year carcinogenicity studies are performed in rodents to determine dose and exposure conditions that may result in a carcinogenic risk to humans. Cancer risk assessment methods are dependent upon the extrapolation of the data obtained in rodent experiments performed at maximum tolerated dose to humans exposed at environmental or therapeutic levels. This requires two leaps of faith; one a belief that rodents are good models for understanding humans and two that high dose exposures predict risk at lower exposures. Appropriate interpretation of cancer bioassay data developed under this paradigm requires that the black box between rodent exposure and response data and human exposure conditions be illuminated. The detection of a carcinogenic potential in the rat provides a mechanism to predict mode of action. Rodent models provide the opportunity to examine the biomarkers and patterns of alteration that occur early in the cancer development process. In addition, rodent models of cancer development can be used to examine the pathways that contribute to cancer development under various conditions. The ability to examine gene and protein expression patterns in a parallel manner permits one to examine human cancer development in the tissue of interest through microarray and proteomic analysis. Alternatively, one can develop biomarkers of cancer in biofluids through metabolomic and proteomic approaches. The utility of OMIC approaches lies in clarification of the dose response and the ability to extrapolate across species.

**1030** CANCER BIOMARKERS: CAN THEIR APPLICATION IN CLINICAL TRIALS IMPROVE THE SAFETY ASSESSMENT OF NEW PHARMACEUTICALS?

R. J. Albertini, *Genetic Toxicology Lab., University of Vermont, Burlington, VT.*  
Sponsor: M. Moore.

Biomarkers for evaluating *in vivo* genotoxicity in humans are conventionally classified as indicators of exposure, effect or individual susceptibility. Their practical utility depends on their validation in terms of intended purpose, i.e. (exposure) sensitivity in reflecting internal doses, (effect) accurate reflection of mechanisms or, if used as a surrogate for clinical outcome, ability to predict disease and (susceptibility) heightened predisposition to deleterious outcomes. Validation requires rigorous field studies and is more highly developed for biomarkers of exposure than for those of effect or susceptibility. However, it is the latter that are most likely to be useful in clinical trials. Traditionally, chromosome aberrations and somatic mutations indicating irreversible changes in DNA are the prototypic effect biomarkers. These changes can be in reporter regions of the genome, being related to cancer by molecular analogy, or in regions directly associated with carcinogenesis. Cell, flow and molecular based assays are available, some with high throughput capabilities. DNA adducts are also potential biomarkers of effect; their utility in safety assessments being dependent on thresholds before irreversible DNA alterations occur. Methods for assessing cellular responses in terms of gene expression (genomics, proteomics) are also available. Susceptibility biomarkers include the genotypes for metabolic, repair or other functions. Validated biomarkers of effect and susceptibility, with the latter serving as modifiers of the former, will have the potential for improving the safety assessment of new pharmaceuticals in clinical trials, especially those biomarkers previously studied in animals in the context of cancer bioassays. Clinical trials can assign relative risks for biomarker responses in treated versus untreated subjects with cancer inferences being dependent on biomarker validation and confidence that the underlying biological changes truly reflect carcinogenic mechanisms.

**1031** MOLECULAR PATHWAYS TO TOXICANT-INDUCED OSTEOPOROSIS.

M. H. Bhattacharyya<sup>1</sup>, E. Puzas<sup>2</sup>, J. B. Lian<sup>3</sup>, D. J. Novack<sup>4</sup> and M. J. Ronis<sup>5</sup>.  
<sup>1</sup>*Biosciences Division, Argonne National Laboratory, Argonne, IL*, <sup>2</sup>*University of Rochester School of Medicine, Rochester, NY*, <sup>3</sup>*University of Massachusetts Medical School, Worcester, MA*, <sup>4</sup>*Washington University School of Medicine, St. Louis, MO* and <sup>5</sup>*University of Arkansas Medical Sciences, Little Rock, AR*.

Osteoporosis is a disease characterized by low bone mass and structural deterioration of bone tissue, leading to bone fragility and an increased susceptibility to fractures of the hip, spine, and wrist. This disease is responsible for more than 1.5 million fractures annually; 44 million Americans have low bone mass such that they either have osteoporosis or are at significant risk of developing the disease. Of the 10 million who actually have osteoporosis, 80 percent are women. Men suffer one-third of all hip fractures that occur, and approximately one-third of these men will not survive more than one year after the fracture. Our population is increasing in the fraction of elderly persons faster than at any other time in human history. Understanding ways in which toxicants contribute to the development of osteoporosis is an important undertaking. In this workshop, we will provide 1) basic information on pathways of bone formation and bone resorption and their role in the development of osteoporosis, 2) new insights into how the important metals, lead and cadmium, affect bone cell pathways and contribute to metabolic bone disease, 3) the role that alcohol consumption may play in the development of osteoporosis, and 4) discussion by workshop participants of the results presented with respect to their application and relevance to human health.

**1032** MOLECULAR PATHWAYS REGULATING OSTEOBLAST GROWTH AND DIFFERENTIATION.

J. B. Lian and G. S. Stein, *Department of Cell Biology and Cancer Center, University of Massachusetts Medical School, Worcester, MA.* Sponsor: M. Bhattacharyya.

Bone remodeling in the adult skeleton is essential to respond to physical stresses on bone and to support calcium homeostasis for regulating vital cellular functions. Bone remodeling requires a perfect balance between bone resorption and bone formation events in order to maintain skeletal mass in the adult. Much has been learned related to the multiple pathways that result in formation of the skeleton in the developing embryo and it is now clear that the morphogenetic, growth factor signaling and gene transcriptional controls essential for the induction of bone formation can be recapitulated for bone renewal in the adult skeleton. Runx2/Cbfa is a central regulator of bone formation and osteogenic differentiation. Ablation of Runx2 in the mouse results in complete absence of a mineralized skeleton. Numerous properties of Runx2 have now been characterized that further establish

this transcription factor as a master regulatory switch for bone formation. Runx2 functions as a platform protein to integrate the signals from developmental signal transduction cascades for positive and negative regulation of gene transcription required for progression of mesenchymal stem cells through the osteoblast lineage for formation of a mineralized bone extracellular matrix. A balance between positive and negative regulation of skeletal turnover in the adult can be achieved through the gene regulatory activities of Runx2. Runx interaction with numerous coregulatory proteins that mediate the responses to developmental signals including BMP, TGF $\beta$ , and c-Src, regulates osteoblast expressed genes that control both bone formation and bone resorption. An overview of the unique properties of Runx2 and the potential for the translation of these properties into therapeutic strategies for the treatment of osteoporosis will be discussed.

### **1033** MOLECULAR PATHWAYS REGULATING OSTEOCLAST DIFFERENTIATION AND FUNCTION.

D. Novack. *Medicine/Bone and Mineral, Washington University School of Medicine, St. Louis, MO.* Sponsor: M. Bhattacharyya.

Osteoclasts (OCs), the multinucleated, monocyte lineage cells responsible for normal and pathological bone resorption, differentiate under the influence of macrophage colony stimulating factor (M-CSF) and receptor activator of NF- $\kappa$ B (RANKL), expressed in membrane-bound and soluble forms by osteoblasts and their precursors. Other soluble cytokines, (eg, TNF, IL-1, and PTH) modulate osteoclastogenesis, particularly in pathologic states. RANKL is a TNF family cytokine that binds to the transmembrane receptor RANK, present on OCs. Ligation of RANK leads to recruitment of TRAFs and initiates signaling through NF- $\kappa$ B, all 3 major MAPKs (p38, ERK, and JNK), as well as PI3K/Akt and src-family kinases. Inhibitor and murine knockout experiments have demonstrated that all these events are required for optimal osteoclastogenesis. Activation of NF- $\kappa$ B occurs via the classical pathway involving degradation of I $\kappa$ B $\alpha$ , as well as through the alternative pathway downstream of NIK and IKK $\alpha$ . TNF $\alpha$  is osteoclastogenic, but only in the presence of RANKL. TNF $\alpha$  activates all of the same signaling pathways as RANKL with the exception of the alternative NF- $\kappa$ B pathway. RANKL is required at all stages of osteoclastogenesis, providing differentiation, resorptive and survival signals. M-CSF, also known as colony stimulating factor (CSF-1), binds to c-fms, a receptor tyrosine kinase related to the PDGF family receptors. Activation of c-fms also leads to signaling via PI3K/Akt and src-family kinases, as well as through MAPKs (particularly ERK). M-CSF is also required at all stages, stimulating proliferation of precursors, survival, and motility. In some circumstances related cytokines such as VEGF and HGF have similar roles, and can substitute for M-CSF. The activities of OCs are linked to those of osteoblasts (OBs), a process known as coupling. In normal bone turnover, OCs remove a portion of bone, and OBs are rapidly recruited to these sites to add new bone matrix. This coordination is often disrupted in disease states, leading to an imbalance in favor of either resorption or formation.

### **1034** LEAD-INDUCED OSTEOFOPOROSIS.

J. E. Puzas. *Department of Orthopaedics, University of Rochester School of Medicine, Rochester, NY.*

Greater than 90% of the body burden of lead resides in the skeleton. In bone, this toxic element has a half life that has been estimated to be over 20 years. Yet bone cells have been an unappreciated target for the effects of this agent. Our laboratory and others have shown that bone cells (i.e. osteoblasts and osteoclasts) exposed to low levels of lead are adversely affected in their function. Both cell types are blunted in their ability to carry out normal bone formation and resorption processes, respectively. However, it appears that the effects of lead are more pronounced on osteoblasts. At all concentrations of lead, *in vitro*, the effect on osteoblast function (such as collagen synthesis, osteocalcin production, alkaline phosphatase activity, etc) is greater than the effect on osteoclast activity (i.e. pit formation on cortical bone wafers). If these findings persist *in vivo*, they would contribute to an accelerated rate of bone loss later in life. In the historical medical literature, lead exposure has never been considered a risk factor for osteoporosis. This is because densitometric studies utilizing DEXA technology have shown no significant decreases in bone mineral density in patients with a large lead burden. However, and most importantly, our recent findings suggest that these historical studies may be in error. We have found that the early generation DEXA scanners overestimate bone mineral density when trace amounts of lead are present in the bone being measured. At the lead levels found in the general population (i.e. 10-100  $\mu$ g/g bone) the overestimates range from 4-11%. This effect is due to the presence of a heavy metal atom in the x-ray beam. The newer generation DEXA scanners do not appear to have this problem. Thus, lead induces two serious situations in the skeleton. It affects bone

cells in a way that would predispose an individual to more rapid bone loss and, in early studies, it masked the detection of low bone density. Given these considerations, our conclusion is that lead exposure is a new risk factor for osteoporosis.

### **1035** FROM ITAI-ITAI TO OSTEOCLASTS: PATHWAYS TO CADMIUM-INDUCED BONE LOSS.

M. H. Bhattacharyya, A. Regunathan and D. A. Glesne. *Biosciences Division, Argonne National Laboratory, Argonne, IL.*

To identify pathways to Cd-induced bone loss, a whole-mouse genome c-DNA microarray was used to analyze bone cell gene expression after a single Cd gavage to mice. Time points were 2h and 4h after gavage for a Cd dose that released calcium from bone starting at 8h. Three mouse strains were studied to identify a robust mechanism that applied across mouse strains. Results showed that ~18 genes increased significantly in mRNA levels. High to low in Cd responsiveness were: cysteine-rich protein 61 (Cyr61), metallothionein 2 (MT2), transferrin receptor, glutamine synthetase pseudogene 1, MT1, acidic chitinase, RIKEN cDNA 3930401B19, src-like adaptor protein, vacuolar proton pump ATPase, integrin alpha v, aquaporin 1, and p38 MAPK. No genes showed analogous decreases. Increases in expression were small but were validated by Northern analyses. Gene changes fit into the following hypothesis: Cd could, perhaps by estrogen receptor activation, increase expression of Cyr61 in osteoblasts (OBs). Cyr61, shown to be high in OBs, is a secreted extracellular matrix-associated protein that binds the integrin dimer, alpha-v beta-3. Cyr-61 secretion from OBs could attract osteoclasts (OCs) and bind to their surface integrin molecules, stimulating OC migration, adhesion and activation. Activation would stimulate expression of other OC genes, including integrin alpha-v (signaling molecule involved in activation), vacuolar proton pump ATPase (provides acid to dissolve bone calcium and activate acid proteases that degrade bone matrix), transferrin receptor (provides needed iron), acid chitinase (acidic polyglucosamine hydrolase, involved in bone matrix degradation?), and aquaporin 1 (water channel for osmotic regulation?). OC precursors contain high levels of p38 MAPK compared to other bone cells, and this kinase is required for OC formation. The latter pathways could explain existing evidence that Cd stimulates bone loss by increasing both OC formation and activation.

### **1036** MECHANISMS OF ETHANOL-INDUCED BONE LOSS DIFFER WITH PHYSIOLOGICAL STATE.

M. J. Ronis<sup>1,2</sup>, T. Badger<sup>1,2</sup>, C. Lumpkin<sup>1</sup>, J. Aronson<sup>1</sup>, M. Hidstrand<sup>1</sup>, K. Shankar<sup>1</sup> and R. Haley<sup>1,2, 3</sup>. <sup>1</sup>*University of Arkansas for Medical Sciences, Little Rock, AR* and <sup>2</sup>*Arkansas Children's Nutrition Center, Little Rock, AR.*

Epidemiological studies indicate that ethanol abuse is a risk factor for the development of osteoporosis. However, the molecular mechanisms underlying ethanol-induced bone loss remain the subject of dispute with some studies showing ethanol effects on osteoblastogenesis and others on bone resorption. In these series of investigations, we have demonstrated dose-dependent ethanol-induced bone loss in both pregnant and cycling female Sprague-Dawley rats. However, the severity of skeletal toxicity was substantially greater in cycling rats at an equitoxic urine ethanol concentration and the molecular mechanism of bone loss was shown to differ. Peripheral computerized tomography, histomorphometry and molecular markers of bone turnover demonstrated that in pregnant rats bone formation was inhibited with no effects on osteoclasts while in cycling females bone resorption was stimulated by ethanol accompanied by increased expression of osteoclast specific genes including tartarate resistant acid phosphatase (TRAP) and a 70 kD vacuolar ATPase. Estradiol treatment of cycling rats to produce plasma concentrations comparable to those found in pregnancy significantly protected against ethanol-induced bone loss. In addition, in physiological conditions where anabolic bone formation predominates such as in female rats following weaning and in rats undergoing distraction osteogenesis, ethanol was found to selectively block osteoblastogenesis. Ethanol-induced bone loss was accompanied by disrupted vitamin D homeostasis. In addition, effects on bone formation were blocked by inhibitors of TNF alpha. These studies illustrate that alternative pathways of ethanol-induced bone loss exist and that the effects of ethanol on the skeleton are multifactorial and dependent on physiological and endocrine status. (This work was supported by NIAAA AA12928 M.R. and AA012223 C.K.L.)

### **1037** TOXICITIES OF NANOMATERIALS.

C. Quan<sup>1</sup>, S. Wilson<sup>2</sup> and L. Chen<sup>1</sup>. <sup>1</sup>*School of Medicine, NYU, Tuxedo, NY* and <sup>2</sup>*New York University, New York, NY.*

Manufactured nanomaterials have fascinating properties and may have great impact to our society, but the environmental risks of the use of these materials are mostly unknown. Although toxic effects after instillation of carbon nanotubes in rats had

been reported, the mechanism of their toxicity is uncertain. In this study, we seek to understand the toxic mechanisms of nanoparticles using an *in vitro* model and relevant biological endpoints to provide a foundation to further investigate the toxicity of nanotubes *in vivo*. Representative fullerene and carbon nanoparticle samples were obtained and characterized, including impurity profiles. BEAS-2B cells were exposed to different doses of nanoparticles ranging from 0.1 $\mu$ g/ml to 1000 $\mu$ g/ml suspended in DMEM medium by sonication. Cell viability measurements using neutral red uptake and trypan blue exclusion, and nuclear factor kB (NF-kB) activation measurement by luminometer were conducted immediately after 24 hrs of exposure. The *in-vitro* study shows that the combustion fullerene soot (98% fullerene) produced greater NF-kB activation than Arc fullerene soot (7% fullerene), while both of these particles produced greater response than fine or ultrafine TiO<sub>2</sub>, and less than that produced by crystalline SiO<sub>2</sub>. Based on the dose-response obtained by *in-vitro* study, each kind of particles were intratracheally instilled to CAF1/J mice at 100 and 250 $\mu$ g/mice suspended in 50 $\mu$ l 1% DMSO in normal saline. Lung lavage parameters were measured 24 hrs post exposure. In both arc and combustion fullerene exposed mice at both doses, total cell counts and protein levels were elevated comparing to saline-exposed control mice, although no differences were observed between arc and combustion fullerene exposed mice for these parameters. These data suggest that exposure to fullerene soot may lead to pulmonary toxicity and that the response appeared to depend on the types of nano-material used and their impurities.

### 1038

#### EXPOSURE TO ULTRAFINE ELEMENTAL CARBON PARTICLES (UCP) SIGNIFICANTLY INCREASE THROMBOGENESIS.

V. M. Silva, N. Corson, A. Elder, R. Gelein and G. Oberdorster. *Environmental Medicine, University of Rochester, Rochester, NY.*

Recent studies indicate that exposure to ultrafine particles (UFP) can increase thrombogenesis. We have established a non-invasive model of *in vivo* thrombo-genesis in ear veins of rats. We have shown with this model that intravenous (IV) and intratracheal (IT) administration of ultrafine aminated-polystyrene particles significantly induced thrombus formation. The objective of this study was to determine if environmentally-relevant UFP such as ultrafine elemental carbon particles (UCP), affect coagulation when administered to rats through IV, IT and inhalation routes. For this purpose, UCP were generated as an aerosol with count median diameters (CMDs) of 25-35 nm (GSD=1.7) using electric spark discharge of ultra-pure graphite electrodes in argon. Particles were collected on filters, and then suspended in saline (250 $\mu$ l) at various concentrations (4, 20, 100 and 500 g/kg) for IV or IT administration into rats. In inhalation studies animals were exposed to UCP for 30 min to 3 hrs in whole-body inhalation chambers at concentrations of 200 and 70 $\mu$ g/m<sup>3</sup>. Our data show that IV doses as low as 4 $\mu$ g UCP/kg (-1g/rat) significantly shorten the time of thrombus formation in rat ear veins. Although the effect of the different IV doses is not significantly different from each other, there seems to be a trend indicating that the lower the dose of UFP in the system the better the response. Similar results were obtained when particles were IT instilled into rats. In this case, even a lower dose (0.8 $\mu$ g/kg or 0.2 $\mu$ g/rat) of UCP enhanced thrombus formation. Inhalation of 25-35nm carbon particles significantly induced coagulation regardless of the dose. Interestingly, this response did not change with time of exposure. These results are consistent with the hypothesis that UCP deposited in the lung translocate to the blood circulation and can activate platelets directly. Furthermore, these data demonstrate that the non-invasive ear vein model is useful to study UFP-induced thrombogenic effects after inhalation exposure.

### 1039

#### NANOPARTICLE DEPOSITION EFFICIENCY IN HUMAN NASAL AIRWAY REPLICAS.

B. A. Wong<sup>1</sup>, J. T. Kelly<sup>2</sup>, J. S. Kimball<sup>1</sup> and B. Asgharian<sup>1</sup>. <sup>1</sup>*CIIT CHR, Research Triangle Park, NC* and <sup>2</sup>*University of California, Davis, CA.*

Particles in the nanometer size range (1-100 nm) may be released to the environment by various means, including escape during manufacturing processes, production during combustion processes, and formation by condensation from gaseous precursors. Toxicologists have long been concerned with, and have studied the respiratory toxicology of particles in the micrometer and submicrometer size range. However, less is known about potential toxicity associated with nanoparticles. The overall deposition efficiency of nanoparticles in the nasal airways provides information about the relative dose between the upper and lower respiratory tract. We studied nanometer particle deposition efficiency in plastic replicas of nasal airways and compared our results with other models. Two replicas were manufactured using stereolithography techniques with morphological data from an MRI scan of a human nose. Monodisperse particles of nanometer and ultrafine sizes from 5 to 150 nm were generated into a constant air flow of 10 and 20 L/min. Deposition efficiency was determined by measuring the particle concentration at the entrance and

outlet of the nasal replica. Deposition efficiency was less than 10% for particles > 30 nm and increased for particles < 30 nm. The increase was attributed to increased particle diffusivity. Our deposition measurements were comparable with deposition values reported for other models. These results suggest that differences in nasal airway morphometry do not significantly affect the overall deposition efficiency of nanometer-sized particles.

### 1040

#### ELECTRON MICROSCOPIC STUDY ON THE TRANSLOCATION OF ULTRAFINE CARBON BLACK PARTICLES AT THE AIRWAY-CAPILLARY BARRIER IN LUNG.

A. Shimada, N. Kawamura, T. Kaewamatawong, M. Okajima, M. Sawada and T. Morita. *Veterinary Pathology, Tottori University, Tottori-shi, Japan.*

Ultrafine particles (UFP) may induce adverse respiratory and cardiovascular effects. Inhaled UFP have been shown to translocate to systemic circulation. Precise mechanisms of the anatomical translocation (crossing the airway-capillary barrier) of inhaled UFP are not fully understood. We examined anatomical location of the intratracheally instilled UFP at light and electron microscopic levels. Ultrafine carbon black (UFCB) particles, printex 90 (Degussa, Frankfurt, Germany), 14nm in diameter, were instilled to the trachea of 10-week-old ICR female mouse at a concentration of 1mg/0.05ml/body. Lung, regional pulmonary lymph nodes, liver and spleen were removed at 0, 5, 10, 30 min, 1, 2, 6, 12 and 24 hrs after instillation (n = 3 at each time point). Paraffin sections cut at 2um were processed for Factor VIII-immunohistochemistry to define blood vessels. Formalin-fixed lung samples were processed for electron microscopy. Aggregates of UFCB were observed in the capillaries of the alveolar walls soon after instillation. UFCB particles, which confined to the cytoplasm of the mononuclear cells with morphological appearance of dendritic cells, appeared in the regional pulmonary lymph nodes at 24 hrs after instillation. No UFCB particles were observed in the liver and spleen. Electron microscopy demonstrated aggregates of UFCB between the edges (epithelial pores) of the elongated thin cytoplasm of type I alveolar epithelial cells. Endothelial cells of the alveolar capillaries appeared to be highly activated with extensive folding, a large number of pinocytic vesicles and ribosomes. The thickness of the basement membrane (BM) varied from point to point. Occasional UFCB were observed in the matrix of the BM, pinocytic vesicles and in the spaces between the folded cytoplasm (fenestration) of the endothelial cells. These results suggest that pro-inflammatory chemokines responsible for the morphological alterations observed may be involved in the passage of the instilled UFCB through the airway-capillary barrier.

### 1041

#### PULMONARY TOXICITY OF CARBON NANOTUBES.

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Carbon nanotubes (CNT) are new members of carbon allotropes similar to fullerenes and graphite. Because of their unique electrical, mechanical and thermal properties, carbon nanotubes are being evaluated for novel applications in the electronics, aerospace and computer industries. Previously, we have observed that exposure of human bronchial epithelial cells to CNT induced iron-dependent oxidative stress, depletion of antioxidants, morphological changes, cytotoxicity, and apoptosis. In the current study, we investigated pulmonary toxicity of CNT in C57BL/6 mice after pharyngeal aspiration. End points were examined on days 1, 3, 7, 14, 28, and 60 post-exposure. We found that CNT caused dose-dependent formation of granulomatous bronchointerstitial pneumonia, fibrosis, and altered pulmonary function. Administration of CNT to C57BL/6 mice also resulted in a dose-dependent augmentation of inflammation biomarkers quantified by cell counts, total protein, lactate dehydrogenase (LDH) and  $\gamma$ -glutamyltranspeptidase (GGT) activities in bronchoalveolar lavage (BAL) fluid samples. Markers of pulmonary cytotoxicity were associated with the development of inflammation, collagen accumulation, and pulmonary fibrosis. TGF- $\beta$  was maximally increased in BAL fluid of mice 7 days after CNT exposure and correlated with morphometric evidence of collagen formation as well as pulmonary function changes. Mice exposed to an equal mass of ultrafine carbon black or fine crystalline silica exhibited less PMN recruitment and cytotoxicity than mice receiving CNT. Our data suggest that exposure to CNT leads to pulmonary toxicity involving inflammation and oxidative stress, which culminates in the development of multifocal granulomatous pneumonia and fibrosis.

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## RESPONSES OF LUNG PARENCHYMA TO CARBON NANOTUBES.

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An increasing number of applications have been and are being developed for new carbon allotropes such as carbon nanotubes (CNT). Pharyngeal aspiration by C57BL/6 mice was used to determine the pulmonary toxicity of CNT. Morphometry of paraffin sections from fixed lung tissue was used to determine the size of deposited CNT and the size of granulomatous lesions produced in response to the aspiration. Measurement of the Sirius red staining in sections was used to assess the connective tissue response. Lung responses were studied at 1 day, 7 days, 1 month and 2 months after a single CNT exposure of 0, 10, 20 or 40 µg/mouse. Examination of lung sections 1 day after aspiration, demonstrated that deposition of the CNT mass was generally in the first or second alveolar ducts proximal to the terminal bronchiole with an average diameter of 15.2±0.6 µm (mean±SE, n=12). At 1 day, CNT deposits were infiltrated with alveolar macrophages. At 7 days significant connective tissue accumulation was apparent within the CNT deposits. At 1 and 2 months, the granulomatous masses were encased in cuboidal epithelial cells. At 2 months, the granulomatous lesions accounted for 0, 0.7±0.1, 2.4±0.2 and 4.6±0.6 % of the alveolar parenchyma at doses of 0, 10, 20 and 40 µg/mouse, respectively. In addition to the granulomatous lesions there were also changes in the alveolar walls. For instance, the average thickness of Sirius red stained connective tissue in alveolar regions, excluding the granulomatous areas, was 0.10±0.03, 0.20±0.09, 0.3±0.09 and 0.5±0.1 µm at doses of 0, 10, 20 and 40 µg/mouse, respectively. The results demonstrate that CNT produce a rapid response in the alveolar region with both focal granulomatous lesions and a more generalized fibrotic response that is dose dependent.

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## PULMONARY TOXICITY SCREENING STUDIES WITH NANO VS. FINE-SIZED QUARTZ AND TIO2 PARTICLES IN RATS.

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For most low solubility dusts such as titanium dioxide, the limited database suggests that fine-sized particles (e.g. pigment-grade) are less toxic than nano-sized particles of the same chemical composition. Fine-sized Min-U-Sil quartz particles are known to be extreme pulmonary toxicants and are classified as IARC Category 1 carcinogens, i.e., human carcinogens. Thus, is it the case that nanoquartz particles could be even more potent than fine-sized quartz particles? This study was designed as a preliminary screen to test 1) the nanoparticle vs. fine-size hypothesis of pulmonary toxicity. In this regard, we assessed whether the Min-U-Sil quartz particles, a known cytotoxic dust, impart significantly greater pulmonary toxicity in the lungs of rats when compared to nano-size quartz particles. In the first experiment with quartz, fine sized quartz (Min-U-Sil) (average diameter = 1.6 µm) particles or nanoscale quartz particles (mean particle size = 50 nm) were instilled into the lungs of rats at doses of 1 or 5 mg/kg. Postexposure evaluations of bronchoalveolar lavage fluids were conducted at 1 day, 1 week, 1 and 3 months postexposure. Exposures to the Min-U-Sil quartz particles produced a significantly greater pulmonary inflammatory response. However, in a second experiment, following exposures to 1 or 5 mg/kg of Min-U-Sil quartz, another nanoquartz sample (10 nm) or fine-sized, sub-micron quartz particles (400 nm), the results suggested that the order of inflammatory potency was nano-Min-U-Sil-fine-sized quartz particles. In a third pulmonary bioassay study, rats were exposed to fine-sized TiO<sub>2</sub> particles, TiO<sub>2</sub> nanorods, and TiO<sub>2</sub> nanodots at 1 or 5 mg/kg with postexposure evaluations at 1 day, 1 week, 1 and 3 months. No significant differences were measured in the inflammatory responses among any of the groups at any postexposure time periods. Our interim results suggest that the pulmonary toxicity of each fine-sized and nano-sized particle type needs to be evaluated on a case-by-case basis.

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## DEVELOPMENT OF ANIMAL MODELS OF INHALATION FEVER USING FINE AND NANOPARTICLE ZINC OXIDE EXPOSURES.

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Occupational fume fever is characterized by a transient flu-like syndrome associated with the inhalation of freshly formed metal ultrafine particles (UFP), notably zinc oxide (ZnO). The aims of this study were to 1) develop an animal model of metal fume fever in rats using either fine-sized (i.e. > 100 nm) or nano (NP - < 100 nm) ZnO particles. These studies are designed to better define the conditions and properties of ZnO nanoparticles (NP) vs. fine-sized ZnO particles that may be associated

with lung injury and fume fever. Successful development of animal models could provide important scientific insights into fever development. Rats were exposed by inhalation for 1 or 3 hours to aerosols of fine-sized zinc oxide particles at 25, 35, or 50 mg/m<sup>3</sup>. Following recovery periods of 24 hrs, 72 hrs or 1 week, the lungs of ZnO and sham-exposed rats were lavaged and cells and BAL fluids were measured for cellular indicators (i.e., inflammatory cells) or noncellular mediators of inflammation and cell injury (e.g. BAL fluid levels of LDH, microprotein, or alkaline phosphatase). Our preliminary results with fine-sized ZnO demonstrated transient, pulmonary inflammatory effects. In this regard, animals exposed for 1 hr to ZnO at 25 or 35 mg/m<sup>3</sup> demonstrated no inflammatory and very mild inflammatory response, respectively. Rats exposed for 3 hours to ZnO particles at concentrations ranging from 25 to 50 mg/m<sup>3</sup> demonstrated transient pulmonary inflammatory responses which were evident after 24 and 72 hours but returned to control levels by 1 week postexposure. In subsequent experiments, groups of rats were exposed for 1 or 3 hrs to nano zinc oxide particles (mean particle size = 65 nm) at 25 mg/m<sup>3</sup> and evaluated at 24, 72 and 168 hrs postexposure. The transient inflammatory effects of nano ZnO particles were not significantly different from exposures to fine-sized ZnO particles. Studies are ongoing to conduct experiments in rats with nano ZnO particles for 1 and 3 hrs at 35 and 50 mg/m<sup>3</sup>. Thusfar, we have not observed differences between the pulmonary responses to nano or fine-sized ZnO particles.

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## PULMONARY EXPOSURE TO CARBON NANOTUBES INDUCES VASCULAR TOXICITY.

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Cardiovascular diseases, which in majority of cases stem from atherosclerosis, continue to be the principal cause of death in the United States. In addition to personal factors like hyperlipidemia and obesity, some environmental factors including cigarette smoking and air pollution, have been associated with cardiovascular diseases. Engineered nanosized particles, such as carbon nanotubes (CNT), are new materials of emerging technological importance in different industries. The unique physical characteristics of these particles raise concerns that they may have not only pulmonary toxicity but also extra-pulmonary toxicity. In the present study, we hypothesized that CNT pulmonary exposure is associated with oxidative and inflammatory responses in the vascular system, which might be a prerequisite of atherosclerosis. C57BL/6 mice were exposed to CNT in doses (0.5; 1; 2 mg/kg) by single intra-pharyngeal installation and the mice were sacrificed at different time points (1; 7; 28; 60 days) after the exposure (the experimental settings have been related to pulmonary toxicity). By extra long quantitative PCR of mitochondrial (mt) DNA, we found that CNT exposure induced a dose-dependent aortic mtDNA damage, an oxidative stress dependable parameter, at day 7, 28 and 60 after exposure. Furthermore, by real-time PCR, we demonstrated that the CNT-induced oxidative changes are accompanied by altered expression of inflammatory genes, including MCP-1 and VCAM-1, in the heart. These effects might be a direct result from CNT which penetrate to the circulation or an indirect result of the lung inflammation. The direct effects of CNT were evaluated *in vitro* in human aortic endothelial cells (HAEC). After 2 hours exposure to CNT, we observed an increase of MCP-1, VCAM-1 and IL-8 mRNA levels in HAECs. CNT also dose-dependently induced low density lipoproteins (LDL) oxidation in the presence of HAECs. In conclusion, CNT induces direct or indirect toxic effects which might be predisposing factors for atherosclerosis.

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## TOXICOGENETIC AND TOXICOGENOMIC ANALYSIS OF ALCOHOL-INDUCED LIVER INJURY.

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Severe steatohepatitis is a hallmark of continuous exposure to alcoholic beverages and a significant health concern due to the development of fibrosis, cirrhosis and hepatocellular carcinoma. The mode of action for alcohol-induced liver damage is thought to involve multiple cell types and mediators with reactive oxygen species and inflammatory cytokines playing the major role. Despite the measurable progress in alcohol research in liver, little is known about the genetic factors that may contribute to a large variability in susceptibility to liver disease in humans. The development of a mouse intragastric model of alcohol-induced liver injury, an excellent model for human disease, allowed a number of important discoveries with help from genetically engineered animals. Here, we tested the hypothesis that by combining a state-of-the-art *in vivo* model of liver toxicity, our prior knowledge of the mechanisms of alcohol-induced liver injury, novel genomic and toxicologic analyses with knowledge of the genetic diversity in mouse inbred strains, a liver toxicity susceptibility state may be defined. A panel of six mouse inbred strains (male

mice) was subjected to acute (5 g/kg, 6 hrs), or sub-chronic (17-24 g/kg/day, 28 days) exposure to alcohol. Liver tissue and serum were collected from naive and alcohol-treated mice. The analysis of our results produced mouse strain-specific baseline and alcohol-induced toxicological and gene expression matrices in liver. This unique dataset allowed us to develop and test novel computational strategies for integration of toxicity data with other multi-dimensional data types such as gene expression profiles. The phenotype-specific genotypic anchoring of alcohol-induced expression changes with liver injury provides invaluable insight into genetically-determined individual variability in response to liver toxicants. Supported by grants from NIH AA11605, ES11391, ES11660, and ES10126.

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**EVIDENCE FOR OSTEOPONTIN INDUCTION *IN VIVO* AND *IN VITRO*: A POSSIBLE MECHANISM FOR HIGHER NEUTROPHIL TRANSMIGRATION AND HIGHER LIVER INJURY IN FEMALE ALCOHOLICS.**

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Alcoholic liver disease (ALD) accounts for more than 450, 000 deaths and public health costs are more than \$184.6 billion per year in the US. In recent years, a three-fold increase in ALD has been observed in the US, and of them 13-33% are women. Although women are known to be more susceptible to ALD, the precise mechanism for increased susceptibility of females to ALD is not completely understood. The present study is based on the hypothesis that increased hepatic neutrophil infiltration and higher liver injury in females during alcoholic steatohepatitis (ASH) is due to enhanced hepatic OPN expression. ASH was induced in the male and female SD rats (weight matched) by feeding ethanol (EtOH) containing Lieber-DeCarli diet (36% calories by EtOH) for 6 weeks followed by a single injection of lipopolysaccharide (LPS, 10 mg/kg, ip). The control rats received an isocaloric diet with maltose-dextrin. Liver injury as measured by plasma transaminase elevations (ALT and AST), and confirmed by H&E stained liver sections, revealed a significantly higher liver injury (>20-fold) in the female ASH model compared to the males. Although steatosis, neutrophilic infiltration, and multifocal coagulative oncotic necrosis was evident both in the male and female rats, hepatic neutrophilic necrotic foci were noted much earlier and more frequently (-> 2-fold) in the females than in the male rats. Hepatic neutrophilic infiltration correlated with higher expression of cleaved OPN (cOPN) in the female EtOH-treated rats compared to their male counterparts. The *in vivo* rat OPN induction data was confirmed with HEPG2 cells exposed to different EtOH and a combination of EtOH+LPS concentrations. In conclusion, these data suggest that EtOH ingestion induces enhanced expression of cOPN *in vivo* in female rats and in HEPG2 cells. Furthermore, higher expression of OPN is likely playing a mechanistic role in the induction of ASH making females more susceptible to ALD (partially supported by NIEHS Grant# ES09106)

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**ACETAMINOPHEN (APAP) USE IN ALCOHOLICS: HEPATIC FUNCTION DURING AND FOLLOWING THERAPEUTIC DOSING.**

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**BACKGROUND:** Therapeutic dosing of APAP is considered safe. Retrospective reports suggest that therapeutic use of APAP in alcoholics may be associated with liver injury, but this phenomenon has not been investigated in prospective clinical trials. This study evaluated hepatic effects in alcoholics during and following 4 g/day APAP dosing for 2 and 3 days. **METHODS:** Two prospective, double-blind, randomized, placebo-controlled trials of alcoholics were conducted at 2 detoxification centers. Patients in the first trial were randomized 1:1 APAP (1g every 4-hrs for 4 doses) or placebo for 2 days. Randomization in the second trial was 2:1 (APAP:placebo) with study drug administered for 3 days. Serum ALT was measured at baseline, day 2 of study drug administration, and 36 h following last dose. Exclusion criteria included elevated baseline AST, ALT or INR. **RESULTS:** There were no differences in demographics, nutritional status or baseline laboratory measures between groups in 586 patients (364 APAP, 222 placebo). ALT means on day 2 of study drug administration were 40±29 IU/L for the APAP group and 42±31 IU/L for placebo group ( $p=0.419$ ). ALT levels 36 h after last study dose were 52±42 IU/L APAP group and 50±44 IU/L placebo ( $p=0.654$ ). 213 placebo group patients and 359 APAP group patients qualified for within group analysis. The ALT mean of the APAP group went from 44 IU/L at baseline to 40 IU/L on day 2 to 52 IU/L 36 h after last dose ( $p<0.001$ ). The ALT mean of the placebo group went from 42 IU/L at baseline and day 2 to 50 IU/L 36 h after last dose ( $p<0.001$ ). A total of 8 patients (5 APAP, 3 placebo;  $p=0.999$ ) developed an ALT ≥200 IU/L (range 203–

312 IU/L) 36 h after last study dose. **CONCLUSIONS:** No differences were found in ALT between the placebo and APAP treatment groups at baseline, day 2 or 36 h after last study dose. ALT fluctuations are common in alcoholics. APAP does not appear to affect hepatic function in alcoholics during and following 2 or 3 days of maximal therapeutic dosing.

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**PHENOBARBITAL AND PHENYTOIN INDUCED ACETAMINOPHEN HEPATOTOXICITY DUE TO DIRECT INHIBITION OF UGT1A6 AND UGT1A9.**

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Here we present a preclinical model, using cultured human hepatocytes, to assess drug-drug interactions leading to toxicity. Treatment with the antiepileptics phenobarbital (PB) or phenytoin (PH) has been associated with increased incidence of acetaminophen (APAP) hepatotoxicity in patients. This was previously attributed to induction of CYPs. In human hepatocytes, we found that pretreatment with PB for 48 h prior to APAP treatment did not increase APAP toxicity. However, the toxicity of APAP was increased by simultaneous treatment with phenobarbital or phenytoin. Cells treated with APAP in combination with PB or PH experienced decreases in protein synthesis as early as 1 hr, ultrastructural damage to mitochondria by 24 h, and release of liver enzymes by 48 h. Toxicity developed with no induction of CYPs. Maximum toxicity occurred at 2 mM PB or 0.2 mM PH combined with 5 mM APAP and correlated with inhibition of APAP glucuronidation and increased accumulation of APAP-glutathione. Inhibition of APAP glucuronidation was not competitive since PB and PH were not glucuronidated. PB or PH inhibited APAP glucuronidation in rat and human liver microsomes. We analyzed the kinetics of PB and PH inhibition of APAP glucuronidation by expressed forms of human UGTs. The IC50 of inhibition of APAP glucuronidation by PB and PH were 1.7 mM and 0.28 mM for UGT1A6, and 3.7 mM and 0.145 mM for UGT1A9, respectively. As with intact hepatocytes, PB and PH were not glucuronidated, suggesting non-competitive inhibition. Our findings suggest that in multiple drug therapies, an inhibitory complex between UGT and one of the drugs can lead to decreased glucuronidation and increased toxicity of a co-administered drug. This human hepatocyte system provides an *in vitro* screen to detect such interactions.

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**GENDER DIFFERENCE OF ACETAMINOPHEN-INDUCED HEPATOTOXICITY IN MOUSE.**

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Although acetaminophen (APAP) hepatotoxicity has been extensively investigated, there is no report on gender difference of hepatotoxicity induced by overdose of APAP. In this study, both male and female mice were injected with a toxic dosage of APAP (i.p., 500 mg/kg). By examining serum alanine aminotransferase (ALT) and sorbitol dehydrogenase (SDH) activities and liver histology, our data showed that female mice were resistant to the hepatotoxic effects of APAP. To investigate the mechanism underlying the gender difference in APAP hepatotoxicity, the expression of genes involved in APAP detoxification, hepatic glutathione (GSH), and APAP metabolites were examined. Female mice showed higher basal Cyp2e1 mRNA levels than males. However, the levels of Cyp2e1 and Cyp3a11 mRNAs were significantly reduced in female mice, but not in male mice, after APAP treatment. Consistent with these findings, free APAP levels in the liver were higher in female than male mice one hour after APAP injection. Surprisingly, glutathione (GSH) levels were lower in female than male mice after starvation overnight and before APAP treatment. Female mice have significantly lower Gst pi mRNA levels compared with male mice. This finding concurred with a previous report which showed that Gst pi knockout mice were also protected from APAP-induced liver toxicity. However, unlike Gst pi knockout mice which have elevated mRNA levels of the antioxidant protein heme oxygenase-1 (HO-1), HO-1 mRNA expression was significantly lower in female than male mice. Among other phase II enzymes, liver Ugt1a1 mRNA levels were higher in females than males. There were no significant gender differences on the APAP-glucuronide and APAP-sulfate concentrations in bile, urine, and serum. Taken together, these data suggest that the conversion of APAP into N-acetyl-p-benzoquinone imine (NAPQI), the toxic metabolite of APAP, is slower in female than male mice, which might in part explain why females are protected from APAP-induced liver toxicity.

ALTERED HEPATOBILIARY DISPOSITION OF ACETAMINOPHEN GLUCURONIDE IN MRP3 KNOCKOUT MICE AND THEIR SUSCEPTIBILITY TO HEPATOTOXICITY.

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Administration of the organic anion indocyanine green (ICG) to male mice reduces the biliary excretion of acetaminophen (APAP) conjugates, suggesting that these metabolites share hepatobiliary transport with ICG. Studies with transport-deficient (TR<sup>-</sup>) hyperbilirubinemic rats showed the involvement of the multidrug resistance protein 2 (Mrp2) in the biliary excretion of APAP conjugates. The urinary output of acetaminophen-glucuronide (APAP-GLUC) was significantly higher in TR<sup>-</sup> rats than in normal rats. This indicates that enhanced expression of the basolateral transporter Mrp3 in TR<sup>-</sup> rats results in greater hepatic excretion of APAP-GLUC into blood. To further investigate the role of Mrp3 in the hepatobiliary disposition of APAP and its metabolites, male Mrp3 knockout and wild-type mice were fasted overnight and the bile duct was cannulated. APAP (150 mg/kg) was administered and bile was collected at 20 min intervals for 120 min. Other groups of mice received 400 mg APAP/kg for assessment of hepatotoxicity at 24 hr. The results show that the biliary excretion of APAP and most conjugates was not altered in Mrp3 null mice. However, the cumulative amount of APAP-GLUC in bile increased from 0.5% in wild-types to 6% of the administered dose in null mice. Concomitantly, there was a dramatic accumulation of APAP-GLUC in the liver of null mice to approximately 20% of the dose in comparison to less than 1% in wild-types. Plasma content of APAP-GLUC in null mice was also consistent with the reduced basolateral excretion of this conjugate. In toxicity studies, Mrp3 knockouts had 70% lower plasma levels of liver transaminases ALT and AST than wild-types. This was confirmed by histopathology. These studies show that the basolateral excretion of APAP-GLUC is almost exclusively dependent on the presence of Mrp3 and that this altered disposition is associated with a lower risk for hepatotoxicity.

RAPID ACTIVATION OF WNT/β-CATENIN PATHWAY FOLLOWING ACETAMINOPHEN-INDUCED LIVER INJURY AND REGENERATION.

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Over dose of acetaminophen (APAP), the leading analgesic and antipyretic agent causes acute liver failure. Understanding the mechanism of surviving hepatocytes and comprehending its molecular basis would have strong clinical relevance. Previously we have demonstrated Wnt/β-catenin pathway activation in liver regeneration following partial hepatectomy. The current study was designed to investigate role of Wnt/β-catenin pathway in regeneration following APAP-induced liver injury. Male CD-1 mice were injected with a sublethal dose (500 mg/kg) of APAP and markers of liver injury (plasma ALT and histopathology) and liver regeneration (proliferation analysis by PCNA) were assessed over a time course of 0-48 hr. Liver injury increased within 6 hr, peaked at 12 hr, and decreased by 48 hr. Increase in hepatocyte proliferation was observed at 6 hr and continued till 24 hr after APAP treatment. Western blot analysis showed an increase in total and activated β-catenin protein within 1 hr after APAP treatment, which remained higher till 6 hr, returning to control levels by 12 hr. A concomitant increase in cyclin D1 was observed between 1-6 hr post-APAP treatments. There was no change in total glycogen synthase kinase 3-β (GSK-3β) protein, a negative regulator of β-catenin, however a significant increase was noted in its serine phosphorylation (inactivation) at 1 hr after APAP treatment. Thus, we demonstrate a rapid activation of Wnt/β-catenin pathway following APAP-induced liver injury and subsequent regeneration that appears to be mediated via GSK-3β inactivation. These data indicate that Wnt/β-catenin pathway might play a crucial role in initiation of liver regeneration following APAP-induced liver injury (supported by RSG-03-141-01-CNE and NIH -1R01DK62277 to SPSM).<sup>β</sup>

ROLE OF MITOCHONDRIAL BAX TRANSLOCATION IN ACETAMINOPHEN-INDUCED HEPATIC NECROSIS IN MICE.

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Acetaminophen (AAP) overdose causes centrilobular necrosis in human and mouse livers. Mitochondria are the main source of oxidant stress and peroxynitrite formation after AAP (reviewed in Toxicology Lett 144:279, 2003) and the mitochondrial membrane permeability transition (MPT) pore opening is a critical event in AAP-

induced liver cell death (Hepatology, in press, 2004). Translocation of the pro-apoptotic Bcl-2 family member Bax to the mitochondria has been observed (Mol Pharmacol 60:907, 2001) but the pathophysiological significance of this event remained unclear. To address this issue, C57BL/6 wildtype and Bax-deficient mice (Bax<sup>-/-</sup>) were treated with 300 mg/kg AAP. In wildtype mice, Bax translocation to mitochondria was observed as early as 2 h after AAP and persisted up to 6 h. Bax translocation to the mitochondria correlated with the release of cytochrome c and Smad from the intermitochondrial membrane space. However, consistent with our previous observations, no activation of caspase-3 was detected. Nitrotyrosine staining as indicator of mitochondrial oxidant stress and peroxynitrite formation, DNA fragmentation and an increase in plasma ALT levels as indicator of cell injury were observed at the earliest at 3 h with a further increase of all parameters at 6 h after AAP overdose. In contrast, in Bax<sup>-/-</sup> mice the AAP-induced translocation of cytochrome c and Smad into the cytosol, nitrotyrosine staining, DNA fragmentation and the increase in plasma ALT activities were significantly attenuated but not completely eliminated. Conclusion: Our findings suggest that the early translocation of Bax to mitochondria precedes mitochondrial dysfunction and oxidant stress, DNA fragmentation and liver cell necrosis. The fact that these events were attenuated in Bax<sup>-/-</sup> mice suggests that Bax is involved in inducing mitochondrial dysfunction leading to cell necrosis after AAP overdose.

ACETAMINOPHEN-INDUCED UPREGULATION OF PI-3K AND NF-κB IN MOUSE LIVER: REGULATION BY TNFα AND CAVEOLIN-1.

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Increasing evidence suggests that macrophages and inflammatory mediators contribute to acetaminophen (AA)-induced tissue injury. Of particular interest is TNFα which appears to play a dual role in the pathogenic process, initially promoting injury and subsequently inducing hepatocyte proliferation and tissue repair. The present studies analyzed mechanisms mediating the actions of TNFα in the liver. Treatment of wild type control mice with acetaminophen (300 mg/kg, i.p.) resulted in a time-dependent induction of centrilobular hepatic necrosis and increased serum transaminase levels, which reached a maximum after 18 hr. This was associated with a biphasic induction of TNFα at 3 hr and 18 hr post treatment, consistent with its role in injury and repair. TNFα signaling involves the phosphatidylinositol 3'-kinase/protein kinase B (PI3K/PKB) pathway, which is important in NF-κB activation and the generation of pro- and anti-inflammatory mediators, as well as anti-oxidants, growth factors and proteins regulating tissue repair. AA administration resulted in increased phospho-PI3-K expression (within 3 hr) followed by increased NF-κB binding activity (6-12 hr) in the liver. These responses were attenuated in TNFR1 (p55) knockout mice, which exhibit increased AA-induced tissue injury, reduced hepatocyte proliferation and delayed expression of matrix remodeling enzymes suggesting that TNFα signaling through p55 is involved in tissue repair. Caveolin-1 (Cav-1) is an integral membrane protein known to negatively regulate PI3-K and NF-κB activation. Western blot and immunohistochemical analysis revealed significant expression of Cav-1 in the livers of control animals. AA treatment of mice markedly down regulated Cav-1 expression within 3 hr. These data, together with our findings that TNFα suppresses Cav-1 expression in cultured macrophages, suggest that TNFα-induced signaling molecules activated when Cav-1 is down regulated play an important role in AA toxicity and tissue repair. (NIH GM34310, ES04738, and ES05022).

EXPOSURE TO METHYLMERCURY AND PCB153 DURING PREGNANCY AND LACTATION. EFFECTS ON BRAIN AND LYMPHOCYTE CHOLINERGIC MUSCARINIC RECEPTORS IN RAT DAMS AND PUPS.

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The cholinergic muscarinic receptors (MRs) are a likely target for the food neurodevelopmental contaminants methylmercury (MeHg) and the ortho-substituted polychlorinated biphenyls (e.g., PCB153). The same receptors, expressed in the lymphocytes, are considered a potential peripheral marker of central nervous system function. The effects of the oral perinatal administration of MeHg (1 mg/kg/day, GD7-PND7) and/or PCB153 (20 mg/kg/day, GD10-GD16) were investigated on the density (Bmax) of cerebral and lymphocyte MRs in the rat offspring and dams on day 21 post-partum by saturation binding studies. Brain and blood Hg levels were also measured by cold-vapor atomic absorption. A common finding to the cerebral cortex of dams, male and female pups was the significant increase in MR density (27-60%) caused by MeHg and PCB153, either alone or combined (respective control Bmax: 583±13, 503±31, 545±10 fmol/mg protein).

In cerebellum, MeHg still augmented MRs in dams (87%; control Bmax: 83±17 fmol/mg prot) and pups (27%; control Bmax 119-127 fmol/mg protein) while PCB153 reduced it by ~30%. After the combined treatment, the MR changes were similar to those caused by PCB153 alone. Brain Hg levels (μg/g) were 7-9 in dams, 1.67±0.43 in males and 1.52±0.33 in females. In blood Hg values (μg/l) were 11330±2258 in dams, 1351±395 in males and 1313±397 in female pups. As observed in the brain, in the lymphocytes MeHg given alone always augmented the MR Bmax (control Bmax: 30-60 fmol/million cells). Similarly to the cerebellum, MeHg affected more markedly the adult (+139%) than the pup lymphocyte receptors (+45-85%) and PCB153 totally masked MeHg effect. MeHg enhanced cerebral and lymphocyte MRs more in dams than in pups in accordance with the higher Hg levels detected in the adult tissues. The trend of MR changes caused by MeHg and PCB153 in the lymphocytes partially mirrored that of the cerebellum (EU Grants: QLK4-CT-2001-00186; FOOD-CT-2003-506543).

## 1056 DEVELOPMENTAL NEUROPATHOLOGY OF METHYL MERCURY CHLORIDE.

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The susceptibility of the brain to chemicals and drugs appears to depend on its developmental stage at the time of exposure. Adverse effects on brain morphology (abnormal proliferation, differentiation and/or migration of cells) can arise, often resulting in quantitative changes, e.g. changes in neuron numbers at their final destination. Excessive neuron loss in the developing brain has negative consequences for the mental and physical abilities of the adult individual, becoming even more pronounced during senescence. However, such quantitative developmental morphological changes may go unrecognized by the morphological approach, proposed in current test guidelines for regulatory Neurotoxicity Testing, as demonstrated in the present study (American Chemistry Council, Ref.nr. 1847). A Developmental Neurotoxicity study was carried out in rats with methyl mercury chloride (MeHg) (5 dose levels). A tiered morphological approach (gross macroscopy, brain weight, slide reading, morphometry (brain layer width) was applied (EPA Guideline OPPTS 870.6300), and stereology (brain region volume, neuron number) as final step, in addition. A significant loss of cerebellar granular neurons and reduction of granular layer volume by MeHg was demonstrated using stereology. These effects, or any other effect on brain morphology, could not be depicted with previous steps in the tiered approach. The results indicate that powerful neuropathology endpoints are required to identify early effects of toxicants on developing brain morphology and suggest that stereology may provide a valuable, additional tool.

## 1057 PRENATAL EXPOSURE TO METHYL MERCURY OR METHYLAZOXY METHANOL: EFFECTS ON EARLY POSTNATAL MOTOR ACTIVITY.

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Developmental Neurotoxicity testing according to specific test guidelines is required for disclosure of the potential adverse effects of industrial chemicals and agricultural pesticides on the developing nervous system. Motor activity is included in these guidelines as an apical behavioural endpoint with the intent to evaluate a range of potential effects on the developing nervous system. It is known that different activity measures may be differently affected by chemicals and drugs, depending on the dose and type of agent. Also, other nervous system functions may be changed and may lead to changes in motor activity. In addition, motor activity may be indirectly affected as a result of systemic toxicity (e.g. adverse effects on body temperature or body weight). In research, supported by the American Chemistry Council (ACC Ref.nr. 1847), we studied motor activity in rats, prenatally exposed to methyl mercury chloride (MeHg) or methylazoxymethanol acetate (MAM) (5 dose levels each). MAM and MeHg were chosen as model neurotoxicants. They both affect brain morphology during development. MeHg, however, primarily causes systemic toxicity. Motor activity was tested on PN 13, 17, 21 and 60-62, as indicated in the EPA guideline OPPTS 870.6300 for Developmental Neurotoxicity testing. The results showed that prenatal exposure to MAM or MeHg affected early postnatal motor activity differently. The results will be shown and the relevance of the effects with regard to developmental neurotoxicity will be discussed in relation to the results obtained for other endpoints (maternal and developmental in-life data, and pathology).

## 1058 METHYLMERCURY (MEHG) ELICITS ACUTE AND LONG-TERM EFFECTS ON NEWBORN RAT HIPPOCAMPAL NEUROGENESIS THROUGH CELL CYCLE MACHINERY.

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While high MeHg exposure causes gross brain defects, effects of lower levels on development are undefined. Acute changes in early neuron production, or neurogenesis, may influence later brain formation. Previous studies indicate injected factors stimulate or inhibit proliferation by regulating cell cycle progression (Cheng, 2002; Carey, 2002). To examine acute neurotoxicant effects on DNA synthesis, we injected P7 rats with MeHg and [3H]thymidine 6h later, measuring incorporation 2h later. At 8h DNA synthesis was reduced 16% at 0.1, 50% at 3, and 80% at 30μg/g in hippocampus, indicating effects on DNA synthesis. Two weeks later, rats injected with MeHg at P7 exhibited reduced hippocampal cell number, indicated by a 17% decrease in total DNA at 3μg/g. In contrast, MeHg did not affect DNA synthesis or cell number in cerebellum though blood flow and Hg content were similar in both regions, and DNA synthesis in cultured granule precursors was inhibited 25%. To examine cell cycle mechanisms, we defined effects on E14.5 cortical precursors: At 6h DNA synthesis was reduced 49% at 3 and 98% at 10μM MeHg, with no change in cell number, suggesting a G1/S block. MeHg elicited a 75% reduction in cyclin E, the stimulatory subunit of CDK2, whose levels were unchanged. Further, MeHg did not increase either p27 or p57, CDK inhibitors commonly stimulated by endogenous anti-mitogens, such as PACAP (Carey, 2002). These studies identify the cell cycle machinery as new MeHg targets, specifically cyclin E. The decreases in DNA synthesis at 6h in hippocampus *in vivo* and cortical precursor cultures suggest MeHg inhibits neurogenesis by interfering with cell cycle progression. Furthermore, the studies indicate that MeHg rapidly and directly alters brain development through modulating regional neurogenesis. NIEHS 11256, USEPA R82939101

## 1059 MEHG DIFFERENTIALLY AFFECTS KINETICS OF sIPSCS OF RAT CEREBELLAR PURKINJE AND GRANULE CELLS.

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We previously showed that the GABAergic spontaneous inhibitory postsynaptic currents (sIPSCs) in Purkinje and granule cells in cerebellar slices exhibited differential sensitivity to methylmercury (MeHg). Because of the differential expression of α1 and α6 subunits of GABA<sub>A</sub> receptor in Purkinje and granule cells, and different kinetics between α1- and α6-containing GABA<sub>A</sub> receptors, we hypothesized that MeHg affects kinetics of sIPSCs in Purkinje and granule cells differently. To test this, effects of MeHg on kinetics of sIPSCs in Purkinje and granule cells in cerebellar slices were examined using whole cell recording techniques. In the control, the mean 10-90% rise time of sIPSCs in Purkinje cells was 0.84 ± 0.03 ms. For currents fitted with two exponentials, the mean fast and slow decay time constants were 9.8 ± 0.6 and 83.9 ± 46.6 ms, respectively. The fraction of the slow component to the peak current of sIPSCs in Purkinje cells was small. After MeHg exposure (10-100 μM), the mean 10-90% rise time of sIPSCs in Purkinje cells did not change significantly. Similarly, neither the mean for the fast nor the slow decay time constant was affected by MeHg. The mean fraction of the slow component to the peak current was virtually unchanged. Thus, MeHg did not appear to affect kinetics of sIPSCs in Purkinje cells. In granule cells, MeHg first transiently shortened and then prolonged the 10-90% rise time of sIPSCs. Effects of MeHg on sIPSC decay phase in granule cells were inconsistent. In most granule cells, MeHg slowed slightly sIPSC decay phase and prolonged the slow decay time constant. However in some granule cells, MeHg actually hastened the decay phase. The inconsistency in responses to MeHg may be due to different composition of GABA<sub>A</sub> receptor subunits in individual granule cells tested. In addition, the mean fraction of slow component to the peak current was decreased by MeHg. Thus, these data suggest that MeHg also differentially affects kinetics of GABAergic sIPSCs of cerebellar Purkinje and granule cells. Supported by NIH grants R01ES033299 and R01ES11662.

## 1060 GESTATIONAL LEAD EXPOSURE PRODUCES ROD-MEDIATED ELECTRORETINOGRAPHIC (ERG) SUPERNORMALITY AND DECREASES IN RETINAL DOPAMINE METABOLISM OF ADULT RATS.

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Children with blood lead concentrations ([BPb]) at or below 10 μg/dL have persistent impairments in retinal, auditory, cognitive and visual-motor function. Our recent study of 7-10 year old children with gestational and continuous postnatal

lead exposure revealed that they have SUPERNORMAL rod-mediated (scotopic) ERGs (Rothenberg et al., IOVS 2002). These unique ERGs are characterized by increased a-wave and b-wave amplitude and sensitivity. Only maternal [BPb] during the first trimester, from 4 to 14  $\mu$ g/dL, had a dose-response relation with these ERGs. Similar persistent supernormal ERGs occur in monkeys with high lifetime lead exposure. Our goal was to determine if similar dose-response scotopic ERG changes occurred in 90 day old (PN90) Long-Evans hooded rats following low-level lead exposure from conception through PN10 (equivalent human gestation period). Retinal histology and dopamine (DA) metabolism also were examined at PN90: the latter to examine one mechanism underlying supernormal ERGs. At PN10, [BPb] in controls and two lead groups were 0.5-1, 10-15 and 20-25  $\mu$ g/dL, respectively. At PN90, [BPb] were similar in all three groups. Retinal histology appeared similar in all three groups, which was interesting since lead exposure during lactation (PN0-21) at similar [BPb] produced rod selective apoptosis and subnormal ERGs. In contrast, our gestationally lead-exposed PN90 rats had significant dose-response scotopic a-wave and b-wave ERG supernormality, and significant decreases in retinal DA metabolism. Thus, the lead-induced supernormal scotopic ERGs occurred in the absence of elevated [BPb] and histological abnormalities. These results suggest that the decreases in DA activity, via a loss of DA amacrine cells and/or an effect on DA metabolism, may underlie the ERG supernormality. These findings further reveal that the early developing retina is a sensitive target site of low-level lead exposure and suggest that long-term retinal alterations may occur in lead-exposed children. Supported by NIH Grant ES012482.

**1061**

**GESTATIONAL LEAD EXPOSURE PRODUCES A SELECTIVE DECREASE IN MOUSE RETINAL DOPAMINERGIC AMACRINE CELL DENSITY AND DISTRIBUTION.**

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Gestational lead exposure produces supernormal rod-mediated electroretinograms (ERGs) in children, monkeys and rats. The latter two also have decreased retinal tyrosine hydroxylase (TH) immunofluorescence (Kohler et al., *Neurotoxicology* 1997) or decreased dopamine (DA) metabolism (Fox and Kala, SOT 2005). Our goal was to develop a clinically relevant model of gestational lead exposure in C57BL/6 mice. Mice were exposed to low levels of lead from conception through postnatal day 10 (PN10): equivalent human gestation period. Retinal histology and immunocytochemical studies with light and confocal microscopy were conducted using PN60 mice. At PN10, the blood lead concentrations [BPb] in controls and two different lead groups were 0.5-1, 10-15 and 20-25  $\mu$ g/dL, respectively. At PN60, the [BPb] were similar in all three groups. In PN60 controls, vertical cryostat sections and whole mounts showed that the density of TH positive DA amacrine cells was low (20-25 cells/sq. mm) and uniform across the retina with dendrites ramifying in sublamina 1 of the innerplexiform layer. At PN60, retinal histology in lead-exposed mice appeared similar to controls. In contrast, in the lead-exposed mice the overall density of DA amacrine cells was decreased. This selective decrease in DA amacrine cells was not uniform across the retina, suggesting quadrant specific effects reminiscent of the rod photoreceptor loss during postnatal only lead exposure (He et al., PNAS 2003). The number and intensity of TH positive dendrites were decreased in regions where DA cell density was lowest. These results are consistent with similarly lead-exposed rats that have persistent scotopic ERG supernormality and decreased retinal DA metabolism. Together, these results suggest that the DA amacrine cells of the early developing mammalian retina are especially vulnerable to low-level lead exposure. These persistent changes in DA cells may underlie the novel scotopic ERG supernormality observed in prenatally lead-exposed children and animals. Supported by NIH Grants ES012482, EY007551 and EY007024.

**1062**

**DEVELOPMENTAL EXPOSURE TO LEAD ELEVATES APP AND  $\beta$ LEVELS IN THE AGING BRAIN BUT DOES NOT ALTER THE PROTEOLYTIC PROCESSING OF APP.**

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Alzheimer's disease (AD) is characterized by excessive deposits of aggregated beta-amyloid peptides ( $\beta$ ), which are snippets of the amyloid precursor protein (APP). The predominately sporadic nature of AD suggests that the environment must play a role in neurodegeneration, however, few environmental risks for AD have been identified.  $\beta$  is a 39-42 residue peptide which is produced following the proteolytic processing of APP at various sites within the molecule by  $\alpha$ -secretase,  $\beta$ -secretase, and  $\gamma$ -secretase. Cleavage of APP by  $\beta$ - and  $\gamma$ -secretases releases the 39-42 amino acid A which aggregates into long filaments outside the cell, and along with fragments of dead and dying neurons, microglia, and astrocytes forms the plaques that are characteristic of AD brains. We found that the developmental exposure

(postnatal day 1-20) to lead (Pb) results in a latent over-expression of APP mRNA and an elevation in APP and A levels in aged animals; however animals exposed to Pb during old age were unresponsive. We also monitored the levels of the activity of enzymes involved in the proteolytic cleavage of APP and found that the activity levels of these enzymes were not altered by Pb-exposure. These results suggest that the increase in A in developmentally Pb-exposed animals is due to de novo synthesis of APP. Therefore we conclude that developmental exposure to Pb causes alterations in the regulation of the APP gene and does not interfere in the enzymatic processing of APP.

**1063**

**DEVELOPMENTAL EXPOSURE TO LEAD MODULATES THE OCT-2 TRANSCRIPTION FACTOR AND ITS TARGET GENES IN THE RAT HIPPOCAMPUS.**

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Lead exposure is highly neurotoxic particularly to the developing central nervous system. A possible mechanism through which lead may cause neuronal damage is by perturbations of brain gene expression through alterations in numerous transcription factors and signal transduction intermediates involved in development and differentiation. We conducted *in vivo* macroarray analysis in an attempt to identify unknown transcription factors that may be involved in the response to lead-exposure during growth and development. We observed that many transcription factors exhibited distinctive temporal patterns of expression. The most prominent changes were exhibited by the Sp and Oct families. These two transcription families appear to play a critical role in mediating lead-induced disturbances in developmental gene expression. While the involvement of the Sp1 zinc finger protein transcription factor in the response to lead exposure has been previously shown, the changes in Oct-2 expression identify the POU domain as a novel target for lead-induced neurotoxicity. To examine whether lead exposure alters the functionality of Oct-2, we performed DNA-binding assays using the electrophoretic mobility shift assay (EMSA). We found that the DNA-binding of Oct-2 was modulated as well as the developmental expression of its target genes: tyrosine hydroxylase, nitric oxide synthase and synapsin. Thus lead exposure may interfere with brain development via alterations in the expression of Oct-2 target genes important for neurotransmission

**1064**

**MODULATION OF BRAIN CYTOKINE GENE EXPRESSION BY LEAD.**

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Lead (Pb) can perturb the central nervous system (CNS) in a number of ways. While its effect on neural transmission has been well documented, its effect on CNS cytokine expression has only begun to be considered. To examine Pb effects on CNS cytokine expression BALB/c mouse litters were exposed to 0.5 mM lead acetate through mothers milk from 0-21 days of age. On day 21 the pups were sacrificed and total RNA was isolated from the brain and spleen by the TRIzol method, followed by further purification using a Qiagen column. The study included 4 litters each, composed of Pb- and non-Pb-exposed mouse pups. Individual cytokine RNAs were quantified by means of real-time RT-PCR using kits developed by Search-LC. Gene expression was measured using a Roche Light-Cycler for cytokines IL-4, IL-5, IL-10, IL-15, IL-16, IL-18, IFN $\gamma$ , and LT $\beta$ . Interestingly, the most highly constitutively expressed cytokines in the CNS of control day 21 pups are IL-18>IL-16>IL-15>IL-5>LT $\beta$ >IFN $\gamma$ >IL-10>IL-1=IL-6>TNF $\alpha$ . IL-4 expression in the brain is negligible. Results indicated that male and female mouse pups had different cytokine gene expression profiles characteristic to their gender. It was noted that Pb had the greatest effect on CNS cytokine expression of females; Pb significantly increased the expression of IL-10, IL-15, and IL-18 ( $p<0.05$ ) and significantly decreased the expression of IL-16 ( $p<0.05$ ). Increased, but not significant, expression was also observed for IL-5, IFN $\gamma$ , and LT $\beta$  in females. Pb had very little effect on the cytokine expression profile of male brains, with the exception of LT $\beta$  where a small decrease in expression was observed. Differential cytokine expression between males and females was also observed in the spleen, but Pb had no significant effect on cytokine gene expression in this organ with respect to the males or females. The results suggest that the primary effect of Pb on cytokine expression occurs in the CNS and that females are more vulnerable to these effects than males. Supported by NIH grant ES11135.

**1065**

**NMDAR SUBUNITS WERE AGGRESSIVELY REDUCED BY PB-EXPOSURE IN THE EARLY DEVELOPMENT OF RAT BRAIN.**

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Chronic Pb exposure has been recognized to induce children memory dysfunction and learning retardation. Our previous studies showed that Pb dose-dependently reduced N-methyl-D-aspartate receptor (NMDAR)-NR1, NR2A and NR2B expression at mRNA and protein levels in primary cultured neuronal cells. The present study was purposed to evaluate effect of Pb exposure on NMDAR subunits in

brain stem (BS), cerebellum (CB), hippocampus (HC) and frontal cortex (FC) in the developing rat brain. Sprague-Dawley (SD) pregnant rats were allowed to drink 0.1% Pb acetate dissolved in distilled deionized water (DDW) from gestation day 6 through 21s postnatal day (PND). Control rats were allowed to drink DDW. Pups were sacrificed on PND 3, 5, 7 and 15. The brain was immediately excised and separated into BS, CB, HC and FC. NMDAR-NR1, NR2A and NR2B on the membrane from brain tissue were examined. Antigens were first enriched with protein A agarose by immunoprecipitation, and later detected by western blotting with specific antibodies. Protein concentration was determined by Lowry's method. Meanwhile, total RNA was extracted from tissue with Trizol. RT-PCR was employed to detect mRNA expressions of NMDAR- NR1, NR2A and NR2B with specific primers. Each experiment was triplicated (n=3). Emerged results indicated that the above NMDAR subunits' protein levels in Pb-treated samples were reduced on FC at PND3. Their mRNA levels were greatly reduced in HC and FC. Furthermore, Pb aggressively reduced both protein and mRNA levels of these subunits on PND5, 7 and 15 in HC and FC, but had a little reduction in BS and CB. Among three examined NMDAR subunits, the reduction of NR1 appeared significant. Starting on PND3, the protein levels of these subunits were gradually decreased from PND 5 and 7 to 15 in BS and CB regions as much as in HC and FC. Our study suggests that Pb aggressively reduces NMDAR-NR1, NR2A and 2B on HC and FC in the early development of rat brain.

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### PRENATAL EXPOSURE TO INHALED MANGANESE ALTERS DOPAMINERGIC SUSCEPTIBILITY TO POSTNATAL METHAMPHETAMINE NEUROTOXICITY.

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Manganese (Mn) is an essential element but neurotoxic when in excess. Unlike ingested Mn, inhaled Mn bypasses all known homeostatic mechanisms. Intake of Mn through nasal passages and lung accumulates within selective brain regions. Therefore, industrial, agricultural and automobile Mn-based emissions place many populations at risk for over-exposure to Mn. Since Mn is necessary for proper brain development, we hypothesize that maternal exposure to low-level inhaled Mn disrupts fetal neurodevelopment that may augment neuro-response to pharmaceuticals and drugs of abuse affecting similar brain regions. Pregnant rats were exposed nose-only to a primary MMT-gasoline combustion by-product manganese sulfate aerosol over a two-day period for 90 minutes per day at a concentration of  $0.70 \pm 0.04 \text{ mg Mn/m}^3$  (MMAD=0.55  $\mu\text{m}$ ,  $\sigma_{\text{g}}=1.5$ ). Modeled with 0.14 total pulmonary deposition fraction and 100% absorption for particles  $< 1 \mu\text{m}$ , the total absorbed dose was  $2.4 \mu\text{g Mn}$ . At one month of age, progeny were administered methamphetamine (Meth). This popular drug of abuse, like Mn, affects dopaminergic transmission. Brain tissues were evaluated for histological augmentation and dopamine concentration. Prenatal Mn exposure greatly reduced Meth-induced swelling of axonal projections through the caudate-putamen. Gestational Mn exposure substantially depleted Meth-induced dopamine levels and altered dopaminergic metabolism when compared to control groups and animals exposed only to Mn or Meth. These results provide evidence that prenatal exposure to aerosolized Mn: 1) alters neurodevelopment of axonal projections into the caudate-putamen and 2) interacts with Meth to enhance dopaminergic toxicity. This Mn-Meth neuronal interaction suggests a drug-environment and gene-environment interference that affect fetal and adolescent neurodevelopment and have incredible potential to impact large populations at high risk for susceptibility to drug addiction and/or neurodegenerative diseases.

## 1067

### EFFECTS OF PRENATAL EXPOSURE OF MICE TO CHROMIUM PICOLINATE, CHROMIUM CHLORIDE, OR PICOLINIC ACID.

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Chromium (III) Picolinate [Cr(Pic)<sub>3</sub>] is a widely marketed dietary supplement. However, Cr(Pic)<sub>3</sub> has recently been associated with oxidative damage to DNA in rats and mutation and DNA fragmentation in cell cultures. In isolated case reports, Cr(Pic)<sub>3</sub> supplementation has been associated with adverse effects, such as anemia, renal failure, liver dysfunction, and neuronal impairment. Studies have shown that not all forms of Cr(III) produce toxic effects or inhibit the rate of development. In *Drosophila*, chromium picolinate, but not chromium chloride, is associated with a dose-related decrease in viability and an increased incidence of developmental delays. In the present study, pregnant mice were fed Cr(Pic)<sub>3</sub>, CrCl<sub>3</sub>, or picolinic acid to determine if either picolinic acid or Cr(Pic)<sub>3</sub> is a developmental toxicant. On gestation days (GD) 6-17, groups of pregnant CD-1 mice were fed a diet containing 200 mg/kg CrCl<sub>3</sub>, 200 mg/kg Cr(Pic)<sub>3</sub>, 174 mg/kg picolinic acid, or the diet

only. The dams were killed on GD 17, and their litters were examined for teratogenic effects. Additional diet-only and Cr(Pic)<sub>3</sub> treated dams were allowed to litter, and their pups were tested for effects on sensory-motor development. A significant increase in bifurcated cervical arches was observed in fetuses from the Cr(Pic)<sub>3</sub> group in comparison with all other groups. Similarly, significant delays in righting reflex and hind limb grasp and deficiencies in motor skills were observed in the offspring of Cr(Pic)<sub>3</sub> fed dams. These results suggest that high maternal exposures to chromium picolinate can cause morphological and neurobehavioral defects in developing offspring. (This research was supported in part by a Howard Hughes Medical Institute Undergraduate Biological Sciences Education Program grant to The University of Alabama)

## 1068

### DEXAMETHASONE ALTERS NEURONAL DEVELOPMENT: CELL ACQUISITION AND DIFFERENTIATION IN PC12 CELLS.

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Dexamethasone (DEX) is used to prevent respiratory distress in preterm infants, but may increase the chance of subsequent cognitive problems. To determine whether this reflects direct effects on neuronal cell replication and differentiation, we exposed undifferentiated and differentiating PC12 cells to DEX (10 nM to 10  $\mu\text{M}$ ) for brief or extended times (24 h to 168 h). DEX progressively reduced cell number (DNA content) in a dose-dependent fashion, reaching deficits >50% by 168 h. DEX had no effect on cell viability (trypan blue exclusion), suggesting that it decreased cell numbers by impairing mitotic activity. This conclusion was supported by the observation that DEX enhanced the rate of neuronal differentiation: DEX augmented cell growth (total protein/DNA) by 25-50% and accelerated appearance of the cholinergic phenotype, as evidenced by a rise in choline acetyltransferase activity per cell (ChAT/DNA). At low concentrations (10 nM), DEX enhanced membrane expansion associated with neurite formation (membrane/total protein) but high concentrations impaired this index. Our results thus suggested that DEX terminates cell replication in favor of differentiation. To test that hypothesis, we assessed the effects of nerve growth factor (NGF), which also initiates differentiation, on the response to DEX. In the presence of NGF, low concentrations of DEX no longer promoted differentiation but concentrations at or above 100 nM still impaired neurite formation and reduced cell numbers. With NGF cotreatment, DEX decreased ChAT instead of increasing it, indicating impairment of the cholinergic phenotype. Our results suggest that low concentrations of DEX promote differentiation at the expense of replication, while high concentrations, in the range of those associated with the therapeutic use of glucocorticoids in preterm delivery, inhibit differentiation and expression of the cholinergic phenotype. Since learning and memory are dependent on cholinergic function, our findings suggest a mechanistic connection between antenatal steroid administration and cognitive dysfunction. NIH HD09713, ES07031

## 1069

### PRENATAL OR NEONATAL GLUCOCORTICOID TREATMENT, AT DOSES COMMENSURATE WITH PHARMACOTHERAPY IN PRETERM INFANTS, DISRUPTS BRAIN DEVELOPMENT AND EVOKE LASTING BEHAVIORAL ABNORMALITIES.

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Glucocorticoid administration is the current consensus treatment for the prevention of respiratory distress syndrome in preterm infants. However, recent evidence indicates that long-term impairments in learning and memory may emerge as a result of this treatment. The purpose of this work is to determine the mechanisms by which glucocorticoid exposure during development results in neurobehavioral abnormalities. We administered dexamethasone in doses ranging from well below clinical usage up to the therapeutic level (0.05, 0.2, or 0.8 mg/kg/day) to pregnant and neonatal rats during three different periods, gestational day (GD) 17-19, or postnatal days 1-3 or 7-9. We then evaluated biomarkers of cholinergic synaptic function and neuronal cell development in forebrain regions both 24 hours after the last injection and in adulthood (PN75). In each case, dexamethasone caused immediate and persistent deficits in neural cell numbers (DNA content) as well as in cholinergic synaptic activity, as indicated by reductions of hemicholinium-3 binding to the choline transporter (a marker for impulse activity) as compared to choline acetyltransferase activity (a constitutive marker for cholinergic nerve terminals). We then assessed the effect of 0.2 mg/kg dexamethasone administered to pregnant rats on GD17-19, on later performance of the offspring in the figure-8 maze, when they were adolescents and reward-based learning in the 8-arm radial maze when they were adults. In the figure-8 maze, dexamethasone exposure reduced initial locomotor activity and impaired habituation in females. In the radial-arm maze, dexamethasone delayed learning. These results indicate that, even at doses at or below those used in preterm delivery, dexamethasone perturbs neuronal development and cholinergic function and impairs learning and memory later in life. NIH HD09713

**1070**

## PERSISTING BEHAVIORAL CONSEQUENCES OF PRENATAL DOMOIC ACID EXPOSURE IN RATS.

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Domoic acid is a neurotoxin produced by Pseudo-nitzschia phytoplankton. Domoic acid is concentrated in fish and shellfish species, which are in turn consumed by humans. Neurotoxic effects of high doses of domoic acid have been demonstrated in adult humans. A hallmark of the neurobehavioral toxicity of domoic acid in adults is cognitive impairment. However, little is known about the possible neurotoxic risks of domoic acid exposure in developing organisms. To investigate the persisting behavioral effects of prenatal exposure to domoic acid, pregnant female Sprague-Dawley rats were injected (sc) with 0, 0.3, 0.6, or 1.2 mg/kg of domoic acid on gestational day 13. No overt clinical signs of toxicity were seen with these doses. The offspring were then run through behavioral testing to determine the developmental effects of the domoic acid on locomotor activity, spontaneous alternation in a T-maze, and working memory function in a radial-arm maze. No significant differences on test performance were found in the T-maze spontaneous alternation. Locomotor activity measured in the Figure-8 maze detected a persisting effect of the 1.2 mg/kg domoic acid dose, which significantly ( $p < 0.05$ ) increased the rate of habituation over the hour-long activity test session. This was characterized by higher initial activity followed by greater decline in activity. In the radial-arm maze the control vehicle treated rats showed the normal sex-related difference in spatial learning and memory with males outperforming females. Developmental domoic acid exposure decreased this effect such that the normal sex difference in spatial memory was not seen with the 1.2 mg/kg domoic acid dose. This study demonstrates persisting neurobehavioral effects of acute prenatal exposure to domoic acid at doses that do not cause overt clinical signs of toxicity.

**1071**

## PERINATAL PERCHLORATE EXPOSURE IN THE RAT: DOES THYROID STATUS AFFECT BRAIN MORPHOMETRY?

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Perchlorate inhibits iodide uptake by the thyroid and excessive perchlorate exposure can reduce thyroid hormone (TH) levels. In rats, severe reductions in maternal TH levels during development have been linked to adverse neurological outcomes, such as reduced myelination and small brain size. To assess some of the evidence for such effects from perinatal perchlorate exposures, we analyzed methods and findings from a recent study in rats exposed to perchlorate *in utero* and via lactation (York 2001). We focused on changes in brain morphometry, specifically size of the posterior corpus callosum (CC) and striatum. York observed an inverted U-shaped dose-response curve for the CC, with an increase in size of about 20% at the middle dose level of perchlorate, and an increase of about 12% at both the highest and lowest dose levels. For the striatum, York also observed size increases of 4% to 9% but with no evidence of dose-response for perchlorate exposure. However, these CC and striatum size increases are inconsistent with a reduction in TH levels, which are generally associated with reduced, not increased, brain size. This may be due to the use of coronal rather than sagittal cross-sections for reviewing brain morphometry. Moreover, there were no accompanying histopathological alterations in the brain (*e.g.* no increase in cell size or cell number), as might be expected if the brain morphometry effects were related to perchlorate. Lastly, there was no apparent dose-response for the changes in brain morphometry and thyroxine ( $T_4$ ) levels in the dams or pups, as would be expected if the observed changes were due to perchlorate-induced TH changes. Thus, given the paradoxical increase in size of the CC and striatum, the absence of accompanying histopathological changes, and the lack of association between changes in  $T_4$  and alterations in brain morphometry, we conclude that it is unlikely that the observed changes are related to perchlorate exposure. Rather, we suggest that the observed changes may be due to issues related to sample analysis.

**1072**

## BEHAVIORAL EFFECTS ASSOCIATED WITH CHRONIC DEVELOPMENTAL EXPOSURE TO KETAMINE OR REMACEMIDE IN RATS.

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The effects of chronic exposure to ketamine or remacemide on the acquisition and performance of complex, food-reinforced behaviors were assessed in developing female Sprague-Dawley rats. Ketamine is an N-methyl-D-aspartate (NMDA) receptor antagonist, whereas remacemide has both NMDA receptor antagonist and sodium channel blocking properties. Learning (LRN), audio/visual discrimination (AVD) and motivation were modeled using incremental repeated acquisition, conditioned position responding and progressive ratio tasks, respectively. Ketamine (10 or 100 mg/kg/day), remacemide (100 or 150 mg/kg/day) or water alone was administered daily (7 days/week) via orogastric gavage beginning on postnatal day (PND) 23 and Monday through Friday behavioral assessments began on PND 27. Chronic treatment with either dose of ketamine or remacemide significantly decreased response rates in all tasks, suggesting decreased motivation and/or motoric capabilities. Chronic treatment with the high dose of either ketamine or remacemide significantly decreased accuracy of AVD task performance. However, neither ketamine nor remacemide had an effect on accuracy of LRN task performance at any dose tested. These findings for remacemide are in marked contrast with those in young monkeys where chronic remacemide had profound disruptive effects on the acquisition of LRN task performance and suggest important species differences. In addition, these findings for ketamine were quite different from those of MK-801 (the prototypic NMDA receptor antagonist) in a previous rat study in which MK-801 severely disrupted the acquisition of both AVD and LRN task performance. These observations suggest important differences in the mechanism of action between ketamine and MK-801.

During the brain growth spurt (BGS) the brain grows and develops at an accelerated rate. In mice and rats this period spans the first 3–4 weeks of life whereas in human it begins in the third trimester of pregnancy and continues throughout the first 2 years of life. Apoptosis or physiological programmed cell death is the natural process by which biologically redundant or unsuccessful neurones are deleted from the developing central nervous system. Anaesthetic agents appear to enhance the apoptosis during the BGS, which may result in deficits in brain development. The aim of this study was to examine the influence on apoptosis of the anaesthetic agent ketamine at different days during the BGS. Rate of neurodegeneration in different brain regions corresponding to the day of exposure and finally, consequences on spontaneous motor activity and learning in adulthood. Neonatal mice aged 3, 10 or 19 days were administered ketamine 25 mg/kg b.wt. or vehicle (0.9% NaCl) by subcutaneous injection. Neurodegeneration was examined 24 hours after treatment using the Fluoro-Jade staining technique and by measuring the expression of brain-derived neurotrophic factor (BDNF). BDNF is an important regulator of naturally occurring cell death. Behavioural tests were initiated at PND (postnatal day) 55 and consisted of spontaneous motor activity and radial arm maze. Ketamine significantly triggered apoptotic neurodegeneration in hippocampus and striatum in mice exposed on PND 3 and PND 10. These two groups also showed hypoactivity, lack of habituation and less learning ability during the behavioural tests. The effects were most pronounced in mice exposed on PND 10. BDNF levels were significantly decreased in frontal cortex and hippocampus in the ketamine group exposed PND 10 compared to vehicle group exposed the same day. The results show that the time of exposure during the brain development is critical for the amounts of neurodegeneration and behavioural deficits produced.

**1073**

## EXPOSURE TO KETAMINE DURING NEONATAL LIFE INDUCES TRIGGERED APOPTOTIC NEURODEGENERATION AND BEHAVIOURAL DEFICITS IN ADULTHOOD.

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During the brain growth spurt (BGS) the brain grows and develops at an accelerated rate. In mice and rats this period spans the first 3–4 weeks of life whereas in human it begins in the third trimester of pregnancy and continues throughout the first 2 years of life. Apoptosis or physiological programmed cell death is the natural process by which biologically redundant or unsuccessful neurones are deleted from the developing central nervous system. Anaesthetic agents appear to enhance the apoptosis during the BGS, which may result in deficits in brain development. The aim of this study was to examine the influence on apoptosis of the anaesthetic agent ketamine at different days during the BGS. Rate of neurodegeneration in different brain regions corresponding to the day of exposure and finally, consequences on spontaneous motor activity and learning in adulthood. Neonatal mice aged 3, 10 or 19 days were administered ketamine 25 mg/kg b.wt. or vehicle (0.9% NaCl) by subcutaneous injection. Neurodegeneration was examined 24 hours after treatment using the Fluoro-Jade staining technique and by measuring the expression of brain-derived neurotrophic factor (BDNF). BDNF is an important regulator of naturally occurring cell death. Behavioural tests were initiated at PND (postnatal day) 55 and consisted of spontaneous motor activity and radial arm maze. Ketamine significantly triggered apoptotic neurodegeneration in hippocampus and striatum in mice exposed on PND 3 and PND 10. These two groups also showed hypoactivity, lack of habituation and less learning ability during the behavioural tests. The effects were most pronounced in mice exposed on PND 10. BDNF levels were significantly decreased in frontal cortex and hippocampus in the ketamine group exposed PND 10 compared to vehicle group exposed the same day. The results show that the time of exposure during the brain development is critical for the amounts of neurodegeneration and behavioural deficits produced.

**1074**

## DEVELOPMENTAL TOXICOLOGY IN THE NEONATAL MOUSE: THE USE OF RANDOMLY SELECTED INDIVIDUALS AS STATISTICAL UNIT COMPARED TO THE LITTER IN MICE NEONATALLY EXPOSED TO PBDE 99.

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In traditional behavioural teratology and/or developmental toxicology the chemical to be tested is given to the mother. The exposure period can include both the gestational and lactation period. In such a case the litter generally should be considered the statistical unit. In several reports we have shown that low-dose exposure of environmental toxic agents such as PCB, DDT, brominated flame-retardants, pyrethroids, organophosphates, and nicotine, can cause persistent developmental neurotoxic effects when administered directly to pups during a defined critical period of neonatal brain development in the mouse. In the present study the use of the litter or randomly selected individuals as a statistical unit in developmental toxicology in the neonate is evaluated. In this investigation 18 different litters were

used. Pregnant NMRI mice were randomly selected and purchased from a commercial breeder. Male pups at the age of 10 days were given either one single oral dose of the brominated flame-retardant, 2, 2', 4, 4', 5-pentabromo diphenyl ether (PBDE 99) 1.4  $\mu$ mol/kg bw, or a vehicle (20% fat emulsion). Spontaneous motor behaviour was studied in 2-month-old-mice. Statistical evaluation was made by both using the litter (n=9) as a statistical unit and randomly selected individuals (n=9, 3 mice randomly selected from 3 different litters). Both statistical evaluations, using ANOVA, showed a significant deranged behaviour in the PBDE 99 exposed mice compared to controls, defects that we earlier have reported on. This study shows that there is no difference whether the litter or the randomly selected individuals are used as the statistical unit. This study also shows that randomly selected animals from different litters (at least 3) have the same statistical effect and power compared to the use of litter based studies to evaluate developmental neurotoxicity in neonatal mice.

## 1075

### POSTNATAL DEVELOPMENTAL NEUROTOXICITY (DNT): EVALUATION OF P-MENTHANE 3, 8, DIOL BY DERMAL ADMINISTRATION IN NEONATAL RATS.

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The potential neurotoxicity of p-menthane 3, 8 diol, an EPA registered component of dermally applied insect repellants, was evaluated in neonatal rats using occluded dermal application. Three hundred and twenty rat pups/sex were assigned to four dose groups (80 pups/sex/group) at doses of 0, 400, 800 and 1000 mg/kg/day. P-menthane 3, 8 diol or water (0) was applied to the backs of the pups on postnatal day (PND) 10 through 21. Doses were adjusted daily for body weight changes and administered at approximately the same time each day. The skin application site was occluded for the 6 hour exposure period to prevent oral ingestion and minimize the loss of the test substance formulation. Pups were separated from the dams for the 6 hour exposure period. One male pup and one female pup from each litter were evaluated as follows: PND 22 brain weights and neurohistology; watermaze and passive avoidance; motor activity and acoustic startle habituation; and adult brain weights and neuropathology examinations. Small but statistically significant reductions in body weight gain occurred at various points early in the study in rat pups of either sex in the 800 and 1000 mg/kg/day dosage groups. However, p-menthane 3, 8 diol did not cause any specific effects (developmental neurotoxicity) as measured by these neurobehavioral evaluations. Additionally, the study design is appropriate for investigating the potential effects of test article or test substance exposure to neonatal rat pups as early as PND 10.

## 1076

### WATER DISINFECTANT BYPRODUCTS (DBP) AND ADVERSE PREGNANCY OUTCOMES (APO): CHLOROACETONITRILE (CAN) INDUCES GROWTH RESTRICTION AND ALTERS GENE EXPRESSION IN FETAL BRAIN.

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Disruption of developmental processes; cell proliferation, differentiation and apoptosis result in reduced proliferation leading to growth restrictions and APO. There is an association between DBP and susceptibility to APO such as skeletal defects, growth retardation and CNS anomalies. Correlation between spontaneous abortion and consumption of chlorinated tap water was also demonstrated. Our objective is to examine the mechanism of DBP-induced APO, using brain development as a predisposing factor. Pregnant mice at gestation day 6 (GD6) were treated with the DBP; CAN (25 mg/kg/day, p.o.) until GD18. Animals were then anesthetized and fetuses were collected and weighed. Fetal brains were extracted, weighed and RNA was isolated. cDNA synthesis was performed and biotin labeled target cRNA was prepared. Target cRNA was hybridized on a mouse genome chip and biotin-cRNA was quantitated using Gene Array Scanner and Affymetrix software. Genes with two-fold change in hybridized target intensity were identified. The results indicated that CAN treatment caused a significant decrease in fetal weight (78% of control) and in fetal brain weight (80% of control). Concordance analysis identified 39 genes with unidirectional change (increase/decrease). Five genes demonstrated an increased RNA expression and 34 genes showed decreased level of expression as compared to control. Thirteen of the down-regulated genes belonged to the crystalline family. Four-fold decrease was indicated in parathyroid hormone related peptide (PTHrP) and cartilage matrix protein genes. RT-PCR analysis indicated that mRNA levels of PTHrP were significantly lower in treated mouse brain. In

conclusion, as PTHrP regulates fetal development, down regulation of PTHrP and matrilin genes may be a factor in the observed decrease in fetal weight. The current study provides a general outlook on the mechanism of the fetal changes that occurred following CAN treatment. Future studies will explore the molecular cascades of DBP-induced APO.

## 1077

### METHAMPHETAMINE-INITIATED NEURODEVELOPMENTAL DEFICITS ARE ENHANCED IN COCKAYNE SYNDROME B (CSB) KNOCKOUT MICE.

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Reactive oxygen species (ROS) increase 8-oxoguanine (8-oxoG), a form of oxidative DNA damage that causes mutations and transcription blockage. 8-OxoG is repaired on the nontranscribed strand of DNA by oxoguanine glycosylase (Ogg1), and on the transcribed strand via the transcription-coupled repair (TCR) pathway, catalyzed by the Cockayne Syndrome group B (CSB) protein. CSB may also regulate Ogg1. We have shown that treatment of pregnant *ogg1* knockout mice with the ROS-initiating neurotoxin methamphetamine (METH) causes enhanced 8-oxoG levels in fetal brain [Toxicologist 72(S-1): 342 (No. 1663), 2003] and postnatal neurodevelopmental deficits [Toxicologist 78(S-1):379 (No. 1843), 2004]. Similarly, the fetal brains of METH-exposed CSB knockout mice exhibited enhanced 8-oxoG levels [Toxicologist 78(S-1):380 (No. 1844), 2004]. Here we investigated the developmental role of CSB in protecting the fetus from postnatal functional deficits following *in utero* METH exposure. METH (40 mg/kg ip) or its saline vehicle was administered on gestational day 17 to pregnant heterozygous (+/-) CSB females (mated to CSB +/- males). Beginning at 6 weeks after birth, the offspring were tested for motor coordination using the rotarod apparatus. Female CSB +/- offspring exposed *in utero* to METH had enhanced coordination deficits compared to +/+ littermates at 8 and 12 weeks (p=0.01 and p=0.02, respectively), and compared to +/- littermates at 8, 10 and 12 weeks (p=0.002, p= 0.004 and p=0.002, respectively). *In utero* exposure of male +/- offspring to METH also resulted in enhanced coordination deficits compared to +/+ and +/- littermates at 10 weeks (p<0.05). This is the first evidence of enhanced neurodevelopmental deficits in CSB-deficient offspring exposed *in utero* to ROS-initiating toxins, and suggests that fetal CSB, in addition to Ogg1, protects against METH-initiated developmental neurotoxicity. (Support: Canadian Institutes of Health Research; SOT Covance Fellowship [WJ])

## 1078

### PERINATAL DIMETHYLTIN EXPOSURE ALTERS SPATIAL LEARNING ABILITY IN ADULT RATS.

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Dimethyltin (DMT) is widely used as a heat and light stabilizer in PVC and CPVC piping, and has been detected in domestic water supplies. Due to the lack of developmental neurotoxicity data on DMT, we initiated studies to evaluate long-term neurobehavioral changes in offspring following perinatal exposure. In the first study, female Sprague-Dawley rats were exposed via drinking water to DMT (0, 3, 15, 74 ppm) before mating and throughout gestation and lactation. As adults, male offspring were tested for spatial learning and retention in the Morris water maze. Acquisition of the platform location occurred over 9 days (2 trials/day), and on the 10th day, the platform was removed to assess retention in a probe trial. The middle dose group (15 ppm) took significantly longer to find the platform during Week 1; however, by Week 2, the 15 ppm males reached asymptotic performance though they continued to spend less time in the middle zone (near the platform), and more time searching in the outer zone. In a second study, DMT exposure, at the same doses, occurred from gestational day 7 to weaning. Learning deficits in the 15 ppm dose group were confirmed in both the male and female offspring tested as adults. The 15 ppm dose group displayed increased latencies throughout the entire acquisition period, and both sexes spent less time in the middle zone and more time in the outer zone. Neither study revealed retention deficits among the 15 ppm group; however, in the second study, they continued to display the same search strategy (i.e. more time in the outer zone). The results of both studies demonstrate a reproducible effect of 15 ppm perinatal DMT exposure on spatial learning. Additionally, in both studies, 74 ppm DMT caused decreased dam and litter weights, but no changes in water maze performance. Compensatory mechanisms might be activated as a result of exposure to the high dose, partially explaining learning deficits at 15 ppm but not at 74 ppm. This is an abstract of a proposed presentation and does not necessarily reflect EPA policy.

PERTURBATION OF NEURAL CELL DAMAGE  
RESPONSE SENSITIZES NRF2 KO MICE, INCREASING  
THE DURATION AND SEVERITY OF KAINEATE-  
INDUCED SEIZURES.

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Systemic administration of kainate is known to cause a selective loss of hippocampal neurons, increased GFAP immunoreactivity, and a seizure phenotype reminiscent of human temporal lobe epilepsy. In response to various stressors of the cellular environment, the transcription factor Nrf2 facilitates the induction of a battery of detoxification genes via the antioxidant response element (ARE) promoter. Interestingly, ARE-driven genes such as NQO1 and HO-1 are known to be increased subsequent to toxins such as kainate and MPTP, and are more highly expressed in diseases of neurodegeneration. These Nrf2-mediated genetic changes to the cellular reducing potential may reflect an intrinsic damage response of neurons or glia in the brain to harmful toxicants. To test this hypothesis, mice containing an hPAP reporter construct expressed in concert with activation of the ARE (ARE-hPAP mice), Nrf2 knockout (KO) mice, or wild type (WT) mice were exposed to varying doses of kainate via intraperitoneal injection. This led to a robust increase in ARE-mediated hPAP reporter activation in the cerebellum, as well as a specific increase in the CA3 subfield of kainate-injected mice. Additionally, Nrf2 KO mice were more sensitive to kainate toxicity as exhibited by increased seizure scores, prolonged seizure duration, and altered GFAP levels. Further, analysis of the hippocampus and cerebellum revealed a region-specific decrease in HO-1 and NQO1 mRNA in the KO mice compared to WT animals, both basally and after kainate injection. Two-by-two microarray analyses of six-month KO vs WT mice revealed not only the expected changes to detoxification and metabolism-related genes, but also changes associated with ion sensitivity and myelination. These results suggest a pivotal role for Nrf2 in the neural cell defense response of the adult brain against toxins that alter synaptic function and conductivity. (Support: ES08089 ES10042-NIEHS)

## 1080

EFFECTS OF PERINATAL EXPOSURE TO PCB 153 ON  
THE TURNOVER OF BRAIN NEUROTRANSMITTERS  
OF OFFSPRING RATS.

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Sponsor: M. Chiba.

Previous reports have suggested that exposure to PCB mixtures at perinatal period may affect the development of the central nervous system both in humans and animals, however, developmental neurotoxicity of each congener remains still unclarified. To investigate the effects of *in utero* exposure to PCB 153 (2, 2', 4, 4', 5, 5'-hexachlorobiphenyl), a representative non-planer PCB congener, on hormonal and neurobehavioral development in the offspring of rats, pregnant CD (SD) IGS rats were given PCB 153 (0, 16, 64 mg/kg/day) orally on Gestation Day 10 (GD 10) to Day 16 (GD 16). Offspring rats were examined at different weeks of age regarding the effects of PCB 153 on (1) endocrine system (thyroid and testis), (2) brain neuroactive substances, and (3) cognitive functions (learning and memory). PCB exposure did not produce any significant effects on the body weights of dams during gestation and lactation. There were no dose-dependent changes in body weight, body length, tail length, liver weight, and kidneys weight in offspring of all ages and both sexes. Anogenital distance (AGD) or AGD indices were increased in females in the PCB 153-exposed groups. Plasma concentrations of thyroxine (T4) and tri-iodothyronine (T3) were decreased in the 64 mg/kg group of both sexes. At 36-week, the blood testosterone concentration was significantly lower in the high PCB dose group, and in comparison, the LH in this group was at high level. In this paper, we described the effects of PCB 153 on the turnover of the brain neuroactive monoamines of the offspring. On the post natal day (PND) 7, the ratio of 5-hydroxyindoleacetic acid (5HIAA, serotonin metabolite) content to serotonin (5HT) content of the brain of male rats increased in a dose-dependent manner. These results suggest that 5HT turnover was accelerated in male offspring following perinatal exposure to PCB 153. The exposure to relatively high-dose of PCB 153 at the early stage of life may induce neurochemical changes in the brain of offspring rats.

## 1081

CHANGES IN GENE EXPRESSION PROFILE IN THE  
CEREBELLUM AND THE HIPPOCAMPUS FOLLOWING  
DEVELOPMENTAL EXPOSURE TO A  
NEUROTOXICANT.

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The vast literature on the group of chemicals known as polychlorinated biphenyls (PCBs) makes it a unique model to understand major issues related to environmental mixtures of persistent chemicals. At background levels of exposure, PCBs have

been shown to affect human health including learning and memory dysfunction. Although the cellular and molecular basis for PCB-induced developmental neurotoxicity is still unclear, a series of *in vitro* and *in vivo* studies have revealed that the disruption of Ca<sup>2+</sup> homeostasis and Ca<sup>2+</sup> mediated signal transduction play a significant role. The culminating event in a variety of signal transduction pathways is the regulation of gene expression, which ultimately has an effect on the growth and function of the nervous system. Therefore, we examined the gene expression profile in cerebellum and hippocampus coupled with toxicity pathways following PCB developmental exposure. Pregnant rats (Long Evans) were dosed perinatally with 0 or 6 mg/kg/day of Aroclor 1254 (AccuStandard Inc., Lot # 124-191) from gestation day 6 through postnatal day (PND) 21. For the present study, only the cerebellum and the hippocampus from PNDs 7 and 14 animals were analyzed. RNA was extracted for analysis on Affymetrix mouse genome 430 2.0 chips. Preliminary analysis of the data indicated that expression levels of ~1600 genes in the cerebellum and ~1200 genes in the hippocampus were changed by >1.5 fold from PND7 to 14. Of those genes, 370 were common in these two brain regions. Following exposure to Aroclor 1254, the number of genes affected > 1.5 fold were greater at PND 7 (~150 genes) compared to PND 14 (~ 100 genes) in hippocampus and cerebellum. The greatest proportions of genes altered were related to signal transduction, cell communication, and transcription factors. These results suggest that changes in intracellular signaling and related gene expression may be involved in mediating PCB-induced neurotoxic effects. (The abstract does not necessarily reflect USEPA policy).

## 1082

EFFECTS OF PCB 84 ATROPISOMERS ON [<sup>3</sup>H]  
PHORBOL ESTER BINDING IN RAT CEREBELLAR  
GRANULE CELLS AND <sup>45</sup>Ca<sup>2+</sup>-UPTAKE IN RAT  
CEREBELLUM.

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There is evidence that Polychlorinated biphenyl (PCB) congeners with ortho substituents have the potential to cause neurotoxicity. Many PCB congeners implicated in these neurotoxic effects are chiral. It is currently unknown if the enantiomers of a chiral PCB congeners have different neurotoxic effects. We herein report the effect of racemic 2, 2', 3, 3', 6-pentachlorobiphenyl (PCB 84) and its enantiomers on two neurochemical measures, PKC translocation as determined by [<sup>3</sup>H]phorbol ester binding in cerebellar granule cells and Ca<sup>2+</sup> sequestration as determined by <sup>45</sup>Ca<sup>2+</sup> uptake by microsomes isolated from adult rat cerebellum. Milligram quantities of the PCB 84 enantiomers were separated using HPLC. The (+)- and (-)-PCB samples were enantiomerically pure as determined by chiral gas chromatography. Both (+)- and (-)-PCB 84 increased [<sup>3</sup>H]phorbol ester binding in a concentration-dependent manner with (-)-PCB 84 being slightly more potent. Racemic PCB 84 was significantly more potent compared to the pure enantiomers alone. This suggests a synergistic increase of [<sup>3</sup>H]phorbol ester binding by the PCB 84 enantiomers. (-)- and (+)-PCB 84 both inhibited microsomal <sup>45</sup>Ca<sup>2+</sup> uptake to a similar extent, whereas racemic PCB 84 was more potent at inhibiting <sup>45</sup>Ca<sup>2+</sup> uptake compared to either enantiomer. In microsomes both PCB 84 enantiomers apparently cause a synergistic inhibition of <sup>45</sup>Ca<sup>2+</sup> uptake. These results indicate that PCB 84 enantiomers alone have similar potencies in both assays whereas a synergistic effect can be observed for the racemic mixture. These findings may have important implications for understanding the mechanism of neurotoxicity of chiral PCB congeners (Supported by ES 012475 and ES 07380 from NIH; this abstract does not necessarily reflect USEPA policy).

## 1083

PCBs REDUCE THE NUMBER OF DOPAMINE  
NEURONS AND INDUCE NEURONAL CELL DEATH IN  
ORGANOTYPIC CO-CULTURES OF DEVELOPING RAT  
STRIATUM AND SUBSTANTIA NIGRA.

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We used an organotypic co-culture system of embryonic rat striatum (E21) and substantia nigra (SN) (E14) to examine the effects of developmental exposure to polychlorinated biphenyls (PCBs), an environmental neurotoxicant, on dopamine (DA) function and cell death. Co-cultures were maintained for 3 days prior to the addition of either 2 or 8 μM PCBs. Co-cultures were then maintained for an additional 7 days, with PCB-supplemented media changed 3 times per week. After exposure, cultures were either examined immunohistochemically or the striatum and

SN were separated and DA concentrations were determined using HPLC with electrochemical detection. Immunohistochemical results indicate a significant reduction (45%) in tyrosine hydroxylase positive (TH+) neuronal cell bodies (DA neurons) in the SN exposed to 8 $\mu$ M PCBs, compared to vehicle-exposed tissue. Additionally, Fluoro Jade B (FJB), a fluorescent marker of neuronal cell death, indicated significant increases in fluorescent intensity in both the striatum and SN of 8 $\mu$ M exposures and in the striatum of 2 $\mu$ M exposure, compared to control tissue. Significant reductions in striatal DA concentrations were seen after PCB exposure, with a 45% reduction at 2 $\mu$ M and a 55% reduction at the 8 $\mu$ M exposure level, while no significant changes were seen in the SN. These results demonstrate that PCBs: (1) reduce the number of DA neurons in the SN; (2) increase neuronal cell death; and (3) reduce striatal tissue DA concentrations at concentrations as low as 2 $\mu$ M. These findings are similar to those seen in continuous cell lines and adult animals exposed to PCBs and validate this *in vitro* model of nigrostriatal development as a means of examining toxicant effects on neuronal development. Supported in part by NIH grant 1P01ES11263-01 and EPA grant R-82939001.

**1084** ALTERED COGNITIVE FUNCTION AND DENDRITIC GROWTH IN WEANLING RATS EXPOSED DEVELOPMENTALLY TO AROCLOR 1254.

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Polychlorinated biphenyls (PCBs) are persistent environmental contaminants. Epidemiological evidence suggests an association between perinatal exposure to low levels of PCBs and non-persistent cognitive deficits in children. These observations have not been tested in juvenile animal models. Risk assessments are further complicated by a paucity of information regarding specific neurodevelopmental events targeted by PCBs. To address these issues, we tested: 1) the feasibility of using weanling rats to assess the impact of developmental PCB exposures on spatial learning and memory using the Morris water maze; and 2) the hypothesis that developmental PCB exposure alters dendritic growth and maturation as measured by quantitative RT-PCR analysis of dendrite-specific proteins and morphometric analysis of layer widths in the brain. Gestational and lactational exposure to Aroclor 1254 at 1mg/kg, but not 6mg/kg, in the maternal diet causes subtle but significant deficits in spatial learning and memory in weanling rats (postnatal day 24, PN24). These effects were not gender specific, and did not persist into adulthood. Aroclor 1254 significantly increased expression of RC3/Neurogranin and spinophilin mRNA in the cerebellum and cortex, but not hippocampus, of PN4 rats. This effect was greatest in the 1 mg/kg treatment group. Aroclor 1254 significantly decreased the width of molecular layers in the cerebellum, but not the hippocampus and cortex, of PN31 rats. Again, 1 mg/kg caused a greater effect than did 6 mg/kg. These results suggest the validity of using this model to evaluate PCB effects on cognitive development, and implicate PCB-induced alterations in dendritogenesis as an underlying cause of cognitive deficits.

**1085** DEVELOPMENTAL EXPOSURE TO PCBs CAUSES AUDITORY IMPAIRMENTS IN RATS.

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It has been reported that developmental exposure to PCBs can result in hearing impairments. A cochlear site of action is supported by recent research from our laboratory, showing compromises in the mechanical integrity of the cochlea of PCB-exposed rats through the measurement of distortion product otoacoustic emissions (DPOAEs). In the current study, we utilized a unique PCB mixture, which was formulated to model the congener profile of PCBs found in fish consumed by a human cohort we are studying in northeastern Wisconsin. The goal is to determine whether developmental exposure to PCBs through maternal consumption of contaminated fish can induce auditory impairments. Female Long-Evans rats were dosed orally with PCBs beginning 28 days prior to breeding and continuing until pups were weaned. Dams were fed one-half of a cookie onto which was pipetted 0, 1, 3, or 6 mg/kg of the PCB mixture dissolved in corn oil vehicle. On PND 21 pups were weaned and one male and one female from each litter were randomly selected for auditory assessment. DPOAE testing was conducted under sedation at approximately 100 days of age. The DPOAEs were generated by simultaneously presenting two sinusoids differing in frequency (the lower frequency labeled f1 and the higher frequency f2) into the sealed ear canal. Seven stimulus pairs were used for DPOAE testing which included f2 = 1, 2, 3, 4, 6, 8, and 12 kHz (f2/f1 = 1.2). The amplitude of the 2f1-f2 distortion product was measured by recording the pressure in the sealed ear canal, and DPOAE thresholds were determined by reducing the f1 and f2 primaries in 5 dB steps. PCB-exposure caused a dose-dependent reduction in DPOAE amplitude as well as an increase in DPOAE threshold across the entire range of frequencies tested. The results are indicative of damage to the outer hair cells of the cochlea. Our future studies will use similar methodologies to assess auditory function in newborn infants born to women consuming contaminated fish.

**1086** PCB INDUCED HYPOTHYROIDISM ALTERS OLIGODENDROCYTE NUMBERS IN TWO WHITE MATTER TRACTS OF THE DEVELOPING RAT BRAIN.

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Developmental polychlorinated biphenyl (PCB) exposure is associated with a number of neurological deficits that overlap with deficits associated with maternal and fetal thyroid dysfunction. These similarities have prompted a number of investigators to propose that the adverse effects of PCBs may be attributed, in part, to their ability to interfere with thyroid hormone (TH) action during development. One target of TH during development is the timing of generation and maturation of oligodendrocytes. There are *in vitro* and *in vivo* data supporting the hypothesis that TH plays a role in controlling the number of oligodendrocytes that ultimately populate white matter tracts. To test the hypothesis that developmental exposure PCBs alters oligodendrocyte generation, pregnant rats were fed either PCBs (Aroclor1254; 5mg/kg/day), methimazole and perchlorate (MMI/Per) *ad libitum* in drinking water to induce hypothyroidism, or a combination of PCB and MMI/Per, starting at gestational day-6 (G6) until sacrifice at postnatal day-15 (P15). Using *in situ* hybridization directed against the oligodendrocyte marker, myelin associated glycoprotein (MAG), the number of MAG-positive cells and the cellular level of MAG mRNA levels were determined in the anterior commissure (AC) and corpus callosum (CC) of experimental animals. The results presented here show that perinatal PCB exposure and MMI/Per treatment cause a significant reduction in circulating thyroxine (T4) levels and a reduction in the number of MAG-positive cells that populate the AC/CC. Cellular levels of MAG mRNA were not affected by PCB exposure but were significantly reduced in animals that received MMI/Per. These studies suggest that PCBs have an effect similar to MMI/Per on serum T4 levels, on the total number of cells that populate the AC/CC, and on the generation of oligodendrocytes. However, cellular levels of MAG mRNA indicate that the effects of PCBs on thyroid hormone mediated oligodendrocyte maturation may not be due to lower serum T4.

**1087** VITAMIN A HOMEOSTASIS IS DISRUPTED IN OFFSPRING OF RAT DAMS EXPOSED TO PBDES DURING GESTATION AND LACTATION.

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Vitamin A and thyroid hormones are critical mediators of nervous system development. Polybrominated diphenyl ether (PBDE) exposure can disrupt nervous system function and alter adult and neonatal thyroid hormone concentrations. PBDE exposure can also decrease adult hepatic vitamin A levels. We tested the hypothesis that vitamin A homeostasis and thyroid hormones are more severely disrupted in DE-71 (a commercial PBDE mixture) exposed dams and offspring under conditions of suboptimal vitamin A nutriture. Female Sprague-Dawley rats (controls) were fed a vitamin A sufficient (VAS) diet throughout the study. To induce a marginal vitamin A status rats were fed a vitamin A deficient diet for 5 weeks prior to mating and through gestation day (GD) 5, and then fed a vitamin A marginal diet (VAM) from GD 6 through the remainder of the study. Within each diet group, subsets of rats were administered daily intragastric corn oil (vehicle) or 18 mg/kg DE-71, from GD 6 through lactation day (LD) 18. Plasma and tissues were collected from dams on LD 19 and pups on postnatal day (PND) 3, 12, 18 and 30. DE-71 exposure significantly decreased liver retinol in both diet groups but only reduced maternal and pup plasma retinol and retinol binding protein (RBP) levels under VAM conditions. Plasma thyroxine (T4) was decreased in both diet groups dosed with DE-71, while thyroid-stimulating hormone was elevated only in the VAM group dosed with DE-71. There was no difference in transthyretin (TTR) levels between the groups. A reduction in T4 binding to plasma TTR from only the animals dosed with DE-71 suggests an interaction between PBDEs and the TTR-RBP complex. Ongoing studies are investigating PBDE-induced alterations in embryonic vitamin A signalling and nervous system development. These data support the concept that women with marginal vitamin A status are at an increased risk of adverse pregnancy outcomes upon exposure to PBDEs. Research supported by: NIH Predoctoral Training Fellowship T32DK007355, Sigma Xi Grant In Aid of Research, Jastro-Shields Graduate Research.

**1088** TCDD MODULATES GENE EXPRESSION ASSOCIATED WITH CEREBELLAR GRANULE NEURON MIGRATION AND DIFFERENTIATION.

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2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) is a ubiquitous and persistent environmental contaminant known to exert developmental toxicity in many species by binding to the aryl hydrocarbon receptor (AhR), a transcription factor that regu-

lates the expression of drug metabolizing enzymes, inflammatory mediators, and genes that participate in cell cycle regulation. Epidemiological and neurobehavioral studies have suggested that the cerebellum is a target for TCDD induced developmental neurotoxicity. We have previously determined that AhR is expressed and transcriptionally active in cerebellar granule neurons during critical developmental periods. These observations led us to hypothesize that TCDD exposure interferes with granule neuron developmental programs by disrupting normal gene expression patterns. In this study, gene profiling experiments indicated that oral exposure to 1 $\mu$ g/kg TCDD increased the expression of several differentiation and migration markers in mouse granule neuroblasts 8 hours after exposure on postnatal day 5 (P5). Immunohistochemical staining demonstrated enhanced expression of the early onset differentiation marker TAG-1 and aberrant cerebellar granule neuron migration patterns on P6 and P10 following TCDD exposure on P3 and P6, respectively. In granule neuroblast cultures, 10nM TCDD accelerated neurite outgrowth and fasciculation, as evidenced by altered TuJ1 expression patterns. Additionally, TCDD produced a concentration dependent increase in the number of PSA-NCAM positive neurons 72 hours after treatment. Taken together these findings suggest that TCDD disrupts cerebellar granule neuron maturation by precociously activating the spatiotemporal gene expression program that regulates differentiation. Therefore, TCDD might exert neurotoxicity by hijacking AhR from its intrinsic roles, thus interfering with genetic programs that potentially control cell cycle regulation, differentiation, and migration during granule neuron development. Supported by NIH ES00375, ES07026, and ES01247.

## 1089

### DEVELOPMENT OF A QUANTITATIVE ASSAY FOR DETERMINATION OF TCDD TOXIC EQUIVALENTS IN F1 GENERATION CNS TISSUES.

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There is a critical need for the development of assays that quantify 2, 3, 7, 8, -tetrachlorodibenzo-p-dioxin (TCDD) toxic equivalents (TEQ) in central nervous system tissues as an alternative to the very expensive mass spectrophotometric methods. TCDD is a prototypical halogenated aromatic hydrocarbon environmental contaminant that is structurally distinguishable from the class of chemicals known as polychlorinated biphenyls (PCBs). TCDD is of particular relevance to human health because of its body burden and its potential to produce a wide range of toxicological responses. Here we describe the development of a quantitative *in vitro* assay that will be used to determine the residual levels of TCDD in F1 fetal brain after exposure on gestational day 15. The overall objective of developing this *in vitro* assay is to quantify the perinatal dose-dependent disposition of TCDD to critical central nervous system structures. HEPA-1c1 cells were grown to confluence and dosed to final TCDD concentrations of 1, 10, 100 fMol; 1, 10, 100pMol; 1, 10 and 100nMol; and finally 1 $\mu$ Mol from a stock solution in DMSO. HEPA cells were incubated in TCDD for 24 hours then total RNA was isolated, and semi-quantitative PCR was performed where the levels of CYP1A1mRNA/18s RNA were quantified. The results from this HEPA cell assay revealed a concentration-dependent increase in CYP1A1 mRNA as a result of TCDD exposure. TCDD TEQ values from F1 generation CNS tissue were calculated by comparing the up-regulated CYP1A1 mRNA induced by F1 generation CNS tissue against the dose-response curve generated in HEPA cells. From a cumulative risk-assessment perspective, development of this assay will allow for the quantitation of perinatal levels of TCDD TEQ that give rise to brain burdens that can be correlated with plasticity/behavioral learning and memory deficits in adult animals.

## 1090

### THE RETURN OF THE "DARK" NEURON. A HISTOLOGICAL ARTIFACT COMPLICATING NEUROTOXICOLOGIC INTERPRETATION.

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It has been known for years that the "dark" neuron is a histological artifact due to post-mortem trauma of unfixed or poorly fixed brain or spinal cord (Cammermayer J- in Structure and Function of Nervous Tissue 6:131-251, 1972, Bourne GH, ed). When studied histologically using the routine hematoxylin and eosin (H&E) staining, these cells are shrunken, hyperchromatic and basophilic, and often seen among populations of histologically normal neurons. Although the artifactual nature of these "dark" cells is clear, a spate of recent papers interpret such cells as representing dying/degenerating neurons (see J. Toxicol. Environment Health. 67:163 and 67:331, 2004 and Exper. Neurol. 172:153, 2001 for examples). This change was thought to be related to neurotoxic effects of agents such as pyridostigmine bromide, D $\delta$ ET, permethrin and malathion, alone or in combination. To help assess the validity of this interpretation, brain tissue was obtained

from young adult control male Sprague-Dawley rats (n=4) following CO<sub>2</sub> euthanasia and gentle blunt dissection to obtain samples for regional neurochemistry (for another study). The residual brain tissue was immersion-fixed in formalin, embedded in paraffin, sectioned and stained with H&E. Light microscopy revealed many "dark" neurons in multiple brain regions, similar to those illustrated in the above indicated papers. The generation of such cells by gentle post-mortem trauma in brains of normal rats raises questions as to the validity of the neuropathologic findings in cited (and similar) works. It is recommended that toxicologists, journal editors, risk assessors and regulatory agency officials acquaint themselves with this common artifact when making scientific judgments.

## 1091

### TEN-DAY EXPOSURE TO CARBONYL SULFIDE PRODUCE BRAINSTEM LESIONS AND CHANGES IN BRAINSTEM AUDITORY EVOKED RESPONSES IN FISCHER 344N RATS.

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Carbonyl sulfide (COS) is an intermediate in the production of pesticides and herbicides, a metabolite of carbon disulfide, a byproduct of combustion of organic material, and occurs naturally. COS was included in a Toxic Substances Control Act data request, and was listed as a Clean Air Act hazardous air pollutant. We have reported that 12-week exposure to 400 ppm COS produces brainstem lesions and altered brainstem auditory evoked responses (BAERs). To determine if shorter duration exposures produce similar effects, and to examine additional behavioral endpoints, male Fischer 344N rats were exposed by inhalation to 0, 300, or 400 ppm COS for 6 h/day, 5 days/week, for 2 weeks. Animals were tested 5-11 days after the last exposure using a functional observational battery and white noise prepulse inhibition (PPI) of the startle response. About 19 days after COS exposure, subjects were implanted with electrodes over the cerebellum and visual cortex and were allowed to recover for 1 week. Animals were presented with auditory stimuli consisting of rarefaction clicks (50, 65, 80 dB SPL), tone pips (4 and 16 kHz at 80 dB SPL), and flash stimuli (15 and 146 lux-s). Rats exposed to 400 ppm COS had lower motor activity and grip strengths, a slightly altered gait, and a few animals lacked the forelimb proprioceptive placing response. No changes were observed in sensory responses. White noise PPI of the startle response was not altered in COS-treated animals. Exposure to COS decreased BAER amplitudes (peak P<sub>4</sub> region). Histopathology confirmed brainstem lesions in the anterior olfactory complex and posterior colliculus. Flash evoked responses were not altered by COS exposure. We have shown that short-term exposure to COS produces mild motor deficits, brainstem lesions, and alters BAERs (without changes in PPI of the startle response), with no change in visual evoked responses. This is an abstract of a proposed presentation and does not necessarily reflect EPA policy.

## 1092

### SELECTIVE NEURONAL DEGENERATION IN THE CENTRAL NERVOUS SYSTEM OF RATS EXPOSED TO 3, 3'-IMINODIPROPIONITRILE (IDPN), ALLYLNITRILE (AN), CIS-CROTONONITRILE (CIS-CN), TRANS-CROTONONITRILE (TRANS-CN) AND 2, 4-HEXADIENENITRILE (HDN).

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IDPN, AN and *cis*-CN damage the vestibular, auditory, visual and olfactory systems of the rat. IDPN also causes a proximal neurofilamentous axonopathy in both central and peripheral axons. However, the possibility that neuronal degeneration in the central nervous system contributes to the behavioral effects of IDPN and AN remains a controversial issue. In addition, recent data indicate that the *trans* isomer of CN spares the sensory systems but induces selective central neurodegeneration. Adult male Long-Evans rats (250-350 g) received saline (control, n=2), IDPN (400 mg/kg/day, n=2), AN (60 mg/kg/day, n=3), *cis*-CN (110 mg/kg/day, n=3), *trans*-CN (250 mg/kg/day, n=2) or HDN (300 mg/kg/day, n=3) administered i.p. for 3 consecutive days. Tissues were examined at 7 days post-dosing. Fixed whole brain and representative segments of the spinal cord were sectioned at 50  $\mu$ m, and every third section was processed for Fluoro-Jade B staining, which selectively stains degenerating neurons. In rats exposed to IDPN or *cis*-CN no Fluoro-Jade B labeling was apparent other than that of nerve terminals in the glomeruli of the olfactory bulbs, indicating degeneration of the olfactory mucosa. AN rats showed a similar olfactory staining. Rats exposed to *trans*-CN and HDN showed a common pattern of selective neurotoxicity; major targets were the inferior olive and the piriform cortex. In three other areas (entorhinal, frontal cingulated, and motor frontal cortex) the effect of HDN was more pronounced than that of *trans*-crotononitrile. In conclusion, the nitriles that are toxic to sensory systems (IDPN, *cis*-CN and AN) cause

no selective neuronal degeneration in the central nervous system. In contrast, a distinct pattern of selective neuronal degeneration is induced by *trans*-CN and HDN, which spare the sensory systems and cause different behavioral effects. Supported by MCyT, Spain (BFI 2003-016060).

**1093**

**NORMAL VISUAL CONTRAST SENSITIVITY (VCS) AND COLOR VISION OF ADULTS AND SCHOOL-AGE CHILDREN FOUR TO FIVE YEARS AFTER EXPOSURE TO PERCHLOROETHYLENE (PERC).**

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In 1998 NYS DOH found levels of 1, 800-2, 400 ug/m<sup>3</sup> perc in a daycare center located adjacent to a dry cleaner. Seven weeks after perc levels were reduced to < 50 ug/m<sup>3</sup>, 9 employees at the daycare center had significantly decreased VCS compared to 9 unexposed adults although VCS of both groups was within the normal range; color vision was decreased, but not significantly. Children attending the daycare center were not given vision tests because they were too young in 1998 to perform them reliably. Follow-up evaluations of this population were conducted in 2001-2002 to assess if: 1) VCS among employees was still decreased; 2) a color vision deficit had developed among employees; and, 3) VCS or color vision deficits were evident among the children. Participants were identified from among daycare center employees and attendees enrolled in the NYS Volatile Organic Compounds Exposure Registry. Participants completed FACT VCS and Farnsworth D15 and Lanthony's Desaturated 15 Hue color vision tests, as well as a thorough ophthalmologic exam. Five employees participated in the follow-up evaluation; none had deficits in VCS or color vision that can be attributed solely to perc exposure. Seventeen children who attended the daycare center prior to 1998 participated and 13 of them were age- and gender-matched to 13 children with no documented exposure to perc. Wilcoxon matched-pairs signed-rank tests indicated no difference between matched groups of children in VCS scores or color confusion indices. McNemar's Exact Test for Correlated Proportions indicated no differences in numbers of pairs of children with discordant clinical judgements (one abnormal, one normal color vision) or with discordant numbers of major errors (one with no errors, one with at least one error) on color vision tests. This investigation did not detect any long term effect on vision among adults or children previously exposed to perc. Funded through USEPA STAR Grant R827446010.

**1094**

**NEW ASSESSMENT OF NEUROBEHAVIORAL CHANGES USING FUNCTIONAL OBSERVATIONAL BATTERY (FOB) IN MONKEYS.**

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[Background and Purpose] The FOB is now widely used to identify potential neurotoxicity of new and existing chemicals in rats, but not in primates. The purpose of this study was to develop the assessment of behavioral and neurologic functions in cynomolgus monkeys, and to investigate differences between monkeys and rats in neurobehavioral evaluation. [Methods] Six male cynomolgus monkeys and Crj:CD(SD)IGS rats were used. The FOB using monkeys consisted of home-cage observations and manipulative measurements of spontaneous activity/excitability, sensorimotor, neuromuscular and autonomic nervous functions. The FOB test was conducted after the administration of chlorpromazine, haloperidol, diazepam and caffeine. [Results] In the FOB test in monkeys, chlorpromazine and haloperidol produced dose-related changes in parameters of behavioral and neurologic functions. The effective dosage that induced neurobehavioral toxicity on the central nervous system (CNS), such as catalepsy, was lower in monkeys than in rats. Diazepam appeared to have an antianxiety effect, including sociability and taming, at a low dosage in monkeys, which did not induce excessive sedation. However, diazepam induced only sedation in rats. Caffeine induced hypoactivity (low-dosage) and hyperactivity (high-dosage) in rats, but only hyperactivity in monkeys. The symptoms observed in monkeys resembled known clinical effects of these drugs. [Conclusion] These findings suggest that neurobehavioral evaluation in primates can be useful in preclinical studies in predicting the clinical effects of new CNS active compounds.

**1095**

**NEUROBEHAVIORAL ASSESSMENT OF SIX NEUROTOXICANTS IN MALE SPRAGUE-DAWLEY RATS.**

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Sprague-Dawley (SD) rats are widely used for safety assessments, however there are few reports on Functional Observational Battery (FOB) and Motor Activity (MA) assays using SD rats. The goal of this study was to establish baseline data in male

SD rats after treatment with six model neurotoxicants: imidodipropionitrile (IDPN; 3 x 300 mg/kg, 10d prior to FOB), amphetamine (AMP 3-6 mg/kg), chlorpromazine (CPZ 3, 6 mg/kg), carbaryl (CRB 50-100 mg/kg), ethanol (ETH 1500 mg/kg), and 5-methoxy-N-N-dimethyltryptamine (MDMT 10 mg/kg). Rats were administered a single oral dose of each compound, and FOB and MA were performed 15-60 min after treatment (except as described for IDPN). All compounds induced behavioral changes consistent with previous reports. IDPN induced changes consistent with brain damage including head weaving, altered gait, and poor righting reflex. AMP induced changes consistent with CNS stimulants including increased reactivity to the observer, darting, increased rearing, repetitive head and jaw movements and exophthalmia. CPZ induced locomotor debilitation including markedly abnormal gait, little interest in surroundings, and reduced response to external stimuli. CRB induced behaviors consistent with acetylcholinesterase inhibition including salivation, twitches, flattened body posture, repetitive jaw movements (chewing), and whole body tremors. ETH induced intoxication evidenced by altered balance and gait as well as slow reaction time. MDMT induced changes for serotonergic agonists including hind-limb abduction, forepaw treading (slithering), and altered reflexes. AMP induced dramatic increases in all MA parameters: ambulation (14-fold), rearing (11-fold), and fine motor movement (5-fold). None of the other test articles yielded significant MA changes relative to controls. This study demonstrates that these compounds induced behavioral changes in male SD rats similar to changes reported in other rat strains, such as the Hooded Long-Evans rat, which have been reported in the literature more frequently for neurobehavioral studies. (Supported by NIAID Contract N01-AI-05417)

**1096**

**ASSESSMENT OF NEUROTOXICITY USING ZEBRAFISH AS A MODEL ORGANISM.**

N. Roy, C. Ton, Y. Lin and C. Parng. *Phylonix Pharmaceuticals, Inc., Cambridge, MA.* Sponsor: D. Monteith.

Neurotoxicity occurs when exposure to toxicants alters the normal activities of the nervous system, including neural transmission, connection and survival. Neurotoxicity can result from exposure to substances used in chemotherapy, radiation treatment, drug therapy, and organ transplant as well as food additives and environmental toxicants. Numerous approved drugs have incomplete neurotoxicity profiles and cause neurotoxic side effects in patients. Moreover, there is no effective cure for many neuropathies. To establish an effective screening platform to understand potential neuropathy after drug treatment, we used zebrafish as a model organism. The transparency of zebrafish is a unique attribute for assessing drug effects directly on the nervous system using whole mount antibody staining and histochemistry. To assess pharmacological effects of drugs on the nerve systems, motor neurons, dopaminergic neurons, and optic nerves were visualized and assessed in zebrafish using whole mount immunostaining. Additionally, *in vivo* dye staining, histochemistry and *in situ* hybridization were used to assess neuronal apoptosis, proliferation, myelin structure and gene expression. Here we showed that compounds inducing neurotoxicity in human also caused similar neurotoxicity in zebrafish including: dopaminergic neuron injury caused by 6-hydroxydopamine, MPTP, and reserpine; optic nerve loss caused by alcohol; demyelination caused by acrylamide and L-2-hydroxyglutaric acid; oxidative apoptosis caused by Angel's salt; peripheral axonal loss caused by anticancer drugs. These results show comparable pharmacologic effects in zebrafish and humans and support the use of zebrafish in assessing neurotoxicity. Conventional neurotoxicity assessment using histological, neurophysiologic and behavior studies are laborious and time consuming. Zebrafish can be used to screen potential neurotoxicity of compounds during drug development. This simple, rapid, whole animal zebrafish assay format will bridge the gap between cell-based and mammalian testing, streamlining the pre-clinical drug development process.

**1097**

**SENSITIVE DETECTION OF BEHAVIORAL IMPAIRMENTS IN MODERATELY LESIONED MPTP MICE BY AUTOMATED GAIT ANALYSIS.**

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Overt behavioral symptoms of Parkinson's disease (PD) are typically not observed until there is over 80% loss of striatal dopamine. Similarly, behavioral manifestations in the 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) mouse model of PD require large doses of MPTP and are often transient. Previously, using novel behavioral tests, we identified alterations in stride length and gait parameters in mice administered low doses of MPTP. These behaviors were highly correlated with decreases of striatal dopamine and undetected by standard locomotor and rotarod tests. To provide more detailed measures of MPTP-induced deficits in stride length and gait, we have utilized a sophisticated gait-analysis system from Mouse Specifics, Inc. In this system, a mouse walks on a transparent conveyor belt and the

paw placement is captured digitally from below with a video camera which allows for sensitive analysis of gait dynamics. Eight week old C57 BL/6j mice were administered a moderate dose MPTP (2x10 mg/kg s.c.). Seven days after MPTP treatment, patterns of gait dynamics for each limb were analyzed for stride length and time, stance time, braking time, and propulsion were analyzed using Cleversys software. MPTP treatment decreased striatal dopamine levels by 45-50% and dopamine transporter and tyrosine hydroxylase levels by 75%. Gait analysis revealed significant decreases in forepaw (10%) and hindpaw (11%) stride length and hindpaw stance (8%) and stride time (9%). The most robust effects were observed with forepaw and hindpaw brake time which were increased and decreased by 20%, respectively. These data suggest that automated gait analysis is a sensitive and detailed method for detecting behavioral deficits in mice with moderate dopaminergic lesions and may aid in testing of therapeutics for PD. (Supported by DAMD 00267036, NIH U54ES012068, F32ES013457, and EPA STAR Fellowship #91643701-0).

## 1098

### ALTERATIONS OF GENE EXPRESSION IN MICE INDUCED BY MPTP ADMINISTRATION: APPLICATION OF MICROARRAY ANALYSIS.

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1-methyl-4-phenyl-1, 2, 4, 6, -tetrahydropyridine (MPTP) is a selective neurotoxin which produces dopamine depletion and causes parkinsonism like symptoms in humans and is used to generate animal models for Parkinson's disease (PD). In this study, C57BL/6N mice were dosed daily with 10 mg/kg (i.p.) MPTP for 5 consecutive days. Mice were sacrificed 72 hours after the last dose, and the brain tissue was collected. Agilent mouse oligo 22K microarrays were used to examine alterations of gene expression in substantia nigra. Bioinformatics tools were used for data analysis and data mining. A list of over 400 genes were determined to be expressed differentially (down- or up-regulated) using a 1.5-fold cut-off and false discovery rate at 0.05. GOFFA found that most of the genes are involved in molecular functions, biological-process and cellular-component. Pathway finding tools indicated that multiple biological pathways are involved in the mechanism of MPTP-induced neurotoxicity in mice. These include, but not limited to, the MAPK pathways, neuroactive ligand-receptor reactions, ubiquitin mediated proteolysis, Wnt signaling pathways, apoptosis, and cytokine-cytokine interactions. A group of genes that are related to dopaminergic pathways were also identified in the gene list that were altered with MPTP treatment. These include tyrosine hydroxylase (TH), dopa decarboxylase (AAAD), dopamine transporter (DAT) and vesicular monoamine transporter 2 (VMAT2), indicating the disorder of dopaminergic systems. However, dopamine metabolic enzymes (MAOa, MAOb, COMT) were not found, suggesting these genes are not affected by MPTP. These results were consistent with our previous findings with acute MPTP treatment using real-time PCR. Further analyses on the pathways detected in this study are needed to elucidate the mechanism of MPTP-induced neurotoxicity.

## 1099

### MICROARRAY ANALYSIS DETECTED GENE EXPRESSION CHANGES IN PC12 CELLS AFTER EXPOSURE TO THE NEUROTOXIN MPP<sup>+</sup>

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MPP<sup>+</sup> is the active metabolite of the neurotoxin 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) and it has been shown to deplete dopamine and elicit cell death in PC12 neuronal cell cultures. However, the mechanism of MPP<sup>+</sup>-induced neurotoxicity has not been adequately elucidated. Agilent rat oligo 22K microarrays were used to examine alterations in gene expression of PC12 cells after 500  $\mu$ M MPP<sup>+</sup> exposure. Bioinformatics tools were used to analyze gene expression of control and treated cells. High correlation ( $R^2 = 0.98$ ) between the two dye channels (Cy3:Cy5) using self-self hybridization analysis (technical replicates) indicated a good quality microarray experiment. Raw data from each microarray slide scan were input into the ArrayTrack database, and data quality for each microarray slide was monitored using the ArrayTrack quality control tool. The raw data was then normalized using a Lowess normalization method to eliminate the dye factor before further statistical analysis. ArrayTrack visualization tools, Spotfire and JMP statistical software were used for further data quality control. The relative expression values of each gene are the average of the Cy3 and Cy5 log intensity from the dye-flipped slides. SAM (Significance Analysis of Microarray Data) was used to pick the genes with 2-fold changes and statistical significance (False discovery rate<0.05). After analysis, above 200 genes showed highly differential expression (up- or down- regulation) with MPP<sup>+</sup> treatment. Biological significance of these genes was analyzed using the ArraytrackTM linked libraries, including Kegg, Pathart pathway library and GOFFA library.

## 1100

### COMPARISON OF MEMBRANE POTENTIAL-SENSITIVE FLUORESCENT DYES FOR MEASUREMENT OF TOXIN-INDUCED DEPOLARIZATION IN MURINE NEOCORTICAL NEURONS: CHARACTERIZATION OF BREVETOXIN-AND KCL-INDUCED DEPOLARIZATION.

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Brevetoxins (PbTx1-10) represent a series of structurally related polyether, lipophilic neurotoxins that are produced by the dinoflagellate *Karenia brevis* and have been implicated in numerous epizootics off the coasts of Florida, Texas, and North Carolina. Brevetoxins also pose a threat to humans as those who consume shellfish contaminated with the toxin can develop Neurotoxic Shellfish Poisoning, a syndrome involving gastroenteritis along with a range of neurological sequelae. Brevetoxin exerts its effects by binding to voltage-gated sodium channels in the brain and maintaining them in an open state, thereby propagating continuous action potentials and causing subsequent excitotoxicity in postsynaptic neurons. Due to brevetoxin's ability to depolarize neurons, this depolarization may be measured using membrane potential-sensitive fluorescent dyes. We have accordingly optimized assay conditions for two such dyes using murine neocortical cells in primary culture. We compared the two dyes, DiBAC and the Membrane Potential Assay Kit (Molecular Devices Inc., blue dye) in an effort to optimize these types of assays for intact neurons. We found with DiBAC, a dye concentration of 2.5  $\mu$ M provided the optimum signal, whereas the blue dye gave the greatest signal at one-half the manufacturer suggested concentration. Using these techniques, we further developed standard conditions that allowed us to quantify the magnitude of PbTx-2-induced depolarization through a comparison to that produced by KCl in neocortical cells. The use of concentrations of brevetoxin and KCl that produce equivalent levels of depolarization will further permit mechanistic studies exploring brevetoxin's influence on neuronal signaling.

## 1101

### BREVENOL, A NATURAL BREVETOXIN ANTAGONIST: COMPETITIVE ANTAGONIST OR ALLOSTERIC MODULATOR?

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Polyether brevetoxins (PbTx<sub>s</sub>) and analogs are bioactive compounds produced by the dinoflagellate *Karenia brevis*, which bind to site 5 of mammalian voltage-gated sodium channels and cause membrane depolarization. Prior studies have also shown that site 5 is allosterically-linked to other receptor sites located on voltage-gated sodium channels. PbTx<sub>s</sub> themselves have been used as tools in neurotoxicology to understand the molecular structure and conformational changes of sodium channels. Brevenal, a natural antagonist of PbTx with a 6-7-6-7-7 ring motif, has been shown to compete for tritiated brevetoxin (<sup>3</sup>H-PbTx-3) binding sites in rat brain synaptosomes (Bourdelais et al., 2004). In this present study, the nature of specific brevenal binding is characterized. Tritium labeled brevenal is produced using tritiated NaBH<sub>4</sub> in the presence of cerium chloride (CeCl<sub>3</sub>) in order to provide a radiolabeled probe for specific receptor binding studies. Brevenal (reduced brevenal), radioactive and unlabeled, were purified using HPLC and the identity of brevenol was confirmed using NMR. Displacement receptor binding studies showed <sup>3</sup>H-brevenol is displaced by brevenal or its analogs. The calculated dissociation constant ( $K_d$ ) for <sup>3</sup>H-brevenol is 4.8nM which correlates favorably with the calculated PbTx  $K_d$  of 1.8nM. Conversely, PbTx-3 was unable to displace <sup>3</sup>H-brevenol from rat brain synaptosomes. Since it has been shown that brevenal exhibits a dose-dependent displacement of <sup>3</sup>H-PbTx-3, we postulate that brevenal may bind to an allosterically-linked receptor site on sodium channels peripheral to site 5. Future research will further characterize the brevenal binding site, a potential new pharmacophore located on voltage-gated sodium channels. Reference: Bourdelais, AJ; Campbell, S; Jacocks, H; Naar, J; Wright, JLC; Carsi, J and DG Baden. Brevenal is a natural inhibitor of brevetoxin action in sodium channel receptor binding assays. *Cell. Mol. Neurobiol.* 2004. 24(4): 553-563.

## 1102

### FEASIBILITY OF CONTINUOUS INTRACEREBRO-VENTRICULAR (ICV) INFUSION IN THE RAT FOR 28 DAYS

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This study was performed to determine the feasibility of drug administration by continuous intracerebro-ventricular (ICV) infusion to the Sprague-Dawley rat for 28 consecutive days. Five males and 4 females were cannulated into the lateral ventricle and assigned to the study. Following arrival, CTBR's standard infusion jacket was placed on each animal and an Infusdisk<sup>TM</sup> infusion pump attached to the

jacket using a Velcro® patch. The catheters were filled with 0.9% Sodium Chloride, U.S.P., connected and tucked under the jacket to prevent damage. The infusion rate of 0.03 mL/h was selected based on the available rate of the pump and the rate of CSF production/flow. During the study, the reservoir was changed every 7 days to maximize use of each pump and pre and post weights of the infusate were performed for dose accountability purposes. Clinical signs (daily) and body weights (weekly) were monitored following receipt. Verification of the catheter placement and integrity of the catheter were performed at necropsy. Infusion was considered overall well-tolerated. Infusion-related clinical signs included swelling of the cranium in 2/9 animals, noted between Days 2 and 24, and single occurrences of convulsions when handled (sustained or non-sustained on Days 2 or 13) in 2/9 animals, which were short in duration. While body weights were lower when compared to expected ranges for non-catheterized and catheterized rats of a similar age, percent gain was higher than expected ranges in both sexes. At necropsy, the catheter tip was found in the left ventricle of the brain in 9/9 animals, and the integrity was confirmed in 8/9 animals. Macroscopic changes that could have been related to the experimental procedure (catheterization) were observed subcutaneously along the catheter in the dorsal cervical region and/or dorsal aspect of the head and described as thickening (8/9), swelling (1/9) and/or mass (1/9). In conclusion, continuous intracerebro-ventricular (ICV) infusion in the rat for 28 days was considered to be a feasible design for safety toxicity or efficacy assessments.

**1103** TEMPORAL ANALYSIS OF OPIATE-INDUCED INTRATHECAL GRANULOMATOUS MASSES PROGRESSION AND REGRESSION USING MRI.

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Continuous intrathecal (IT) delivery of morphine sulfate (MS) for 28 days can produce aseptic subdural inflammatory masses (granulomas) localized at the catheter tip. The rate at which these IT masses develop, and if they regress after MS infusions are terminated are unknown. To assess this, we performed serial MRI scans with gadolinium (Gd) enhancement in dogs. Beagle dogs were implanted with chronic IT lumbar catheters and were infused at 0.96 ml/day for 28 days. Dogs received a 100% granuloma inducing concentration of MS (12.5 mg/ml) or the maximum tolerable concentration of fentanyl HCl (2 mg/ml). *Ex vivo* studies with dog dura suggest that MS, but not fentanyl, produce activation of duram mast cell. Before MS infusions, MRI scans were obtained with a Siemens Symphony 1.5 Tesla system while dogs were anesthetized with propofol. Dogs had repeated MRI scans at intervals ranging from 3 to 14 days, for up to 45 days, with IV Gd (Optimark, 0.2 mmol/kg). One group of dogs had saline substituted for MS at the first appearance of a mass by MRI. A second group of dogs continued to receive MS for 28-days, when saline was substituted for MS. On the day of the final scan, 1 ml of Gd (Magnevist) 1:400 in saline was injected IT. With MRI, masses in some MS dogs were apparent as early as 3 days and in all by 14 days. Termination of MS resulted in a rapid regression of the masses. This was greater in dogs receiving MS for 14 days vs. 28 days. Fentanyl did not produce IT granulomas. Repeat MRI scans with propofol anesthesia and IV Gd were well tolerated in dogs with no lasting negative effects noted. Morphine-induced IT masses form and regress quickly. Histology of the masses correlated well with MRI images. Mass size and behavioral were not well correlated but faster growing masses produced greater behavioral signs. IT fentanyl as predicted by lack of dural mast cell activation, did not produce IT masses and may be a therapeutic alternative to MS. Supported in part by DA-15353.

**1104** A COMPARATIVE EVALUATION OF ANIMAL CHARACTERISTICS AND DEVELOPMENT OF CHOROIDAL NEOVASCULARIZATION (CNV) IN A LASER-INDUCED NON-HUMAN PRIMATE MODEL OF MACULAR DEGENERATION.

M. Wills. *Development and Discovery Services, Charles River Laboratories, Sparks, NV*. This study evaluated the relationship of animal characteristics (age, sex, study history, origin and weight) and location of laser lesion to development of CNV. 34 cynomolgus monkeys were evaluated. Laser lesions were placed in the perimacular region. Lesions that developed a clear luminescent circle on fluorescein angiograms were counted as CNV. Animals were divided into age groups (2-5 years and over 5 years) and weight groups (2-3.5 kg and 3.5-5.2 kg). Incidence was compared for males vs. females, naive vs. non-naive and Chinese vs. Indonesian origin. 25/27 animals aged 2-5 years developed CNV. 10/10 animals aged 2-3.5 developed CNV in at least one eye; 15/17 animals aged 3.6-5 years developed CNV in at least one eye. 6/7 animals over the age of 5 developed CNV in at least one eye. 26/29 males developed CNV in at least one eye, 5/5 females develop CNV in at least one eye. 1/19 non-naive animals did not develop CNV in either eye. 2/15 naive animals did not develop CNV at least one eye. 26/28 Chinese animals developed CNV; 5/6

Indonesian animals developed CNV. 18/19 animals weighing 2-3.5 kg developed CNV in at least one eye. 13/15 animals weighing 3.6 to 5.2 developed CNV. There was no clear evidence of age- or weight-related differences in the propensity to develop CNV. An insufficient number of females were available to conclusively determine a sex-related difference, although this has been noted in rodent species. Non-naive animals developed CNV at a similar rate as naive animals, indicating that reuse of animals that have not been on previous ocular studies is acceptable. Laser lesions placed adjacent to or directly above the macula and in lesions placed nasal and superio-nasal to the macula appear to be most likely to develop CNV.

**1105** ALZHEIMER'S DISEASE: THE CYNOMOLGUS MONKEY AS A MODEL IN TOXICOLOGICAL RESEARCH?

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Alzheimer's disease describes a human degenerative brain disorder with an age-related increasing incidence. The present work was undertaken to evaluate whether aged cynomolgus monkeys (*Macaca fascicularis*) exhibit alterations indicative of Alzheimer's disease. Twenty animals, aged between 2 and > 20 years, were examined. Specifically, the beta amyloid proteins 1-40 (Ab40) and 1-42 (Ab42) as well as the hyperphosphorylated Tau protein were studied. Examinations were conducted in both cerebrospinal fluid and formalin-fixed and frozen brain tissue. Evaluations considered the 1. similarity of parameters between the cynomolgus monkey and humans, 2. age as a factor relative to the degree of parameter expression and 3. suitability of parameters in toxicological research. With advancing age, progression of Ab42 content and change of the Ab42/Ab40 ratio in cerebrospinal fluid were observed in primates older than 15 years. These proteins were also detected in the brain and were found to be comparable to a great degree with those of ageing human beings. In the respective primate brain tissue, development of plaque burden and Tau pathology were detected using immunocytochemical methods as well as silver and standard staining procedures. Hyperphosphorylated Tau (paired helical filaments) was present in both nerve cells and oligodendrocytes. Furthermore, vascular amyloidosis could also be diagnosed. In conclusion, a close principal relationship of Alzheimer's disease parameters between cynomolgus monkeys and humans was found. The in-life access to humoral parameters via sampling of cerebrospinal fluid during an extended period of the individual primate development, the similarity of primate and human brain pathology, as well as the possibility to histologically confirm the diagnosis post mortem in the primate brain tissue suggest that the cynomolgus monkey can serve as a potential relevant model for development of drugs against Alzheimer's disease.

**1106** EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS (EAE) IN THE FEMALE LEWIS RAT.

S. Rowton and S. Grainger. *Covance Laboratories Ltd., Harrogate, United Kingdom*. Sponsor: D. Everett.

EAE is an established and well-documented animal model used for investigating autoimmune disease of the central nervous system (CNS). In this model, clinical signs exhibited by the animals, histopathology of the CNS and the progression of the disease, are comparable in many ways to multiple sclerosis in humans. EAE can be induced by a number of agents and in several different species and strains of animal. We have validated this model in the female Lewis rat, using whole guinea pig spinal cord and carbonyl iron as the encephalitogenic agent. On Day 1, each rat received a 1mL intraperitoneal injection of guinea-pig CNS material:carbonyl iron (1:1 ratio). On Day 1 to Day 15, rats were dosed intraperitoneally once daily with vehicle, prazosin (4 mg/kg) or cyclosporin (4 mg/kg) in 20% Dalivit (a multivitamin). In addition, a fourth group of rats received 1mL (300mg) of whole guinea pig spinal cord intraperitoneally on Day 3 and 4. Administration of large doses of CNS material was expected to act upon the T cells, suppressing the EAE response by causing the T cells to self-destruct. Animals were observed daily from Day 1 to Day 26 for signs of EAE. All vehicle-treated rats successfully developed EAE (66% exhibited a minimum of a completely limp tail (a score of 23)), followed by spontaneous recovery. EAE signs in this group were first apparent on Day 8, peaked on Day 11 and by Day 17 had completely disappeared. Cyclosporin in Dalivit prevented EAE development in 58% of rats, minimal signs were seen in the remaining animals, with only one animal displaying a completely limp tail (a score of 3). Rats treated with CNS material prevented EAE in 25% of rats, with 50% scoring at least 3. All prazosin-treated rats developed EAE, with 83% scoring 3 or more. Subsequent studies, which examined EAE histopathologically, have shown a close correlation between the degree of perivascular cuffing, leukocyte infiltration and EAE severity in the female Lewis rat. In conclusion, EAE in the Lewis rat provides a useful model to investigate the potential immunosuppressant properties of a compound.

ANALYSIS OF MYELIN LOSS USING SCANNING ELECTRON MICROSCOPY (SEM) UNDER FULLY HYDRATED CONDITIONS: VALIDATION IN A MOUSE MODEL OF EXPERIMENTAL AUTOIMMUNE ENCEPHALITIS.

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Experimental allergic encephalomyelitis (EAE), induced in the Biozzi AB/H (anti-body high) mouse by sensitization with spinal cord homogenate (SCH) in adjuvant, is a model for the human disease of multiple sclerosis (MS). Histopathologically, the disease is characterized by inflammation and axonal degeneration. We investigated the utility of a newly developed technology of scanning electron microscopy (SEM) designed for analysis of fully wet tissue specimens. We used 21, 7-9-wk-old Biozzi female mice; 5 were untreated, and in the other 16, relapsing remitting (RR) EAE was induced. Animals were sacrificed 60 days after disease induction. The clinical signs were scored, and following deep anesthesia and perfusion, spinal cord tissues were dissected out and processed either for conventional histopathology or analysis by wet SEM. Histologic evaluation was performed on H&E-stained sections cut adjacent to those analyzed by wet SEM. Four anatomical sides (dorsal, lateral right, lateral left, ventral) of the spinal cord (SC) white-matter were viewed at X400, and in each side, two fixed areas of interest, each one of 90, 000 micron<sup>2</sup> were selected at random and photographed. The relative number of axons having rings with irregular thickness and fragmentation or loss of myelin sheath was scored. Our investigation indicated that the wet SEM methodology rendered myelin sheaths, axons, and inflammatory cells simple to differentiate. The identity of normal and pathological components was confirmed and correlated by light microscopy. We conclude that the wet SEM technology provides a quick, accurate, and detailed structural evaluation of the SC and can be applied as a routine test for potential new MS therapies.

NEONATAL/PREPUBERTAL EXPOSURE TO BUTYL BENZYL PHTHALATE, BUT NOT BISPHENOL A, CAUSES SUBTLE ALTERATIONS TO THE RAT FEMALE REPRODUCTIVE TRACT.

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Exposure to environmental chemicals during critical periods of early development may play an important role in cancer susceptibility later in life. We hypothesized that exposure to estrogenically-active chemicals alone, and in combination, during early critical periods of development will alter predisposition for breast cancer. We exposed rats to bisphenol A (BPA) or butyl benzyl phthalate (BBP) during the neonatal/prepubertal periods only. Lactating dams were gavaged with 250 ug BPA/kg body weight (BW) or 500 mg BBP/kg BW or an equivalent volume of the vehicle (sesame oil) on days 1-21 (Mondays-Fridays only). On day 21, offspring were weaned and the treatments were discontinued. Mammary tissue was collected for genomic, proteomic, gland differentiation and cell proliferation analysis. Here, we report on morphometric determinations. Neonatal/prepubertal exposure to BBP, but not to BPA, resulted in precocious vaginal opening compared to controls. Also, neonatal/prepubertal exposure to BBP resulted in 3/14 female rats as compared to 1/14 in control or PBA exposed rats to have irregular estrous cycles (diestrus phase). Neonatal/prepubertal exposure to BPA and BBP did not significantly alter BWs or uterine wet weights at 21, 35, 50 or 100 days from controls. However, when the data was expressed as uterine to BW ratio BBP-, but not BPA-, exposed females had significantly increased ratio at day 21 only. These results demonstrate that neonatal/prepubertal exposure to BBP, but not to the selected dose of BPA, resulted in subtle alterations to the developing female offspring. It will be interesting to see how these treatments will affect genomics, proteomics, gland morphology and cell proliferation in the mammary gland of the developing offspring, and susceptibility for mammary cancer.

THE ARYL HYDROCARBON RECEPTOR (AHR) IS REQUIRED FOR NORMAL OVULATION IN THE MOUSE.

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The AhR is ligand-activated transcription factor that mediates the toxicity of a variety of environmental chemicals. Although little is known about the physiological role of the AhR, studies suggest that it might play an important role in female reproduction. Female AhR deficient (AhRKO) mice have impaired folliculogenesis and reduced fertility compared to wild-type (WT) mice. Because alterations in folliculogenesis can affect the ability to ovulate, the objective of this study was to test

the hypothesis that AhR deficiency interferes with the ability of follicles to grow to the ovulatory stage and to ovulate in response to gonadotropin treatment. To test this hypothesis, WT and AhRKO mice were dosed with the pregnant mare serum gonadotropin (PMSG; 5 IU) to induce follicular growth and after 72 hours, ovaries were collected and processed for histological evaluation of the number of corpora lutea (an indicator of ovulations). In addition, WT and AhRKO mice were dosed with PMSG (5 IU) to induce follicular growth and after 48 hours, the mice were dosed with either 2.5 or 5 IU of human chorionic gonadotropin (hCG) to induce the ovulation. After 24 hours, oocytes were collected from the oviducts and counted. The data indicate that AhRKO ovaries contain fewer corpora lutea compared to WT ovaries after PMSG treatment (WT=6.5±0.4, AhRKO=3.2±0.8, n=5, p≤0.02), suggesting that AhRKO ovaries have a reduced capacity to ovulate compared to WT ovaries in response to PMSG. The data also indicate that both WT and AhRKO mice ovulate similar numbers of eggs in response to 5 IU hCG, but that AhRKO mice ovulate fewer eggs than WT mice in response to 2.5 IU hCG (WT=25.8±2.0, AhRKO=18.2±1.2, n=5, p≤0.02), suggesting that AhRKO ovaries are less sensitive to gonadotropins than WT ovaries. Collectively, these data indicate that AhRKO follicles have a reduced capacity to ovulate compared to WT follicles. Thus, in addition to mediating toxicity of environmental chemicals, the AhR is required for normal ovulation. (Supported by NIH HD38955 and T32HD07170)

EFFECTS OF ESTROGEN RECEPTOR MODULATORS ON UTERINE PATHOLOGY AND GENE EXPRESSION.

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Tamoxifen, a selective estrogen receptor modulator (SERM), is an effective treatment for breast cancers. In the CD-1 mouse model, neonatal oral dosing with tamoxifen leads to the development of adenomyosis. Subcutaneous dosing with tamoxifen or 4-hydroxyestradiol leads to adenocarcinomas after 12 to 18 months. Both 4-hydroxyestradiol and tamoxifen can form DNA-reactive metabolites and may be involved in carcinogenesis of the uterus. We compared the uterotrophic response of these compounds and their effects on uterine pathology and gene expression. 14-day-old CD-1 female mice were orally dosed daily for 3 days with estradiol benzoate (1-100µg/kg), tamoxifen (250-5000µg/kg), or 4-hydroxyestradiol (76.3-1920µg/kg). After 4 days uterine weights were recorded, then sections processed for PCNA staining. Maximal uterotrophic doses for weight increase over controls (fold change ± s.d.) were: estradiol (100µg/kg) 2.6 ± 0.003, 4-hydroxyestradiol (385µg/kg) 6.7 ± 0.03 and tamoxifen (250µg/kg) 2.6 ± 0.005. PCNA staining showed maximal cell proliferative effects at this time were not related to uterine weight increases. Estradiol inhibited the development of glandular epithelium, whereas tamoxifen was able significantly to increase the number of glands. Maximal uterotrophic dose of estradiol (100µg/kg) was given orally to newborn CD1 mice on days 2 to 5 after birth and gene and pathological changes examined in the uterus at 3 months after dosing. No adenomyosis was seen. Gene expression changes showed up-regulation of typical estrogenic response genes including *actb*, *sp1* while other genes such as *ngfa* and *spp1* were down-regulated. This study shows that neonatal estradiol treatment brings about long-term changes in gene expression in the absence of apparent pathological changes.

THE ARYL HYDROCARBON RECEPTOR (AHR) REDUCES EXPRESSION OF ENZYMES IN THE ESTROGEN BIOSYNTHESIS PATHWAY.

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The AhR is a ligand-activated transcription factor that mediates the toxic effects of environmental contaminants. Previous studies have shown that AhR deficient (AhRKO) mice have reduced fertility compared to wild-type (WT) mice. The reduced fertility in AhRKO mice is due, in part, to a reduced number of ovarian antral follicles and reduced levels of estradiol (E2). The reasons that AhRKO mice have decreased levels of E2 are unknown, however, it is possible that they stem from defects in estrogen biosynthesis. During estrogen biosynthesis, cholesterol is taken across the mitochondrial membrane by the steroidogenic acute regulatory protein (StAR). Cholesterol is then converted into androgens and finally into E2 by a series of enzymes including cytochrome P450 side chain cleavage (CYP 450ccc), CYP17α (CYP 17), aromatase, 17-beta hydroxysteroid dehydrogenase (17β-HSD), and 3-beta hydroxysteroid dehydrogenase (3β-HSD). To determine whether levels of these enzymes are altered in AhRKO ovaries compared to WT ovaries, ovaries were collected from WT and AhRKO mice on postnatal day (PD) 90. Total RNA was extracted, reverse transcribed to cDNA, and subjected to both semi-quantitative and real time polymerase chain reaction (PCR). The results show that

the levels of P450scc, 3 $\beta$ -HSD, and 17 $\beta$ -HSD were similar in WT and AhRKO ovaries (n=3). In contrast, real-time PCR analysis shows that the levels of CYP17 in AhRKO ovaries were lower than those of WT ovaries (WT=1.23 $\pm$ 0.02, AhRKO=0.05 $\pm$ 0.0005 genomic units, n=3, p<0.0001). Further, the levels of StAR were lower in AhRKO ovaries compared to WT ovaries (WT=0.7 $\pm$ 0.09, AhRKO=0.38 $\pm$ 0.06 genomic units, n=5, p<0.02). Collectively, these data suggest that AhR deficiency reduces the levels of key steroidogenic enzymes in the mouse ovary. Support provided by the Howard Hughes Medical Institute Undergraduate Scholars Program at UMBC, HHMI Foundation, and NIH grants GM072195-01 and NIH HD38955.

## 1112 THE ARYL HYDROCARBON RECEPTOR (AHR) REGULATES OVARIAN FOLLICLE GROWTH *IN VITRO*.

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The AhR plays an important role in mediating the toxicity of various environmental toxicants that cause adverse effects on the development and function of the female reproductive tract. Studies using AhR-deficient (AhRKO) mice have shown that the AhR has an important physiological role in the mouse ovary. Previous studies in our lab have demonstrated that AhRKO ovaries have a decreased number of antral follicles compared to wild-type (WT) ovaries. Since our previous studies also indicate that AhR deficiency does not affect atresia (follicle death via apoptosis) of antral follicles, the purpose of these studies was to determine whether AhR deficiency reduces follicle numbers by slowing follicular growth. Further, since antral follicles produce estradiol (E2) and E2 is required for normal follicular growth, these studies also tested whether AhR deficiency results in decreased synthesis of E2 by antral follicles. To test these hypotheses, antral follicles were isolated from AhRKO and WT ovaries and cultured for 168 hours. During culture, follicle growth was assessed by daily measurements of follicular diameter. After culture, media was collected and E2 levels were measured using an enzyme-linked immunoassay (ELISA). AhRKO and WT ovaries were also subjected to measurements of proliferation using immunohistochemistry (IHC) for proliferating cell nuclear antigen (PCNA) antibody. Our results show that WT follicles grew significantly larger than AhRKO follicles by 168 hours of culture (WT: 615.5 $\pm$ 17.15 mm; AhRKO: 489 $\pm$ 17.03 mm; p<0.001; n=3 mice per genotype, 10 follicles per mouse). The results also show that WT follicles produced significantly more E2 compared to AhRKO follicles (WT: 2463 $\pm$ 508 pg/ml, n=15 follicles; AhRKO: 971 $\pm$ 316 pg/ml, n=9 follicles; p=0.007). Further results from IHC show that AhRKO follicles had less PCNA staining in granulosa cells compared to WT follicles. These data suggest that in addition to mediating toxicity of environmental chemicals, the AhR is an important regulator of ovarian follicle growth and E2 production. Supported by NIH grants GM072195-01, HD38955, and R25-GM55036.

## 1113 ACTIVATION OF TH1 PRO-INFLAMMATORY CYTOKINE EXPRESSION BY ARYL HYDROCARBON RECEPTOR (AHR) LIGANDS IN HUMAN UTERINE ENDOMETRIAL CELLS.

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The environmental contaminants polychlorinated biphenyls and dioxins are known to modulate immune function and have been linked to infertility, uterine disease and low birth weight in animal and human studies. This study used the human uterine endometrial adenocarcinoma RL95-2 cell line as a model for investigation of the effects of prototype environmental organochlorines on pro-inflammatory Th1 cytokine expression. Cells were cultured in the presence of three coplanar ligands for the aryl hydrocarbon receptor (AhR), 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), poly-chlorobiphenyl CB-77 (3, 3■, 4, 4■) and CB-126 (3, 3■, 4, 4■, 5), respectively, as well as the AhR non-ligand CB-153 (2, 2■, 4, 4■, 5, 5■). Analysis of mRNA levels by quantitative real-time PCR showed that TCDD, CB-77 and CB-126 significantly increased expression of IL-1 $\alpha$ , IL-1B, and IL-8. The AhR antagonist alpha-naphthoflavone (aNF) partially or completely blocked the PCB-126 mediated-induction of IL-1 $\alpha$ , IL-1B and IL-8. In this regard, the chemokine IL-8 is known to be a key attractant for cells during inflammatory immune responses. An ELISA assay for IL-8 showed that the three AhR ligands significantly increased IL-8 secreted protein levels, which was found to be suppressed by aNF, the AhR antagonist. In addition, a correlation was observed between CYP1A1 protein levels and IL-8 secretion for TCDD and 9 PCB congeners, including several hydroxy-metabolites. In summary, the AhR appears to mediate coordinate changes in the expression of multiple pro-inflammatory cytokines in the RL95-2 uterine endometrial cell line. A shift to enhanced expression of pro-inflammatory Th1 cytokine pathways by AhR ligands may be a fundamental mechanism for adverse reproductive effects associated with infertility and uterine disease. (Supported by NIH ES07375)

## 1114 STIMULATORY AND INHIBITORY EFFECTS OF GENISTEIN ON UTERINE LEIOMYOMA CELL GROWTH *IN VITRO* IS DETERMINED BY DOSE.

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Genistein, a phytoestrogen, is largely found in soy products commonly consumed by women in the USA. Due to dietary exposure of women to genistein and the estrogen responsiveness of a common gynecologic tumor of women, uterine leiomyoma (UtLM) "fibroid", we were interested in determining the response of cultured UtLM cells to genistein. We evaluated the effects of genistein (0, 0.001, 0.01, 0.1, 1, 10, 50 and 100  $\mu$ g/ml) on the growth, morphology and cell cycle progression of UtLM cells compared to normal uterine smooth muscle cells (UtSMC) following 24h and/or 7 days of treatment. Also, analysis of DNA content by flow cytometry was done to determine whether UtLM cell growth was altered due to apoptosis. We found that overall low doses of genistein (0.001, 0.1, and 1  $\mu$ g/ml) had a significant (p<0.01) stimulatory effect on the growth of UtLM cells, but not UtSMC; whereas, 10, 50, and 100  $\mu$ g/ml genistein significantly (p<0.01) inhibited UtSMC and UtLM cell growth. Morphologically, all cells given higher concentrations of genistein were fewer in number and had decreased cytoplasm and small nuclei. At lower concentrations cells maintained their normal elongated appearance. Cell cycle analysis of UtLM cells treated with 1  $\mu$ g/ml genistein for 24h showed an increase in the percentage of cells in the S phase compared to controls; however, at 50  $\mu$ g/ml genistein, there was a higher percentage of cells in the G2/M phase with decreased number of cells in the G1 phase compared to low dose and control groups. These effects were not seen with UtSMC. There was a 2.4-fold increase in the percentage of apoptotic cells detected in UtLM cells exposed to 50  $\mu$ g/ml genistein for 24h compared to controls. In summary, low doses of genistein stimulate UtLM tumor cell growth, but have no effect on normal UtSMC. At high doses, genistein has an inhibitory effect on both cell types. The inhibition observed with high doses of genistein in the UtLM cells may be due, in part, to altered progression of cells through the cell cycle and increased apoptosis.

## 1115 SUPEROXIDE ANION, HYDROGEN PEROXIDE, AND HYDROXYL RADICALS IN CIGARETTE SMOKE INHIBIT HAMSTER OOCYTE CUMULUS COMPLEX PICKUP.

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The hamster oviduct and oocyte cumulus complex (OCC) are targets of cigarette smoke. Pickup of the OCC by the ciliated oviduct is inhibited in hamsters during acute *in vitro* exposures of oviducts or OCCs to mainstream and sidestream smoke solutions. Smoke solutions inhibit OCC pickup by increasing adhesion between the OCC and oviduct. Cigarette smoke generates many reactive oxygen species including superoxide anion, hydrogen peroxide, and the hydroxyl radical. Our initial studies showed that superoxide anion and hydrogen peroxide are at least partially responsible for inhibiting OCC pickup. The antioxidants, superoxide dismutase and/or catalase, protected the hamster oviduct and oocyte cumulus complex from the inhibitory effects of cigarette smoke on OCC pickup rate and adhesion. When oviducts or OCCs were exposed to mainstream or sidestream whole, gas, or particulate smoke solution plus either superoxide dismutase or catalase, most adverse effects on OCC pickup rate and adhesion were eliminated. Mannitol, a hydroxyl radical scavenger, was used to determine if inhibition of OCC pickup was due to the direct action of superoxide anion or hydrogen peroxide or was due to their role in the formation of hydroxyl radicals. When oviducts were exposed to mainstream particulate, sidestream particulate or sidestream whole smoke solutions plus mannitol, the adverse effects on both OCC pickup rate and adhesion were eliminated. Mannitol also eliminated the adverse effect of mainstream whole on adhesion, but not OCC pickup rate. When OCCs were exposed to smoke solutions plus mannitol, the adverse effects of mainstream whole, mainstream particulate, sidestream whole, and sidestream gas on OCC pickup rate and adhesion were eliminated. Mannitol also eliminated the adverse effect of mainstream gas on adhesion, but not OCC pickup rate. These results clearly show that the hydroxyl radical is a major contributor to the adverse effect that cigarette smoke has on OCC pickup.

## 1116 CHARACTERIZING THE OVOTOXICITY OF CYCLOPHOSPHAMIDE METABOLITES ON CULTURED MOUSE OVARIES *IN VITRO*.

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Toxic metabolites of cyclophosphamide (CP), a chemotherapeutic agent, are reported to target dormant primordial ovarian follicles in rodents and humans. Such damage is permanent, because this oocyte population cannot be replenished. CP is

activated by the liver to 4-hydroxy-CP, which spontaneously forms acrolein (ACR) and the ovotoxicant, phosphoramide mustard (PM). In order to investigate the specificity of PM-induced follicle loss, the sensitivity of ovaries in an *in vitro* system was characterized. Multiple products of CP were added to *in vitro* tissue cultures of intact postnatal-day-4 mouse ovaries on d0 of culture. Chemicals were removed on d2, cultures were continued until d8, then histology and follicle counts were done on each ovary. Whereas small numbers of follicles were continuously activated to grow and develop up to the secondary follicle stage in these ovaries, approximately 70% of all follicles remained dormant throughout the culture period. Exposures to either 4-hydroperoxy-CP (an activated form of 4-hydroxy-CP) or PM specifically depleted primordial and smallest primary follicles in a concentration-dependent manner ( $\geq 3\mu\text{M}$ ,  $p < 0.05$  for both), but did not alter populations of larger primary or secondary follicles ( $p > 0.05$ ). Follicle loss also occurred in unexposed ovaries cultured next to those exposed to 10 $\mu\text{M}$  PM, implicating a volatile breakdown product. PM-induced follicle loss became significant ( $p < 0.05$ ) by d2 or d6 following exposures to 10 $\mu\text{M}$  or 3 $\mu\text{M}$  PM, respectively. In contrast to PM, toxicity of ACR (1-100 $\mu\text{M}$ ) did not specifically deplete ovarian follicles, even when exposures were continued throughout the 8d culture. Thus, results suggest that 1) brief exposures to 4-hydroperoxy-CP or PM are sufficient to induce permanent follicle loss in ovaries, 2) that PM and/or a volatile breakdown product are likely the ultimate ovotoxicant, and 3) this *in vitro* system mimics effects reported *in vivo*. Further, the equal sensitivity of ovaries to 4-hydroperoxy-CP and PM suggests little detoxification of 4-hydroperoxy-CP occurred in ovarian cultures.

## 1117

### EFFECTS OF BUTHIONINE SULFOXIMINE TREATMENT ON THE EXPRESSION OF GLUTAMATE CYSTEINE LIGASE SUBUNITS IN THE RAT OVARY.

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Glutathione (GSH), an antioxidant tripeptide, is found in moderately high levels in the ovary. GSH is important for detoxifying reactive oxygen species and exogenous chemicals. Its synthesis occurs in two steps, the first of which is controlled by the rate-limiting enzyme, glutamate cysteine ligase (GCL). GCL is a heterodimer consisting of a catalytic (GCLc) and modulatory (GCLm) subunit. Buthionine sulfoximine (BSO) depletes GSH by blocking the active site on GCL, inhibiting GSH synthesis. BSO treatment up-regulates GCL subunit expression in several cell culture models. We therefore hypothesized that BSO treatment would increase ovarian mRNA and protein levels of the GCL subunits *in vivo*. Adult female cycling Sprague-Dawley rats were given either BSO (5 mmol/kg) in saline vehicle or saline alone intraperitoneally on proestrous morning, and ovaries were harvested 8, 12, or 24 hours following a single injection or 12 h after two injections administered 12 h apart. Ovarian GSH concentrations were depleted by about 50% at 8h after a single dose of BSO and increased gradually at subsequent time points. Northern analysis showed that GCLc and GCLm mRNA levels were slightly increased at 8h after BSO ( $p < 0.05$ ), but there was no significant effect of BSO at the other time points. Western analysis showed no significant increases in GCLc or GCLm protein at 8, 12, or 24 h after a single injection of BSO. However, there was a modest, statistically significant increase in GCLc protein 12h after a second BSO injection. GCL enzymatic activity was not significantly increased at 24h after a single BSO injection compared to controls. These data indicate that the ovary is minimally able to up-regulate GSH synthesis in response to GSH depletion by BSO. This work was supported by NIH K08 ES10963.

## 1118

### CUMULATIVE EFFECTS OF THIRAM AND AMITRAZ ON PREGNANCY MAINTENANCE AND DEVELOPMENT IN THE RAT.

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Amitraz and thiram are pesticides that have been shown to disrupt luteinizing hormone (LH) secretion in rats, albeit by different mechanisms. Amitraz acts via  $\alpha$ -noradrenergic antagonism; whereas thiram inhibits norepinephrine synthesis. Here, we sought to evaluate the cumulative effects of amitraz and thiram on pregnancy maintenance when administered during the LH-dependent period of pregnancy (days 7-10). To assess dose additivity, in Experiment 1, F344 rats were administered amitraz at 40 mg/kg (A40), thiram at 25 mg/kg (T25), or the two combined (AT-high) at 20 and 12.5 mg/kg, respectively, by gavage on gestation days (GD) 6-10. Controls received vehicle (1% methylcellulose). In Experiment 2, all doses were halved to yield treatments of A20, T12.5, and AT-low (A10 + T6.25). Tail-blood samples were taken from selected dams for assay of serum LH. Litters were examined on postnatal days (PD) 1 and 6. Full-litter resorption (FLR) was observed in 23 (79%), 8 (33%), and 18 (64%) of the dams in the A40, T25, and AT-high

groups, respectively, and in 7 (41%), 3 (25%), and 1 (7%) of the dams in the A20, T12.5, and AT-low groups, respectively. One (5%) control dam had full-litter loss, but, unlike the other cases, the loss appeared to occur near term. Reduced serum LH levels were observed on GD 10 in A40, T25, and AT-high dams with FLR, and in A40 dams with live litters. For dams with surviving litters, delayed parturition was noted in the A40, T25, and AT-high groups. Surviving A40 litters had increased resorption rates and increased incidences of eye defects (microphthalmia, anophthalmia). Pup weights were reduced on PD 1 at A40 and T25. Thus, both chemicals, alone and in combination at the high doses, caused FLR and delayed parturition; these findings are consistent with dose additivity. Whereas the low doses of each chemical alone also caused FLR, dose additivity was not clearly demonstrated by the AT-low combination. [This abstract does not necessarily reflect EPA policy.]

## 1119

### DIBROMOACETIC ACID ATTENUATES A DITHIOCARBAMATE-INDUCED SUPPRESSION OF THE LH SURGE IN THE RAT.

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Elevated concentrations of the haloacetic acid, dibromoacetic acid (DBA) can act as a reproductive toxicant in male and female rats. One effect in females is an increase in serum estradiol (E2) that may be due to a suppression in hepatic catabolism. In the rat, the normal rise in E2 beginning on diestrus up-regulates the brain mechanisms involved in triggering the proestrous luteinizing hormone surge (LH-S) that stimulates the process of ovulation. The present study investigated whether DBA is able to augment the LH-S and affect the established ability of the neurotoxicant sodium dimethylthiocarbamate (DMDC) to block the surge in ovariectomized (ovx), steroid-primed rats. Two experiments were performed: (1) to establish a dose-related DMDC suppression in the surge under exposure to a single concentration of DBA (60 mg/kg) & (2) to determine a dose-related effect of DBA combined with a single dose of DMDC. Young-adult S-D rats were gavaged daily for 2 wks with DBA. They were ovx on d11 and implanted with an estradiol benzoate capsule to generate daily LH-Ss. DMDC was injected i.p. on d14 (1300h) & blood sampled at 1400, 1600, 1800 & 2000h. DMDC (range 0-0.2 mM) caused a dose-related suppression in the surge. As a result, 0.1 mM DMDC was employed in Exp. 2, using DBA doses of 0, 37.5, 75 or 150 mg/kg. Since the areas under the LH curve (AUC) over the sampling times for the two lower doses (0 & 37.5 mg/kg) were almost identical, as were the AUC for the 2 higher doses (75 & 150 mg/kg), they were combined, forming high & low DBA groups that were significantly different from one another. Also, the number of rats showing a detectable LH-S was increased at 150 mg/kg. At 150 DBA/0.1 DMDC, the timing of an identifiable LH peak was comparable to non-DMDC females, unlike the 37.5 DBA/0.1 DMDC group. The results indicate that DBA can cause an attenuation in a dithiocarbamate blockade of the LH-S via a likely augmentation in the E2-associated up-regulation in the brain mechanisms stimulating the surge. (This abstract does not reflect USEPA policy)

## 1120

### A SINGLE DOSE OF 8-METHOXYPSORALEN REDUCES LUTEINIZING HORMONE LEVELS IN FEMALE RATS.

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Although mammalian females are endowed with a large supply of primordial follicles at birth, only a limited number of these follicles reach the antral stage for ovulation. Several environmental chemicals, including the psoralsen, have been shown to further deplete this pool of follicles that reach ovulation. The psoralsen occur naturally in plants. Further, synthetic forms of 8-methoxysoralen (8-MOP or methoxsalen) are used in combination with UVA irradiation to treat the skin condition psoriasis in the medical procedure referred to as PUVA. This drug has been implicated in a number of adverse reproductive effects in the female Wistar rat including decrease in ovulations, number of corpora lutea, levels of circulating 17 $\beta$ estradiol, granulosa cell population in antral follicles, and decrease in weights of the pituitary, ovaries, and full as well as empty uteri. This study was initiated to test the hypothesis that 8-MOP compromises reproduction in adult female rats by affecting the hypothalamic-pituitary axis. Thus, the effects of 8-MOP on circulating levels of gonadotropins were monitored during the post-dosing period. Adult female Wistar rats were given 200 mg/kg of 8-MOP (i.p.) or DMSO vehicle during diestrus and monitored at 6-hour intervals for up to 30 hours (6, 12, 18, 24, and 30 hrs). At those time points, rats were euthanized if they were in proestrus. Serum was collected to quantify levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH). Exposure to 8-MOP significantly reduced LH production by female rats at 6 hours after dosing (18.65 ng/mL, 8-MOP, n = 10; 31.75 vehicle control, n = 6;  $p < 0.05$ ) and 12 hours after dosing (15.78 ng/mL, 8-MOP, n = 5; 21.95, control vehicle, n = 4;  $p < 0.05$ ). Dosing did not significantly affect FSH levels at these time points. The reduction in LH production is consistent with our previous

reports suggesting that 8-MOP induces an effect on the pituitary with a subsequent disruption of the pituitary-hypothalamic-ovarian axis. (Supported by NIH grant # 2S06 GM008197-20 MPRC-B to MMD)

## 1121 EFFECTS OF FEED RESTRICTION ON FERTILITY AND EARLY EMBRYONIC DEVELOPMENT IN FEMALE RATS.

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Pharmaceuticals intended to reduce appetite and promote weight loss will generate safety data that will be challenging to interpret. To aid with this, the effects of feed restriction on rat fertility and early embryonic development were investigated. Purina Certified Rodent Diet 5002 was provided to female Sprague-Dawley rats (~60 days old and weighing ~200 g, n=20) ad libitum (control), 20, 15, 10, or 7.5 g/day (g/d). This feeding paradigm was administered 14 d prior to mating, during mating, and ended on gestation day (GD) 7 (plug/sperm positive=GD 0). From GD 8-14, ad libitum diet was provided to animals in all groups. On GD 14, uterine contents were evaluated. Vaginal cytology was analyzed daily up to GD 0 to monitor estrous cycle progression. For the 14 d of premating, control rats consumed ~21 g/d. On d 7, mean body weights in the 20, 15, 10, and 7.5 g/d groups were reduced by 5%, 12%, 18%, and 21% compared to control, respectively. On d 14, mean body weights were reduced by 5%, 16%, 27%, and 33% compared to control in those same respective groups. During the premating period, estrous cycle length was unchanged in groups receiving  $\geq 15$  g/d. In the 10 and 7.5 g/d groups, persistent diestrus reduced the number of occurrences of estrus by 16% and 45%, respectively. By d 14, all animals receiving 7.5 g/day were moribund and were euthanized. Mean time to mate, copulation and pregnancy rates were unaffected in the 20 and 15 g/d groups. At 10 g/d, mean time to mate was 3.6 times longer than control, and copulation and pregnancy rates decreased by ~40%. Control rats consumed ~28 g/d from GD 0-7. Compared to the control, mean body weight gain from GD 0-7 was reduced by 76% in the 20 g/d group, and body weight loss occurred in groups receiving 15 and 10 g/d. These data indicate an adverse effect on female rat fertility when food consumption was  $\leq 50\%$  of ad libitum values. When consumption remained  $> 50\%$  compared to ad libitum, maternal body weight gain was negatively impacted although fertility was unaffected.

## 1122 LATE GESTATIONAL ATRAZINE EXPOSURE ALTERS MATERNAL NURSING BEHAVIOR IN RATS.

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Atrazine, ATR, is a widely used herbicide shown to delay early mammary development and reduce body weight in female offspring of exposed Long Evans (LE) rat dams. To determine if ATR affects maternal behavior, dams were gestationally dosed and evaluated following parturition. Timed-pregnant LE rats were gavaged gestational days (GD) 17-19, 15-19, or 13-19 with 100 mg ATR/kg body weight (BW), an environmentally based mixture (EBM) of ATR and its metabolites (8.7 mg/kg total, 25% ATR) GD13-19, or vehicle (controls, C) GD13-19 (these treatments shown to have no effect on fetal weight). Pup BW was recorded on postnatal days (PND) 4 and 11 (analyzed by litter). By PND 4, female pup BW in ATR GD17-19 and 13-19 groups were decreased from control ( $p < 0.001$ ), and male pup BW in EBM, and ATR GD17-19 and 13-19 groups were decreased from control ( $p < 0.02$ ), leading us to believe there may be an effect of ATR on lactation. To assess maternal nursing behavior on PND 11, dams were removed from their litters for 3 hours to allow for milk accumulation. Pups were weighed immediately before dams were placed back with their litters. Pups in all groups were allowed to suckle for 30 consecutive minutes and then removed from their dam for weighing. Dams were sacrificed immediately. Maternal behavior was defined as crouching on litters for nursing, in an arched-back position. There was a significant effect of treatment on this maternal behavior, ( $p < 0.001$ ). Dams exposed to 100 mg/kg ATR GD17-19 and 15-19 took more than 2- and 3-fold longer to nest upon their litters, respectively, than control dams ( $p < 0.005$ ). Female pup BW in the ATR GD13-19 group was still decreased from control ( $p < 0.001$  pre-suckling,  $p < 0.02$  post-suckling). These results show that brief gestational exposures to ATR can alter maternal behavior specifically related to nursing. (This abstract is of a proposed presentation and does not reflect EPA policy; Supported by NHEERL-DESE, USEPA CT826513.)

## 1123 PERIPHERAL BENZODIAZEPINE RECEPTOR LIGAND PK11195 AFFECTS FEMALE FERTILITY AS DEMONSTRATED IN AN IN-VITRO MOUSE FOLLICLE BIOASSAY.

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The isoquinoline carboxamide PK11195 is a high affinity ligand of the peripheral benzodiazepine receptor (PBR) with potential anti-inflammatory properties. PBR are expressed in the somatic compartment of the ovary and play a role in the regu-

lation of steroidogenesis. To investigate possible effects of PK11195 on female fertility the multiparametric in-vitro mouse follicle bioassay was used, allowing the simultaneous multiparametric evaluation of the ovarian function and oocyte quality. Early preantral mouse ovarian follicles were cultured individually under physiological conditions during 12 days up to the pre-ovulatory stage. *In vitro* ovulation was triggered with hCG. Follicles were continuously exposed to a PK11195 dose range (1, 2.5, 5, 10, 15  $\mu$ g/mL). Results are reported from 460 follicles in 3 independent repeat experiments. The effect of PK11195 was determined on folliculogenesis, oogenesis and steroidogenesis. No effect was observed on follicle survival at any treatment dose, however the highest dose lowered the follicle differentiation capacity (antrum formation rate) to  $72 \pm 21\%$  versus  $98 \pm 4\%$  in the controls. PK11195 significantly decreased the oocyte nuclear maturation rate at doses of 5, 10 and 15  $\mu$ g/mL ( $P \leq 0.05$ ). Respectively  $65 \pm 10\%$ ,  $61 \pm 23\%$ ,  $38 \pm 18\%$  of the long-term exposed oocytes were able to extrude the first polar body versus  $95 \pm 6\%$  in the controls. PK11195 exposure did not affect testosterone and estradiol secretion patterns, but did elevate the progesterone output at the end of the follicular phase in a dose dependent way, while the hCG-induced progesterone remained unaltered in all treatment groups compared to controls. The multiparametric follicle bioassay demonstrated that long-term exposure to PK 11195 affects clearly oocyte quality in a dose dependent way, while folliculogenesis and steroidogenesis were only weakly affected. These data suggest that PK11195 can disrupt female fertility through the PBR pathway.

## 1124 THE CHEMICAL FORM OF ALUMINUM AFFECTS ITS PARACELLULAR FLUX ACROSS AND UPTAKE INTO CACO-2 CELLS.

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Aluminum (Al) has been suggested to contribute to dialysis encephalopathy, a low-turnover osteomalacia and Alzheimer's disease. This study investigated the influence of the chemical form (species) of Al on its flux across and uptake into Caco-2 cells, to further understand the mechanisms of Al absorption from the gastrointestinal tract. Al, as the ion, fluoride, citrate, maltolate and hydroxide, was used. For the flux study, Caco-2 cell monolayers grown on porous membranes were mounted in vertical diffusion chambers. The 5 Al species were added to the medium on the apical side of the cells and the Al concentration determined on the basolateral side. Permeability of Lucifer yellow, a marker of paracellular flux, was concurrently measured. To study Al uptake, Caco-2 cells grown in 35 mm dishes were incubated with the 5 Al species for up to 240 min at 37 deg C or at various temperatures to create an Arrhenius plot. Lumogallion staining imaged by confocal laser microscope was used to localize Al in the Caco-2 cells. The apparent permeability (Papp) of Al ion, fluoride, citrate and maltolate correlated highly with Lucifer yellow Papp, whereas Al hydroxide, which has the lowest Papp, did not. The uptake kinetics of each of the 5 Al species was different from the others. The uptake rate of Al fluoride was  $>$  Al ion  $>$  Al maltolate  $>$  Al citrate  $>$  Al hydroxide. The activation energy of the Al ion, fluoride, and citrate, calculated from the Arrhenius plot, was 13-22 KJ/mol. The confocal images showed similar intracellular Al distribution after exposure to Al ion, fluoride, and citrate. The cell nuclei showed a high Al concentration. The results suggest soluble Al species diffuse through the paracellular pathway. Caco-2 cell uptake of Al is a diffusion process. The ligand may influence the rate of cellular Al uptake. Intracellular Al localization was heterogeneous. Funded by EPA STAR Grant 829783.

## 1125 ORAL ALUMINUM (AL) BIOAVAILABILITY FROM A FOOD CONTAINING AN AL FOOD ADDITIVE: RELEVANCE TO THE MAJOR SOURCES OF AL EXPOSURE FOR HUMANS.

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Aluminum, a neurotoxicant, has been controversially implicated in the etiology of Alzheimer's disease. Food provides ~ 95% and drinking water 1 to 2% of typical daily Al intake. The primary food source of Al is approved food additives. The objective was to prepare a representative food containing the approved food additive, acidic sodium aluminum phosphate (SAIP), a leavening agent, and determine oral Al bioavailability from the food. To study Al absorption following consumption of an amount that models food consumption, 26Al was incorporated into the food. Extremely low levels of 26Al can be quantified by accelerator mass spectrometry. We prepared 26Al-containing Al(OH)<sub>3</sub> and incorporated it into the synthesis of 26Al-containing acidic SAIP (McDonald, US Patent #2550490, 1951). The 26Al-containing Al(OH)<sub>3</sub> and acidic SAIP were characterized by near infrared spectroscopy and x-ray powder diffraction. The 26Al-containing SAIP, also character-

ized by elemental analysis of Na, Al and P and thermogravimetric analysis, was incorporated into a biscuit, as 1 and 2% SALP (~ 1 nCi [50 ng] 26Al/gm biscuit). To determine oral Al bioavailability from this food, Fisher rats, acclimated to dietary conditions that resulted in no food in the stomach at the time of biscuit presentation and consumption of biscuit when presented, were given 1 gm of 26Al-containing biscuit. Concurrently studied rats received 26Al in water, replicating a previous study, or no treatment, to determine cross-contamination with 26Al. The bioavailability of Al from this representative food compared to water, in light of the daily intake of Al from food and water, suggests the major source of Al that reaches systemic circulation, thereby creating the greater potential for Al toxicity in the human. Supported by NIH Grant R01 ES11305.

## 1126

### DOES ALUMINUM WELDING FUME CAUSE CLINICALLY SIGNIFICANT PNEUMOCONIOSIS AND LUNG CANCER? - AN ANALYSIS OF SPECIFIC CAUSATION.

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We were asked to evaluate whether there was merit in a tort claim that a man's 22-year work history as an aluminum welder caused "aluminum pneumoconiosis" and ultimately caused or contributed to lung cancer from which he died. To assess "specific causation", we reviewed the available medical records, published scientific literature, and other information, then ask five questions: 1) evidence of exposure?; 2) known toxicology?; 3) onset of claimed symptoms consistent with known toxicology?; 4) resolution of claimed symptoms consistent with known toxicology?; and 5) relevance of alternative causes? Microscopic and chemical analysis of lung tissue confirmed extremely high levels of welding fume particles. Opposing experts argued that these particles caused pulmonary fibrosis that severely restricted the full expansion of the man's lung (i.e., a restrictive lung disease) and lung cancer, citing published data of lung fibrosis in aluminum flake workers (which they referred to generically as "aluminum pneumoconiosis") and lung cancer in aluminum smelter workers. After reviewing the medical records and published literature, it was demonstrated 1) that the exposure characteristics of aluminum welders are substantially different from those of aluminum flake workers and aluminum smelter workers; 2) that the Plaintiff's pulmonary problems were primarily obstructive lung disease and bronchogenic carcinoma most likely due to his long history of heavy cigarette smoking; and 3) that the Plaintiff's development of a "barrel chest" (hyper-expansion of the lungs secondary to his emphysema) confirms that aluminum welding fume (like iron welding fume) does not cause pulmonary fibrosis formation to an extent that results in a clinically significant pneumoconiosis that restricts lung expansion.

## 1127

### PHYSICAL AND CHEMICAL CHARACTERIZATION OF BERYLLIUM PARTICLES FROM SEVERAL WORKPLACES IN QUEBEC - PART A: DETERMINING METHODS FOR THE ANALYSIS OF LOW LEVELS OF BERYLLIUM.

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Chemical and physical characterizations of beryllium particles found in dust samples from four industries based in Quebec were attempted using a variety of analytical methods. These analyses were deemed important based on the hypotheses that: different chemical forms of Be do not present the same risks; different morphologies lead to different risks; the 2 ug/m3 standard value does not adequately protect workers from all chemical forms and furthermore the 2 ug/m3 standard value does not adequately protect the workers from all PM sizes and forms. Standards were used to prove the adequacy of the chosen methods prior to the analyses of industrial samples. However, low concentrations of beryllium in samples were a limiting factor for most methods: few detected Be in industrial samples. Only time-of-flight secondary ion mass spectrometry (TOF-SIMS) was able to detect Be in industrial samples as well as inductively coupled plasma-mass spectroscopy and flame atomic absorption spectrophotometry. Quantification of Be and characterization of Be particles determined that particle numbers are very high, even for concentrations below 100 ppm. Furthermore, Be seems to be present as fine particles of Be metal, possibly mechanically aggregated to larger particles or compounds such as cryolite. Other major elements present in the samples are limited to Na, Al, Ca, and F. We conclude that particle number represents an important exposure metric and that TOF-SIMS is a new valid method for characterization studies of Be. Funding for this research was provided by the Institut de recherche Robert-Sauve en sante et en securite du travail (IRSST).

## 1128

### PHYSICAL AND CHEMICAL CHARACTERIZATION OF BERYLLIUM PARTICLES FROM SEVERAL WORKPLACES IN QUEBEC, CANADA. PART B: TIME-OF-FLIGHT SECONDARY ION MASS SPECTROSCOPY.

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The problematic of detecting and characterizing beryllium particles in industrial samples from Quebec (Canada) was already addressed by our research group. The present study is a continuation of the work aimed at redefining the current occupational exposure level for beryllium. The goals are to determine the principal chemical forms and the principal physical characteristics of Be particles sampled in four Quebec industries, using time-of-flight secondary ion mass spectroscopy (TOF-SIMS). Most of the samples gave similar results. For dust samples with concentrations of Be varying from 60 to 140 ug/g, the physical characteristics are convergent: numerous fine beryllium particles or aggregates evenly dispersed throughout the samples. Beryllium particles or aggregates are spread out homogeneously. Beryllium does not appear to be concentrated in large particles, but evenly distributed in numerous fine particles. However we were not able to confirm if these fine particles are combined to specific compounds, chemically or physically, or independent Be particles. Most of the particles containing Be are fine, with diameters less than 10 um, which is important from an occupational health and safety standpoint. TOF-SIMS is an appropriate technique for qualitative characterization of Be particles, a complement to the recognized quantitative methods ICP-MS and SAAF. Funding for this research was provided by the Institut de recherche Robert-Sauve en sante et en securite du travail.

## 1129

### HUMAN SUSCEPTIBILITY GENE CONFFERS BERYLLIUM HYPERSENSITIVITY ON FVB MICE.

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Beryllium (Be) is an indispensable metal for many national defense programs in aerospace, electronics, and weaponry due to its unique physico-chemical properties. Exposure to beryllium is an occupational hazard that can cause Chronic Beryllium Disease (CBD), an irreversible, debilitating granulomatous lung disease, in as many as 3-5% of exposed workers. CBD begins as an MHC Class II-restricted, Th1 hypersensitivity, and the Human Leukocyte Antigen, *HLA-DPB1 E<sup>69</sup>*, is associated with risk of developing CBD. Because inbred strains of mice are unable to contract CBD, we developed *HLA-DPB1 E<sup>69</sup>* transgenic mice. Each transgenic contains a single haplotype of *HLA-DPB1* that confers a different magnitude of risk for CBD: *HLA-DPB1\*0401* (OR = 0.2), *HLA-DPB1\*0201* (OR = 15), and *HLA-DPB1\*1701* (OR = 240). We employed the mouse ear swelling test to determine if these genes would support a hypersensitivity response to beryllium. The wild type FVB inbred strain provided the control. In three separate experiments, mice were placed into one of three groups: control/control (skin sensitized with vehicle and challenged with vehicle), control/Be (skin sensitized with vehicle and challenged with beryllium sulfate), and Be/Be (skin sensitized with beryllium sulfate and challenged with beryllium sulfate). In the *HLA-DPB1\*1701* mice, the strain with the highest risk transgene, the Be/Be group displayed increased ear thickness 24h post-challenge, an average of 12.3% over the baseline measurement within group and compared to 2.8 and 2.4% in the control/control and control/beryllium groups, respectively. No significant changes were observed in the other strains for any treatment condition. These results suggest that the *HLA-DPB1\*1701* transgene product is responsible for inducing a beryllium-sensitive phenotype and that this model will be a useful tool for investigating beryllium sensitization.

## 1130

### THE ROLE OF HYPOXIA INDUCIBLE FACTOR 1 $\alpha$ IN COBALT CHLORIDE INDUCED CELL DEATH IN MOUSE EMBRYONIC FIBROBLASTS.

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Cobalt has been widely used in the production of alloys, paints and batteries and in the treatment of anemia and as a hypoxia mimic in cell culture. It is known to activate hypoxic signaling by stabilizing the hypoxia inducible transcription factor 1 $\alpha$  (HIF1 $\alpha$ ) and cobalt exposure can lead to tissue and cellular toxicity. The present

study was conducted to determine the role of HIF1 $\alpha$  in mediating cobalt-induced toxicity. HIF1 $\alpha$  null mouse embryonic fibroblasts (MEFs) were used to show that HIF1 $\alpha$  protein can influence cobalt-induced cytotoxicity. In addition, the BH3 domain containing cell death gene, BNip3, is upregulated by cobalt chloride treatment in a HIF1 $\alpha$ -dependent manner. The cobalt-induced upregulation of BNip3 expression is time and dose dependent and directly correlated with an increase in BNIP3 protein levels. The time and dose dependent increase in BNIP3 levels also correlates with the presence of chromatin condensation, one marker for apoptosis. Interestingly, this increased chromosomal condensation was not coupled to caspase-3 activation as usually seen in a typical apoptotic response. Modulation of BNip3 expression levels correlated with toxicity upon cobalt chloride exposure. These results show that HIF1 $\alpha$  is playing a major role in mediating cobalt-induced toxicity in mouse embryonic fibroblasts and may offer a possible mechanism for the underlying pathology of injuries seen in workers exposed to environmental contaminants such as cobalt.

### 1131 STUDIES OF MECHANISMS OF POTASSIUM DICHROMATE-INDUCED NEPHROTOXICITY IN ISOLATED RAT RENAL CORTICAL SLICES.

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The kidney is considered to be one of the target organs for Cr(VI) toxicity. The information regarding the mechanisms of Cr(VI)-induced nephrotoxicity is at present very limited. The present study was carried out to investigate the mechanisms of Cr6+(using potassium dichromate)-induced toxicity in isolated rat renal cortical slices. The dichromate (Cr6+) showed both concentration (0-4 mM) and time (0-3 hr) dependent toxicity, as measured by the MTT assay. The dichromate (1 mM) produced both time-dependent significant depletion of nonprotein sulfhydryl contents and significant increase in lipid peroxidation (LPO), which appeared much later than the former. Pretreatment of cortical slices with either mannitol or dimethylthiourea failed to reduce Cr6+-induced toxicity but reduced LPO. Similarly, pretreatment with either catalase or superoxide dismutase failed to reduce such toxicity. However, treatment with either excess glutathione (GSH) or pretreatment with excess ascorbic acid (Vit.C) reduced both the toxicity and LPO. Although Cr6+ induced mitochondrial permeability transition (MPT), cyclosporin A, or carnitine, or trifluoperazine (inhibitors of MPT) failed to prevent such toxicity, suggesting a non-significant role of MPT in such toxicity. Cotreatment with some modulators of intracellular calcium fluxes failed to prevent such toxicity indicating a nonsignificant role of calcium homeostasis. Pretreatment with carmustine failed to prevent Cr6+-induced toxicity suggesting a nonsignificant role of glutathione reductase activity in such toxicity. The results suggest that depletion of GSH might be a determinant step in the oxidative stress and subsequent nephrotoxicity and that Vit.C is a powerful antioxidant to prevent such toxicity.

### 1132 PROTECTIVE EFFECT OF ALPHA-TOCOPHEROL ON RENAL INJURY CAUSED BY CHROMATE EXPOSURE.

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Chromate Cr(VI) is a common industrial waste product and environmental pollutant. It is also a recognized human carcinogen. Some toxic effects of Cr(VI) exposure have been related with its oxidative capacity. In the kidney, exposure to Cr(VI) has been associated with proximal tubular necrosis, although its mechanism remains unclear. Our aim was to examine acute renal damage caused by the exposure of Cr(VI), to disclose the degree of lipid peroxidation (LPO) caused by this metal and the functional consequences on different nephron segments (glomeruli, proximal and distal tubules). We also assessed the segmental selectivity of the protective effect of  $\alpha$ -tocopherol on these alterations. Wistar female rats (200 g bw) received potassium dichromate (15 mg/kg, sc, single dose). LPO and renal function were monitored on days 0, 1, 2, 3, 7, 10 y 14 after Cr(VI) administration. A second group received  $\alpha$ -tocopherol (125 mg/kg/day, gavage), 5 days before and 7 days after Cr(VI), and were monitored on 0, 2 and 7 days of Cr(VI) exposure. LPO was quantified in renal tissue; creatinine clearance, glucose and sodium fractional excretions, free-water and osmolal clearances were also measured. Results showed oxidative damage 48 h after exposure to Cr(VI) with spontaneous recovery after day 7. Renal function was maximally altered on day 3 and thereafter recovery ensued. Glomerular filtration decreased, glucose and sodium fractional excretions increased and free water and osmolal were affected. These changes showed a similar time-course to that of the oxidative damage.  $\alpha$ -tocopherol-treated group showed less oxidative damage and preservation of proximal tubule function. In contrast, glomerular and distal function did not show difference with the group that received Cr(VI) alone. These results suggest that Cr(VI) causes alterations along different segments

of the nephron and the antioxidant treatment had protective effect only on the injury caused by Cr(VI) on the proximal tubular function. (Funded in part by G34511 Conacyt).

### 1133 CHROMIUM(VI) ACTIVATES STAT3 IN BRONCHIAL EPITHELIAL CELLS.

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Inhaled hexavalent chromium (Cr(VI)) promotes pulmonary diseases through poorly defined mechanisms, and modification of signaling proteins may play a significant role in this etiology. Although several transcription factors have been demonstrated to respond to Cr(VI) exposures, most studies have focused solely on acute changes in NF- $\kappa$ B or NF- $\kappa$ B in combination with activator protein-1 (AP-1). Thus, we hypothesized that non-cytotoxic levels of Cr(VI) may promote pulmonary disease by increasing Stat activity in bronchial epithelial cells (BEAS-2B). A 24 h Cr(VI) (5  $\mu$ M) exposure caused an 8.5-fold increase in nuclear protein binding to the Stat5 *cis*-response element. To validate this finding, BEAS-2B cells were exposed to Cr(VI) for 1, 4 and 24 h. Cr(VI) caused a transient activation of Stat1 at 1 h that was diminished by 4 h. Stat3 remained phosphorylated after 4 h while Stat5 was not activated at any of the time-points. Sub-chronic investigations were initiated to mimic realistic exposure times. After 24, 48, and 72 h, Cr(VI) increased nuclear levels of phosphorylated Stat3, minimally activated Stat1, and had no effect on Stat5. Immunofluorescence microscopy confirmed that Cr(VI) increased nuclear levels of phosphorylated Stat3. Inhibition of Lck, a Src family kinase, with specific siRNA sequences caused a 2-fold reduction in Cr(VI)-induced Stat3 phosphorylation. These data suggest that Cr(VI) activates tyrosine kinases to effect long-term shifts in transcription factor signaling and gene expression, which may underlie disease prone phenotypes in lung epithelium. Supported by NIEHS grant ES10638.

### 1134 CARCINOGENIC LEAD CHROMATE INDUCES DNA DOUBLE-STRAND BREAKS AND ACTIVATES ATM KINASE IN HUMAN LUNG CELLS.

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Hexavalent chromium (Cr(VI)) is a widespread environmental contaminant and a known human carcinogen, generally causing bronchial cancer. Studies have shown that it is the particulate forms of Cr(VI) that are the potent carcinogens. Particulate Cr(VI) is known to induce a spectrum of DNA damage such as DNA single strand breaks, Cr-DNA adducts, DNA-protein crosslinks and chromosomal aberrations. However, particulate Cr(VI)-induced DNA double strand breaks (DSBs) have not been reported. Thus the aim of this study is to determine if particulate Cr(VI)-induces DSB in human lung cells. Using the single cell gel electrophoresis assay (comet assay), we found that lead chromate (LC) induced concentration dependent increases in DSBs with 0.1, 0.5, 1, 5 and 10  $\mu$ g/cm<sup>2</sup> lead chromate inducing a 0.21, 0.50, 0.62, 1.10 and 1.68-fold relative increase in tail integrated intensity ratio, respectively. To begin to understand the mechanisms in the repair of these lesions, we detected H2AX foci formation in cells treated with LC. H2AX becomes rapidly phosphorylated (r-H2AX) on residue serine 139 and forms foci surrounding damaged DNA in response to DSBs. r-H2AX foci formation is believed to be a sensitive signal for the existence of DSBs. By using immunofluorescence, we found that lead chromate induced concentration-dependent increases in r-H2AX foci formation with 0.1, 0.5, 1, 5 and 10  $\mu$ g/cm<sup>2</sup> LC inducing a 1.4, 1.5, 2.1, 2.2, 2.4 fold increase relative to control, respectively. The ataxia telangiectasia mutated (ATM) protein plays a central role in early stages of DSBs detection and controls cellular responses to this damage. We also found that LC activates ATM at concentrations that are both genotoxic and cytotoxic. Future experiments will focus on elucidating the role of ATM and related genes in the mechanism of DSBs repair. This work was supported by NIEHS grant ES10838 (J.P.W.).

### 1135 REPAIR OF CHROMATE-INDUCED CHROMOSOME DAMAGE IS MEDIATED BY XRCC1.

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Chromium (VI) compounds are established human carcinogens that induce chromosomal aberrations and DNA damage. The mechanisms by which this damage is repaired is unknown. DNA repair mechanisms include several proteins necessary

for the successful repair of DNA damage. XRCC1 is a protein involved in the repair of DNA single strand breaks (SSB). In this study we examined the role of XRCC1 in cell survival and repair of chromosomal aberrations induced by particulate and soluble chromium compounds in Chinese hamster ovary (CHO) cells. Three different cell lines were used: AA8 (parental), EM9 (XRCC1 mutant) and H9T3 (EM9 complemented with human XRCC1 gene). Results show a concentration-dependant decrease in relative survival in all cell lines. Relative survival in AA8 cells treated with 0.1, 0.5, 1.0, 5.0  $\mu\text{g}/\text{cm}^2$  lead chromate (LC) was 103, 98, 98 and 83%, and was 89, 82, 69 and 37% in XRCC1 deficient (EM9) cells, respectively. However, relative survival was not different in EM9 compared to AA8 and H9T3 cells treated with 1.0, 2.5, 5.0 and 10.0  $\mu\text{M}$  sodium chromate (SC). LC was clastogenic inducing 0.25, 0.78 and 0.52 aberrations/cell at 0.1, 0.5 and 1  $\mu\text{g}/\text{cm}^2$  respectively in AA8 cells. In EM9 cells, 0.1, 0.5 and 1  $\mu\text{g}/\text{cm}^2$  LC caused 0.61, 0.85 and 0.96 aberrations/cell respectively. Recovery experiments show that repair was efficient in the parental cell line and the number of chromosomal aberrations was not increased, while in EM9 cells total damage was dramatically increased, especially at higher concentrations. Results for the H9T3 cells were similar to those for parental cell line. These data indicate that XRCC1 is important for cell survival of particulate chromate treated cells, but not in sodium chromate treated cells. XRCC1 is required for repair of particulate and soluble chromate induced chromosomal aberrations. Future studies will be directed towards further examining the functional role of XRCC1 in repair of chromium(VI)-induced DNA SSB. This work was supported by NIEHS grant ES10838(JPW).

### 1136

#### IMPAIRMENT OF CHROMIUM-DNA ADDUCT FORMATION BY HYPOXIA.

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The reduction of hexavalent chromium [Cr(VI)] generates several genetic lesions. While mechanisms include both direct metal-DNA interactions and the generation of radical intermediates, the precise role of oxygen in this process is unclear. We studied the formation of Cr-induced lesions in DNA *in vitro* reacted with either Cr(VI) and ascorbic acid (Asc), or Cr(III), under both ambient and hypoxic conditions. Hypoxia had no effect on the kinetics of Cr(VI) reduction by Asc, but decreased, by ~50%, the levels of Cr(VI)/Asc-induced Cr-DNA adducts and DNA polymerase-arresting lesions (PALs) following a 2 hour treatment. In addition, hypoxia impaired the formation of Cr-induced DNA interstrand crosslinks by ~2-fold in plasmid DNA treated with Cr(VI)/Asc. Although the effects of oxygen deprivation were pronounced after 2 hours; following a 20 hour incubation of DNA with Cr(VI)/Asc, Cr-DNA adduct levels were again similar under both ambient and hypoxic conditions indicating that hypoxia reduced the rate at which Cr interacted with DNA, but not the ultimate steady state level of Cr-DNA binding. The inhibitory effect of hypoxia on Cr(VI)/Asc genotoxicity could not be explained by alterations in the reactivity of intermediate Cr species [i.e. Cr(V)] because Cr(III)-induced DNA damage (adducts, PALs, ICLs) was also impaired by hypoxia. Moreover, the production of Cr(V), as detected by electron spin resonance (ESR), was similar in ambient and hypoxic reactions. These effects were specific for Cr(III) because hypoxia did not affect the generation of ICLs by cisplatin, another inorganic DNA crosslinking agent. Taken together, the results from this study reveal a novel oxygen-dependent step in Cr genotoxicity wherein molecular oxygen facilitates the formation of coordinate Cr(III)-DNA complexes.

### 1137

#### ACUTE INHALATION TOXICITY OF TRIMETHYL GALLIUM IN RATS.

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Trimethyl gallium (TMG) is a pyrophoric liquid used in the semiconductor industry. TMG produces a flame or visible aerosol (smoke) when exposed to air, but can produce an invisible aerosol containing TMG degradation products at lower concentrations. There are no inhalation data available on TMG and therefore a screening inhalation study was performed using groups of 10 Crl:CD(SD)IGS BR rats (5/sex/group). Rats were exposed once for 30 minutes in a nose-only exposure system to either TMG (highest non-smoking concentration in air) or air only on Day 0. Gallium (Ga) levels were measured in blood and urine samples collected on Days 1 and 14 and in lung and kidney specimens collected at necropsy on Day 14. Ga was analyzed using Inductive Coupled Plasma Mass Spectrometry. There were no significant clinical observations, body weight effects or gross necropsy findings in

either the control or the exposed groups. On Day 1, exposed group blood and urine Ga levels ( $21 \pm 2$  and  $45 \pm 5 \mu\text{g}/\text{L}$ ) were significantly higher ( $p < 0.05$ ) than those of the control group ( $2 \pm 0.1$  and  $1 \pm 0.3 \mu\text{g}/\text{L}$ ). On Day 14, exposed group blood and urine Ga levels ( $3 \pm 0.2$  and  $8 \pm 1 \mu\text{g}/\text{L}$ ) were still significantly higher ( $p < 0.05$ ) than those of the control group ( $2 \pm 0.1$  and  $0.5 \pm 0.0 \mu\text{g}/\text{L}$ ), however, between day 1 and day 14, blood and urine Ga levels in the exposed group had decreased by approximately 80%. Exposed group lung and kidney samples contained  $66 \pm 12$  and  $47 \pm 11 \text{ ng/g}$  of Ga, respectively. Ga was not detected in control animal lung and kidney samples. The results of this screening study showed that acute inhalation exposure to TMG did not induce observable signs of toxicity. Measurable amounts of Ga in kidney indicated systemic uptake of Ga from the lung, however, this study suggests that the urine is an important excretion route for clearance of inhaled Ga from the body.

### 1138

#### OAT1 TRANSPORTS N-ACETYL CYSTEINE (NAC)-S CONJUGATES OF BOTH METHYLMERCURY AND INORGANIC MERCURY IN MDCK CELLS.

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The renal proximal tubule has been shown to be one of the primary targets where inorganic and organic forms of mercury ( $\text{Hg}^{2+}$ ) accumulate and induce pathologic effects *in vivo*. We recently demonstrated that type II Madin-Darby Canine Kidney (MDCK) cells stably transfected with human organic anion transporter 1 (hOAT1) gain the ability to transport certain thiol-conjugates of  $\text{Hg}^{2+}$ . Based on these findings, the present study was designed to investigate role of OAT1 and amino acid transporters in the basolateral uptake of the potentially relevant NAC conjugate of  $\text{Hg}^{2+}$  (NAC-Hg-NAC) and  $\text{CH}_3\text{Hg}^+$  ( $\text{CH}_3\text{Hg-NAC}$ ). Uptake of both NAC-Hg-NAC and  $\text{CH}_3\text{Hg-NAC}$  was significantly greater in the hOAT1-transfected cells than the control MDCK cells. The  $K_m$  values for transport of NAC-Hg-NAC and  $\text{CH}_3\text{Hg-NAC}$  in hOAT1-transfected cells were  $144 \pm 29 \mu\text{M}$  and  $35.8 \pm 2.77 \mu\text{M}$ , respectively. Transport of both conjugates was also time-dependent and temperature-sensitive. In the presence of well-established inhibitors of OAT1, the uptake of NAC-Hg-NAC and  $\text{CH}_3\text{Hg-NAC}$  were significantly inhibited only in the hOAT1-transfected cells. Significant amount of uptake of  $\text{CH}_3\text{Hg-NAC}$  (but not NAC-Hg-NAC) was also noted in control MDCK cells. In the presence of L-type amino acids (such as leucine, isoleucine, cysteine, phenylalanine, histidine and tyrosine), the uptake of  $\text{CH}_3\text{Hg-NAC}$  was significantly inhibited in both control MDCK and hOAT1-transfected cells. The uptake of  $\text{CH}_3\text{Hg-NAC}$  was further decreased (in an additive manner) only in hOAT1-transfected cells in the presence of leucine & glutarate or leucine & adipate as compare to that in corresponding cells exposed to leucine, glutarate or adipate alone. Overall these findings indicate that the activity of OAT1 is likely responsible for basolateral uptake of both NAC-Hg-NAC and  $\text{CH}_3\text{Hg-NAC}$ . Our data also indicate that amino acid transporters are also involved in uptake of  $\text{CH}_3\text{Hg-NAC}$ .

### 1139

#### MERCURIC CHLORIDE ALTERS BAX-BCL2 RATIO IN LEYDIG CELLS TOWARD APOPTOTIC PATHWAY.

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Bax (Bcl2-associated X protein) and Bcl2 (B-cell CLL/lymphoma 2) are well known countermolecules, which regulate apoptosis with Bax acting as a promoter of apoptosis and Bcl2 as anti-apoptosis. Also, Bax has been shown to form heterodimers with Bcl2. The ratio of Bax to Bcl2 determines death or survival of cells following apoptotic stimuli such as exposure to toxic chemicals. In this study, Leydig cells were exposed *in vitro* to mercuric chloride ( $\text{HgCl}_2$ ) at a dose of 27  $\mu\text{m}/\text{ml}$  for 0, 1, 3, 12, and 24 hours. The ratio of Bax to Bcl2 was then determined by RT-PCR. The results showed that  $\text{HgCl}_2$  significantly up-regulated Bax in a time-dependent manner when compared to the control. In contrast, Bcl2 showed no dose response when compared to the control. The data suggest that  $\text{HgCl}_2$  may induce apoptosis in Leydig cells through the mitochondrial pathway. Furthermore, death of Leydig cells by apoptosis may consequently reduce the amount of testosterone secreted by these cells. Hence, reduced fertility *in vivo* could result from decreased testosterone production due to environmental exposure to mercury. Supported by DHHS/NIH/NCRR/RCMI grant (G12RR03059-17).

### 1140

#### EFFECTS OF SUBCHRONIC EXPOSURE TO MERCURIC CHLORIDE ON RAT FERTILITY AND REPRODUCTIVE PERFORMANCE: A CROSSOVER MATING TRIAL STUDY.

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Our previous multigenerational reproductive and fertility study indicated that exposure of male and female rats to mercuric chloride (MC) resulted in significant differences in fertility indices. The objectives of this study were 1) To determine

which sex, male, female or both, contributed most to the reproductive and fertility effects and 2) To evaluate semen quality by computer-assisted sperm motion analysis (CASA). Three combinations of breeding pairs were used in a crossover mating trial study. The treatment groups were control males (0.0 mg Hg/kg/day) x control females (0.0 mg Hg/kg/day), control males (0.0 mg Hg/kg/day) x high dose females (2.5 mg Hg/kg/day) and control females (0.0 mg Hg/kg/day) x high dose males (2.0 mg Hg/kg/day). The results showed that the fertility and 4-day survival indices were reduced in the combination of treated female x control male to 57% and 88%, respectively. In contrast, the fertility in treated male x control female was only reduced to 75%. The male body weights in the treated group were significantly decreased during the entire treatment period (91 days) when compared to the control. There were no statistical difference between the body weights of exposed and control females except in week two. Testicular, epididymal and prostate weights were significantly decreased when compared with the control. Conversely, uterine and ovarian weights were not different from the control. Motile sperm and progressive motility percentage were significantly decreased when compared with the control. Parameters indicative of sperm motion showed no dose response. The experimental results presented in this study would suggest that subchronic MC exposure in rats affects the reproductive and fertility performance of both male and female rats but the reduction of fertility indices was more severe in the treated female x control male pair. Supported by DHHS/NIH/NCRR/RCMI grant (G12RR03059-17).

**1141 MN INDUCED PULMONARY VEGF-EXPRESSION IN VIVO: A POTENTIAL MECHANISM OF PULMONARY TOXICITY.**

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Toxic effects of pulmonary Manganese (Mn) exposure have largely escaped notice given the invariable overlap with more dominant neurological symptoms. We hypothesize that chronic Mn exposure affects pulmonary Vascular Endothelial Growth Factor (VEGF), whose altered expression over time changes lung vasculature, potentially enhancing pulmonary disease susceptibility. In normal lung, the role of VEGF in vascular function remains to be clarified. VEGF is a potent positive regulator of angiogenesis, the sprouting and growth of new blood vessels from existing vasculature. Its expression is tightly controlled; expression levels are usually low, elevated levels reflect excessive angiogenesis, which is linked to many diseases. In murine lung 3 major VEGF isoforms have been identified. VEGF<sub>120</sub> and VEGF<sub>164</sub> normally have low expression levels. VEGF<sub>188</sub>, the physiologically important isoform, increases during development and remains elevated in adulthood. Here we demonstrate increased pulmonary VEGF<sub>188</sub> and VEGF<sub>164</sub> mRNA levels from exposure to an environmentally relevant Mn concentration. Female FVB/N mice (11-12 weeks) were exposed to MnCl<sub>2</sub> (concentration: 2 ± 0.36 mg/m<sup>3</sup>; particle size: 1.98 ± 0.12 µm) via nose-only inhalation for 6 hours/day. A 5-day exposure produced 2.5 and 2-fold increases in VEGF<sub>188</sub> and VEGF<sub>164</sub> mRNA levels (determined by RT-PCR) respectively. This increase was still detectable 2 and 7 days after exposure cessation. No histological evidence of inflammation was observed in exposed mouse lungs. Mn-induced changes in vasculature seem likely: Endoglin, an endothelial cell surface marker whose expression in the vasculature under normal physiological conditions is low, was also upregulated 2.5-fold. The vasculature in diseased tissues displays multiple abnormalities, which are potentially reversible by therapeutic intervention. Elucidating vascular alterations in response to metals such as Mn may provide insight into the pathophysiology of environmentally relevant pulmonary diseases such as asthma and other chronic airway inflammation diseases.

**1142 A POTENTIAL MECHANISM FOR PULMONARY MANGANESE-TOXICITY: MANGANESE INDUCES PULMONARY VEGF EXPRESSION IN VITRO.**

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The respiratory tract constitutes a major route of entry and absorption for airborne Manganese (Mn) dust and fume particles. Although chronic Mn-exposure causes toxic responses in lung, little is known about the underlying mechanisms that mediate these effects. In non-pulmonary cell lines Mn induces cellular expression of Vascular Endothelial Growth Factor (VEGF) *in vitro*. VEGF is perhaps the most important positive regulator of angiogenesis, the sprouting and growth of new blood vessels from the existing vasculature. Angiogenic activity, which is usually low under normal physiological conditions, contributes to the pathogenesis of many diseases, and elevated VEGF levels frequently correlate with poor prognosis and disease outcome. Here we demonstrate that Mn increases VEGF expression *in vitro* in several human pulmonary epithelial cell lines (A549, Calu-3, NCI-H292). Cells were transiently transfected with a reporter plasmid containing the gene for firefly luciferase under the control of the VEGF wild type-promoter. Twenty-eight hours later, MnCl<sub>2</sub> was directly added to the medium in concentrations ranging from 50

to 1000 µM. The cells were incubated for another 20 hours and then lysed. Analysis of the cell lysates for firefly activity revealed cell- and dose-dependent increases in promoter activity between 1.5 and 3.5-fold. Interestingly in comparison to non-treated controls, exposure to 0.25 mM MnCl<sub>2</sub> for 20 hours increases promoter activity 2-fold for up to 24 hours after Mn is removed. Further, growing the cells in the presence of 0.25 mM MnCl<sub>2</sub> for 2 weeks did not affect their viability. These data suggest that Mn might promote changes in pulmonary angiogenic growth factor expression, which, over time, could affect lung vasculature morphology, leading to enhanced susceptibility to disease. Further studies may provide an insight into the pathogenesis of, and therapeutic targets for, lung diseases such as asthma and other chronic inflammatory airway diseases.

**1143 THE DISTURBANCE OF CELLULAR IRON REGULATION BY SOLUBLE NICKEL EXPOSURE AND ITS POSSIBLE MECHANISMS.**

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Proper cellular iron level is maintained by coordinated actions of different iron metabolic proteins, most of which are post-transcriptionally regulated by iron regulatory protein 1 (IRP-1). IRP-1 gains its binding activity under iron-deplete condition or some stimuli, such as H2O2, nitric oxide or activated protein kinase C. When iron is replete, IRP-1 loses its RNA binding activity and is converted to cytosolic aconitase. Here, we found that nickel exposure decreases cellular aconitase activity and increases IRP-1 binding activity. The increased activity of IRP-1 after nickel exposure resulted in the increase of transferrin receptor mRNA and antagonized the induction of ferritin light chain protein by iron. Direct incubation of cellular extract with nickel does not cause a decrease in aconitase activity, suggesting nickel needs some mediators for its effect. Although nickel is able to induce hypoxia inducible factor 1 alpha and activate protein kinase C, these factors were not found to be responsible for the effects of nickel on aconitase and IRP-1. It was found that nickel exposure decreased total cellular iron in a dose- and time- dependent manner, which likely caused the observed decrease in aconitase activity and the increase of IRP-1 activity. Cotreatment with iron reverted the effect of nickel on cytosolic aconitase and iron regulatory protein 1. Since iron plays important roles in cell growth and energy metabolism, it is expected that the disturbance of cellular iron homeostasis by nickel may have a great impact on the ability of the cell to function properly.

**1144 SOLUBLE NICKEL ALTERS IRON HOMEOSTASIS.**

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Nickel compounds have been identified as human carcinogens/cocarcinogens in both *in vivo* studies and epidemiological evaluations. Exposure of cells to soluble nickel turns on the hypoxic response pathway, which may be involved in carcinogenesis, by stabilizing hypoxia inducible factor-1a (HIF-1α). The stability of the HIF-1α protein is directly controlled by a family of prolyl hydroxylases, whose activity is dependent on iron, ascorbic acid, and 2-oxoglutarate. Here, we study the ability of nickel to affect iron homeostasis by competing with iron for entry into the cell and inhibiting the activity of iron dependent enzymes. Using a human cell line (HEK293) with a tetracycline inducible vector expressing divalent metal ion transporter-1 (DMT1), we show that nickel is transported into the cell via DMT1. In addition, we demonstrate that soluble nickel can compete with iron for DMT1-dependent entry into the cell. Levels of total iron in A549 cells, a human lung cancer line, were then analyzed using graphite furnace atomic absorption after exposure to soluble nickel. Exposure to soluble nickel decreased total iron levels in a dose and time dependent manner. Since total iron levels were lower, we expected that the activity of iron dependent enzymes would also be affected. The enzyme activity of aconitase, catalase and prolyl hydroxylase were all decreased by exposure to soluble nickel. Interference with iron dependent processes may be involved in nickel-induced carcinogenesis. Our data may give new insight into the mechanisms of nickel-induced carcinogenesis, as well as, contribute important information for the treatment and prevention of occupational diseases.

**1145 CHRONIC EXPOSURE TO LEAD IONS FROM LEAD CHROMATE INTERFERES WITH NORMAL MITOTIC PROGRESSION.**

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Hexavalent chromium (Cr(VI)) compounds are established human lung carcinogens. Solubility plays a key role in the carcinogenicity of Cr(VI), with the most potent carcinogens being the particulate Cr(VI) compounds, however, it remains un-

known why. Lead chromate(LC) and sodium chromate(SC) were used as model particulate and soluble Cr(VI) compounds, respectively. We tested the hypothesis that the Pb cation may disrupt normal mitotic progression after chronic exposures to LC, by determining the presence of c-anaphase cells after exposure to LC, SC and lead glutamate (LG). LC induced concentration and time-dependent increases in intracellular Cr ions, but only concentration-dependent increases in intracellular Pb ions. LC induced concentration-dependent increases in chromosome damage after a 24 h exposure, but only a small increase in c-anaphase cells. However, more chronic exposures to LC induced a greater increase in c-anaphase cells. Specifically, after exposure to 0.5  $\mu\text{g}/\text{cm}^2$  LC for 24, 48 and 72 h, c-anaphase cells increased from 1 to 6 to 16%, respectively, with none in the control. Concentrations of 0.5, 1 and 2.5  $\mu\text{M}$  SC, which have similar intracellular Cr levels as the LC treatments, did not induce c-anaphase cells indicating that the Cr ions are not causing this effect. We next selected soluble Pb ion treatments (50 and 100  $\mu\text{M}$  LG), that mimicked the amount of Pb ions inside the cell after LC exposure and found that Pb ions did induce an increase in c-anaphase cells. Specifically, 50 and 100  $\mu\text{M}$  LG induced a 3.5 and 6.5% increase in c-anaphase cells, respectively. These data indicate that chronic exposure to Pb ions from LC disrupts normal mitotic progression in human lung cells and may be exerting an epigenetic effect as part of the overall carcinogenic mechanism of lead chromate. This work was supported by NIEHS grant ES10838 (J.P.W.).

## 1146

### MICRONUCLEUS FREQUENCY IN BLOOD LYMPHOCYTES FROM CHILDREN ENVIRONMENTALLY EXPOSED TO LEAD.

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Lead has been classified as a possible human carcinogen by the EPA since results from animal models had shown genotoxic effects. However, in adult humans results are controversial and studies to determine genotoxic effects in children environmentally exposed are not available. Thus, we evaluated micronucleus (MN) frequencies in peripheral blood lymphocytes of 47 children from a rural area in a mining zone in Mexico. We used a cross-sectional study design in boys and girls aged 6 to 10 years environmentally exposed to different levels of Pb. Information on socio-demographic status and factors affecting exposure were obtained by parent interviews. Blood lead concentration (PbB) was evaluated using a Zeeman graphite furnace atomic absorption spectrophotometer. Total arsenic (tAs) was determined in urine by hydride generation atomic absorption spectrophotometry as a possible confounding factor. MN were determined in binuclear cultured lymphocytes. Children had PbB values ranging from 2.8 to 20.4  $\mu\text{g}/\text{dl}$  (mean of 9.7  $\mu\text{g}/\text{dl} \pm 3.8$ ). The average tAs concentration was 15.6  $\mu\text{g}/\text{L}$  (ranging from 4.2 to 64.2  $\mu\text{g}/\text{L}$ ). Four percent of the population had urinary tAs levels above 50  $\mu\text{g}/\text{L}$ . MN found in studied children ranged from 0 to 7 MN, within the range of values found in non-exposed children. Preliminary statistical analysis showed no significant correlations between MN frequencies and lead or arsenic levels. This work was partially supported by FOMIX GUE-2002-C01-5456.

## 1147

### SPERM NUCLEUS UPTAKES LEAD FROM TESTIS AND EPIDIDYMIS, ALTERING SPERM CHROMATIN CONDENSATION.

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Sperm chromatin is reorganized during late stages of spermatogenesis when protamines, basic proteins rich in cysteine residues, replace histones to bind DNA. During epididymal transport, thiol groups of sperm protamines become oxidized into disulfide bonds condensing the DNA into a compact structure. Lead (Pb) has been associated with detrimental effects on sperm chromatin condensation, but this is still under investigation. The aim of this study was to evaluate sperm Pb uptake during testicular development and epididymal maturation and the effects on sperm chromatin. After a chronic exposure (0.075% PbCl<sub>2</sub>/16 weeks, drinking water), adult male mice (NMRI, n=8) were sacrificed and sperm from caput epididymis (CE) and cauda epididymis-vas deferens (E-VD) were obtained. Chromatin condensation in sperm from E-VD was measured by the SCSA parameter: High DNA Stainability (HDS). Lead in sperm, in reproductive tissues and in blood (PbB) was measured by AAS. Lead levels in sperm from E-VD were significantly higher (28%) than those in sperm from CE, showing that sperm uptakes Pb from testis, but also during the passage through the epididymis, where disulfide cross-links occur to condense sperm chromatin. A significant decrease (43%) in HDS was showed in E-CD sperm from the Pb-exposed group, indicating an increase in chromatin con-

densation, maybe by Pb binding to protamine-thiol groups. EDTA-treatment of Pb-exposed sperm decreased the chromatin condensation, indicating that the sperm nucleus accumulates Pb. Lead distribution in the reproductive tissues was: coagulating glands>prostate>seminal vesicles>testes, suggesting that sexual glands may be additional sources of Pb during ejaculation. Finally, PbB levels in Pb-exposed mice were 75.62  $\mu\text{g}/\text{dL}$ , and no alterations in sperm quality were observed. This study shows that epididymis is an additional Pb source to sperm nucleus altering sperm chromatin, probably by the interaction with thiol groups, since Pb binding to sulphydryl groups explains many of its toxic effects.

## 1148

### ASSOCIATIONS AMONG URIC ACID, LEAD BIOMARKERS, RENAL FUNCTION AND GENETIC POLYMORPHISMS IN KOREAN LEAD WORKERS.

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Increasing evidence suggests that both lead and uric acid (UA) may be nephrotoxic at lower levels than previously recognized. Lead is also associated with increased UA; therefore UA may be involved in lead-related nephrotoxicity. UA, blood urea nitrogen, serum creatinine, measured and calculated creatinine clearances, urinary N-acetyl- $\beta$ -D-glucosaminidase (NAG) and retinol-binding protein and lead biomarkers were measured in 803 lead workers. Genetic polymorphisms included  $\delta$ -aminolevulinic acid dehydratase (ALAD), endothelial nitric oxide synthase and the vitamin D receptor. Mean (SD) tibia and blood lead were 37.2 (40.4)  $\mu\text{g}/\text{g}$  bone mineral and 32.0 (15.0)  $\mu\text{g}/\text{dl}$ , respectively. After adjustment, none of the lead biomarkers was associated with UA. However, when effect modification by age on these relations was examined, blood lead was a significant predictor of UA among workers in the oldest age tertile (age > 46.0 years;  $\beta = 0.0111$ ;  $p < 0.01$ ). Effect modification by ALAD genotype on associations between lead dose and UA was also observed in older workers (age > median). Next, the six renal function measures were modeled as outcomes; UA was significantly ( $p < 0.05$ ) associated with all except NAG. Finally, previously published analyses in this population showed tibia and blood lead to be predictors of worse renal function, primarily among workers in the oldest age tertile. In models restricted to the oldest tertile of workers, fewer associations between the lead biomarkers and the clinical renal outcomes were significant ( $p \leq 0.05$ ) following adjustment for UA. These data suggest that older workers comprise a susceptible population for increased UA due to lead; ALAD genotype could be a factor as well. UA may be one, but not the only, mechanism for lead-related nephrotoxicity.

## 1149

### PBPK MODELING OF THE EFFECT OF MICROGRAVITY ON THE RELEASE OF LEAD FROM BONE INTO BLOOD.

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Although ~95% of the body burden of lead (Pb) is stored in the bones and ~1% is in the blood, the adverse cognitive and other systemic effects of Pb exposure correlate with the levels of Pb in blood (PbB). If the release of stored Pb from bone due to microgravity-induced accelerated osteoporosis were to significantly increase astronauts' PbB, it could be a health issue for astronauts. Crewmembers on the International Space Station (ISS) lose an average of ~1% of bone mass/month and may stay on orbit 6 months or more. Measured PbDW levels on ISS normally range from <1 to <10  $\mu\text{g Pb/L}$ , while water from drinking fountains at NASA's Johnson Space Center contains 2 - 7  $\mu\text{g Pb/L}$ . The lead PBPK model developed by Ellen O'Flaherty (Environment Health Persp. 1998, 106: 1495-1503) was modified to calculate PbB resulting from various rates of osteoporosis while exposed to various levels of Pb in drinking water (PbDW). O'Flaherty's default input parameters were modified to reduce the calculated current PbB levels to equal the measured PbB values reported by the National Health And Nutrition Evaluation Survey III (NHANES III) (EPA OSWER #9258.7-52, 2002). NHANES reported an average PbB for the 36 - 45 year-old US population of about 2  $\mu\text{g}/\text{dL}$ . Using the modified PBPK model, estimated astronaut PbB levels were calculated for 1000 days of microgravity and exposure to drinking water containing 0, 1, 5, 10, 15, 25, 50, and 100  $\mu\text{g Pb/L}$ . Predicted astronaut PbB levels increased rapidly when exposed to high levels of PbDW but rapidly decrease again when PbDW levels are reduced. However, the predicted PbB levels for astronauts in microgravity decreased below pre-launch levels when the drinking water contained 0 or 1  $\mu\text{g Pb/L}$ , but the decline was less than it would have been for a person at full gravity exposed to the same PbDW. For US astronauts, the predicted increase of PbB due to microgravity-induced acceleration of osteoporosis does not reach harmful levels and can be completely cancelled by minor reductions in PbDW levels.

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PHARMACOKINETICS OF LEAD (PB) IN SALIVA OF RATS FOLLOWING AN ACUTE ORAL EXPOSURE.

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Biological monitoring for Pb is based upon a determination of blood concentration; however, efforts are underway to use saliva as a non-invasive biomatrix for assessing exposure. The pharmacokinetics of Pb were evaluated in whole blood (WB), red blood cells (RBC), plasma, parotid gland, and saliva following a single exposure to Pb-acetate in rats. Saliva volume, pH, total saliva protein and amylase activity were also determined to evaluate salivary gland function. Five male Sprague-Dawley rats per time-point (0, 0.5, 1, 5, 12 and 24 hr post-dosing) were gavaged with 100 mg Pb-acetate/kg and the animals were anesthetized (ip; ketamine/xylazine) and administered pilocarpine (ip; 1 mg/kg) to induce salivation. Saliva was collected, the animals were humanely sacrificed and samples of blood, parotid gland and saliva were collected, weighed, and processed for Pb analysis by ICP-MS. Saliva protein and amylase were determined spectrophotometrically. Pb was detectable in all samples by 30 min post-dosing. In blood components the average peak Pb concentrations were 8.8 and 6.7  $\mu\text{g}/\text{ml}$  at 1 hr post-dosing for WB and RBC, respectively, and 0.2  $\mu\text{g}/\text{ml}$  at 5 hr post-dosing in plasma. Peak saliva Pb concentration (~0.2  $\mu\text{g}/\text{ml}$ ; 5 hr post-dosing) was comparable to plasma. Overall, the relative concentration of Pb followed the order: WB>RBC>>plasma=saliva. The average amount of Pb in the parotid gland was ~0.04  $\mu\text{g}$  at 30 min and increased to 0.12  $\mu\text{g}$  by 24 hr post-dosing. The average saliva pH decrease to a minimum (8.12) at 12 hr, but returned to control levels (8.45) by 24 hr post-dosing. Average saliva amylase activity likewise decreased from 18 U/ml (control) to 10 U/ml by 24 hr post-dosing; whereas, total saliva protein did not appreciably change over time. These results demonstrate a feasibility to rapidly detect Pb in saliva, and suggest that a high dose of Pb may impact salivary gland function. Future studies will evaluate a prolonged Pb exposure on Pb clearance and salivary gland function. (Supported by NIH/NIESH grant 1 R01 ES010976-01A2)

1151

LEAD DISTRIBUTION FOLLOWING RESPIRATORY EXPOSURE TO LEAD-CONTAINING MATERIALS IN SPRAGUE-DAWLEY RATS.

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Lead (Pb) is a ubiquitous environmental contaminant that especially threatens the health of pre-school age children in the US. Although the major route of Pb exposure is ingestion, inhalation of air-borne Pb also occurs and little is known about the toxicokinetics of Pb following this type of exposure. Two preliminary experiments were conducted with objectives of determining the disposition of Pb in tissues following tracheal instillation (TI) of a Pb-containing soil or lead acetate (PbAc) and comparing the distribution of Pb between TI and oral gavage (GI) routes of exposure. In the first experiment, 54 anesthetized male rats underwent TI of double distilled water (dd water) or 225  $\mu\text{g}$  of Pb/kg of body weight (BW) as either Pb-containing soil or PbAc, and were sacrificed at 0, 3, 6, 12, 24, 48, 72, or 96 hours post-TI. Based on the results of the first experiment, another 50 rats were exposed to dd water, Pb-containing soil (100 or 200  $\mu\text{g}/\text{kg}$  BW), or PbAc (112.5 or 225  $\mu\text{g}/\text{kg}$  BW), either by TI or GI, and were sacrificed at 96-hours post exposure. In each of these experiments, blood, lung, liver, kidney, and bone were collected and analyzed for Pb using atomic absorption spectrophotometry, at a detection limit (dl) of 10 ppb in blood and tissues and .5 ppm in bone. In the first experiment all tissues had measurable Pb concentrations at 96 hours post-exposure and the kidney cortex had the highest Pb concentration of the tissues collected. In the second experiment, all lung Pb concentrations were below the dl and all other tissue Pb concentrations were higher in the TI versus GI groups, with the kidney cortex Pb concentration being approximately 20-fold higher in the TI groups. These preliminary results indicate that respiratory exposure to Pb, even at low doses, will result in elevated tissue and blood Pb concentrations and the kidney is the site of lead accumulation following respiratory tract exposure.

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ROLE OF PLASMA GLUTATHIONE IN HEPATIC TOXICITY CAUSED BY VANADIUM COMPOUNDS IN MICE.

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Ammonium metavanadate (V[5]; pentavalent vanadium compound) is more toxic than vanadyl sulfate (V[4]; tetravalent vanadium compound). It is known that the pentavalent vanadium is reduced to the tetravalent vanadium form by antioxidants. Therefore, the reduction process is regarded as a mechanism for the detoxifi-

cation of vanadium. The present work was undertaken to elucidate the role of plasma reduce glutathione (GSH) in hepatic toxicity caused by vanadium compounds in animals. Male ICR mice were injected with V[5] (0.1-0.3 mmol/kg, s.c.) or V[4] (0.1-0.9 mmol/kg, s.c.) and sacrificed 6 h after the injection. Activities of AST and ALT were increased dose-dependently with both compounds. The hepatic toxicity caused by V[5] was higher than the toxicity by V[4]. The role of GSH in the prevention of toxicity caused by the vanadium compounds, was explored in mice treated with BSO, a specific inhibitor of GSH synthesis. The depression of both intracellular and extracellular (plasma) GSH levels in mice pretreated with BSO, markedly enhanced the hepatic toxicity by V[5] and hepatic vanadium content. In animals treated with V[4], the toxicity and vanadium content was not affected by BSO pretreatment. To gain some insight into the mechanism of enhancement of vanadium toxicity, we examined the effect of GSH administration on the BSO-enhanced hepatic toxicity of V[5]. Contents of GSH in tissues of mice 1 h after administration of V[5] were measured. Levels of GSH in plasma and liver were decreased by the administration of V[5] or BSO. Pretreatment by GSH protected against the decrease of GSH level in plasma by the administrations, but not affected hepatic GSH level. Furthermore the pre-administration of GSH to BSO-treated mice protected against hepatic toxicity of V[5], and decreased the height vanadium accumulation in the liver. These data suggest that plasma GSH plays a protective role by reducing V[5] to V[4], resulting in the decreased hepatic vanadium uptake and toxicity.

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SPLEEN REPERCUSSIONS AFTER CHRONIC INHALATION OF VANADIUM PENTOXIDE (V2O5) EVALUATED IN MICE.

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A previous report from our group informed the presence of thrombocytosis as a consequence of Vanadium inhalation. This time, we decided to evaluate the effects of this element on the mice spleen, to identify the morphological modifications in this hematopoietic organ. CD-1 mice inhaled V2O5 0.02M one hour, twice a week during 12 weeks. Spleens were extracted, weighed, and processed by the usual histological technique. The main changes observed were: an increase in the white pulp and in the amount of megakaryocytes in those mice exposed to V (Figs. 1, 2). These findings explain in part, the thrombocytosis reported previously, and the question about platelets function remains to be elucidated. DGAPA IN\_204304 P.O.S. Institute

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ROLE OF OXIDATIVE STRESS IN THE MODULATION OF AHR-REGULATED GENE EXPRESSION BY HEAVY METALS.

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Since heavy metals, mainly arsenite ( $\text{As}^{3+}$ ), cadmium ( $\text{Cd}^{2+}$ ) and chromium ( $\text{Cr}^{6+}$ ), have been shown to exert their toxicity, at least partly, by the generation of reactive oxygen species (ROS), we evaluated the role of metal-induced ROS on the expression of AHR-regulated genes: Cyp1a1, NAD(P)H:quinone oxidoreductase (QOR), and glutathione S-transferase Ya (GST Ya). For this purpose, Hepa 1c1c7 cells were treated with 5  $\mu\text{M}$  of  $\text{As}^{3+}$ ,  $\text{Cd}^{2+}$  or  $\text{Cr}^{6+}$  in the presence or absence of TCDD (1 nM), an AHR ligand. All three metals caused perturbations in cellular glutathione redox status, but only  $\text{Cd}^{2+}$  and  $\text{Cr}^{6+}$  increased the production of ROS. Although all three metals potentiated the induction of Cyp1a1 mRNA by TCDD, they decreased the stability of the induced protein and inhibited the induction of its activity. Pre-treatment with the thiol antioxidant N-acetylcysteine (NAC) did not alter Cyp1a1 mRNA levels but completely abrogated the inhibition of Cyp1a1 activity induction by all three metals. In parallel, Cyp1a1 mRNA expression was further potentiated whereas Cyp1a1 activity was further inhibited when cells were pretreated with the glutathione synthase inhibitor, L-buthionine-[S, R]-sulfoximine (BSO). Furthermore, all three metals induced the expression of heme oxygenase-1 (HO-1) which was further potentiated with BSO but inhibited with NAC pretreatments. Additionally, metals alone induced Cyp1a1 mRNA expression, which was superinduced in the presence of the protein synthesis inhibitor, cycloheximide. All three metals, alone or in the presence of TCDD, enhanced QOR and GST Ya activities and mRNA levels, an effect that was completely abrogated with NAC pre-treatment and markedly potentiated with BSO. Pre-treatment with the DNA transcription suppressor, actinomycin-D, abolished the induction of QOR and GST Ya mRNA levels by the three metals. Our data clearly show that  $\text{As}^{3+}$ ,  $\text{Cd}^{2+}$ , and  $\text{Cr}^{6+}$

induced ROS production modulates Cyp1a1 activity by decreasing Cyp1a1 protein stability and inducing HO-1, but induces QOR and GST Ya activities at the transcriptional level, likely regulated by the antioxidant response element.

1155

INTEGRATIVE ANALYSIS OF GENOME-WIDE GENE EXPRESSION AND PATHWAY MAPPING IN MOUSE EMBRYONIC FIBROBLAST (MEF) EXPOSED TO CADMIUM, ARSENIC AND METHYLMERCURY: INDUCTION OF OXIDATIVE STRESS, DISRUPTION OF UBIQUITIN-PROTEASOME SYSTEM AND CELL CYCLE REGULATION.

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Environmental and occupational exposures to heavy metals including Methylmercury (MeHg), arsenic (As) and cadmium (Cd) pose significant health risks to humans. Although many toxicological impacts have been associated with these metals exposure, the underlying mechanisms of toxicity have not been clearly defined. We conducted gene expression array analysis by using the CodeLink Mouse UniSet 10K oligonucleotide-based platform to explore the gene alterations caused by metals. We treated mouse embryonic fibroblast cells (MEF) with environmentally relevant doses of MeHg (2.5 $\mu$ M), Cd (5.0 $\mu$ M) and As (5.0  $\mu$ M) for 24 h. A classical proteasome inhibitor, MG132 (0.5  $\mu$ M), was included to compare its pattern of gene expression with metals to determine if disruption of ubiquitin-proteasome system (UPS) is a critical mechanism of metal-induced toxicity. The doses selected were consistent with our previous studies where minimal impacts were observed on stress signaling and apoptotic pathways. Following normalization of the array data, we have employed multi-level analysis tools to explore the data, including group comparisons, cluster analysis and gene annotations analysis using MAPPFinder. Furthermore we applied knowledge-based pathway mapping of our gene expression data by using GenMAPP. Using these integrated approaches, we identified significant alterations in UPS, antioxidant and phase II enzymes, DNA repair and damage pathway and cell cycle regulation pathways across the three metals. The results suggest the critical roles of above pathways in metal-induced toxicity. Furthermore, this study demonstrated that the integration of toxicogenomic and knowledge-based pathway mapping is a powerful approach to elucidate molecular mechanism of toxicity. (Funded by EPA: R826886 and NIEHS: ES09601, ES07033, ES11387 and ES10613)

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EFFECT OF COMBINED LEAD, CADMIUM AND ARSENIC EXPOSURE ON THE EXPRESSION OF CELLULAR PROTECTIVE FACTORS IN RAT KIDNEYS AT LOEL DOSE LEVELS.

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Lead (Pb), Cadmium (Cd) and Arsenic (As) are ubiquitous toxic metals/metalloids in the environment. We have previously demonstrated the oxidative DNA adduct, 8-hydroxy-2-deoxyguanosine (8-OHdG), and induction of heme oxygenase-1 (HO-1), a sensitive indicator of oxidative stress, in rat kidneys in the Cd x As and Pb x Cd x As 180-day treatment group, respectively. We also found that rats displayed compensatory responses when exposed to these elements for 90 days, as compared to 30- and 180- day exposure. The present studies were undertaken to further investigate cellular and molecular mechanisms contributing to these phenomena focusing on the mRNA expression of metallothionein (MT), heat shock proteins (HSPs) and anti-oxidant enzymes. Male Sprague-Dawley rats (15/group) were exposed to deionized drinking water containing Pb (25ppm), Cd (10 ppm), As (5 ppm), or PbxCd, PbxAx, CdxAx or PbxCdAx mixtures for 30, 90, or 180-days. At each time point kidneys were collected and subjected to real-time polymerase chain reactions (RT-PCR) for MT1 and MT2, HSP 32 and HSP60, glutathione peroxidase (GPx) and superoxide dismutase (SOD). At 30 days MT1 and MT2 mRNA expression were down-regulated in the PbxAx group; HSP60 mRNA was up-regulated in the PbxAx group, but down-regulated in the Cd xAs group; GPx mRNA expression was up-regulated in the As, Cd xAs and Pb x Cd xAs groups. At 90 days GPx mRNA expression was up-regulated in the PbxAx, Cd xAs and Pb x Cd xAs groups and SOD mRNA expression was down-regulated in all treatment groups; Further, HSP60 expression was decreased in the Pb and Pb x Cd groups, and MT2 expression was decreased in Cd and As groups. By 180 days SOD was up-regulated in the Cd, Cd xAs and Pb x Cd xAs groups and GPx was up-regulated in the PbxAx group and HSP60 was decreased in the Pb treatment group. These results indicate that single and combined exposure to Pb, Cd and As alters the expression patterns of protective enzymes/proteins in the rat kidneys. (Supported in part by USEPA STAR Grant # 827161)

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COMPARISON OF METALS IN CYTOTOXICITY, FREE RADICAL GENERATION, AND HEAT SHOCK PROTEIN EXPRESSION IN A HUMAN BRONCHIAL EPITHELIAL CELL LINE, BEAS-2B.

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A number of toxic heavy metals such as arsenic (As), chromium (Cr), cadmium (Cd), mercury (Hg), nickel (Ni), vanadium (V), and manganese (Mn) are widely used in occupational settings, and exposure to these metals is associated with the development of pulmonary disease. Cytotoxicity, apoptosis, and reactive oxygen species (ROS) generation were tested to compare the biological reactivity of these heavy metals using a human bronchial epithelial cell line, BEAS-2B. Also, heat shock protein 70 (Hsp70) expression was observed as an early and sensitive biomarker of cellular stress. Exposure to metals (50 microM) for 24 hr caused significant cytotoxicity for all the metals tested. Among the metals tested, As (20%), Cr (10%), Cd (30%), and Mn (44%) showed less than 50% survival rate compared to control cells. Apoptosis was significantly increased in the cells exposed to 50 microM of As (2.2-fold), Cr (4.5-fold), and Cd (2.5-fold). Intracellular ROS generation has the capacity to induce DNA damage, alter signal transduction, and cause lipid peroxidation leading to either apoptosis or carcinogenesis. Electron spin resonance (ESR) was used to detect short-lived free radical intermediates generated in the reaction of metal with cells. Hydroxyl radical generation was greater in the presence of As, Cr, Cd, and Hg compared to the other metals. As, Cd, and Hg showed a high expression of Hsp70 protein in Western blotting and ELISA while Cr, Ni, V, and Mn did not show any significant increase of Hsp70 protein. These results suggest that both cytotoxicity and apoptosis were significant with all metals tested; however As, Cd, Cr, and Hg were relatively most toxic metals tested. Generation of ROS may be involved in metal induced lung cell damage. Metal-induced Hsp70 expression could be a sensitive indicator of lung cell injury by As, Cd, and Hg.

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METAL-INDUCED OXIDATION OF THIOREDOXIN-1 AND THIOREDOXIN-2.

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Exposure to metals can elicit numerous deleterious effects leading to apoptosis, carcinogenesis and a variety of other diseases. Some metals have been described as being reactive and binding to free sulfhydryls en route to toxicity. While much data has been collected regarding the effects of metals on glutathione, the primary intracellular antioxidant, very little is known about the effects of metals on another important intracellular antioxidant system, the thioredoxin system. Here, we evaluate the effects of arsenic, cadmium, cesium, copper, iron, mercury, nickel and zinc on cytoplasmic/nuclear thioredoxin-1 (Trx1) and mitochondrial thioredoxin-2 (Trx2) redox states. HeLa cells were grown to 80% confluence and then treated with either 10 or 100  $\mu$ M of each metal listed above for 4 h. Cells were collected and derivatized with iodoacetic acid for Trx1 and 4-acetamido-4'-maleimidylstilbene-2, 2'-disulfonic acid (AMS) for Trx2 and analyzed following separation of the reduced and oxidized forms via redox Western techniques. Trx1 was significantly oxidized up to +30 mV from control (-278 mV) with arsenic, cadmium and mercury. The other metals tested showed no Trx1 effects. Similarly, arsenic, cadmium and mercury significantly oxidized Trx2 (-352 mV in controls) but significant oxidation was also noted with cesium as well. Copper, iron, nickel and zinc showed no Trx2 effects. Mitochondria are believed to be protected by metallothionein during the toxic, oxidative effects of some metals, including cadmium and mercury. Our findings show that metal exposure can cause both cytoplasmic/nuclear and mitochondrial oxidation as principal effects of metal toxicity.

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INDUCTION OF OXIDATIVE STRESS IN RESPONSE TO INGESTION OF LEAD, CADMIUM AND ARSENIC MIXTURES.

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Human populations are commonly exposed to mixtures of chemicals. To date, relatively few studies have examined potential interactive effects using a statistical factorial design. Multiple drinking water studies were performed to test the hypothesis that exposure to arsenic, lead, or cadmium (or their combinations) for 30, 90, or 180 days at lowest-observed-effect levels (LOELs) results in increased levels of oxidative stress in the kidney, which is a target organ for trace element-induced toxicity. Male Sprague-Dawley rats were exposed to lead, cadmium, arsenic, or mixtures of these three trace elements for 30, 90, or 180 days via drinking water. Oxidative stress levels (as measured by increases in kidney carbonyls) were generally increased at 30 days and decreased at 90 and 180 days. At 30 and 180 days, cadmium appeared to attenuate carbonyl increases among mixture groups. Among all treatment

groups, increases in kidney carbonyls were lowest among the PbxCdxAs group at all three timepoints. Cellular adaptation to trace element-induced oxidative stress is suggested by the attenuation of increases in kidney carbonyls at the 90 and 180 day timepoints. Statistically significant increases in kidney glutathione levels (measured as nonprotein thiols) were measured after 30 and 180 days of exposure among most treatment groups, with some of the greatest increases measured among the four combination groups at the 30 day timepoint (96%-145% increase) and the 180 day timepoint (20%-70% increase). In contrast, kidney non-protein thiols were statistically significantly decreased in 4 of 7 treatment groups after 90 days of exposure (28%-33% decrease). These data demonstrate that low-level exposure to trace elements or their mixtures results in measurable increases in oxidative stress and up-regulation of cellular defensive mechanisms [Supported by USEPA Star Grant R827161-01-0].

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### CHANGING METAL ACCUMULATION IN NEW ORLEANS: DIFFERENCES BETWEEN SURVEY I (1992) AND SURVEY II (2000).

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New Orleans soils have been surveyed and mapped twice. Survey I was completed in 1992 and Survey II was completed in 2000. This study evaluates the possibility for change between the two surveys. All samples were collected in residential neighborhoods at least one block from a busy street. The Survey I collection had 4, 026 samples stratified by 283 census tracts and Survey II had 4, 389 samples stratified by 286 census tracts. The extraction methods used the same protocol for pH (1 M Nitric acid), room temperature and 2 hour shaker time. They differed in amount of sample extracted, 4.0 g (Survey I) compared with 0.4 g (Survey II). ICP-AES techniques were used to measure 8 metals. The analytical results of the extraction methods were evaluated with homogenized soil samples from the Wageningen Evaluating Programs for Analytical Laboratories, International Soil-analytical Exchange (WEPAI; ISE). All correlation results were linear and the Survey I results were converted to make them equivalent with the Survey II results. Geographic Information Science (GIS) evaluation was done by assigning a median soil metal result to the centroid of each census tract; Kriging interpolation of the above data with Surfer; Importing ASCII grids into ArcView GIS using the grid machine extension; Forming a new grid by Survey II divided by Survey I grid cells. Changes in metals were observed. Most prominent were increases in the inner city Pb, Zn, Cu. Amounts of Cr, Cd and Mn appear to be about the same, and V, and Ni decreased. Reasons for increases of Pb and Zn include: Power sanding of New Orleans old wood homes with Pb-based paint; Zn is a constituent of tires and paints. The suburb lacks similar metal sources and new soils are commonly added for landscaping purposes. Overall, residential soil metals appear to undergo a relatively high rate of change. Pb, Zn and Cu appear to continue accumulating in the inner-city.

## 1161

### TOXICOGENOMICAL STUDY ON HUMAN BLADDER EPITHELIUM EXPOSED TO ARSENIC.

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In this study, human urothelium (UROtsa) cells were exposed to arsenite [As(III)-30  $\mu$ M], monomethylarsenous acid [MMA(III) 1-5  $\mu$ M], or buthionine sulfoximine [BSO-25  $\mu$ M] followed by [As(III)-1  $\mu$ M] for up to 18 hr and the changes in gene expression determined by using a human oligonucleotide chip (18, 861 genes). The hybridizations were performed at least three times using independent total RNA preparations to ensure reproducibility. Differentially expressed genes were identified based on 2-fold cut off and gene significantly different from the control cells (t-test analysis,  $p<0.05$ ). Only up-regulated genes have been assessed. Both As(III) and MMA(III) treatments produce an oxidative stress response. Interestingly, MMA(III) exposure for 6 hr did not induce any metallothionein genes but induced numerous unique genes [such as dual specificity phosphatase (DUSP1, DUSP2); CDC like kinase (CLK1); DNA damage inducible transcript (DDIT3)] and even caused greater increases in stress genes [e.g. heat shock protein (HSP46, HSPA1A); DnaJ(HSP40)]. Reduction in cellular GSH content via BSO treatment followed by As(III) exposure for 6 hr exacerbate the gene expression-modifying effects of arsenite on UROtsa cells. The induction of heat shock protein (HSP1A1, HSPA6); metallothionein (MT1G); and solute carrier family (SLC30A) genes revealed synergistic effects of cytotoxicity for both As(III) and BSO. These results indicate that oxidative stress must be a common pathway in cellular response to exposure to different arsenicals. Furthermore, As(III) and MMA(III) induce genes involved in similar but different pathways. The absence of metallothionein gene induction by MMA (III) exposure may demonstrate different mechanism of recognition by the UROtsa cells or mechanism of toxicity (NIEHS 04940, SWEHSC 06694 and NCI 023074).

## 1162

### DIFFERENTIAL EFFECTS OF CHRONIC LOW LEVEL ARSENIC EXPOSURES ON TRANSCRIPTION FACTOR BINDING IN CARDIOVASCULAR TISSUES.

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Chronic exposure to low levels of trivalent arsenic in drinking water promotes differential induction of cardiovascular genes, vascular remodeling, and vascular disease. To resolve the signaling pathways involved in arsenic-induced phenotypic change, proteomic analysis of transcription factor DNA binding arrays in nuclear extracts from mouse hearts or cultured porcine vascular smooth muscle cells were examined. *In vivo*, mice fed 10-50 ppb of sodium arsenite (AsIII) in their drinking water for 5 weeks had increases in nuclear proteins binding to AP-1, Ets, FKHR, GATA, HIF and Stat consensus DNA cis elements. Nuclear extracts from smooth muscle cells had similar, but more limited increases in the binding of these transcription factors. In contrast to previous reports for higher levels of exposure, there were no significant increases in stress response factors, NF- $\kappa$ B, or p53 DNA binding in either model with these AsIII exposures. AsIII exposures also decreased nuclear levels of a significant number of transcription factors. Gel mobility shift assays and western analysis of proteins from multiple animals and cell cultures were used to confirm increases seen in the array analyses. Genes induced by these AsIII exposures include vascular endothelial cell growth factor, plasminogen activator inhibitor-1, endothelin-1. These data demonstrate that chronic, low dose AsIII exposures activate multiple interacting signaling cascades to transcriptionally promote cardiovascular vascular remodeling and response genes. Supported by NIEHS SBRP grant ES07373

## 1163

### EUKARYOTIC TRANSLATION INITIATION FACTOR 4E IS A CELLULAR TARGET FOR ARSENIC BUT NOT CHROMIUM TOXICITY.

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The mRNA cap binding translation factor, eukaryotic translation initiation factor 4E (eIF4E), was studied as a potential cellular target for the toxicity and cell death induced by arsenic and chromium. Human cell lines HCT15, PLC/PR/5, HeLa and Chang, were treated with sodium arsenite (As) and potassium dichromate (Cr). Exposure to both As and Cr resulted in significant cytotoxicity and cell death in all four cell lines. In all the cell lines treated with As and Cr, the transcript for eIF4E did not exhibit any change compared with the corresponding control cells. However, in the cells treated with As alone, the cellular expression level of eIF4E protein was significantly lower compared to the corresponding control cells. Further studies revealed that exposure of cells to As, but not to Cr, resulted in significant induction of ubiquitination of eIF4E protein. Results of the experiments involving inhibitors for the cellular proteasome pathway confirmed that the exposure of As but not Cr activated the proteolysis of eIF4E mediated through the ubiquitin/proteasome pathway. Whether the As-induced cytotoxicity and cell death were due to the inhibition of eIF4E was studied by specifically silencing the expression of eIF4E gene using a small interfering RNA (SiRNA) targeting eIF4E gene expression. The SiRNA-mediated silencing of eIF4E gene resulted in cytotoxicity and cell death suggesting that eIF4E is a potential cellular target for cytotoxicity and cell death due to exposure to As.

## 1164

### EFFECT OF ARSENITE ON PU.1, C/EBPS, AND NFKB ACTIVATION IN U937 PROMONOCYTIC LEUKEMIA CELLS.

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Arsenite has been reported to exert dose-dependent dual effect; triggering apoptosis at relatively high concentration, whereas it induces partial differentiation at low concentration in leukemia cells. However its bilateral character regarding the molecular mechanisms remains to be clearly defined. We examined the effect of arsenite on key transcription factors for macrophage differentiation such as PU.1 and C/EBPs to find out how arsenite interacts with the signaling pathways for differentiation in U937 promonocytic leukemia cells. Electrophoretic mobility shift assays were used to analyze the interaction between arsenite induced signaling and these transcription factors. In addition the phorbol ester TPA which activates PKCs was compared with arsenite as a second type of differentiation inducer for leukemia cells. The PU.1 activation was not changed in the presence of 1  $\mu$ M or 10  $\mu$ M arsenite for 3, 6, or 24-hrs exposure. On the other hand, activation pattern of PU.1

was drastically changed in the presence of 10 nM TPA after 24-hrs exposure. Among C/EBPs, C/EBP $\alpha$  and C/EBP $\epsilon$  were slightly affected by arsenite, but their activation were suppressed by TPA after 24-hrs exposure. NF- $\kappa$ B was constitutively activated by serum free conditions in U937 cells and this activation was suppressed by arsenite. These results indicate that arsenite and TPA interact with key regulators by different pathway in monocytic differentiation of U937 cells.

## 1165 IDENTIFICATION OF MOUSE SLC39A8 AS THE TRANSPORTER RESPONSIBLE FOR CADMIUM-INDUCED TOXICITY IN THE TESTIS.

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Testicular necrosis is a sensitive endpoint for cadmium (Cd++, Cd) toxicity across all species tested. Resistance to Cd-induced testicular damage is a recessive trait assigned to the Cdm locus on mouse chromosome 3. We first narrowed the Cdm gene-containing region to 880 kb. SNP analysis of this region from two sensitive and two resistant inbred strains demonstrated a 400-kb haplotype block consistent with the Cd-induced toxicity phenotype; in this region is the Slc39a8 gene encoding a member of the solute-carrier superfamily. Slc39a8 encodes SLC39A8(ZIP8), whose homologs in plant and yeast are putative zinc transporters. We show here that ZIP8 expression in cultured mouse fetal fibroblasts leads to a >10-fold increase in the rate of intracellular Cd influx and accumulation and a 30-fold increase in sensitivity to Cd-induced cell death. The complete ZIP8 mRNA and intron-exon splice junctions have no nucleotide differences between two sensitive and two resistant strains of mice; using *in situ* hybridization we found that ZIP8 mRNA is prominent in the vascular endothelial cells of the testis of the sensitive strains of mice, but absent in these cells of resistant strains. Slc39a8 is therefore the Cdm gene, defining sensitivity to Cd toxicity specifically in vascular endothelial cells of the testis.

## 1166 ROLE OF EUKARYOTIC TRANSLATION INITIATION FACTOR 4E (eIF4E) IN CADMIUM-INDUCED CYTOTOXICITY AND CELL DEATH.

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The role of eukaryotic translation initiation factor 4E (eIF4E), in cadmium-induced cytotoxicity and cell death was investigated. Exposure of human cell lines HCT15, PLC/PR/5, HeLa and Chang, to cadmium chloride (Cd) resulted in a dose-dependent toxicity and death. Western blot analysis of the cells demonstrated a significant inhibition of eIF4E gene (protein) in response to Cd exposure. Whether the inhibition of eIF4E was responsible for the observed toxicity and death was studied by silencing the cellular expression of eIF4E gene by employing a small interfering RNA (SiRNA) specifically targeting the eIF4E gene. The SiRNA-mediated silencing of eIF4E gene expression resulted in significant cytotoxicity and cell death suggesting that the cytotoxicity and cell death noticed among the Cd-treated cells were probably due to the chemical-induced inhibition of eIF4E gene expression. Transgenic Chinese hamster ovary cell lines overexpressing eIF4E were resistant to Cd-induced cytotoxicity and cell death. Results of Western blot analysis and immunoprecipitation experiments demonstrated a significant induction of ubiquitination of eIF4E in the Cd treated cells. Pre-exposure of cells to proteasome inhibitors blocked the Cd-induced inhibition of eIF4E gene expression as well as the resulting cytotoxicity and cell death. Furthermore, exposure of cells to Cd resulted in a significant inhibition of expression of the cell cycle and growth regulating gene, cyclin D1. Transfection of cells with SiRNA specifically targeting eIF4E gene expression also resulted in a significant inhibition of cyclin D1 gene expression suggesting that the observed inhibition of cyclin D1 gene in the Cd-treated cells is most likely mediated through inhibition of eIF4E gene. Taken together, our results demonstrate that the exposure of cells to cadmium chloride resulted in cytotoxicity and cell death due to the enhanced ubiquitination and proteolysis and the consequent inhibition of eIF4E gene expression leading to diminished cellular level of critical genes such as cyclin D1.

## 1167 MECHANISMS OF ARSENITE-STIMULATED HEMOXYGENASE-1 UPREGULATION IN HUMAN KERATINOCYTES.

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Hemeoxygenase-1 (HO-1) is the rate-limiting enzyme involved in heme catabolism. It is an oxidative stress responsive gene upregulated by various physiological and exogenous stimuli including heme, ultraviolet irradiation, heat shock, inflam-

matory cytokines, heavy metals and arsenite. HO-1 has many stress-activated recognition sites in the promoter region of its gene. These include NF- $\kappa$ B, heme response elements, antioxidant response elements and activator protein 1 (AP-1). Arsenic is a known dermatotoxin and chronic exposure has been associated with increased incidence of keratinocytic tumors. The mechanism of arsenic-mediated skin carcinogenesis is not well-understood, but activation of mitogen-activated protein kinases (MAPKs) and generation of reactive oxygen species (ROS) may contribute to tumor promotion and progression. We have reported that arsenite (AsIII)-dependent activation of ERK, but not p38, is dependent upon EGF receptor activity. In this study we investigated the potential contributions of ROS generation and AP-1 activation to AsIII-dependent regulation of HO-1 in HaCat cells, a spontaneously immortalized human keratinocyte cell line. Both EGF and AsIII induced ROS as observed via dihydroethidium (DHE) staining and fluorescence microscopy. Western blotting showed arsenite-induced sustained upregulation of HO-1 in a time-dependent (0-72h) and concentration-dependent (3-30 $\mu$ M) manner. Inhibition of EGF receptor, MEK I/II, and p38 activation moderately reduced HO-1 expression, but none completely abrogated the arsenite-induced response suggesting that the signaling proteins (EGF receptor, ERK and p38) were necessary but not sufficient for AsIII-induced HO-1 upregulation. Inhibition of Src-family kinases also slightly reduced HO-1 induction indicating a small contribution to the observed induction. The superoxide scavenger MnTTPyP also partially decreased the arsenite-induced expression of HO-1. These results suggest that both the stress-activated and EGF receptor pathway are involved in regulation of HO-1 expression in response to arsenite in keratinocytes.

## 1168 MERCURY, CADMIUM, ZINC, AND ARSENITE INHIBIT PAX3 DNA BINDING VIA THE PAIRED DOMAIN.

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Mercury, cadmium, zinc, and arsenite are common metallic environmental contaminants that have high affinities for endogenous thiols, such as glutathione and protein thiols. Pax3 is a murine transcription factor that is involved in a number of developmental processes, including neural tube closure. Using the Splotch mouse model, it has been shown that Pax3 haploinsufficiency confers added sensitivity to neural tube defects (NTDs) induced by arsenite. Previous studies have also shown that cysteine residues in the Pax3 paired domain (PD) are required for binding to PD-specific DNA target sites and that such binding is reduced or blocked entirely by pro-oxidants. The current study was undertaken to test whether the presence of an environmentally relevant metal is capable of abrogating Pax3 binding to a PD-specific DNA target, the e5 segment from the *Drosophila* even-skipped gene. Electrophoretic mobility shift assays (EMSA) were performed with 32P labelled e5 in the presence of 2mM glutathione, a cellularly relevant concentration. Mercuric chloride, cadmium chloride, zinc sulfate, and sodium arsenite were each able to dose-dependently inhibit Pax3 DNA binding with EC50s of 10, 50, 60, and 450  $\mu$ M, respectively. Thus far, this work demonstrates that, like the sensitivity to pro-oxidants, Pax3 binding to DNA targets via the PD is sensitive to the presence of thiophilic metals. Experiments are underway to examine the effect of the one-electron redox cycling metals, copper and iron, in the presence and absence of the one-electron reductant, ascorbate, to determine to what extent redox chemistry plays a role in mediating Pax3 DNA-binding inhibition. We are also examining the effect of GSH:GSSG ratios in the presence and absence of thiophilic or redox cycling metals to determine the role of redox environment in the inhibition of Pax3 DNA binding via the PD.

## 1169 NF $\kappa$ B MEDIATES ZINC-INDUCED COX-2 EXPRESSION IN HUMAN BRONCHIAL EPITHELIAL CELLS.

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Upregulation of COX-2 expression is a pivotal event in inflammatory reactions induced by a variety of stimuli. Divalent zinc (Zn<sup>2+</sup>) component has recently been implicated as a causative agent in airway inflammation induced by exposure to ambient particulate matter. Our recent studies have shown that Zn<sup>2+</sup> exposure increases COX-2 expression through both transcriptional and posttranscriptional mechanisms in human bronchial epithelial cells. This study aims to determine whether the transcription factor nuclear factor  $\kappa$ B (NF $\kappa$ B) mediates Zn<sup>2+</sup>-induced COX-2 mRNA expression in these cells. Exposure to Zn<sup>2+</sup> had a minimal effect on I $\kappa$ B $\alpha$  breakdown and nuclear translocation of NF $\kappa$ B. However, Zn<sup>2+</sup> exposure caused a time-dependent phosphorylation of NF $\kappa$ B (p65), a component of the NF $\kappa$ B transcription factor, suggesting a transactivation mechanism activated by Zn<sup>2+</sup> exposure. Consistent with this, exposure of cells to Zn<sup>2+</sup> resulted in a time-dependent increase in NF $\kappa$ B reporter activity. Inclusion of human specific NF $\kappa$ B

p65 siRNA by means of transfection reagents selectively inhibited NF $\kappa$ B p65 expression as well as Zn<sup>2+</sup>-induced COX-2 mRNA expression with minimal effect on p42 MAPK expression. These findings suggest that exposure to Zn<sup>2+</sup> triggers NF $\kappa$ B transactivation through phosphorylation of p65, which is required for subsequent upregulation of COX-2 gene transcription in human bronchial epithelial cells.

**1170** COMPARISON OF BCNU AND SARCNU TOXICITY IN LONG-TERM CULTURES OF PRECISION-CUT LUNG SLICES.

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Interstitial pneumonitis is a common histopathological manifestation of anticancer drug-induced injury to the lung and a precursor to dose-limiting pulmonary fibrosis in the clinic. *In vitro* systems from human lung tissue that model these responses would allow detailed mechanistic studies to help develop safer drugs and assays to select the most promising and effective clinical candidates. Since only primary multicellular systems comprising appropriate lung cell types and numbers in their native orientation and configuration would seem to have the best prospects for meeting this need, our laboratory has been reexamining precision-cut slices as a potentially useful model for this application. In this work, precision-cut slices were prepared from male F-344 rat lungs using a Krumdieck slicer and incubated in M-199 media under air/5% CO<sub>2</sub> in a roller organ culture system for up to 28 days. ED-1 immunochemical staining was used to document BCNU (carmustine)-induced changes in the number of activated macrophages (AM) in the slices and the results were compared with AM induced by SarCNU [(2-chloroethyl)3-sarcosinamide-1-nitrosourea], a less potent analogue. ED-1 counts in the slices were increased in a concentration-dependent manner following exposure to 1-100  $\mu$ M BCNU. There was an increase in collagen deposition with BCNU at 100  $\mu$ M that was evident only after 28 days of exposure whereas increased numbers of AM were seen as early as 7 days. In addition, there was an associated reduction in slice ALP and LDH. SarCNU in the same concentration range as BCNU was less potent as it exhibited relatively fewer AM, less change in slice ALP and LDH content, and no collagen deposition. These results confirm that long-term cultures of precision-cut lung slices are feasible and encouraging as a model system for ranking the relative potency of lung toxicants for these effects and for mechanistic studies. Supported by NIH grant CA097438.

**1171** A HIGH-THROUGHPUT *IN VITRO* MODEL OF HUMAN TRACHEAL/BRONCHIAL EPITHELIUM (EPIAIRWAY) FOR PRECLINICAL SAFETY AND EFFICACY TESTING OF PHARMACEUTICALS.

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A growing need exists for high throughput *in vitro* models which can provide rapid, reliable safety and efficacy screening in preclinical drug development applications. The current poster describes development of an *in vitro* model of human tracheal/bronchial epithelium (TBE) in a 96-well high-throughput screening (HTS) format compatible with robotic manipulation. The HTS model is derived from normal human cells cultured at the air/liquid interface in 96-well microporous membrane plates to produce three-dimensional organotypic cultures. Histology of the culture model was evaluated by H&E staining of formalin fixed paraffin sections. The 96-well TBE culture is pseudostratified, and displays a differentiated mucociliary phenotype with barrier properties similar to native tracheal/bronchial or nasal epithelium, including development of transepithelial electrical resistance (TEER), conferred by functional tight junctions. Over the course of several consecutive culture lots, the intraplate and interlot barrier function reproducibility of the 96-HTS TBE cultures was determined by measurement of TEER. In initial studies to date, the average baseline TEER readings of all wells in a given 96-well plate (intraplate average) ranged from 388.6 to 445.0  $\Omega\text{-cm}^2$ . The average coefficient of variation between wells on the same plate (i.e. intraplate variability) was 19.5 %, while the variability between plates (i.e. interlot variability) was 6.8 %. The utility of the HTS TBE model was demonstrated by evaluation of the effects of various cytokine treatments on 15-lipoxygenase gene expression and TARC secretion. PCR analysis of total RNA isolated from the cultures showed that TNF- $\alpha$  plus IL-4 induced 15-lipoxygenase-2 expression. ELISA experiments revealed the same treatments also induced TARC secretion. The HTS airway model should find utility for *in vitro* irritation/toxicity screening, high content cell and molecular biology assays, as well as drug permeation studies. This work was supported by NIEHS grant #5R44 ES010237-03.

**1172** VASCULAR PERMEABILITY IN THE RAT.

S. Rowton and P. Robinson, *Covance Laboratories Ltd, Harrogate, United Kingdom*. Sponsor: D. Everett.

The ability of a compound to induce, or prevent an acute inflammatory response can be assessed in a number of ways. For screening purposes one of the simplest *in vivo* models for such investigation is the vascular permeability model in the rat. Intradermal administration of a phlogistic agent such as the mast cell degranulator compound 48/80 induces a localised inflammatory response with the release of histamine, prostaglandins and leukotrienes. These mediators cause dilation of arterioles and venules and an increase in vascular permeability. The increase in permeability allows for the passage of larger molecules such as plasma proteins to leak out of the vessels resulting in oedema. In this model, Evan's Blue given intravenously prior to the phlogistic agent, enables visualisation of permeability. As permeability increases the dye is able to seep out of the vessels in the vicinity of the intradermal injection sites. The degree of bluing is proportionate to permeability and hence the inflammatory response. When assessing the inflammatory potential of a test article, it is the test article that is administered intradermally. For anti-inflammatory assessment, the test article is administered by the intended clinical route of administration, at an appropriate time prior to intradermal injection of a known phlogistic agent. For validation purposes, the inflammatory effects of known phlogistic agents (histamine and compound 48/80) were examined. When compared to control injection sites, histamine and compound 48/80 produced a statistically significant increase in permeability as anticipated. Validation of the anti-inflammatory effect of test articles using this model employed the H1 anti-histamine, promethazine. Pretreatment of animals with promethazine (50, 150 or 300 mg/kg po) produced a statistically significant inhibition of compound 48/80-induced inflammation. In conclusion, the vascular permeability model in the rat provides a reliable and useful screening model for investigating the potential inflammatory or anti-inflammatory properties of novel test articles.

**1173** REDUCED STRESS IN GROUP HOUSED NON-HUMAN PRIMATES.

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Group/pair housing of non-human primates undergoing toxicity studies is clearly of benefit to the animals, allowing the expression of a greater variety of natural behaviours. This type of housing is also claimed to have benefits to the 'science' of such studies in that the animals are exhibiting a more normal state with regard to behavioural and physiological parameters as a result of lower stress levels. However, little hard evidence of such reduction in stress levels has been satisfactorily demonstrated due to difficulties in measuring meaningful stress markers when animals are physically restrained for sampling, which in itself results in stress. Also there has been little data presented to substantiate claims that beneficial changes in physiological parameters are achieved by pair/group housing. By using a reliable method of determining faecal cortisol levels we have been able to confirm a significant reduction in the levels of cortisol present in group housed animals when compared with individually housed animals. These data suggest that group housed animals are subject to less stress than their individually housed counterparts. This reduces the potential for stress-exacerbated clinical signs and interactions with test materials which may manifest themselves during toxicity studies. Also, using radio telemetry to measure key physiological functions in fully conscious animals we have been able to demonstrate significant beneficial differences in values for pair housed cynomolgus macaques over those recorded for singly housed animals. Again, this reduces the potential for housing based influences to exacerbate or modify clinical reactions to test materials.

**1174** PREDICTING ANTI-EPILEPTIC DRUG TOXICITY USING HEPATIC PROTEIN COVALENT BINDING AND GENE EXPRESSION POTENTIAL.

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The formation of one or more reactive metabolites is common to many anti-epileptic drugs associated with idiosyncratic drug reactions (IDR). The reactive metabolites may be detoxified or may cause deleterious effects through protein covalent binding or oxidative stress. To evaluate the IDR potential, six anti-epileptic drugs (AED), felbamate, carbamazepine, gabapentin, phenobarbital, phenytoin and valproic acid, were tested for hepatic protein covalent binding and hepatic gene expression changes. The covalent binding potential of AED was tested by incubation radiolabeled AED with rat liver and human microsomes and hepatocytes, and by *in*

*in vivo* treatment of radiolabeled AED to rats. cDNA microarrays were also used to characterize hepatic gene expression changes induced by the treatment of AED. Carbamazepine exhibited the strongest covalent binding potential *in vitro*, while valproic acid *in vivo*. The gene expression data showed both compound and dose related effects, but was generally consistent with a previously established oxidative stress / reactive metabolite profile. These results demonstrated that neither protein covalent binding nor changes in gene expression alone were sufficient to detect anti-epileptic drugs with known idiosyncratic potential. However, by combining the two techniques, the risk assessment was improved. Therefore, measurement of protein covalent binding and biomarkers of oxidative stress appear to be helpful supplemental tests that could be used early in drug development as part of the selection process for candidate anti-epileptic drugs.

**1175**

**IN VIVO AND IN VITRO NON-CLINICAL DRUG SAFETY ASSESSMENT: A KEY PARTNER FOR ANTICANCER DRUG DEVELOPMENT.**

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Hematopoietic progenitor clonogenic assays are interesting tools for assessing the potential for bone marrow anticancer drugs toxicity. We evaluated the hematotoxic potential of a new cytotoxic antitumor compound, acting as an alkylating agent, both *in vivo* in mice, rats and dogs and *in vitro* on mice granulocyte-macrophage (CFU-GM) progenitors and human CFU-GM and megakaryocyte progenitors (CFU-MK). The maximal tolerated dose following single intravenous dose was 12, 4.5 and 6 mg/m<sup>2</sup> for mice, rats and dogs, respectively, and exclusively related to hematotoxicity. Total leucocyte count was reduced by about 50% on Days 4 or 7 after dosing at 3 mg/m<sup>2</sup> in rats and from 1.5 mg/m<sup>2</sup> in dogs. Corresponding maximal plasma exposure to the test item were 4.86 and 3.13 10-7 M for rats and dogs, respectively. Platelet count was reduced by 40% on Day 14 at 3 mg/m<sup>2</sup> in dogs, with corresponding maximal plasma exposure of 6.02 10-7 M. These changes correlated with concomitant bone marrow hypoplasia, thymic lymphoid atrophy and few active follicles in lymph nodes and tonsils. None of them were noted on Day 32, suggesting their full recovery. An inhibition of mice bone marrow cloning efficiency CFU-GM progenitors was observed *in vitro*, with an IC 90 equal to 3.44 10-7 M, which is well correlated with the *in vivo* toxicokinetic data. To assess the potential risk to human, we performed *in vitro* clonogenic assays on granulocyte-macrophage (CFU-GM) and megakaryocyte (CFU-MK) human progenitors. An inhibition of human megakaryocyte progenitors cloning efficiency was observed with an IC 90 equal to 6.43 10-8 M. Inhibitory effects on human CFU-GM were similar to those observed in mice, with an IC 90 equal to 1.38 10-7 M. These *in vitro* results were associated with *in vivo* data to anticipate hematotoxicity and the possible maximal tolerated dose in man, as previously accurately demonstrated for some other anticancer therapeutics.

**1176**

**PHARMACOKINETICS AND ENHANCED ORAL BIOAVAILABILITY OF NANOSTRUCTURED DRUG CRYSTALS: COMPARISON OF PARTICLE ENGINEERING TECHNOLOGIES FOR DANAZOL, KETOCONAZOLE AND NAPROXEN IN BEAGLE DOGS.**

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Reports quote anywhere from 30-60% of drug candidates exhibit poor water solubility in physicochemical testing and poor absorption from the GI tract. Poor oral absorption limits the usefulness of a drug. In order to overcome this limitation, an emerging approach is to increase the surface area-to-volume ratio of drug crystals by applying particle engineering technologies and add excipients to prevent reaggregation. The Dow Chemical Company has developed a number of particle engineering technologies to produce novel nanostructured drug particles that enhance oral bioavailability of poorly water soluble drugs. Early determination of ways to enhance oral bioavailability of a drug candidate is important in order to generate accurate kinetic and toxicological data. Poorly absorbed drugs (danazol, ketoconazole and naproxen) were processed by various technologies, stabilized with various pharmaceutically acceptable excipients, and evaluated in beagle dogs. Different doses of the drugs were orally administered to dogs in capsule or tablet form and blood collected at various time-points post-dosing. Concentrations of parent drugs were determined in plasma by LC/MS/MS. WinNonlin was used for kinetic analysis. The technologies evaluated showed significantly higher oral bioavailability than the micronized drug as received or commercial products. The oral bioavailability of nanostructured naproxen, ketoconazole and danazol was 1.3, 3 and up to 64 fold

higher than the drug as received, respectively. Increase in oral bioavailability of nanostructured naproxen was 17-29% and of ketoconazole ~300% compared to the commercial products. Particle engineering did not cause much change in the  $T_{max}$  or plasma  $t^{1/2}$ . However, the  $C_{max}$  of the nanostructured drugs was several fold higher than the commercial or micronized drug as received.

**1177**

**REDUCTION AND REFINEMENT IN RADIOLABELLED PRIMATE METABOLISM STUDIES.**

J. Kelly, J. Hedley and R. Allen. *Covance Laboratories Ltd., Harrogate, United Kingdom*. Sponsor: D. Everett.

Traditionally, primate metabolism studies are conducted by performing each phase separately. In a typical intravenous/oral crossover study, animals are dosed up to four times - twice for the pharmacokinetic (PK) phase and twice for the mass balance phase. To minimise stress to the animals undergoing metabolism studies, the PK phase is often conducted in tandem with the excretion balance phase. The main draw back to this is that the monkeys have to be removed from their metabolism cages for sampling. This could be up to 12 occasions in the first 24 hours. The potential therefore exists for excreta losses, even though steps are taken to minimise them. In addition to the potential impact on the data generated from studies where animals are frequently removed from their home cages to undergo procedures, the welfare of the animals is not maximised. Development of a new style of caging was undertaken through consultation between experienced animal technicians and scientists. This new cage design allows the animals to be dosed and bled *in situ* without the risk of sample loss. This arrangement allows the different phases of the study to be run concurrently. Handling is therefore minimised thus reducing stress to the animals whilst on study. Consideration was also given to the macro-environment in which the animals were housed and steps taken to provide enrichment strategies designed to help reduce the stress created by the necessary isolation during the study period. The new cage design confers both improved welfare and an improved environment on the animals whilst at the same time allowing good scientific data to be generated from a study.

**1178**

**ADJUVANT ARTHRITIS IN THE MALE LEWIS RAT.**

S. Rowton and P. Robinson. *Covance Laboratories Ltd., Harrogate, United Kingdom*. Sponsor: D. Everett.

Adjuvant arthritis in the rat has many similarities to rheumatoid arthritis in humans and as such, is a frequently used *in vivo* model for the screening of compounds intended to alleviate arthritis. Administration of an adjuvant initially induces inflammation of the injected paw, this is known as the primary lesion. Some days after this response, secondary lesions appear, which are identified as inflammation of untreated areas, such as paws ears, nose and tail. Validation of this study employed both prophylactic or therapeutic study designs, using male Lewis rats. For the prophylactic study, on Day 1 each rat received 0.1mL of adjuvant (*Mycobacterium butyricum*) subcutaneously into the plantar surface of a designated hind paw. Paw oedema of both hind paws was measured using a plethysmometer prior to administration of the adjuvant and at various time-points up to Day 5 post-adjuvant. Animals were dosed orally once daily with vehicle, indomethacin (25mg/kg/day) or cyclosporin (3mg/kg/day) from Day 1 to Day 15. Animals were observed and scored for lesions from Day 5 to Day 22. For the therapeutic study, on Day 1 each animal received 0.1mL of adjuvant as described above. On Day 20, animals were allocated to treatments groups. Only those animals exhibiting lesions were used. Animals were dosed orally once daily with vehicle, indomethacin (25mg/kg/day) or cyclosporin (3mg/kg/day) from Day 20 to Day 34. Animals were observed and scored for lesions from Day 20 to Day 35. Primary and secondary lesions were exhibited by 100% of rats in both study designs. Lesion development in control animals had peaked by Day 20 and diminished thereafter. Cyclosporin and indomethacin, in the prophylactic study design, suppressed lesion development. Whereas in the therapeutic study, alleviation of established arthritic lesions was achieved following cyclosporin treatment, with indomethacin having little or no effect. In conclusion, adjuvant-induced arthritis in the rat provides a reliable and useful model for investigating the potential therapeutic and prophylactic effect of anti-arthritic compounds.

**1179**

**EVALUATION OF GENOTOXICITY AND SENSITIZATION POTENTIAL OF PEPTIDE COUPLING REAGENTS.**

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Advances in biotechnology over the past decade have encouraged increased efforts toward the chemical synthesis of complex peptides. Success in the chemical synthesis of peptides, performed either in solution or via solid-phase techniques, relies

heavily upon efficient peptide coupling reagents. These agents enhance coupling yields, reduce racemization, and allow reduced cycle times during peptide synthesis. Peptide coupling reagents are reactive molecules and are capable of functioning as alkylating agents. In fact, some coupling agents have been reported to be potentially genotoxic and/or sensitizing. The potential for exposure to peptide coupling reagents exists during their use in the synthesis of polypeptides in the chemical and pharmaceutical industries, as well as during protein synthesis in the recombinant DNA industry. Considering the chemical reactivity of peptide coupling agents, it is important to determine the genotoxicity and sensitizing potential of selected peptide coupling agents. This presentation describes the results of short-term mutagenicity and dermal sensitization assays for several peptide coupling reagents and/or their derivatives.

## 1180

### COMPARISON OF PHOTOTOXICITY TESTING METHODS USED TO SCREEN LEAD COMPOUNDS L. LEE, G. STEVENS, AND B. JESSEN, PFIZER GLOBAL R&D.

L. Lee, *Pfizer Global R&D, San Diego, CA*. Sponsor: G. Stevens.

New chemical entities that absorb light within the visible range have the potential to form reactive intermediates. Photoactivation of small molecules can lead to photo-irritation or UV-associated carcinogenesis after topical or systemic administration. Current ICH guidelines recommended assessment for phototoxicity if the molar extinction/absorption coefficient is greater than 10 liter x mol<sup>-1</sup> x cm<sup>-1</sup>. The initial phototoxicity assay recommended by ICH is an *in vitro* viability assessment using murine 3T3 fibroblasts cell. The basis of this assay is to compare the potential phototoxic hazard of a compound in the presence and absence of exposure to simulated solar light at non-cytotoxic concentrations. The 3T3 cell viability is determined by measuring neutral red uptake after incubation with the test article. The assay for neutral red uptake requires washing, fixing, and solubilizing cells to measure the amount of neutral red present in the cell extract. If this assay were to be applied for early screening of compounds that absorb light, increased throughput and efficiency of this assay is required. The purpose of this study was to evaluate a more rapid viability endpoint (intracellular ATP) as an alternative to the neutral red uptake assay. A double-blinded study was performed with twelve known phototoxins and four non-phototoxic compounds to validate each method. The results indicated that the ATP assay was equally sensitive with the advantage of fewer preparation steps. These data suggest that throughput could be increased several fold through implementation of ATP as the viability endpoint and further optimization through automation of the assay.

## 1181

### SUBCHRONIC TOXICITY STUDY OF HEXACHLOROBENZENE (HCB) IN FEMALE HARLAN SPRAGUE-DAWLEY RATS.

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HCB (cas #118-74-1) is used as a fungicide and an intermediate in the manufacture of dyes, pentachlorophenol, and rubber. HCB has some dioxin-like activity thereby necessitating the need to evaluate it for potential inclusion in the toxic equivalency factor scheme used to assess the risk of dioxin-like compounds. HCB, in corn oil/1% acetone, was administered by gavage (10 rats/group) at dosages of 0 (control), 0.03, 0.1, 0.3, 1, 3, 10, or 25 mg/kg/day, five days a week, for 13 weeks. HCB treatment had no effect on survival. Group mean BW was increased (25 mg/kg/day). Total free T4 was decreased ( $\geq$ 10 and  $\geq$ 1 mg/kg/day), T3 was decreased (25 mg/kg/day), and TSH was similar to control. Dose-related increases in hepatic CYP1A1, CYP2B, and CYP1A2 and pulmonary CYP1A1 activities were observed at dosages equal to and greater than 1, 0.03, 0.3, and at 25 mg/kg/day, respectively. Mean maximal observed CYP1A1 activity was 340 pmol/min/mg (25 mg/kg/day), which is markedly lower than that observed previously for animals treated for 13 weeks with 100 ng TCDD/kg (2130 pmol/min/mg). HCB increased liver cell proliferation ( $\geq$ 10 mg/kg/day). Spleen, liver, and lung absolute and relative organ weights were increased ( $\geq$ 3,  $\geq$ 10, and 25 mg/kg/day, respectively). HCB-related microscopic findings included hepatocellular hypertrophy ( $\geq$ 3 mg/kg/day), pulmonary focal interstitial fibrosis and histiocytic infiltration ( $\geq$ 1 mg/kg/day), mammary gland hyperplasia and thymic atrophy ( $\geq$ 10 mg/kg/day), dermal suppurative inflammation and ulceration (25 mg/kg/day), and splenic hematopoietic cell proliferation ( $\geq$ 3 mg/kg/day) and lymphoid hyperplasia ( $\geq$ 10 mg/kg/day). These data indicate that HCB has only weak dioxin-like activity and also exhibits non dioxin-like effects. (Supported by NIEHS Contract No. N01-ES-65406).

## 1182

### SUBCHRONIC TOXICITY OF ALPHA-METHYL-1,3-BENZODIOXOLE-5-PROPIONALDEHYDE IN RATS.

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alpha-Methyl-1,3-benzodioxole-5-propionaldehyde (MMDHCA), a clear yellowish liquid that is an important fragrance ingredient, was evaluated for its dermal toxicity when applied to the dorsal skin of rats for 13 weeks, followed by a 4-week recovery period. Four groups of male and female rats (15/sex/group) were treated topically with the MMDHCA for at least 90 days at dose levels of 0 (control group; treated with reverse osmosis water), 50, 150 or 300 mg/kg/day. Five animals/sex/group were designated as recovery animals and were observed for approximately 4 weeks post treatment. The animals were sacrificed and necropsied during week 14 (terminal sacrifice) and week 18 (recovery sacrifice). The study evaluations included: clinical observations, dermal irritation observation, body weight, food consumption, estrous cycle, ophthalmology, hematology, serum chemistry, organ weights, macroscopic observations, coagulation, histopathology and male reproductive assessment. There was no material-related mortalities or effects on evaluated parameters. The material-related irritation observed in the skin of treated animals at all dose levels with an increased incidence and severity in animals given 300 mg/kg/day improved to slight or resolved completely during the recovery phase. Based on the findings in this study, the non-observable-adverse-effect level (NOAEL) when applied as a dermal application for at least 90 days is less than 50 mg/kg/day for dermal toxicity and the no-observable-effect-level (NOEL) for systemic toxicity is  $>$  300 mg/kg/day.

## 1183

### SUBCHRONIC TOXICITY OF ACETYL CEDRENE IN RATS.

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The subchronic toxicity of acetyl cedrene, a widely used fragrance ingredient, was evaluated in a 13-week dermal toxicity study in rats with a 4-week recovery period. Acetyl cedrene was applied to the intact dorsal skin of male and female rats (15/sex/dose) at dose levels of 0 (control), 50, 150 or 300 mg/kg bodyweight/day. Control animals were treated with reverse osmosis water. Observations included morbidity and mortality assessments, body weights, food consumption, dermal irritation, estrous cycle and ophthalmology. Twenty animals from each group were sacrificed during week 14 and the remaining animals were sacrificed during week 18. A complete necropsy was conducted on all animals. Two females in the control group died on day 33 and one female in the mid-dose group was sacrificed in a moribund condition on day 63. Histopathological examination of this animal revealed a spontaneous fibrosarcoma. Dermal irritation was observed at all doses with higher incidence and severity in higher dose groups. These observations were noted in the recovery group but with lower incidence and severity and had resolved by the recovery sacrifice. There were significant changes in mean body weights, body weight changes and food consumption, but no clear relationship to test material administration. At the terminal sacrifice only, increases in kidney-to-body weight ratios were observed in males in the 2 highest dose groups. In males in the 300 mg/kg group, hyaline droplet formation was noted in the tubular epithelium but this finding had resolved by the recovery sacrifice. This finding was compatible with the diagnosis of alpha-2-micro-globulin nephropathy and was not considered relevant. At the terminal sacrifice, mild chronic inflammation and acanthosis/hyperkeratosis was noted in all dose groups; again, these changes had completely resolved by the recovery sacrifice. Based on these findings, it can be concluded that the no-observable-effect-level (NOEL) for acetyl cedrene when applied as a dermal application for at least 13 weeks is less than 50 mg/kg/day for dermal toxicity and the NOEL for systemic toxicity is 150 mg/kg/day.

## 1184

### CNS AND CARDIORESPIRATORY EFFECTS OF TWO NOVEL BISPYRIDINIUM OXIMES (ICD-39 AND ICD-585).

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Inhibition of acetylcholinesterase (AChE) constitutes a critical toxicity component in nerve agent intoxication. Oximes can restore AChE to a functional level and are therefore considered to play a critical role in nerve agent casualty management. As part of a R&D effort in search of a more effective oxime, we evaluated cardiorespiratory and CNS effects of two novel bispyridinium oximes, ICD-39 and ICD-585 (3 doses at 57 umole/kg/45-min, im) in anesthetized and unanesthetized guinea pigs. ICD-39, in a time- and dose-dependent fashion, caused a positive chronotropic effect in unanesthetized animals and a negative chronotropic effect in anesthetized animals. Blood pressure in these animals typically showed a time- and dose-dependent decrease. In unanesthetized animals, respiratory responses after each oxime dose were biphasic (increase/decrease). In anesthetized animals, respiratory frequency invariably increased after ICD-39. No anomalous ECG waveform attributes were noted following ICD-39 in anesthetized or unanesthetized animals. ICD-585 produced no significant change in heart rate and blood pressure. No ab-

normalities were seen in ECG waveform attributes. Respiratory rate showed either no change (anesthetized animals) or a biphasic response pattern (unanesthetized animals). Somewhat unexpected were the findings that 50% of PYR-pretreated animals and 33% of animals without PYR pretreatment displayed aberrant ECoG waveform attributes and neurobehavioral anomalies indicative of seizures following either the first or the second dose of ICD-585. What can be inferred from this study is twofold. First, in addition to their ability in reactivating nerve agent-inhibited AChE, ICD-39 and ICD-585 both appeared to be able to mediate a variety of changes in cardiorespiratory and CNS activities. Second, from the standpoint of safety/toxicity, our data suggest that ICD-39 appears to engender fewer untoward cardiorespiratory and CNS effects than ICD-585.

#### 1185 TOXICOLOGICAL EVALUATION OF CIGARETTES WITH TWO BANDED CIGARETTE PAPER TECHNOLOGIES.

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A tiered testing strategy has been developed to evaluate the potential of tobacco processes, ingredients, or technological developments to change the biological activity resulting from burning cigarette tobacco. The strategy is based on comparative chemical and biological testing. The introduction of banded cigarette papers in cigarettes to meet the New York State Fire Safety Standards for Cigarettes (NYS cigarettes) constitutes an example of a technological development evaluated in a tiered testing strategy that included a comparison of the chemical and biological effects of cigarettes with and without the banded cigarette paper technologies. Specific testing included mainstream cigarette smoke chemistry studies; *in vitro* genotoxicity (Ames and Sister Chromatid Exchange) and cytotoxicity studies (Neutral Red); and *in vivo* 13-week inhalation study in Sprague-Dawley rats and 30-week dermal tumor promotion study in SENCAR mice. The yields of constituents from cigarettes with or without the banded technologies were generally similar and within the range observed for cigarettes in the US market. Collectively, data indicated that cigarettes with and without banded technologies had a similar toxicological profile in this test battery.

#### 1186 TOXICOLOGICAL EVALUATION OF A CIGARETTE PAPER WITH REDUCED IGNITION PROPENSITY: *IN VITRO* AND *IN VIVO* TESTS.

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A novel print-banded cigarette paper exhibiting reduced propensity to ignite secondary surfaces in a defined test protocol was compared to a conventional un-banded cigarette paper to assess the potential of the band application to affect the toxicity of cigarette mainstream smoke. Experimental filtered cigarettes made with a nominally realistic quantity or approximate 1.2-fold exaggerated level of the applied banding formulation were compared to a matched control cigarette. The tar, nicotine and CO yields of the cigarette made with the normal-banded paper were moderately higher than those of the control cigarette made with an otherwise identical unbanded paper. An Ames assay using a battery of *Salmonella* tester strains indicated no differences in the mutagenic activity of smoke particulate matter of the banded and control cigarettes. The mammalian cell cytotoxicity (Neutral Red Uptake) of both the particulate and gas/vapor phases of the mainstream smoke of the normal- and heavy-banded cigarettes was indistinguishable from that of the unbanded control cigarette. A 13-week subchronic nose-only smoke inhalation bioassay in rats resulted in no substantive or biologically significant differences in the incidence, severity or reversibility of a variety of measured clinical chemistry, hematologic, histopathologic and physiologic indices of cigarette smoke toxicity. A 26-week SENCAR mouse skin painting bioassay revealed no substantive differences in any indices of tumorigenic potential; including tumor incidence, latency or multiplicity of the cigarette smoke condensates from the test and control cigarettes. It is concluded that the presence of nominally realistic or 1.2-fold exaggerated levels of the applied printed banding material does not meaningfully affect the biological activity in a variety of test systems that have traditionally been employed to evaluate cigarette smoke toxicity.

#### 1187 TOXICOLOGICAL EVALUATION OF DIAMMONIUM PHOSPHATE (DAP) AND UREA AS INGREDIENTS ADDED TO CIGARETTE TOBACCO.

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A tiered testing strategy has been developed to evaluate the potential for new ingredients, tobacco processes, and technological developments to alter the biological activity that results from burning cigarette tobacco. The foundation of this evaluation

strategy is comparative testing, typically including chemical and biological components. In the manufacture of cigarettes, DAP and urea have been used as ingredients added to tobacco, to reconstituted tobacco sheet, and to other processed tobaccos. As part of ongoing stewardship efforts, a toxicological assessment of cigarettes with and without DAP and urea was conducted. Chemical and biological analyses were conducted for test cigarettes containing 0.5% DAP and 0.2% urea in the final blend and 1.0% DAP and 0.41% urea in the final blend compared to reference cigarettes containing no DAP or urea. Principal components of this evaluation included a determination of selected mainstream smoke constituent yields, an Ames assay in *Salmonella typhimurium* strains TA98 and TA100, sister chromatid exchange assays in Chinese hamster ovary cells, a 13-week inhalation study of mainstream cigarette smoke in Sprague-Dawley rats, and a 30-week dermal tumor promotion evaluation of mainstream cigarette smoke condensate in SENCAR mice. Comparative evaluations demonstrated that the addition of DAP and urea to cigarettes at up to 1.0% and 0.41%, respectively, does not alter the biological activity compared to reference cigarettes without DAP or urea.

#### 1188 IN VITRO TOXICITY EVALUATION OF TOOTHPASTES USING RECONSTRUCTED HUMAN ORAL AND GINGIVAL MUCOSA MODELS.

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5 different toothpaste formulations (A, B, C, D, E) were evaluated for their toxicity on *in vitro* reconstituted human oral and gingival mucosa (SkinEthic laboratories, Nice, France). Toothpastes A, D and E contained SDS, toothpastes B and C did not. They all did contain fluoride. A 30 % solution was topically applied to triplicate oral and gingival mucosal tissues. H<sub>2</sub>O served as negative control, SDS 0.5% was used as positive control. Tissue viability (MTT), tissue morphology (both LM and TEM) and the release of pro-inflammatory mediator IL-1 $\alpha$  was evaluated after 10 minutes, 1 h and 3 h of exposure. Using gingival mucosa, topical exposure of the toothpastes for 10 min, 1 h or 3 h did not affect MTT values as compared to the negative control. Using oral mucosa however, toothpastes B, E and D induced a significant loss of viability after 1 h (respectively 49.19%, 55.55% and 78.38%). After 3 hours, tissue viability for toothpaste D and E further decreased to 11.12% and 4.53% respectively. Only gingival mucosa tissues exposed to toothpaste D and E show a marked increase of IL-1 $\alpha$  at 1 hour, and to toothpaste A at 3 hours exposure. Oral mucosa tissue exposed for 1 hour resulted in increased levels of IL-1 $\alpha$  for toothpastes A, B, D, & E, which became more important at 3 hours. Morphological analysis of oral mucosa demonstrated partial necrosis after exposure to toothpastes A, B and C, and severe necrosis to D and E. Gingival mucosa exposed to toothpastes D and E showed morphological changes undetected by MTT, indicating that MTT cannot be used as single toxicity parameter and should be confirmed by histology. Both *in vitro* oral and gingival mucosa models are suitable to evaluate the toxicity of toothpaste formulations but show a different sensitivity. The presence of SDS in toothpastes A, D, and E could be responsible for the observed toxicity *in vitro*. These *in vitro* results confirm the clinical inflammatory effects of SDS containing oral care products reported in the literature.

#### 1189 IN VITRO SAFETY EVALUATION STUDIES OF LUTEIN AND ZEAXANTHIN.

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Lutein is a carotenoid found in vegetables and fruits. Dietary lutein and zeaxanthin accumulate in human retina and have been implicated in the prevention of age-related macular degeneration (AMD). Some studies have also indicated that lutein and zeaxanthin inhibit human breast cancer cell proliferation and contribute to protection against UV light. Lutein supplementation for the treatment of AMD is currently under way at some hospitals. For evaluation of the safety of chronic supplementation of lutein and zeaxanthin in primates, dose response studies of lutein and zeaxanthin toxicity were conducted using different available cell lines: normal human epidermal keratinocyte (NHEK) cells and normal rat kidney epithelial (NRK52E) cells. DMSO was used as the dissolving vehicle as well as the vehicle control. The cells were exposed to 0.001 to 0.1 mmol/L lutein and zeaxanthin for 24, 48 and 72 hours. The alamar blue assay was used to assess cellular metabolic toxicity. The results of this study indicate that there is no effect on the viability of NHEK cells with DMSO at concentrations lower than 1.0 %. Lutein at 0.05 mM and 0.1 mM reduced NHEK cell viability to 87 ? 1% and 69 ? 4% respectively after 24 h exposure. Decreases of 10 - 20% of cellular metabolism were found only at the highest concentration zeaxanthin groups (0.10 mM). The same results were found in NRK52E cells. Zeaxanthin at 0.05 mM reduced NRK52E cell viability to 90 ?

9% after 48 h exposure and at 0.1 mM, it reduced the viability to 88 ? 5% and 85 ? 5% after 24 and 48 h exposure respectively. Collecting the data suggests that low dosage lutein and zeaxanthin do not cause toxicity in NHEK and NRK52E cells. (Supported by NEI grant EY 12658-02)

#### 1190 AEROSOL CHARACTERIZATION FOR CONSUMER LAUNDRY ENZYME-CONTAINING STAIN REMOVERS.

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The use of enzymes in laundry trigger-spray stain removers has been common practice for a number of years. These products use different sprayer nozzles that may produce droplets with different size, mass, decay patterns, and enzyme content. Understanding these parameters is important to prevent consumer over-exposure by inhalation and the potential for sensitization of atopic individuals. The objective of this study was to measure the impact of test substance application on airborne aerosol characteristics as well as enzyme levels. Exposure to commercial stain removers was measured by simulating a heavy use scenario in an environmentally controlled simulated residential exposure chamber. The product was applied 6 inches from fabric targets mounted vertically (horizontal application) over a standard washing machine. The tests were conducted in eight replicates for each test substance. Each replicate applied the test substance sequentially on 8 fabric targets with 5 sprays for each. The characteristics of trigger sprayers were evaluated in the adult breathing zone by determining aerosol mass levels, airborne particle and mass distribution, and enzyme concentration. Aerosol concentrations were high in the first 1-3 minutes and returned to baseline within 10 minutes, during which the average mass concentration was in the range of 4.0-67.3  $\mu\text{g}/\text{m}^3$  and the observed enzyme concentration in air was 12.0-46.7  $\text{ng}/\text{m}^3$ . Large particles were deposited in the fabric with particles < 1 micron remaining in the breathing zone. The variation on these parameters was influenced mainly by the trigger type and product physical characteristics (e.g. viscosity range). In general, a normal use of these products does not produce airborne enzyme levels that exceed common industrial practice SDA guidelines or ACGIH workplace limits.

#### 1191 ENHANCING EFFECT OF CHLORINATED ORGANIC SOLVENTS ON INFLAMMATORY MEDIATOR PRODUCTION.

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The prevalence rate of allergic diseases such as bronchial asthma and rhinitis is increasing in industrial areas and countries. There are some reports on epidemiological studies that may suggest a link between the incidence of allergic disease and environmental pollutants, such as diesel exhaust particles (DEPs), formaldehyde and volatile organic compounds (VOC). Chlorinated organic solvents are classified into VOC, and recently have become major environmental pollutants. Previously we observed the enhancing effects of some chlorinated organic solvents, such as tetrachloroethylene (PCE) and trichloroethylene (TCE), on histamine release from rat mast cells *in vitro*, and on passive cutaneous anaphylaxis (PCA) *in vivo*. In the present study, we investigated the effects of low concentration of PCE and TCE on antigen-induced inflammatory mediator production from rat mast cell line RBL-2H3. Cells were incubated with anti-dinitrophenol (DNP) monoclonal IgE antibody, and then stimulated by DNP-BSA in the presence of PCE (0.01, 0.1 and 1 mg/L) or TCE (0.03, 0.3 and 3 mg/L). The conditioned medium and cell lysate were collected, and measured IL-4, TNF- $\alpha$  and MCP-1 by ELISA. Each inflammatory mediator production was increased in a dose-dependent manner. Furthermore, mRNA expression of them was evaluated by semi-quantitative RT-PCR, these mRNA expressions were found to be up-regulated by the exposure of PCE and TCE. Thus, this overexpression of inflammatory mediator from RBL-2H3 exposed to PCE and TCE is associated with inflammatory response in late phase. These results suggest that PCE and TCE, even in a low concentration, may affect the immune response via the modulation of cytokine production in the mast cells, and lead to the augmentation of allergic diseases.

#### 1192 RELATIONSHIP BETWEEN CD86/CD54 EXPRESSION AND CELL VIABILITY IN *IN VITRO* SKIN SENSITIZATION TEST OF WATER-SOLUBLE CHEMICALS USING THP-1 CELLS.

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We have reported that the human monocytic leukemia cell line, THP-1, which shows enhanced CD86 and/or CD54 expression when treated with allergens, can be used as an *in vitro* skin sensitization test to predict the allergenic potential of

chemicals. In our previous study, we optimized our human cell line activation test (h-CLAT) and performed an inter-laboratory study of the protocol between two laboratories. The results suggested that partial cytotoxicity may be necessary for augmentation of CD86/CD54 expression in allergen-treated THP-1 cells. A similar phenomenon has already been reported in dendritic cells. The aim of this study is to clarify the relationship between CD86/CD54 expression and viability of allergen-treated THP-1 cells. Several water-soluble chemicals (nickel sulfate (Ni), p-phenylenediamine (pPD), ammonium tetrachloroplatinate (Pt) and sodium lauryl sulfate (SLS)) were evaluated at multiple doses that covered the range of concentrations with strong cytotoxicity to almost no-cytotoxicity. In the case of Ni (allergen), enhancement of CD86 expression was observed at about 70-80% cell viability and enhancement of CD54 expression was observed at 40-90% cell viability. For Pt and pPD (allergens), enhancement of both CD86 and CD54 expression was seen when the cell viability was in the range of about 50-90%. The concentration which caused maximum induction of these cell surface antigens varied from chemical to chemical. On the other hand, SLS (non-allergen) did not enhance either CD86 or CD54 expression at any concentration in the range giving 30-90% cell viability. These results suggest that partial cytotoxicity is necessary for induction of CD86/CD54 expression in water-soluble allergen-treated THP-1. Furthermore, two or more measurement points at subtoxic concentrations are necessary for accurate evaluation. These findings should be helpful for dose setting in h-CLAT.

#### 1193 THE RELATIONSHIP BETWEEN CD86/CD54 EXPRESSION AND THP-1 CELL VIABILITY IN AN *IN VITRO* SKIN SENSITIZATION TEST FOR WATER-INSOLUBLE CHEMICALS.

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Several *in vitro* skin sensitization methods using human cell lines have been reported. In our previous study, we optimized our human cell line activation test (h-CLAT) using THP-1 cells (monocytic leukemia cell line) and conducted an inter-laboratory study. We found that measuring CD86/CD54 expression may be useful for predicting skin sensitization *in vitro*. Also we showed that a certain level of cytotoxicity may be necessary for up-regulation of CD86/CD54 expression. The aim of this study was to confirm the relationship between CD86/CD54 expression and viability of THP-1 cells in the h-CLAT, especially for water insoluble chemicals. In this study, four allergens (DNCB, Propyl gallate, Isoeugenol, Eugenol) were evaluated. For each chemical, more than 10 concentrations that gave a predicted cell viability range of 20-95% were used to determine the relationship between cell viability and surface marker expression. An up-regulation of CD86 and/or CD54 expression at the 70-90% cell viability range was observed. For DNCB, maximum CD86 and CD54 expression was observed at about 85% and 70% cell viability, respectively, whereas for Eugenol, it was at about 70% and 80% cell viability, respectively. Expression patterns of CD86/CD54 differed depending on chemical. The data suggest that: 1. Some cytotoxicity is needed for enhancement of CD86/CD54 expression in the h-CLAT using THP-1 cells for water insoluble chemicals. 2. Two or more concentrations covering the 70-90% cell viability range would increase reliability in the evaluation of various chemicals. 3. A narrow dose setting range is needed for chemicals with a sharp cell survival decline. These findings seem to be helpful for dose setting in the h-CLAT, which should result in a more robust *in vitro* skin sensitization test.

#### 1194 PHENOTYPIC ALTERATIONS AND CYTOKINE PRODUCTION IN THP-1 CELLS IN RESPONSE TO ALLERGENS.

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Dendritic cells (DC), including Langerhans cells (LC) present in epidermis, play a crucial role in the induction phase of allergic contact hypersensitivity. It has been known that DCs express several surface molecules and produce cytokines in response to an allergen. We have previously shown that THP-1 cells, human acute monocytic leukemia cell line, can discriminate allergens and irritants by measuring expression of surface molecules, CD86 and CD54. At the same time, we have also reported that production of IL-1 $\beta$  was up-regulated in THP-1 cells when treated with an allergen, dinitrochlorobenzene (DNCB). The aim of our present study is to further address phenotypic changes and cytokine production in THP-1 cells when treated with chemicals. THP-1 cells were treated with two known allergens (DNCB and NiSO<sub>4</sub>), an irritant (sodium lauryl sulfate (SLS)) and an immunostimulant (lipopolysaccharide (LPS)) for 24 hours at the concentration that exhibited 70-95% viability. Exposure to DNCB, NiSO<sub>4</sub> and LPS induced significant augmentation of CD40 and CD83 expression as well as CD86 and CD54. On the contrary, SLS did not change or slightly decreased expression of these molecules. Also,

DNCB, NiSO<sub>4</sub> and LPS induced marked increase of TNF-alpha secretion in a dose-dependent manner while SLS did not. In addition, THP-1 cells showed augmented production of IL-6 following incubation with only NiSO<sub>4</sub> and LPS. These results revealed that THP-1 cells, with their wide variety of responses to allergens, may emulate allergen-induced maturation processes as those of immature LCs in epidermis. It has been suggested that THP-1 cells, which could develop several LC-like properties, are capable of identifying sensitizing potential of chemicals based on mechanism of sensitization development.

## 1195

### EVALUATION OF CYTOKINES RELEASE FROM THP-1 CELLS AS PREDICTIVE MARKERS OF CONTACT SENSITIZATION.

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The assays currently recommended for evaluating contact hypersensitivity such as the murine local lymph node assay (LLNA) have inherent limitations such as low throughput, and require the use of animals. The development of a predictive high throughput *in vitro* assay as an alternative to the use of animals may prove useful in hazard assessment, especially for the preliminary screening of new molecules. Our primary goal in this study was to identify specific and sensitive marker(s) to predict the potential of compounds to induce immune-mediated hypersensitivity reactions, using THP-1 cells, a human histiocytic lymphoma cell lines. THP-1 cells were incubated for up to 72 hours with the respiratory allergen trimellitic anhydride (TMA) at 2 and 20 mg/mL, contact allergen 2, 4-dinitrofluorobenzene (DNFB) at 1.25 and 2.5 µg/mL, irritant sodium dodecyl sulfate (SDS) at 35 and 70 mg/mL or vehicle control dimethyl sulfoxide (DMSO). Changes in multiple inflammatory cytokines/chemokines including interleukin-1 alpha (IL-1 $\alpha$ ), interleukin-1 beta (IL-1 $\beta$ ), interleukin-2 (IL-2), interleukin-10 (IL-10), interferon-gamma (IFN $\gamma$ ), tumor necrosis factor-alpha (TNF $\alpha$ ), interleukin-8 (IL-8) and macrophage inflammatory protein-1 beta (MIP-1 $\beta$ ) were evaluated in the media of THP-1 cultures using chemiluminescent-based proteome arrays. Treatment of THP-1 cells with non-toxic concentrations of DNFB for up to 72 hours induced significant increases (1.6- to 10.8-fold) in the levels of IL-1 $\alpha$ , IL-1 $\beta$ , IL-10, IFN $\gamma$ , MIP-1 $\beta$  and TNF $\alpha$ . Interestingly, the concentration of IL-8 was increased by greater than 37-fold in the media of THP-1 cells treated with DNFB for up to 24 hours. Changes in these cytokines/chemokines were not seen at non-toxic doses of SDS or TMA. Our data suggest that measurement of inflammatory cytokines/chemokines and in particular soluble levels of IL-8 in this *in vitro* model may provide a sensitive method for assessing the contact sensitization potential of a chemical.

## 1196

### USE OF HISTORICAL LOCAL LYMPH NODE DATA IN THE DEVELOPMENT OF ALTERNATIVE TEST METHODS FOR SKIN SENSITIZATION.

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In developing new alternative toxicological test methods, the availability of high quality, relevant and reliable *in vivo* data for the endpoint of interest is essential. Ideally, data derived in humans would be the most appropriate as the test methods are attempting to predict a toxicological effect in man. However, a sufficient quantity of such data is most likely not available, so data derived from animal studies usually serves as the basis for comparison. In recent years, the local lymph node assay (LLNA) has emerged as a practical option for assessing the skin sensitization potential of chemicals. In addition to accurately identifying skin sensitizers, the LLNA has also been shown to provide a reliable measure of relative sensitization potency; information that is critical in successful management of human health risks. Therefore, for use in evaluating new alternative test methods for skin sensitization and the development of quantitative structure-activity relationship models, a database of historical LLNA data for 232 different chemicals has been created. This extensive chemical dataset encompasses both the chemical and biological diversity of known chemical allergens. To cover the range of relative allergenic potency, the dataset includes 18 extreme, 31 strong, 76 moderate, 65 weak contact allergens as well as 42 chemicals that are considered to be non-sensitizers. In terms of chemical diversity, aldehydes, ketones, aromatic amines, quinones and acrylates are among the classes represented in the dataset. In addition to 2D chemical structures, the physicochemical parameters included are logK<sub>p</sub>, LogK and molecular weight. It is hoped that this database will accelerate the development, evaluation and eventual validation of new approaches to skin sensitization testing.

## 1197

### DEVELOPMENT OF A PEPTIDE REACTIVITY MODEL FOR SCREENING THE SKIN SENSITIZATION POTENTIAL OF CONTACT ALLERGENS.

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In order for a chemical to function as a sensitizer it must penetrate the skin, react with protein and be recognized as antigenic by immune cells. Protein reactivity correlates with skin sensitization potential and this relationship is attributed to the fact that chemical allergens, either directly or following biotransformation have electrophilic properties that enable them to form covalent bonds with nucleophilic amino acids, like cysteine and lysine. An *in vitro* assay using peptides containing cysteine or lysine, or glutathione as surrogate nucleophiles was developed. More than sixty different chemicals with sensitization potencies ranging from weak to extreme, and nonsensitizers were reacted with the peptide at various concentrations and incubation times, and peptide depletion, which indicated chemical adduction, was measured by HPLC. The average depletion using the cysteine peptide at a ratio of 1:10 (peptide:chemical), and an incubation time of 24 hours at 25°C was 79, 60, 26 and 4% for chemicals in the strong, moderate, weak and nonsensitizer groups, respectively. The correlation between the reactivity of this peptide and sensitization potential was found to be statistically significant (rank correlation = 0.75, p < 0.0001). Similar results were obtained with the lysine peptide and glutathione. These peptide reactivity data were used to develop a prediction model based on classification tree methodology. Additional chemicals with known sensitization potential were analyzed and the peptide reactivity data were used to assess the robustness of the prediction model and to refine it. Collectively, these data support the use of a peptide reactivity prediction model to screen the sensitization potential of new chemicals. This model may lead to a reduction or replacement of animal use in skin sensitization testing.

## 1198

### IDENTIFICATION OF POTENTIAL MARKERS FOR THE PREDICTION OF SKIN SENSITIZATION: REAL-TIME PCR ANALYSIS OF ALLERGEN-TREATED DENDRITIC CELLS.

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Cellular changes within resident skin dendritic cells after allergen uptake and presentation are ideal events to model for study of the allergic immune response. Previous microarray analysis of human peripheral blood-derived dendritic cells (PBMC-DC) revealed many changes in gene expression following dinitrobenzene sulfonic acid (DNBS)-allergen treatment. Analysis of the sensitivity, selectivity, and dynamic range of these genes were evaluated previously by quantitative PCR using a select group of chemicals to determine their usefulness as markers for contact allergy. In this study we developed criteria to select a focused candidate gene list. Per those criteria, subsequent validation of the target genes using an expanded chemical dataset was performed. PBMC-DC were treated for 24 hours with various doses of chemicals and RNA was extracted for use in real-time PCR reactions. Specific primers were designed for selected genes and mean relative fluorescence units (RFU) were calculated and then converted to mean fold changes comparing mean RFU in control (vehicle-treated) samples versus mean RFU in treated samples. Allergen-induced changes in the expression of numerous genes associated with immune function, such as CD43, CCL4, CCL23, SLAM, and the Lectin Receptor, were observed. The dynamic range and sensitivity of these genes were examined using multiple doses of allergens that have various potency classifications such as hydroxycitronellal and dicyclyphenylpropenone. Further prioritization of the targets was carried out by analysis of the same candidate gene list on irritant-treated PBMC-DC to determine their specificity for skin sensitization. Real-time PCR analysis of multiple chemical studies has identified 27 potential genes that fit the selection criteria for sensitivity, dynamic range, and reproducibility and thus will be analyzed further for their usefulness as an endpoint measure for the prediction of contact allergy.

## 1199

### EFFECTS OF LIPOPHILICITY AND VISCOSITY OF SOLVENTS ON DPM/LN BACKGROUND LEVEL IN MURINE LOCAL LYMPH NODE ASSAY (LLNA).

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This poster is the third part of our positive- and vehicle-control validation studies in murine local lymph node assay (LLNA). Based on our vehicle-control tests results, (the results have been presented as two posters at the Society of Toxicology

43rd Annual Meeting, Baltimore, March 21-25, 2004 and 10th International Congress of Toxicology, Tampere, July 11-16, 2004, separately), ethanol-water systems were found to be suitable for LLNA tests. One of the most useful vehicles is the ethanol-water (70/30, v/v) (EtOH 70%) solution. In order to provide sufficient scientific rationale, a further investigation and comparison of the allergenic potency and local clinical signs caused by the positive control substance alpha-hexylcinnamaldehyde (HCA) have been made. In six parallel LLNA validation studies with 96 CBA/CaOlaHsd mice performed at RCC, six different solvent systems, five of them are recommended by OECD Guideline 429, have been assayed. In order to rationalize and quantitatively analyze the tests results, some relevant physicochemical properties of the six organic solvents have been compared, and some statistic analyses have also been performed with the data obtained from six different vehicles systems. In the experimental studies, the EC3 value of HCA in EtOH70% system is lower than that in AOO (11.72), PG (7.35) and DMSO (16.21). The EC3 values of HCA in 2-butanone (1.76) and DMF (5.71) are lower than that in EtOH70%, but there are some clinical signs observed in both tests with 2-butanone and DMF. Based on the tests results and statistical analyses on dose-response relationship, including comparison of the allergenic potential of the positive control substance HCA in the six vehicles, it has been improved and rationalized that EtOH70% is a very suitable vehicle and may be widely used in LLNA st

## 1200 INFLUENCE OF AGEING ON LOCAL LYMPH NODE ASSAY RESPONSES TO HEXYL CINNAMIC ALDEHYDE.

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The murine local lymph node assay (LLNA) is an established method for the identification of chemicals that have the potential to cause skin sensitisation and allergic contact dermatitis. This assay relies on the measurement of induced proliferative responses in draining lymph node cells (LNC) as measured by incorporation *in vivo* of radiolabelled thymidine. In the LLNA, skin sensitizers are defined as materials that provoke a 3-fold or greater increase in proliferation compared with concurrent vehicle treated controls. It has been reported previously that immune responses, including some aspects of allergen-induced lymph node activation, may be affected by age. In the current investigations we have examined the impact of age of CBA mice (the recommended strain for use in the LLNA) on proliferative responses stimulated by exposure to hexyl cinnamic aldehyde (HCA); a recommended positive control for skin sensitisation testing. Mice (n=4 per group) of average age of 8, 15 or 21 weeks were exposed to 25%w/v HCA in acetone:olive oil vehicle (AOO; 4:1 ratio), or to AOO vehicle alone, in 2 or 3 independent experiments. Stimulation indices (SI) were derived for each experiment. These values were very consistent within and between age groups of mice, with SI values of 4.9, 6.9, and 6.6 achieved for 8 week, 15 week and 21 week old mice, respectively (representative data). Dose responses were performed (using 5, 10 and 25% HCA) in order to determine mathematically using linear interpolation the estimated concentration of chemical necessary to induce an SI of 3 (EC3 value). Values obtained for 8, 15 and 21 week old animals were 13.0, 10.7 and 13.6, respectively. These data are consistent with previous publications for this chemical using animals in the age range of 8-12 weeks (EC3 of 10.3 +/- 0.6). The conclusion is that, at least with respect to HCA, a mild to moderate skin sensitiser, responses in the LLNA assay are stable across mice with age ranges differing by 13 weeks.

## 1201 CORRELATION OF LLNA AND HUMAN SKIN SENSITISATION THRESHOLDS.

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For years, methods have been available for the predictive identification of chemicals that possess the intrinsic potential to cause skin sensitization. However, many of these methods have proven less capable in terms of the determination of relative sensitizing potency. In this respect, the local lymph node assay (LLNA) has been shown to have a number of advantages. Through interpolation of LLNA dose response data, the concentration of a chemical required to produce a threshold positive response (the EC3 value) can be measured. The robustness of this measure has been thoroughly demonstrated in terms of inter and intra laboratory reproducibility. Additionally, the relationship between potency estimates from the LLNA and an appreciation of human potency has been reported. In the present work, we have sought to enhance this by undertaking a thorough and extensive analysis of existing human predictive assays, particularly where dose response information is available from historic human repeated insult patch tests (HRIPTs). From this human data, information on the approximate threshold for the induction of skin sensitization in the HRIPT was characterised for 24 chemicals. This was compared to LLNA EC3

values. The results from each assay, (expressed in terms of dose per unit area) showed a clear correlation, substantiating the utility of LLNA EC3 values for prediction of the relative human sensitizing potency of newly identified skin sensitizers.

## 1202 POTENCEY ESTIMATIONS FOR SEVERAL FRAGRANCE MATERIALS IN THE LOCAL LYMPH NODE ASSAY AND THEIR CORRELATION TO HUMAN STUDIES.

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In addition to its application as a method to identify potential contact allergens, the murine Local Lymph Node Assay (LLNA) presents the opportunity for the objective and quantitative measure of relative skin sensitizing potency. These potency estimations are typically based on linear interpolation of the dose response data, which yields an estimated concentration (EC3) required to elicit a positive response. In the present study, the LLNA potency estimations of 29 fragrance materials were compared to those derived from human NOELs for induction determined by the Human Repeated Insult Patch Test (HRIPT) and/or the Human Maximization Test (MAX). For each material the human NOELs and EC3 values were converted to their dose per unit area ( $\mu\text{g}/\text{cm}^2$ ) equivalents to allow for direct comparison. Each value was then used to independently assign both human and murine potency based on an expert classification scheme which groups materials as non-sensitizing, extremely weak, weak, moderate, strong and potent. A good correlation existed between the calculated EC3 values and the NOELs for induction of sensitization in humans. The EC3 values were observed to predict and in some cases slightly over predict the potency classes determined from the human NOELs for approximately 80% of the materials tested. Recently there has been a great deal of attention given to the estimation of potency in the LLNA and its correlation to humans with the aim of utilizing the EC3 value as an important data source for dermal sensitization risk assessments. The results observed show that the EC3 value can provide a useful estimate for contact allergenic potential in humans. The data demonstrate that these estimates can be applied to the risk assessment process in the determination of weight of evidence NOELs for the induction of sensitization. Further, the data show that information obtained in the LLNA can be useful in the design of human studies, where ethics dictate that the design act to confirm a calculated NOEL, rather than identify sensitization potential.

## 1203 ASSESSMENT OF THE LOCAL LYMPH NODE ASSAY USING A MODIFIED LOATS AUTOMATED MICRONUCLEUS ASSAY SYSTEM.

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During sensitization, such as in allergic contact dermatitis, antigen-presenting cells are formed and migrate to the local draining lymph nodes (LN) to activate CD4+ T-cells resulting in lymphocyte proliferation. This is the principle of the local lymph node assay (LLNA) that detects DNA synthesis of lymphocytes in the draining auricular LN. The current study was designed to investigate using a modified Loats Automated Micronucleus Assay System (LAMS) for the LLNA. The modified LAMS distinguishes the proliferative phenotype of the cells by color based on 5-bromo-2-deoxyuridine (BrdU) incorporation; red for proliferating cells or blue for non-proliferating cells. Two trials were conducted in female CD-1 mice sensitized with mercaptobenzothiazole (MBT). In the first trial, Animals were administered BrdU intraperitoneally once-a-day for two days following three consecutive days of topical MBT treatment (7.5%). At the end of the trial, the auricular LNs were excised, lymphocytes were isolated, placed on glass slides, and immunostained against BrdU. As expected, MBT increased LN weight and number of lymphocytes compared to controls. BrdU incorporation (proliferation index) was significantly greater in sensitized animals than in control animals; however the Stimulation Index (SI) was less than 3. In the second trial, each animal served as its own control, such that the right ear was sensitized with MBT, and the left ear was challenged with vehicle. One animal was challenged with vehicle only as a negative control. In 4/5 sensitized animals, there was an increased LN weight and lymphocyte number in the sensitized LNs compared to controls. In addition, BrdU incorporation was significantly greater in the sensitized LNs compared to controls. These data indicate that the modified LAMS can quantitatively measure cell proliferation induced by sensitizers, such as MBT, in the LLNA, thereby reducing the number of animals needed, as well as the assay cost.

## 1204 CYTOKINE EXPRESSION PROFILES OF CD4 AND CD8 CELLS FROM PEANUT ALLERGEN-PRIMED MICE.

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There is a growing interest in the development of approaches for assessing the allergenic potential of proteins. Although most foreign proteins are immunogenic (stimulate IgG antibody responses), relatively few are important food allergens. In

the UK and the USA, peanuts are a common cause of food allergy associated usually with high titer IgE antibody and consistent with the preferential activation of T helper (Th) 2 type cells. We have shown previously that sensitization of BALB/c mice to peanut agglutinin (a minor peanut allergen) results in IgE production and elevated type 2 cytokine expression. We have now examined the relative contributions of CD4 (Th) and CD8 (T cytotoxic; Tc) cells to this cytokine phenotype. Mice were immunized by intradermal injection of 1 mg/ml peanut agglutinin and 14 days following the initiation of exposure, draining auricular lymph nodes were excised and single cell suspensions prepared. Cells were labelled with carboxyfluorescein succinimidyl ester (CFSE) to identify proliferating cells, and restimulated *in vitro* with allergen, or with the T cell mitogen concanavalin A (con A). Cells were subsequently stained with fluorescein-labelled anti-CD8 or anti-CD4 antibodies and intracellular cytokine production was measured after saponin permeabilization using fluorescein-labelled anti-cytokine antibodies. Stimulation of cells from peanut-primed mice with either con A or specific allergen resulted in vigorous proliferation of CD4 and CD8 cells, whereas cells from naive mice responded only to con A. Moreover, the proliferating allergen-specific CD4 population was skewed towards a type 2 phenotype, whereas CD8 cells preferentially expressed a Tc1 phenotype (Interleukin-4 and Interferon- $\gamma$  expressing cells 3.5:1 and 1:0.1, respectively). In contrast, con A-activated CD4 and CD8 cells from both peanut primed and naive mice exhibited a heterogeneous cytokine profile. These data suggest that allergen-specific CD4 cells are the major contributors to the Th2 cytokine profile associated with allergic responses induced in mice by peanut agglutinin.

## 1205

### ORAL AND SUBCUTANEOUS EXPOSURE TO PURIFIED PEANUT ALLERGENS AND PEANUT EXTRACT: THE FOOD MATRIX EFFECT.

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There has been a growing interest in the development of experimental animal models to predict allergenicity of food proteins, but at present no definitive test is available. One of the issues that remains to be elucidated is the effect of the food matrix on immune responses to food proteins. To study the intrinsic immunogenicity of purified allergens, and the influence of the food matrix, purified peanut allergens (Ara h 1, Ara h 2, Ara h 3 or Ara h 6) and a whole peanut extract (PE) were tested in both the murine popliteal lymph node assay (PLNA) and in an established oral model of peanut hypersensitivity. In the PLNA, PE or one of the purified allergens were injected into the hind footpad of BALB/c mice and specific responses were measured on day 7. In the oral exposure experiments, PE (1 or 6 mg/mouse) or allergens (0.1 or 1 mg/mouse) in combination with cholera toxin (10  $\mu$ g) as adjuvant were given by gavage to C3H/HeJ mice on three consecutive days followed by weekly dosing. Specific antibody responses were determined weekly. Upon food injection, none of the Ara h proteins did induce significant changes in cell proliferation, cytokine production, or activation of antigen presenting cells (APCs). In contrast, PE induced a 4-fold increase in cell number and high levels of IL-4, IFN- $\gamma$  and IL-10 upon ex-vivo restimulation with PE or the purified allergens. Furthermore PE induced an increase in expression of costimulatory molecules (CD80, CD86, CD54) on APCs. Oral exposure to PE or the Ara h's induced specific IgG and IgE responses in all cases, and Ara h-specific responses were comparable with those observed following exposure to PE. The results obtained using the subcutaneous route of exposure suggest that purified peanut allergens do not possess intrinsic immunogenicity in contrast to a whole PE. However, oral exposure data imply that in this model allergenicity of individual peanut antigens can be revealed with or without the presence of a food matrix.

## 1206

### INFLUENCE OF BACTERIAL ENDOTOXIN ON IGE ANTIBODY RESPONSES PROVOKED IN MICE BY PROTEIN ALLERGENS.

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Immediate type hypersensitivity reactions including food allergy and asthma are mediated by specific IgE antibody. The endotoxin content of protein allergen preparations has been suggested recently to influence the quality of immune responses. Thus, intranasal exposure of mice to the common allergen ovalbumin (OVA) with low levels of endotoxin (0.1  $\mu$ g/mouse) enhanced type 2 responses, including the production of IgE, whereas high dose endotoxin (100  $\mu$ g/mouse) resulted in a selective type 1 response. We have investigated the impact of endotoxin on IgE antibody responses induced by intraperitoneal (ip) exposure of BALB/c strain mice to a range of allergens under conditions where variable amounts of specific IgE are produced. The proteins comprised OVA, and the major Brazil nut and potato allergens, Ber e 1 and Sol t 1, respectively. Animals (n=5) received 1% of protein supplemented with endotoxin (lipopolysaccharide from *E. coli* serotype 055

B5; 0.25 or 25  $\mu$ g/mouse) by ip injection on days 0 and 7. Seven days later, serum samples were analyzed for specific IgE antibody by homologous passive cutaneous anaphylaxis assay. The majority of animals were IgE responders following administration of OVA or Sol t 1, although considerably higher titers (1 in 8 to 1 in 32) were recorded for OVA compared with Sol t 1 exposure (1 in 2). In both cases, IgE responses were unaffected by exogenous endotoxin (0.25 or 25  $\mu$ g/mouse). Treatment with Ber e 1 failed to stimulate detectable IgE antibody. However, high dose endotoxin provoked a marked increase in anti-Ber e 1 IgE antibody production with a titer of 1 in 8 recorded. These data demonstrate that the influence of bacterial endotoxin on IgE antibody is allergen-dependent, but is not associated with the vigor of the IgE response.

## 1207

### CYTOKINE FINGERPRINTING OF CHEMICAL ALLERGENS: COMPARISONS OF MESSAGE VERSUS PROTEIN.

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Topical exposure of BALB/c strain mice to the contact allergen 2, 4-dinitrochlorobenzene (DNCB), or to the respiratory allergen trimellitic anhydride (TMA) induces selective type 1 or type 2 cytokine secretion profiles, respectively. We have now examined whether these cytokine expression patterns are regulated at the level of mRNA and/or protein production. Thirteen days after the initiation of topical exposure, a single cell suspension of auricular lymph node cells (LNC) was prepared. Total RNA was prepared from freshly isolated cells and cytokine gene expression was analyzed by ribonuclease protection assay (RPA). Cells were cultured for 120 h and supernatants analyzed for cytokine protein by ELISA. DNCB-activated LNC secreted high levels of the type 1 cytokine interferon (IFN)- $\gamma$ , compared with TMA-stimulated LNC, reaching maximal levels after culture for 96–120 h. The converse type 2 pattern was observed following treatment with TMA; with secretion of most type 2 cell products (including interleukins [IL]-5, 10 and 13) reaching maximal levels after 96–120 h of culture. LNC from TMA-treated mice displayed a type 2 cytokine mRNA profile, with high levels of transcripts for IL-4, IL-10 and IL-13 detected and relatively low levels of IFN- $\gamma$ . In contrast, RNA isolated from DNCB-activated LNC displayed a mixed cytokine phenotype with relatively low levels of transcripts for both type 1 and type 2 cell products. The observation that IFN- $\gamma$  mRNA expression is not increased despite robust secretion of this cytokine indicates that production of IFN- $\gamma$  by draining LNC is controlled mainly at the level of secretion or translation of previously transcribed mRNA. Furthermore, the preferential cytokine profile recorded following TMA exposure was more clearly contrasting when measured at the level of protein secretion rather than at the level of mRNA expression. These data suggest that cytokine profiling by RPA is not appropriate for the prospective characterization of chemical respiratory and contact allergens.

## 1208

### IMPACT OF ROUTE AND INTENSITY OF EXPOSURE DURING SENSITIZATION IN BN RATS.

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There are good indications that skin exposure can act as a route for respiratory tract sensitization but less is known about effects of the route on the type of immune reaction evoked and on dose-response relationships. In the present study, both the inhalation and dermal route were used to examine whether allergen-induced effects were dose-related or route-of-induction related. The respiratory allergen trimellitic anhydride (TMA) was given at various concentrations topically to male Brown Norway (BN) rats on days 0 and 7, or by inhalation for 3 h/d on days 0 to 4. Dose levels were comparable on a mg/kg BW basis. All animals were challenged by inhalation to TMA on day 21 and lung function was assessed before, during, shortly and 24 h after challenge. At necropsy on day 23, blood was sampled, bronchoalveolar lavage was performed and the respiratory tract was collected. Lung function changes and an inflammatory reaction predominantly associated with a delayed type, cell-mediated immune reaction were observed following sensitization and challenge by inhalation. Increases in total serum IgE were seen at high TMA concentrations only. Sensitization by the dermal route resulted in an immediate, antibody-mediated type of inflammation, altered lung function changes and increased serum IgE levels at all effective doses. Because changes in lung function were seen at comparable (external) doses it could be concluded that these reactions occurred dose-dependently, independent from the route of sensitization. However, the inflammatory reaction and serum IgE levels appeared predominantly influenced by the sensitization route. This research is funded by the Dutch Ministry of SZW and CEFIC-LRI (Brussels, Belgium).

**1209****TOPICAL EXPOSURE OF MICE TO PHTHALATE IS WITHOUT ADJUVANT EFFECT.**

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It has been suggested that one possible contributor to the increasing prevalence of IgE-mediated allergic diseases (including respiratory allergy and asthma) in Western Europe and the US is the presence in the environment of chemicals that may act as adjuvants. It has been reported previously that certain commonly used phthalate plasticizers, including that used most widely worldwide, di-(2-ethylhexyl) phthalate (DEHP), are able under some circumstances to modify immune responses induced in mice by the common allergen ovalbumin (OVA). Thus, it was reported that subcutaneous administration of DEHP (at 2mg/ml) in the presence of OVA resulted in an increase in anti-OVA IgG1 antibody production compared with mice exposed to OVA alone. Murine IgG1 antibody can be considered a surrogate of IgE antibody as cytokine regulation of this subclass of antibody parallels that of IgE. However, the significance of these observations for human health, and for the development of allergic disease is unclear; not least because the studies have been conducted exclusively using subcutaneous administration of phthalates. We have therefore investigated the ability of DEHP when applied topically to affect anti-OVA IgG1 antibody responses induced by subcutaneous exposure to OVA in BALB/c strain mice. Concentrations of DEHP (500mg/ml) were used that resulted in a marked (approximately 30%) increase in liver weight. Under conditions where anti-OVA IgG1 antibody responses were sub-optimal (immunization with 1mg OVA) topical administration of DEHP was without impact on anti-OVA antibody responses, regardless of whether DEHP was applied local or distant to the site of OVA immunization. These data suggest that the reported adjuvant effect of DEHP is a feature of the subcutaneous route of exposure as topical application of concentrations of DEHP that provoked marked systemic effects were without effect on the induction of immune responses.

**1210****AUGMENTATION OF OVALBUMIN-INDUCED IGE AND AIRWAY HYPERREACTIVITY RESPONSE BY PERFLUOROOCTANOIC ACID (PFOA).**

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Studies were conducted to investigate the role of dermal exposure to Perfluorooctanoic acid (PFOA), an immunosuppressant with widespread use as a carpet and fabric protectant, on the hypersensitivity response to ovalbumin in a murine model. BALB/c mice were exposed dermally to concentrations of PFOA ranging from 0.01-2.0% (0.25-50mg/kg) for 4 days. In hypersensitivity studies, mice were also intraperitoneally injected with 7.5µg ovalbumin and 2mg alum on days 1 and 10 and in some studies, intratracheally challenged with 250µg ovalbumin on days 17 and 26. Endpoints for studies included body and organ weights and cellularities, IgE, airway hyperreactivity, and lung histopathology. Following exposure to PFOA, an increase in liver weights and a decrease in thymus and spleen weights and cellularities were observed. Similar immunomodulatory trends were demonstrated in mice co-administered PFOA and ovalbumin. Greater than a 2-fold increase in total IgE was demonstrated when mice were co-exposed with concentrations of PFOA ranging from 0.75-1.5%, while the ovalbumin-specific IgE response peaked after a 3-fold increase ( $p<0.01$ ) in 0.75% PFOA co-exposed animals as compared to the ovalbumin alone exposed animals. Antigen-specific airway hyperreactivity was increased ( $p<0.05$ ) in the 1.0% PFOA co-exposed group, with a dose-responsive pleiotropic cell response characterized by eosinophilia and mucin production, in animals co-exposed to concentrations of PFOA up to 1.0%, as compared to the ovalbumin alone exposed animals. PFOA was demonstrated to be immunotoxic in a murine model following dermal exposure, with an enhancement of the hypersensitivity response to ovalbumin, suggesting that PFOA exposure may augment the IgE response to environmental allergens.

**1211****EVALUATION OF DERMAL SENSITIZATION TO WESTERN RED CEDAR EXTRACT AND ABIETIC ACID USING THE LOCAL LYMPH NODE ASSAY.**

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Western Red Cedar (WRC, *Thuja plicata*) induces sensitization and asthma after occupational exposure. Plicatic acid, a low molecular weight (LMW) compound present at high concentrations in WRC wood has been implicated as the active agent. Another wood product, abietic acid (ABA), a terpenoid present in the resin of conifer species, has also been identified as a LMW agent causing occupational sensitization and asthma. We used the mouse local lymph node assay to address the question of whether these agents could induce sensitization via the dermal route.

WRC was extracted in water and the extract was lyophilized (WRC Extract). Mass spectrometry was consistent with plicatic acid being a major, but not the only, component of WRC extract. WRC extract and ABA were applied in concentrations of 12.5, 25, and 50% (w/v) to the ears of Balb/C mice. Vehicle treatment was used as a negative control and 30% -hexylcinnamaldehyde was used as a positive control. Neither WRC nor ABA caused increases in ear thickness, suggesting that neither was a dermal irritant. *In vivo* cell proliferation in draining lymph nodes was measured as 3H-thymidine incorporation following 3 days of exposure. Lymph node cell populations were evaluated for the presence of B220+ IgE+ B cells by flow cytometry on day 10, following 4 days of exposure. 3H-thymidine incorporation in draining lymph nodes was significantly increased by exposure to all three concentrations of WRC extract and the highest concentration of ABA (50%). Stimulation indices were: 3.4, 6.9, and 11.0 for 12.5%, 25%, and 50% WRC extract; and 3.0 for 50% ABA. Only the 50% WRC extract caused significant increases in B220+ IgE+ B cell percentage in draining nodes. Serum total IgE was increased 2 weeks after dermal exposure every other day to 25% and 50% WRC extract. Our results show that both WRC extract and ABA can induce sensitization by the dermal route and that WRC extract and ABA are weak dermal sensitizers (EC3 10% and 50%, respectively).

**1212****INDUCTION OF CONTACT SENSITIZATION BY ANNATTO EXTRACT BIXIN BUT NOT BY NORBIXIN IN FEMALE BALB/C MICE.**

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Annatto seeds, also known as 'achiote seeds', are commonly used in South American cooking. Annatto dye is an orange-yellow food coloring extracted from the seeds of the tree *Bixa orellana*. It has been used to color butter, margarine, cheese, smoked fish, snack foods, beverages, and cereals. Reported adverse reactions associated with annatto dye ingestion include urticaria and angioedema. The present studies were carried out to examine a possible contact allergenic effect of two purified carotenoid pigments from annatto extract: bixin (BIX: C25H30O4) and norbixin (NOR: C24H28O4). The experiments were conducted in female BALB/c mice by applying BIX (1-25% w/v) and NOR (1-20% w/v) in acetone topically, and assays performed including the local lymph node assay (LLNA), the irritation assay, the mouse ear swelling test (MEST) and flow cytometric analysis. There was no treatment-related adverse effect on the body weights. However, a three-fold increase in the proliferation of auricular lymph node cells was observed at BIX concentrations of 5%-25% ( $p\leq 0.01$ ). A significant increase in percent ear swelling was also produced in mice treated with BIX (5-10%) at 24 hr after challenge as measured in MEST assay. Further study indicated that BIX was not an irritant at the concentrations tested. Additional study demonstrated that exposure to BIX induced a significant increase in the percentage of B cells (Ig+) in the draining lymph node. In contrast, the results from studies on NOR showed that exposure to NOR at concentration levels of 1-20% did not alter the lymph node cell proliferation in the LLNA assay. Taken together, these results suggested that in annatto extract bixin but not norbixin is a contact sensitizer in female BALB/c mice (Supported by the NIEHS Contract ES 05454).

**1213****DIFFERENTIAL ALLERGIC AND NEUROTROPHIN RESPONSES TO FUNGAL COMPONENT EXTRACTS IN MICE.**

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*Metarhizium anisopliae* mycelium (MYC), conidia (CON) and inducible protease (IND) extracts were combined to produce the antigen MACA to screen for allergenic potential. Involuntary aspiration (IA) exposure to MACA in BALB/c mice has caused immune, inflammatory and physiological responses characteristic of allergic lung disease. Our objective here was to determine if the component extracts have similar allergenic potential. BALB/c mice received 4 IA of 10 µg of MACA, CON, MYC, IND, or BSA (negative control) in 50 µl HBSS, or HBSS alone (vehicle control) over a 4-week period. Additional mice received 1 exposure to agents as non-allergic controls. Serum and bronchoalveolar lavage fluid (BALF) were collected 3 days (D3) after the final exposure. Mice were assessed for immediate responses following IA exposure and airway hyperresponsiveness to methacholine (MCH; D1 & D3) by whole-body plethysmography, an index of pulmonary resistance and bronchoconstriction (PenH). All fungal extract exposures resulted in elevated BALF total protein, LDH, total cell counts, total IgE and IgA and serum IgE compared to HBSS and BSA controls. These responses were highest in MYC exposed mice, but their PenH was significantly lower compared to the other fungal treatments. To investigate this dichotomy, the level of BALF neurotrophins (NGF, NT-3, NT-4) that have been associated with allergic lung disease were evaluated. All extract exposures resulted in increased levels of the neurotrophins compared to controls. The IND treated mice had the lowest levels of the neurotrophins and significantly higher PenH compared to the other fungal treatments. The rank order of

airway responses to the fungal extracts was the inverse of the other endpoints. These data show differences in the magnitude of responses by the *M. anisopliae* extracts. Additionally, the data suggest that the neurotrophins are not the sole source of the airway responses induced by these extracts. (Supported by UNC/EPA Agreement CT826513. This abstract does not reflect EPA policy)

**1214**

EFFECTS OF DIESEL EXHAUST ON PULMONARY RESPONSES DURING ALLERGIC SENSITIZATION TO AEROSOLIZED OVALBUMIN IN BALB/C MICE.

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Inhalation of diesel exhaust (DE) is associated with the development of asthma. Studies have also demonstrated that DE induces pulmonary changes that worsen asthmatic responses to respiratory allergens. We have established a DE inhalation-exposure system with the capability of adjusting from whole exhaust to gas-only exposures and that provides time-of-flight measurements of both size-fractionated particle concentrations and gaseous constituents such as NOX, SOX, O<sub>2</sub>, and CO<sub>2</sub>. In order to understand the effects of DE on early development of allergic airway responses *in vivo*, female BALB/c mice were exposed for 4 hours on a single day to either air or DE (particle concentrations of 0.2 mg/m<sup>3</sup> and 2.0 mg/m<sup>3</sup>) or each day for 5 days followed by a daily 40 min exposure to aerosolized ovalbumin (OVA). Responses were measured in the bronchoalveolar lavage fluid (BALF) and in lung tissue homogenates on days 1 and 5. On day 1, DE exposed mice had increased inflammatory cell influx and raised levels of NF-κB in lung tissues, which correlated with some increases in cytokine release, but no increases in cytotoxicity or pulmonary edema compared to air-exposed mice. Five days of DE exposure followed by OVA did not affect cellular inflammation, but cytotoxicity and pulmonary edema were increased compared to air/OVA exposures. Inhalation of OVA increased MIP-2 levels in the BALF and induced both NF-κB and phosphorylated p38MAP kinase signaling proteins in lung cell lysates. Five days of DE exposure had a synergistic effect on lung injury in OVA sensitized mice but did not potentiate the cytokine response. We conclude that this system will be useful for tracking qualitative and kinetic changes in cellular responses during allergic sensitization with controlled modifications of inhalation exposure conditions. Further studies using this system will make it possible to correlate specific biological responses to individual particulate and gaseous components of DE emissions. (This abstract does not reflect EPA policy.)

**1215**

GLABRIDIN INHIBITS INDUCIBLE NITRIC OXIDE SYNTHASE EXPRESSION BY BLOCKING NUCLEAR FACTOR-κB ACTIVATION IN MURINE MACROPHAGES.

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Glabridin, a flavonoid present in licorice extract, is known to have antimicrobial, anti-inflammatory and cardiovascular protective activities. In the present study, we report the inhibitory effect of glabridin on nitric oxide (NO) production and inducible nitric oxide (iNOS) gene expression in murine macrophages. Glabridin attenuated lipopolysaccharide (LPS)-induced NO production in isolated mouse peritoneal macrophages and RAW 264.7 cells, a mouse macrophage-like cell line. Moreover, iNOS mRNA expression was also blocked by glabridin treatment in LPS-stimulated RAW 264.7 cells. Further study demonstrated that the LPS-induced NF-κB/Rel DNA binding activity and NF-κB/Rel-dependent reporter gene activity were significantly inhibited by glabridin in RAW 264.7 cells and that this effect was mediated through the inhibition of inhibitory factor-κB degradation and p65 nuclear translocation. In animal model, *in vivo* administration of glabridin increased the rate of survival of LPS-treated mice and inhibited LPS-induced increase in plasma concentrations of nitrite/nitrate and tumor necrosis factor-α. Collectively, these data suggest that glabridin inhibits NO production and iNOS gene expression by blocking NF-κB/Rel activation and that this effect was mediated, at least in part, by inhibiting reactive oxygen species generation. Furthermore, *in vivo* anti-inflammatory effect of glabridin suggests a possible therapeutic application of this agent in inflammatory diseases.

**1216**

RHOA/ROCK SIGNALING NEGATIVELY REGULATES NUCLEAR FACTOR KAPPA B (NF-κB) ACTIVATION VIA MODULATION OF IκBα LEVELS IN KIDNEY EPITHELIAL CELLS.

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The transcriptional activator, NF-κB, controls the expression of genes involved in inflammation, proliferation and survival in a variety of cell types. We previously described NF-κB activation in normal rat kidney epithelial (NRK52E) cells by

lipopolysaccharide (LPS) and other agents, and showed that this involves phosphorylation and degradation of the inhibitor protein, IκBα. However, the specific mechanisms mediating this response were not defined. Here, we report findings suggesting the involvement of the Rho family GTPases, RhoA, and its associated kinase, ROCK, in the control of NF-κB activation in kidney cells. Treatment of NRK cells with LPS (1 μg/ml) induced maximal NF-κB-DNA binding intensity within 30 min, which dissipated within 2 hrs. Pretreatment of cells with Y27632 (30 μM), a specific ROCK inhibitor, doubled the maximal intensity of LPS-induced NF-κB-DNA binding and prolonged the binding intensity for up to 4 hrs. Y27 pretreatment also doubled the intensity of LPS-induced NF-κB-dependent transcriptional activity in cells transfected with a 4xNF-κB-luciferase reporter plasmid. Consistent with these observations, nuclear IκBα content declined to <10% of untreated levels by 10 min after LPS treatment but recovered to 100% by 3 hrs. In contrast, when Y27 was administered before LPS, nuclear IκBα declined and remained at <10% of untreated levels with no recovery for up to 4 hrs. Finally, Y27 pretreatment dramatically increased both the amount and duration of IκBα phosphorylation, compared with that seen after LPS alone. Similar effects were observed when cells were pretreated with GGI, an inhibitor of RhoA activation. These findings suggest that Rho/ROCK signaling negatively affects NF-κB activation in kidney epithelial cells by modulating both cytoplasmic and nuclear levels of the NFκB inhibitor, IκBα. Supported by ES04696, ES07032 and ES07033.

**1217**

INDUCTION OF NUCLEAR FACTOR-KB ACTIVATION THROUGH TAK1 AND NIK BY DIESEL EXHAUST PARTICLES IN L2 CELL LINES.

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Diesel exhaust particles (DEPs) are known to induce allergic responses in airway epithelial cells such as the production of various cytokines via nuclear factor-kappa B (NF-κB). However, the intracellular signal transduction pathways underlying this phenomenon have not been fully examined. This study showed that the DEP induced NF-κB activity via transforming growth factor-β activated kinase 1 (TAK1) and NF-κB-inducing kinase (NIK) in L2 rat lung epithelial cells. DEP induced the NF-κB dependent reporter activity approximately 2-3 fold in L2 cells. However, this effect was abolished by the expression of the dominant negative forms of TAK1 or NIK. Furthermore, it was shown that DEP induced TAK1 phosphorylation in the L2 cells. These results suggest that TAK1 and NIK are important mediators of DEP-induced NF-κB activation.

**1218**

APPLICATION OF A HIGH-COVERAGE, FUNCTIONAL GENOMIC SCREEN TO DISSECT THE NFκB SIGNALING PATHWAY.

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While the organization of numerous signal transduction pathways has been extensively studied, the composition of the majority of pathways is still not completely understood. Current estimates indicate that greater than 40% of the predicted human genes do not have a putative molecular function or known role within a signaling network. Closing the knowledge gap by assigning functions to these genes and placing them contextually into signaling pathways will be critical for understanding cellular responses to toxic agents and disease. The NFκB signaling pathway was chosen due to its role in the cellular response to inflammation and acute toxicity. To identify the components of the NFκB signaling pathway, HEK 293T cells were transduced with a lentivirus containing a NFκB luciferase reporter creating a stable cell line with a high level of signal-to-noise (>30-fold induction with TNFα). Using the stable cell line, a high-coverage gain-of-function (GOF) screen was performed by individually expressing approximately 14, 500 full-length mammalian genes and evaluating what genes when added to cells alter NFκB signaling. More than 30 high confidence genes were identified in the preliminary analysis with several of the genes previously shown to play a role in NFκB signaling including lymphotxin α, TNFR10A, and TNFR10B. However, most of the genes identified are not currently known to play a role in NFκB signaling. The results are presently being confirmed in follow-up assays. The results of the GOF screen were stored in a relational database and bioinformatic tools were used to construct an interactive signal transduction network. The structure of the signaling network was refined using known biomolecular interactions and additional signaling information from the literature. The results demonstrate that application of large-scale genomic screens can identify genes that play a functional role in signaling pathways and reveal previously unknown targets of toxicity.

**1219**

TRPV1 ANTAGONISTS INDUCE TRANSLOCATION OF INTRACELLULAR TRPV1 TO THE PLASMA MEMBRANE ENHANCING VANILLOID-INDUCED TOXICITIES.

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Functional regulation of TRPV1 influences the relative sensitivity of lung epithelial cells to toxicities induced by vanilloid receptor agonists, such as capsaicin. An understanding of how TRPV1 is regulated in lung epithelial cells will provide essential information that could facilitate the identification of drug therapies to remediate lung disorders (e.g., asthma, COPD), that are caused and/or aggravated by xenobiotics interacting with TRPV1. Preliminary results have shown that TRPV1 over-expressing BEAS 2B cells preexposed to various TRPV1 antagonists exhibited increased sensitivity to prototypical TRPV1 agonists, as measured by enhanced calcium flux and cytotoxicity. Incubation (4 hrs) with LJO-328 (1  $\mu$ M), a potent and selective TRPV1 antagonist, caused a >250-fold decrease in the LD50 and a doubling in calcium flux following nonivamide administration. In contrast, LJO-328 co-incubated with TRPV1 agonists (i.e. RTX or nonivamide) prevents TRPV1-mediated calcium flux and cell death. Attenuation of the antagonist-induced enhancement of TRPV1-mediated toxicities with brefeldin A (a golgi transport inhibitor), but not cycloheximide (a protein synthesis inhibitor), suggested that exposure to TRPV1 antagonists induced translocation of existing intracellular stores of TRPV1 to the plasma membrane, augmenting TRPV1 agonist sensitivity in these cells. These data indicate an intracellular receptor trafficking mechanism for the regulation of TRPV1 distribution and function to maintain an operative plasma membrane population of TRPV1 in these cells. Supported by: The National Heart, Lung, and Blood Institute (HL069813).

**1220**

INDUCTION OF ORGANIC ANION TRANSPORTING POLYPEPTIDE 2 EXPRESSION BY CYCLIC AMP-DEPENDENT SIGNALING PATHWAY IN MOUSE HEPA-1 CELLS.

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Cyclic AMP (cAMP) is a ubiquitous second messenger involved in many signal transduction pathways. The expression of a number of genes is modulated in response to hormones and other environmental signals via variations in intracellular cAMP. The aim of this study was to determine whether a cAMP-dependent signaling pathway is involved in the regulation of mouse organic anion transporting polypeptide 2 (Oatp2), a hepatic uptake transporter for organic anions, such as cardiac glycosides. Hepa-1 cells (a mouse hepatoma cell line) were treated with four different concentrations of the adenylate cyclase activator forskolin (0.5-10  $\mu$ M) or two cellular membrane-permeable cAMP analogs, dibutyryl cAMP and 8-bromo-cAMP (0.01-1 mM). Mouse Oatp2 mRNA was quantified using the branched DNA signal amplification assay. All four concentrations of forskolin, as well as dibutyryl cAMP and 8-bromo-cAMP induced mouse Oatp2 expression. The Oatp2 mRNA expression was increased at least 3 fold in cells treated with 10  $\mu$ M forskolin, 1 mM dibutyryl cAMP, or 1 mM 8-bromo-cAMP. A luciferase reporter gene construct containing 7.8-kb of the 5'-flanking region of mouse Oatp2 was engineered. Luciferase activity in response to treatment with 5  $\mu$ M forskolin was assayed in Hepa-1 cells transiently transfected with the mouse Oatp2 promoter reporter gene construct. Forskolin treatment induced reporter gene activity by approximately 3 fold. In conclusion, Oatp2 expression is induced by a cAMP-dependent signaling pathway in mouse Hepa-1 cells. (Supported by NIH grants ES-09649)

**1221**

TOBACCO SMOKE-INDUCED EPITHELIAL CELL PROLIFERATION AND SQUAMOUS METAPLASIA IN THE LUNGS OF RATS: ROLE OF MAPK/AP-1 PATHWAY.

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Epidemiological and experimental evidence has demonstrated tobacco smoke (TS) is causally associated with various types of cancers including lung cancer. However, the cellular and molecular mechanisms remain unclear. Sustained epithelial cell hyperplasia and squamous metaplasia are considered as preneoplastic lesions during the formation of lung tumors. Mitogen-activated protein kinases (MAPKs)/activator protein -1 (AP-1) pathway plays central role in the control of cell proliferation and differentiation, and can be activated by various stimuli such as carcinogens, oxidants and inflammatory cytokines, all factors that are produced by TS. To date, information on the response of MAPK/AP-1 pathway during TS-induced hyperplasia and squamous metaplasia is virtually absent. The present study investigated the effects of TS on the development of epithelial hyperplasia and squamous metaplasia, activation of MAPK/AP-1, and expression of AP-1 target genes including cell cycle

regulators and differentiation markers in rats. Exposure to TS (30 mg/m3 and 80 mg/m3, 6h/day x 3days/ week) for 14 weeks dramatically induced epithelial cell proliferation and squamous metaplasia in a dose-dependent manner, effects that paralleled the activation of AP-1 - DNA binding activity. TS significantly increased the levels of phosphorylated ERK1/2, JNK, p38 and ERK5, indicating the activation of these MAPK pathways. Expression of jun and fos proteins were differentially regulated following TS exposure. Moreover, TS upregulated AP-1 -dependent cell cycle proteins including cyclin D1 and PCNA, while AP-1 -dependent cell differentiation markers including keratin 5, 14, loricrin, and filaggrin were altered correspondingly. These findings provide the first report that demonstrated the role of MAPK/AP-1 pathway in TS-induced preneoplastic lesions such as epithelial hyperplasia and squamous metaplasia.

**1222**

MAPPING GENE EXPRESSION NETWORKS: USING WHOLE GENOME EXPRESSION ANALYSIS WITH RNAI TO DEFINE THE HEAT SHOCK SIGNAL TRANSDUCTION CASCADE.

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Gene expression networks (GENs) are collections of linked signal transduction pathways that convert input signals into complex gene expression cascades. In a well known example, exposure of cells to steroid hormones results in a primary wave of gene expression that is regulated by the corresponding ligand-dependent transcription factor. The primary wave of gene expression is enriched in transcription factors that, in turn, activate a secondary wave of gene expression. This cycle is repeated to form the GEN associated with the specific steroid hormone. In this work, we present an approach to "reverse engineer" GENs using a combination of time-course microarray analysis coupled with RNAi-mediated inhibition of key transcription factors in the GEN. Our model for this approach is the heat shock treatment of HeLa cells since it is a general stress response in cells and is present following exposure to chemical toxicants. When HeLa cells are pretreated with an siRNA for the HSF1 transcription factor (siHSF1) we observe a five-fold decrease in the induction of HSP70, indicating that HSP70 is regulated by HSF1. Although this observation has been previously reported, it validates the underlying approach. In the presence of siHSF1, we also detected a significant alteration in a number of other genes such as placental growth factor and PZDK3. Additionally, we have identified two transcription factors that are induced in the primary wave of gene expression, KLF2 and EGRI. Expression of KLF2 was then blocked via siRNA treatment and downstream signaling was compared to both siHSF1 treated cells and untreated cells. Additional genes were identified as KLF2-dependent, allowing a separate branch of the heat shock GEN to be mapped. These results demonstrate that the coupling of whole-genome expression analysis with RNAi can be a powerful tool for clarifying existing signaling branches, and identifying previously unknown branches that play a role in a given network or toxicological endpoint.

**1223**

IDENTIFICATION OF SIGNALING PATHWAYS ACTIVATING REACTIVE GLIOSIS IN MULTIPLE MODELS OF BRAIN INJURY: A GENOMIC, PROTEOMIC AND PROTEIN PHOSPHORYLATION ANALYSIS.

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Reactive gliosis (gliosis, glial activation) is a hallmark of disease-, trauma- and chemical-induced damage to the CNS. This response is characterized by activation of microglia and astrocytes at sites of damage. Despite the regional- and cell-type specific targets of individual nervous system insults, reactive gliosis represents a feature common to all types of neural injury. This implies that there are common 'signals' responsible for its induction, signals that remain to be elucidated. Discovery and characterization of the signaling events that lead to this common response to brain damage would enhance our ability to detect early signatures of the neurotoxic condition. To achieve this, we used various brain injury models [1-Methyl-4-phenyl-1, 2, 3, 6, -tetrahydropyridine (MPTP), methamphetamine (METH), kainic acid (KA), and traumatic brain injury (Stab)] as denervating tools to damage distinct areas of mouse brain and elicit a glial response. Applying genomic (cDNA microarray, real-time PCR) and proteomic (antibody microarray, Ciphergen ProteinChip<sup>®</sup> platform, phosphoprotein immunoblots) analysis, we identified the early induction of several ligands of the gp130 signal transducer family (IL-6, CNTF, LIF, OSM) and other cytokines and chemokines (TNF- $\alpha$ , MCP-1) known to activate the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway. The temporal expression of these ligands and the specific activation of JAK2-STAT3 was selective to the target region and preceded the induction of GFAP and astrocyte hypertrophy across all brain injury models examined. Pharmacological inhibition of JAK2 with tyrophostin B42 (AG490) attenuated STAT3 activation and GFAP expression in the MPTP model. Taken together, our

results suggest that the JAK2-STAT3 pathway is activated prior to induction of astrogliosis in multiple models of brain injury. Ligands and effectors associated with activation of this pathway may serve as early 'biomarkers' of neurological/neurotoxic hazards.

## 1224

### CHANGES IN EXPRESSION OF TOTAL AND PHOSPHORYLATED ERK1/2 IN TCDD-EXPOSED EMBRYONIC MOUSE PALATES.

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2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD) induces cleft palate in mice by preventing fusion of the palatal shelves. TCDD maintains EGF and EGF receptor (EGFR) expression levels in medial epithelial cells of the palate at a time when EGFR levels fall in those of controls. EGFR activates the MAPK - ERK pathway; therefore, MAPK signaling may play a critical role in mediating TCDD-induced cleft palate. In this study, we examined whether TCDD exposure alters MAPK signaling by monitoring the ratio of phosphorylated ERK (pERK) to total ERK (tERK) in embryonic mouse palates. Pregnant mice were dosed with 24 ug/kg TCDD or corn oil (control, CO) on gestational day (GD) 12 and embryos were collected on GD 14. Embryonic palates from 5 control and 5 treated litters were isolated and pooled by litter. Expression of pERK and tERK protein were quantitated by densitometric analysis of Western blots and by ELISA, and the ratio of pERK/tERK was determined. On Western blots, all bands were normalized to Bactin, and ERK appears as 2 bands, ERK1 and ERK2. Western analysis revealed that the pERK/tERK ratio was reduced in the TCDD (0.25 ± 0.05) compared to CO (0.41 ± 0.08; p < 0.01) group for ERK1 but not for ERK2 (0.25 ± 0.09 TCDD vs. 0.33 ± 0.10 CO). There was more tERK1 in the TCDD group compared to CO. ELISA analysis showed the ratio of pERK/tERK was not different between the TCDD and CO groups. However, ELISA analysis does not separate ERKs 1 and 2 and may not be able to detect changes in one isoform. When data from both bands were added together in the Western analysis, no significant difference was found in the ratio of pERK/tERK ratio between TCDD and CO groups. These preliminary results suggest tERK1 is more abundant compared to controls but kinase activity is not high enough to maintain the pERK/tERK ratio in palates exposed to TCDD. Additional data on pERK/tERK at other time points and in specific areas of the palate may more fully illustrate the pathway for TCDD-induced cleft palate. [This abstract does not necessarily reflect EPA policy].

## 1225

### INTERACTIONS WITH THE TGF- $\beta$ SIGNALING PATHWAY: A KEY MECHANISM OF DEVELOPMENTAL TOXICITY?

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The TGF- $\beta$  signaling pathway is responsible for regulation of several cellular processes, including pattern formation and tissue specification during embryo development. Mutations in components of the TGF- $\beta$  pathway at later development stages often result in patterning defects and malformation of embryonic organs in vertebrate animals; similarly, mutations in these components have been linked to several human birth defects. However, interference with this pathway by environmental agents has yet to be defined as a key mechanism of toxicity. To determine if interactions between environmental toxicants and this signaling pathway represent a key mechanism of toxicity, we searched the literature for interactions between agents that cause developmental toxicity and initiating events of the TGF- $\beta$  signaling pathway. These events were then linked to adverse outcomes through a cascade of biochemical and physiological changes that resulted from the initial interactions between the toxicants and the signaling pathway. We found that environmental tobacco smoke, retinoic acid, cyclophosphamide and alcohol all interact with the TGF- $\beta$  signaling pathway in a similar manner to cause adverse outcomes. Moreover, these agents all interact with the pathway to cause a change in either the expression of TGF- $\beta$  or its receptor or the localization pattern of TGF- $\beta$  or its receptor. These results demonstrate that interactions between these agents and the TGF- $\beta$  signaling pathway may constitute a fundamental pathway for developmental toxicity. Defining mechanisms of toxicity will be important for reducing uncertainties and for animal to human data extrapolation in human health risk assessment. Disclaimer: The views expressed are those of the authors and do not necessarily reflect the views or policies of the USEPA.

## 1226

### DIABETES-INDUCED NEPHROPATHY ASSOCIATES WITH AKT AND P38 KINASE ACTIVATION.

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Diabetic nephropathy is related to tubulointerstitial accumulation of extracellular matrix (ECM). Tubular atrophy, initiated from renal tubular epithelial cell (RTC) apoptosis, has repeatedly been shown to be one of the best histological predictors of

renal ECM accumulation in biopsies. The present study tested the hypothesis that alterations of renal signaling pathways under diabetic condition are causative factor for the development of diabetic nephropathy. Firstly, diabetic mice were produced by single dose of streptozotocin (STZ) and were sacrificed at one, three, and six months after hyperglycemia. Results showed that six months of STZ-induced diabetic mice significantly presented renal fibrosis, shown by increases in serum BUN and renal PAS staining. Western blot analysis demonstrated significant increases in renal Akt phosphorylation at one and three months, followed by a decrease at six months after hyperglycemia. Increased Akt phosphorylation at 3 months, resulted in enhanced association of Akt and p38 MAPK as demonstrated by immunoprecipitation studies. These results suggest a cross-talk between Akt/p38 kinase pathways. Immunohistochemical staining of control and 3 months diabetic kidney sections showed enhanced phosphoS<sup>473</sup>-Akt in the RTC. Therefore, we have further studied the effect of high levels of glucose (22 mM) on human RTCs (HCK11 cell line) on the Akt signaling and apoptotic cell death. Exposure of RTCs to 22 mM glucose significantly increased Akt Ser<sup>473</sup> phosphorylation at three and nine hours, and decreasing thereafter. Additionally, hyperglycemia induced p38 phosphorylation at six to twelve hours. Under the same experimental condition, apoptotic cell death, detected by TUNEL positive cells, was significantly increased after 12 hrs. These results suggest that diabetes-induced nephropathy may associate with diabetes-induced RTC cell death through down-regulation of Akt/p38 MAPK pathways, resulting in a tubular atrophy and consequently tubulointerstitial fibrosis (Supported, in part, by ADA and AHA grants).

## 1227

### DISPOSITION OF TCDD IN A MOUSE MODEL OF OBESITY AND TYPE II DIABETES.

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Recent epidemiology studies have shown an association between type II diabetes and exposure to TCDD (2, 3, 7, 8-tetrachlorodibenzo-p-dioxin). A possible explanation is that diabetics have a slower elimination of TCDD than non-diabetics. The objective of the present study was to examine the influence of diabetes and body fat mass on the disposition of TCDD using a mouse model of obesity and Type II diabetes. Male C57BL/6J (C57) and AJ/6 (AJ) mice were placed on a normal diet or a high fat, high simple carbohydrate (HFHSC) diet for 13 wks before the start of the study. The HFHSC diet was used to induce obesity and signs of type II diabetes in C57 mice; while AJ mice on a HFHSC diet became fatter than AJ/6 on a normal diet, but not obese nor hyperglycemic. Mice were then housed in metabolism cages, maintained on their respective diets throughout the study, and given a single po dose of 5  $\mu$ g [ $^3$ H]TCDD/kg. After 40 days, mice (singly housed in metabolism cages with separate collection of urine and feces every 24h) were killed and tissues collected. Disposition in excreta and tissues was quantified. Percentages ( $\pm$ SD) of cumulative administered dose excreted were: in feces 50±5.4, 32±3.6, 48±2.3, 33±3.8; and in urine 22±2.0, 27±5.7, 15±1.5, 21±5.0 for C57 normal diet, C57 fat diet, AJ normal diet, and AJ fat diet, respectively. Percentages ( $\pm$ SD) of administered dose at 40 days post-dosing were: in liver 2.9±0.3, 6.7±1.2, 5.0±0.5, 5.5±1.3; and in adipose tissue 4.7±0.6, 28.3±4.1, 7.6±0.8, 21.7±3.2 for C57 normal diet, C57 fat diet, AJ normal diet, and AJ fat diet, respectively. Data from this study made clear the effects of body fat and diabetes on the deposition of TCDD in excreta and tissues, and showed that a high body fat mass slowed the elimination of TCDD. (This work was funded in part by an Interagency Agreement with the US Air Force #FQ7624-00YA085. This abstract does not represent USEPA policy.)

## 1228

### MATERNAL AND FETAL DISPOSITION OF GENISTEIN GLUCURONIDE AND SULFATE CONJUGATES FOLLOWING 14-DAY *IN UTERO* EXPOSURE TO GENISTEIN.

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Diets high in soy-based products are well known for their estrogenic activity. Genistein, the predominant phytoestrogen present in soy, is known to interact with both estrogen receptor (ER)  $\alpha$  and  $\beta$ . Genistein has been shown to elicit reproductive effects in developing rodents. In the rat, genistein is metabolized predominantly to glucuronide and sulfate conjugates. Neither the glucuronide nor the sulfate conjugate are capable of activating ER. Therefore it is critical to understand how much unconjugated genistein is delivered to the fetus following maternal exposure, and how much remains unconjugated once in the fetus. Genistein (4 and 40 mg/kg) was administered to pregnant Sprague-Dawley rats by oral gavage daily from GD 5-19. Maternal and fetal plasma along with placenta were collected at various time points (0.5, 1, 2, 4, 6, 8, 12 and 24 h) following administration of the final dose on GD 19. Concentrations of genistein, genistein glucuronide and genistein sulfate were quantitated by LC-MS/MS. For both maternal and fetal plasma,

the genistein glucuronide and genistein sulfate were predominant, with unconjugated genistein levels much lower. The ratio of genistein glucuronide to genistein sulfate was higher in the fetal plasma compared to maternal plasma. There was a linear increase in genistein glucuronide and genistein sulfate in fetal plasma with time, while unconjugated genistein concentrations remained relatively constant after an initial appearance in fetal plasma within 1 hour of *in utero* exposure. In the placenta, the concentration of genistein was present at higher levels than the conjugates. It is unclear from these data whether it is maternal transfer, placental formation or fetal liver formation that contributes to the concentrations of conjugates observed in fetal plasma. These data suggest that the concentration of unconjugated genistein available for ER interaction in the fetus is relatively low following maternal exposure to genistein. (Supported by the American Chemistry Council).

**1229**

THE CANALICULAR TRANSPORTER MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN 2 FACILITATES BILIARY EXCRETION OF DIETHYLSТИLBESTROL.

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Diethylstilbestrol (DES) is a synthetic estrogen that is metabolized in liver primarily by conjugation with UDP-glucuronic acid. The multidrug resistance-associated protein 2 (Mrp2) transporter is localized to the canalicular membrane of hepatocytes and is responsible for export of glucuronide conjugates into bile. The purpose of this work was to test the hypothesis that Mrp2 is responsible for the biliary excretion of DES by studying DES excretion in TR(-) rats, which lack functional Mrp2 protein. The femoral artery, femoral vein, and common bile duct of male control Wistar rats and TR(-) rats were cannulated, after which rats received an intravenous dose of 100 µg/kg (25 µCi/kg) of radiolabeled DES. Blood and bile samples were collected over the course of one hour. Chloroform extraction of samples was performed to separate parent lipophilic DES from its water-soluble conjugates, after which DES was quantified by scintillation counting. Plasma disappearance of parent DES was similar in both Wistar and TR(-) rats. In Wistar rats, serum levels of conjugated DES remained low, however, in TR(-) rats, serum levels of conjugated DES increased over the course of one hour. Within 15 min of administration, Wistar rats excreted approximately 12.5 µg of DES per kg body weight into bile; nearly 78% of this DES was in conjugated form. In contrast, in TR(-) rats, only 0.1 µg/kg (63% conjugated) of DES was excreted into bile within 15 min. After 1 hour, 30.3 µg/kg of DES (83% conjugated) was excreted into bile of Wistar rats, whereas in TR(-) rats, only 0.85 µg/kg (84% conjugated) of DES was excreted. Total DES was quantified in liver and kidney 1 hr after administration. TR(-) rats had 3.6- and 3.9- fold more DES in liver and kidney, respectively, compared to Wistar rats. In summary, impaired biliary excretion of DES, increased serum levels of DES conjugates, and accumulation of DES in tissues in TR(-) rats, collectively indicate that Mrp2 is principally responsible for biliary excretion of DES. (Supported by ES-08156, ES-09716 and ES-07079)

**1230**

DISPOSITION OF 2, 2', 4, 4', 5, 5'-HEXBROMODIPHENYL ETHER IN F344 RATS AND B6C3F1 MICE.

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2, 2', 4, 4', 5, 5'-Hexabromodiphenyl ether (BDE-153) is a major congener in the polybrominated diphenyl ether (PBDE) Great Lakes DE-71®. DE-71 is used as a flame retardant in plastics, electronic equipment, textiles, and building materials. DE-71 congeners are present in the environment and have been shown to accumulate in mammalian tissues and fluids. PBDE's are of toxicological interest because of their reported neonatal neurotoxicity in rodents. Toxicity studies on DE-71 are being designed by the National Toxicology Program. The disposition of individual congeners in DE-71 is being determined to investigate possible interactions among them. Thus the disposition of <sup>14</sup>C-BDE-153 was studied in male and female F344 rats and B6C3F1 mice for comparison to similar studies of BDE-99 and 47 conducted in our lab. Results of iv and oral administration of 1 µmol/kg doses, indicate that ca. 70% of a single oral dose is absorbed in rats in contrast to 85% of BDE-99 and 70% of BDE-47 being absorbed. After an oral dose, BDE-153 concentrates in fat (17% of the total dose after 24 hours in rats and 21-27% in mice) and is excreted in the feces (25-30%), with less than 1% in urine. The liver/fat ratio is 0.88 in rats and 0.29 in mice in comparison to 0.22 and 0.14 for BDE-99 and BDE-47 respectively in rats. BDE-153 concentration after an oral dose was higher in all tissues than in blood, with tissue/blood ratios (TBR) over 6 in liver, fat, thymus, and adrenal glands. The adrenal gland TBR ranged from 48 to 244 with the concentration twice as high in female rats and mice as in males. The amount of BDE-153-derived radioactivity in thyroid is not significantly different from other tissues in the rat but is 3 to 4 times higher in mice than rats. The thyroid concentration in rats

was similar to that found in BDE-99 treated rats and 10-fold lower than after BDE-47 treatment. In summary, the tissue distribution of BDE-153 is typical of a lipophilic, polyhalogenated, aromatic chemical and is not as well absorbed as BDE-99, but similar to BDE-47.

**1231**

METABOLISM AND TISSUE DOSIMETRY OF PENTAVALENT AND TRIVALENT MONOMETHYLATED ARSENIC AFTER ORAL ADMINISTRATION IN MICE.

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Humans are exposed to monomethylarsonic acid (MMA(V)) and monomethylarsonous acid (MMA(III)), which are formed as metabolites of inorganic arsenic. The sodium salts of MMA(V) are also used as herbicides. This study examined the metabolism and tissue dosimetry of MMA(V) and MMA(III). Adult female B6C3F1 mice were oral gavaged with either MMA(V) (0.4 or 40 mg As/kg; <sup>14</sup>C or unlabeled) or MMA(III) (0.4 mg As/kg, unlabeled). Mice were sacrificed over 24 hr and tissues (blood, bladder, kidney, liver, lung) were removed and analyzed either for radioactivity or for MMA or dimethylarsinic acid (DMA) by hydride generation-atomic absorption spectrometry. MMA(V)-derived radioactivity (<sup>14</sup>C) was rapidly absorbed and distributed among all tissues examined. Peak tissue concentrations of <sup>14</sup>C were observed between 1 and 4 hr post-dosing. Terminal tissue half-lives of <sup>14</sup>C ranged from 4 (liver) to 15 hr (kidney). For the two dosage levels, differences between the area under the curves (AUC) of the concentration-time profiles of <sup>14</sup>C in the blood and organs were less than 100-fold. Only carcass, which consisted of tissues not removed and intestinal contents, had a 100-fold difference in AUC. These findings suggest that absorption of MMA(V) was dose-dependent. Speciation of arsenicals in mice found levels of DMA that were five-fold more or greater in tissues of MMA(III)-treated mice than in MMA(V)-treated mice. Thus, dose and oxidation state of monomethylated arsenic can affect its disposition. (This abstract does not necessarily reflect EPA policy.)

**1232**

PHARMACOKINETIC EVALUATION OF PERFLUOROOCTANOIC ACID IN THE MOUSE.

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Perfluorooctanoic acid (PFOA) is a stable chemical surfactant with wide industrial and consumer applications. Because PFOA has been detected in both human and wildlife populations, its potential adverse health effects are under active investigation. The pharmacokinetic properties of PFOA are unique: in the rat, there is a pronounced gender difference in its renal clearance such that half-life in females is estimated as 3 h and that in males as 5 days; however, such a major gender difference is absent in humans. The present study is designed to examine the pharmacokinetic properties of PFOA in another rodent species. Young male and female CD-1 mice received a single gavage administration of PFOA at either 1 or 10 mg/kg. Three animals from each group were sacrificed at 4 h, 8 h, 12 h, or 1, 3, 6, 9, 13, 20, 27, 34, 42, 48 days after treatment. Serum was prepared from trunk blood collected from the descending aorta and analyzed for PFOA by HPLC-MS-MS. PFOA was absorbed readily, reaching Cmax between 4-8 h. In contrast to the rat, no discernable sex difference in the disposition of PFOA was observed in the mouse. The terminal serum half-life of PFOA was estimated in the range of 15-20 days. A similar pharmacokinetic profile was seen in both dose groups, suggesting linearity between body burden of the fluorocarbon and administered doses up to 10 mg/kg. These results thus indicate a significant difference in the pharmacokinetic disposition of PFOA between the rat and the mouse, with the profile of the latter species resembling the lack of an apparent sex-dependent elimination in humans. This abstract does not necessarily reflect USEPA policy.

**1233**

INITIAL SKIN PENETRATION STUDY FOR DETERMINING BIS-(2-CHLOROETHOXY)METHANE (CEM) TOXICOKINETICS AFTER A SINGLE DERMAL APPLICATION IN RODENTS.

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CEM is a colorless, liquid, haloethane substance used in manufacturing processes, e.g. polysulfide elastomers. It is not a naturally occurring substance but is found in the environment because of volatilization during manufacturing, translocation in

plants, uptake by animals, and industrial waste from plastic and metal finishing processes. Human exposure to CEM occurs primarily via inhalation and dermal routes and, since CEM-rodent studies identified target organs (heart, thymus, liver, kidney, skin), it is potentially toxic to humans. For these reasons, the present study was conducted to obtain initial information about the time course of CEM's dermal penetration into the systemic circulation, generate biological samples for quantifying blood and tissue concentration ranges, and determine appropriate doses and time points for a definitive TK study. The subscapular dorsal skin, clipped of fur, was used as the application site. Single application dosages were 75 or 300 mg/kg for F344 rats (M/F) and 150 or 600 mg/kg (M) and 100 or 400 mg/kg (F) for B6C3F1 mice. Dosing volumes were 0.5 (r) and 2 (m) mL/kg. Plasma concentration-time point profiles were biphasic, except for the mouse low dose (<LOQ, 2.5 ng/mL). Cmax, ke, and AUCinf values were 1.3 ug/mL, 0.216 hr<sup>-1</sup>, and 1.8 hr.ug/mL at 75 mg/kg for male rats; 10 ug/mL, 0.204 hr<sup>-1</sup>, and 17 hr.ug/mL at 300 mg/kg for male rats; and 1.7 ug/mL, 1.8 hr<sup>-1</sup>, and 2.2 hr.ug/mL at 600 mg/kg for male mice, respectively. Females had similar values. Heart and thymus values were similar to plasma results, except thymus AUC values were elevated. Rat plasma AUC values increased disproportionately with dosage suggesting saturation of elimination. These results indicate that a single dermal application of CEM at 100, 200, or 400 mg/kg (rats) and 400 or 600 mg/kg (mice) will produce plasma/tissue concentrations sufficient to characterize CEM TK parameters within and outside the dose proportional range. [Supported by NIEHS N01-ES-05456]

## 1234

### RODENT PLASMA AND TISSUE CONCENTRATION TIME COURSE DATA FOR BIS (2-CHLOROETHOXY)METHANE: A PRELIMINARY IV STUDY.

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bis-(2-Chloroethoxy)methane (CEM) is used extensively in a variety of manufacturing processes and is a potentially toxic substance to humans based on rodent-study target organs (heart, thymus, liver, and kidney). A metabolite of CEM, thiodyglycolic acid (TGA), inhibits mitochondrial function, thereby possibly contributing to the mechanism of CEM-induced toxicity. For these reasons, generation of time-course data for CEM and TGA are being developed for use in a PBPK-model for the evaluation of dose matrices and characterization of tumor-dose response relationships. The present study was conducted to obtain preliminary TK parameters, generate biological samples for analytical method development and validation, and determine appropriate doses and time points for a definitive TK study. F344 rats and B6C3F<sub>1</sub> mice were given a single intravenous (IV) administration of CEM in cremophor:ethanol:water (1:1:8) at 10 mg/kg (2 mL/kg). Plasma, heart, and thymus CEM concentration time profiles were biphasic (rats) and monophasic (mice). (Profiles following dermal application suggest CEM may be triphasic for rats and biphasic for mice.) The CEM plasma concentration-time profile resulted in C<sub>5 min</sub>, k<sub>e</sub>, and AUC<sub>inf</sub> values of 8.0 ug/mL, 0.0131 min<sup>-1</sup>, and 211 min.ug/mL for male rats and 3.8 ug/mL, 0.118 min<sup>-1</sup>, and 66.6 min.ug/mL for male mice, respectively. Females had similar values. AUC values were increased for the thymus and heart when compared to the plasma AUC. TGA concentrations were below the LOQ (200 ng/mL), thereby precluding construction of plasma/tissue profiles following a single IV CEM administration. These results indicated that a single IV administration of CEM at 10 mg/kg will allow TK parameters to be determined but a higher dosage (non-toxic) or lower LOQ (2.5 ng/mL) are necessary to characterize a gamma phase. Also, a single IV administration of TGA is needed to determine TK parameters of the metabolite. [This study supported by NIEHS Contract No. N01-ES-04546.]

## 1235

### EFFECTS OF PARTICLE SIZE ON THE SYSTEMIC BIOAVAILABILITY IN RATS FOLLOWING INHALATION EXPOSURE.

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Risk assessment of occupational exposure to airborne pollutants encountered in the workplace is commonly extrapolated from results of inhalation toxicology studies using experimental animals. Though such studies provide a linkage between exposure and its associated biological effect, there is not much information provided regarding the absorption of inhaled particles deposited into various regions of the lung. In this study we hypothesized that altering the regional deposition dose within the respiratory tract by inhaling two distinct sizes of particles may result in subsequent changes of their pharmacokinetics following a single nose-only inhalation exposure to test drug MK-0679. Specifically, two groups of young, adult rats were exposed to a test atmosphere containing either 1.4 or 3.5  $\mu$ m MMAD particles of MK-0679 at the target exposure dose level of 6 mg/KgBW for 1 hour. Upon exposure termination, blood samples were collected at six discrete time points (5 rats/time/group), via cardiac puncture, from rats anesthetized with CO<sub>2</sub>. Drug

concentrations in plasma were measured by HPLC for the determination of bioavailability of drug. Area under the curve (AUC), peak plasma concentration (Cmax) and its corresponding time point (Tmax), and plasma elimination half-life (T1/2) were determined for both exposure groups. The results showed that for both groups post-exposure plasma drug levels increased initially, reached to Cmax at approximate 1-hr post-exposure, decreased rapidly to less than 40% of Cmax at 2-hrs post-exposure, and gradually decreased to approximate 10-20% of Cmax at 6-hrs post-exposure. There was a good agreement of Cmax, Tmax, T1/2, and AUC between the two exposure groups, indicating that there was no statistically significant difference in the systemic absorption between the two exposure groups, despite the expected greater fractional deposition of the 3.5  $\mu$ m particles in the upper respiratory tract. The results suggest that the systemic absorption of MK-0679 via inhalation exposure is influenced the most by the dose deposited in the lungs.

## 1236

### EVALUATION OF THE HALF-LIFE (T<sub>1/2</sub>) OF ELIMINATION OF PERFLUOROOCTANOATE (PFOA) FROM HUMAN SERUM.

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The ammonium salt of perfluorooctanoate (C<sub>8</sub>F<sub>15</sub>O<sub>2</sub><sup>-</sup>) is used as an emulsifier and surfactant. PFOA is readily absorbed, distributes primarily to extracellular space (liver and blood), is highly protein (albumin) bound in serum, has no known metabolic conversion and undergoes enterohepatic circulation. Marked sex and species differences occur in the elimination of PFOA. Urine is the primary route of excretion. The T<sub>1/2</sub> of serum elimination in male and female rats is 4-6 days and 2-4 hours and is approximately 21 and 30 days in male and female monkeys, respectively. Sex hormones may modulate differential expression of organic anion transporters involved in the urine elimination of PFOA. To investigate the T<sub>1/2</sub> of serum elimination in humans, 27 retirees (25 males, 2 females) from two fluorocarbon manufacturing plants were followed for 5.5 years for periodic blood collections. The analysis for PFOA used a primary acid extraction in combination with an alkaline back extraction technique. A 5  $\mu$ L injection was introduced to the mass spectrometer through a high performance liquid chromatography system. All quantitative calculations were based on the ion ratios between PFOA and the internal standard (dual substituted <sup>13</sup>C-PFOA). The primary standard was a mixture of linear and branched chain isomers of PFOA. A total of 464 serum samples were analyzed. One retiree's samples were excluded due to the likelihood of occupational exposure to PFOA during follow-up. Initial serum concentrations of PFOA for the remaining 26 retirees ranged between 0.07 to 5.10 mg/mL. Only linear chain PFOA was observed. Individual serum elimination rates were calculated with Pharsight WinNonlin® software. The mean T<sub>1/2</sub> of serum elimination was 1378 days (SD 609) and ranged between 561 and 3334 days. Median was 1257 days (3.5 years). The T<sub>1/2</sub> of serum elimination was not associated with initial PFOA concentrations, age or sex of retiree, years worked at manufacturing facility or the time between retirement and first blood collection.

## 1237

### DISPOSITION OF [<sup>14</sup>C] DECAMETHYLCYCLOPENTASILOXANE ([<sup>14</sup>C]D<sub>5</sub>) IN FISCHER 344 RATS FOLLOWING SINGLE AND MULTIPLE INHALATION EXPOSURE.

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The disposition of D<sub>5</sub> in male and female Fischer 344 rats following single or multiple inhalation exposures was evaluated. Animals were administered a single 6 hour nose-only exposure to 7 or 160 ppm [<sup>14</sup>C]D<sub>5</sub> or fourteen 6-hour nose only exposures to unlabeled D<sub>5</sub> followed on the 15th day by a 6 hour exposure to [<sup>14</sup>C]D<sub>5</sub>. Subgroups of exposed animals were established to evaluate body burden, distribution, and elimination. Samples of blood, fat, liver, lung, feces and expired air were also processed for parent D<sub>5</sub> analysis. Retention of D<sub>5</sub> following single exposures was relatively low (~4-5% of inhaled D<sub>5</sub>), with ~8-10% retained following multiple exposures. Approximately 50-80% of this retained dose was attributed to deposition on the fur for males and ~60-70% for the females. Parent and radioactivity was widely distributed to tissues of both males and females, with maximum concentrations observed in the majority of the tissues by 3 hours post-exposure. D<sub>5</sub> was distributed to fat, with elimination of parent and radioactivity occurring slower than observed for plasma and other tissues. Elimination of retained radioactivity was similar in urine (~12%) and feces (~16%) of both sexes following all exposures. Expired air was similar for both sexes following multiple exposures and females following single exposure (~45%) with significantly higher amounts for the males following a single exposure (~72%). In the plasma, liver and lung, the majority of radioactivity immediately following exposure could be attributed to parent, with this decreasing over time to a small fraction attributable to parent from 24 to 168 hours

post exposure. In the urine samples, several peaks were present, but none corresponded to the retention time of parent D<sub>5</sub>. In contrast, the major peak for the feces corresponded to the retention time for parent D<sub>5</sub>. Supported in part by the Silicones Environmental, Health and Safety Council of North America.

## 1238 TOXICOKINETICS OF 14C-RDX IN MINIATURE PIGS.

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Cyclotrimethylenetrinitramine (RDX), a nitramine compound, has been used extensively as an explosive in military munitions since World War II. The toxicokinetic data in animals are limited. In this study, the toxicokinetics of 14C-RDX-derived radioactivity was examined following administration of a single oral dose formulated as an aqueous suspension in 0.1% carboxymethylcellulose at a target dose level of 43 mg/kg (53  $\mu$ Ci/kg) to male and female Yucatan minipigs. Blood was collected at 1, 6, 12 and 24 hours postdose from each animal. Urine and feces were collected through 24 hours postdose. Animals were sacrificed at 24 hours postdose and selected tissues collected. Blood, plasma, tissues, and excreta were analyzed for total radioactivity. Female minipigs vomited and experienced tremors and convulsions within 1 hour postdose. Only vomiting was observed in male minipigs, occurring within 2 hours postdose. Animals appeared normal by 2.5 hours postdose. Urine was the major route for elimination of 14C-RDX-derived radioactivity, with 17.3 and 17.6% of the radioactive dose, respectively, in males and females. RDX was well absorbed. Feces accounted for less than 1% of administered dose and gastrointestinal contents for about 5.6% of the dose. The distribution of 14C-RDX-derived radioactivity was extensive, with radioactivity observed in all collected tissues. The highest concentrations of radioactivity were observed in liver and kidney. The calculated percent of radioactive dose was high in liver (3.5 to 5.8%) and muscle (2.5 to 4.7%). Profiling and metabolite identification in urine, plasma, and selected tissues are in progress. (Abstract does not reflect US Army policy)

## 1239 TOXICOKINETICS OF TETRABROMOBISPHENOL A IN HUMAN SUBJECTS.

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Tetrabromobisphenol A (TBPA) is a widely used as a flame retardant and is suspected to be stable in the environment with possible widespread human exposures. This study reports the characterization of the toxicokinetics of a low dose of tetrabromobisphenol A in human subjects. A single oral dose of 0.1 mg/kg TBPA was administered to 3 male and 2 female human subjects in a gel capsule. The study was performed with informed consent by the human subjects and approved by the local institutional review board. Urine and blood concentrations of TBPA and its metabolites were determined by LC/MS-MS at different time points. TBPA glucuronide and TBPA sulfate were identified as metabolites of TBPA present in blood or urine of the exposed human subject. TBPA glucuronide was formed as a metabolite in all individuals, whereas TBPA sulfate was only detected in two of the five individuals. Maximum plasma concentrations of TBPA glucuronide (10-15 pmol/mL) were obtained within two hours after administration, the glucuronide was cleared from blood with an apparent half-life of 26 h. In the two individuals where TBPA sulfate was present in blood, maximum concentrations were obtained at the 4 hour sampling point, the concentrations rapidly declined to reach the limit of detection after 8 hours. Parent TBPA was not present in detectable concentrations in any of the plasma samples. In urine of all participating individuals, only TBPA glucuronide was detected. This metabolite was slowly eliminated to reach the limit of detection 96 hours after administration. Only a part (approx. 25 %) of the administered dose was recovered in urine. The obtained result suggest absorption of TBPA from the gastrointestinal tract, rapid metabolism of the absorbed TBPA by glucuronide formation, and a possible involvement of polymorphic sulfotransferase in TBPA biotransformation. Due to the high molecular weight of the TBPA-glucuronide, most of the TBPA-glucuronide is likely excreted with feces. This work was supported by the European Commission in context of the FIRE-project

## 1240 TISSUE DISTRIBUTION OF <sup>14</sup>C-ACETAMINOPHEN IN THE MONKEY USING QUANTITATIVE AUTORADIOGRAPHY OR LIQUID SCINTILLATION COUNTING.

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Distribution studies are an important part of preclinical drug development programs. They also support GLP compliant safety studies, for which both rodents and larger animals are required. In this study, quantitative autoradioluminography

(RLG) and liquid scintillation counting (LSC) were compared in one cynomolgus monkey given a single dose of 20 mg/3.7 MBq/kg of <sup>14</sup>C-acetaminophen intravenously. Forty-five minutes after treatment, the animal was cryopreserved and bisected along its length. One half was sectioned at 30  $\mu$ m with a 3600 Leica cryomacrotome. The evaluation of the sections was performed using a Fuji BAS-2500 scanner linked to AIDA Raytest software, which had previously been validated in accordance with FDA 21 CFR, part 11 guidelines. Quantification was achieved using blood calibration samples processed together with the sections. The other half of the animal was defrosted and 23 tissues/organs were collected, homogenized, combusted using a 307 Packard Oxidizer and counted for total radioactivity. When comparing results obtained with either method, the slope and  $r^2$  values of the linear regression analysis of paired concentrations were 0.9999 and 0.9995, respectively. In addition, 19 out of 23 RLG results ranged between -10.6% and +13.8 % of the reference LSC values. The other 4 results were within -26.1% and +18.4% of the reference LSC values. The organ to blood ratios, a standard parameter for the evaluation of accumulation, were very similar whatever the method used. In conclusion, quantification with RLG gave comparable results to those obtained with LSC. RLG is a reliable method applicable to large animals which allows the collection of data in a GLP-compliant manner.

## 1241 ETHYLENE GLYCOL UPTAKE IN VOLUNTEERS EXPOSED BY INHALATION AND BY THE DERMAL ROUTE.

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Four healthy, non-smoking male volunteers (between 30 and 56 years old) inhaled over 4 h a defined quantity of 13C2-ethylene glycol (13C2-EG) vapor. Separately, the same volunteers were exclusively exposed up to 6 h to liquid 13C2-EG (0.8 ml), applied to 66 cm<sup>2</sup> of the inner forearm skin. Plasma samples and urine were collected up to 30 h after starting the exposures and analyzed by GC/MS for labeled and unlabeled (endogenous) EG, glycolic acid (GA) and oxalic acid (OA). The amounts of inhaled EG (1.34-1.61 mmol) corresponded to inhalation exposures ranging from 7.0-8.4 ppm (4 h; alveolar ventilation 20 l/min). Maximum plasma concentrations ranged from 10-16  $\mu$ mol/l 13C2-EG and from 0.9-2.6  $\mu$ mol/l 13C2-GA. The amounts excreted ranged from 44-130  $\mu$ mol 13C2-EG, from 9.5-13  $\mu$ mol 13C2-GA, and from 0.2-3.8  $\mu$ mol 13C2-OA, corresponding to 3.0-9.3% 13C2-EG, 0.59-0.92% 13C2-GA, and 0.02-0.28% 13C2-OA of the amounts of 13C2-EG inhaled. In the dermal experiments, maximum plasma concentrations of 13C2-EG were 0.5-2.0  $\mu$ mol/l (n=3) and below the quantitation limit (QL=0.5  $\mu$ mol/l; n=1). Neither 13C2-GA (QL=2.9  $\mu$ mol/l) nor 13C2-OA (QL=0.2  $\mu$ mol/l) was quantifiable. The amounts excreted (adjusted for 4-h exposures) ranged from 7.9-15  $\mu$ mol 13C2-EG (n=4). 13C2-GA was 1 and 4  $\mu$ mol in two volunteers and not quantifiable in the two others. 13C2-OA was not detected. Plasma concentration of endogenous GA ranged from 25-55  $\mu$ mol/l and the amounts excreted within 24 h were 90-870  $\mu$ mol GA and 120-240  $\mu$ mol OA. From the urinary 13C2-EG and 13C2-GA analyses it is calculated that systemic EG uptake from continuous dipping of both hands (hand skin area 910 cm<sup>2</sup>) in liquid EG for 4 h and longer is similar to that absorbed during inhalation of 10-30 ppm EG over the same time period. The calculated GA excretion resulting from an 8-h exposure to 30 ppm EG ranges from (70-110  $\mu$ mol) being comparable with that of endogenous GA in 24 h. Financial support by the Ethylene Oxide and Derivatives Sector Group of CEFIC (Brussels, Belgium) is gratefully acknowledged.

## 1242 STRATEGIES TO ASSESS SYSTEMIC EXPOSURE OF TEST MATERIAL IN SUBCHRONIC STUDIES.

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Strategies were developed to estimate systemic bioavailability of test materials, their diurnal variations in blood levels and elimination in subchronic feeding/drinking studies after reaching steady-state levels utilizing minimum number of blood samples. Systemic bioavailability of test materials was determined by calculating area under the blood/plasma concentration curve over 24 h (AUC-24 h) using complete sets of data (5-8 samples) and 3, 2 and 1 selected time-points. Twenty-one sets of data (17 from literature) were analyzed for AUC-24 h. Chemicals included short t<sub>1/2</sub> drinking water disinfectant by-products to intermediate t<sub>1/2</sub> pharmaceutical drugs and pesticides. The best predictions of AUC-24 h were made when three time points were used corresponding to C<sup>max</sup>, a mid-morning sample, and C<sup>min</sup>. These values were 103±10% of the original AUC-24 h, with 13 of 17 values between 96 and 105% of the original. Calculation of AUC-24 h from two samples (C<sup>max</sup> and C<sup>min</sup>) or one mid-morning sample afforded slightly larger variations in the calculated AUC-24 h (69-135% of the original). Following drinking water exposure, prediction of AUC-24 h using 3 time-points (C<sup>max</sup>, mid-morning and C<sup>min</sup>) was very close to actual values (80-100%) among mice. Collection and analysis of 1-3 blood samples per dose level provide insight into dose proportional or non-dose proportional differences in systemic bioavailability that may point towards saturation of absorption or elimination or some other phenomenon, which may need further investigation. In addition, collection of the terminal blood samples from rats which is usually conducted 16 hours after removing

the fortified diet/water be helpful in estimating the blood/plasma half-life of the compound. Amount of the test materials and/or metabolite(s) in excreta and their possible use as biomarkers in predicting the daily systemic exposure evaluated. Determining these parameters in the early stages of the development is critical for appropriate design of other, longer-term toxicity studies.

#### 1243 THE EFFECT OF DOSE ON THE METABOLIC PROFILE OF TRICHLOROETHYLENE.

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The purpose of these experiments is to characterize the effect of dose on the metabolic profile of trichloroethylene (TCE) by monitoring the major metabolites: trichloroacetic acid (TCA), trichloroethanol (TCOH), and chloral hydrate (CH). Fasted male Sprague-Dawley rats received either 10 or 50 mg/kg of TCE as an aqueous Alkumuls emulsion by gavage. Serial blood samples were obtained via a carotid artery cannula. TCE, CH, TCA, and TCOH were quantitated by headspace gas chromatography. The Cmax of TCE increased 15-fold with the 5-fold increase in dose suggesting saturation of first pass metabolism of TCE. The half-lives of CH, TCA, and TCE increased 3-, 2-, and 3- fold with the increase in dose, indicating saturation of metabolism (CH, TCA, and TCE) or renal clearance (TCA). The half-life of TCOH did not change with the dose. The half-lives of TCA and TCOH are significantly longer than that of TCE at both doses indicating that the elimination of TCA and TCOH is the rate-limiting step in their disposition. The half-life of CH is approximately equal to the half-life of TCE indicating it is a formation rate-limited metabolite. Cmaxs for CH and TCA increased 4-fold with the 5-fold increase in dose; whereas, Cmax of TCOH increased 8-fold. These changes in Cmax coupled with the changes in half-life resulted in 16-, 10-, and 12-fold increases in AUC for CH, TCA, and TCOH with a 5-fold increase in TCE dose. The changes in the metabolic profile of TCE as a function of dose suggest nonlinear elimination of TCE as well as the metabolites. These changes in TCA toxicokinetics may significantly impact the carcinogenic potential of TCA. (Supported by EPA STAR Grant R830800).

#### 1244 MATERNAL AND FETAL DISPOSITION OF LAMIVUDINE, ZIDOVUDINE, AND LAMIVUDINE-ZIDOVUDINE IN THE PREGNANT RAT.

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The pharmacokinetics and fetal disposition of lamivudine (3TC) and zidovudine (AZT) monotherapies and lamivudine-zidovudine combination therapy were compared in the pregnant rat. Pregnant Sprague-Dawley rats were anesthetized with ketamine: acepromazine: xylazine (50:3:3:3.4 mg/kg, IM) on day 19 of pregnancy. A jugular catheter was implanted and laparotomy performed. Rats were dosed with 3TC alone, AZT alone, or 3TC-AZT combination at 25mg/kg, IV bolus (n=6). Serial blood, placenta, fetus and amniotic fluid (AF) samples were obtained. 3TC and AZT levels were quantitated by HPLC-MS-MS. Pharmacokinetic parameters and relative exposures (AUC<sub>tissue</sub>/AUC<sub>maternal plasma</sub>) were determined by WinNonlin. The plasma pharmacokinetic parameters for 3TC in combination with AZT were consistent with 3TC monotherapy. For AZT, a decrease in half-life (82.2 ± 12 min to 51.3 ± 2.3 min) and volume of distribution (1.13 ± .15 L/kg to 0.78 ± 0.1 L/kg) was noted when 3TC was coadministered, while no differences were noted in the clearance or AUC. The fetal exposure of amniotic fluid, fetal, and placental tissues to 3TC when AZT was coadministered increased from 0.38 ± 0.07 to 0.84 ± 0.4, 0.19 ± 0.06 to 0.39 ± 0.1, and 0.14 ± 0.08 to 0.19 ± 0.03, respectively. This suggests that the placental transport of 3TC to the fetal compartment was increased by the presence of AZT or that the efflux transport of 3TC out of the fetal compartment was blocked. The relative exposure of amniotic fluid, fetal and placental tissues to AZT when 3TC was coadministered decreased from 0.44 ± 0.06 to 0.37 ± 0.1, 0.48 ± 0.05 to 0.30 ± 0.07, and 0.53 ± 0.05 to 0.43 ± 0.1, respectively. The changes noted in the placenta, fetus, and amniotic fluid suggest that transporters play a complex role in the uptake of 3TC and AZT in these tissues. Thus, combinations of nucleoside drugs can dramatically influence fetal exposure resulting in changes in fetal toxicity.

#### 1245 TOXICOKINETICS OF DELTAMETHRIN IN DIFFERENT VEHICLES IN RATS.

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Deltamethrin (DLM) is a relatively potent neurotoxin and a type II pyrethroid. Crofton et al. (1995) and several reports revealed that DLM administered in methylcellulose or in Emulphor® (EM) was not toxic, whereas DLM in corn oil or

in glycerol formal (GF) produced severe hypomotility, convulsions and motor incoordination. In order to determine if the effects of different vehicles on neurotoxicity might be due to toxicokinetic differences, we characterized the toxicokinetics of DLM in GF or suspended in 5% EM. Male adult Sprague-Dawley rats were given 10 mg DLM/kg by gavage. One mg DLM/kg in GF or 10 mg/kg in EM was delivered intravenously. Serial plasma and tissue samples were collected over 96 hr and analyzed for DLM content by high performance liquid chromatography. Relevant pharmacokinetic parameters were calculated. DLM was trapped in the lung following intravenous doses in both vehicles. The concentrations of DLM in fat and spleen remained quite high over time following oral administration. The absolute bioavailability in GF and EM were  $0.17 \pm 0.05$  (mean ± SD) and  $0.12 \pm 0.04$ . Apparent absorption rate constants for GF and EM were 1.38 and 1.60 hr<sup>-1</sup>, respectively. DLM was absorbed 6-fold more from GF in the GI tract; a peak concentration in plasma of  $0.46 \pm 0.03$  µg/ml was reached within 1.3 hr after oral dosing in GF, whereas a Cmax of  $0.072 \pm 0.020$  µg/ml was reached within 1 hr in EM. Average areas under the curve (AUC) for plasma were  $3.88 \pm 1.05$  and  $0.59 \pm 0.19$  µg·hr/ml, respectively. Therefore, it appears that the greater neurotoxicity of DLM in GF can largely be attributed to its greater bioavailability in that vehicle. (Supported by EPA STAR Grant R830800)

#### 1246 PHARMACOKINETICS AND TOXICOKINETICS OF PHOSPHORODIAMIDATE MORPHOLINO OLIGOMERS AGAINST HEPATITIS C VIRUS AFTER SINGLE INTRAVENOUS INJECTION.

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Modulation of gene expression via nucleic acid sequence-specific intervention represents a new approach for drug discovery and development. Phosphorodiamidate morpholino oligomers (PMOs) represent third generation antisense agents, with translational inhibition as the mechanism of action. The objective of this study was to examine the toxicokinetics and bioavailability of HCV-IRES and HCV-AUG designed against the IRES region and the translational start site of HCV RNA. PMO concentrations were measured by the "HPLC duplex assay" following single, bolus intravenous administration of 15, 50 and 150 mg/kg. Serum chemistry data indicated no significant difference between treated and control group. Complete blood count data showed decreased hematocrit in both the HCV-IRES and HCV-AUG treated groups and a decrease in leukocyte and platelet count only in the group that received the 150mg/kg dose of HCV-AUG. Plasma and tissue disposition of both compounds were characterized by a rapid distribution into all tissues examined; distribution half-life of 8-15 min. The apparent elimination half-life increased with dose to over 48 hrs. The kidney had the highest concentration followed by the liver, with lower concentrations observed in the spleen, lung, heart, brain and skeletal muscle. Plasma and tissue concentration generally increased linearly with increased dose. No degraded PMO was observed in any blood, tissue or urine. Overall, maximal urinary excretion of both compounds occurred within 4 hours following administration. Excretion of intact HCV-IRES increased as a function of dose from 0.06mg to 20mg over a 10-fold increase in dose (15mg/kg to 150 mg/kg). HCV-AUG excretion increased from 0.83mg to 6mg from 15mg/kg to 50mg/kg. The study provides new and unique data regarding pharmacokinetic and toxicokinetics of phosphorodiamidate morpholino oligomers targeting hepatitis C virus.

#### 1247 TOXICOKINETIC STUDY FOR DELTAMETHRIN AND ITS METABOLITE, 3-PHENOXYBENZOIC ACID, IN IMMATURE AND ADULT RATS.

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Deltamethrin (DLM) is a widely-used type II pyrethroid insecticide with relatively potent neurotoxicity. Previous studies have shown that immature rats are more susceptible to acute DLM neurotoxicity than adults. The primary objective of this investigation was to determine whether the susceptibility of immature rats to acute DLM neurotoxicity was due to age-dependent toxicokinetics of DLM. Male adult Sprague-Dawley (S-D) rats with an indwelling arterial cannula were dosed with 1 mg DLM/kg intravenously in glycerol formal or with 10 mg/kg by gavage. Serial blood and tissue samples were collected over 96 hr and analyzed for DLM and 3-phenoxybenzoic acid (PBA) content by high performance liquid chromatography. Ten-day-old (D10) male S-D rats received 10 mg/kg by gavage. Salivation was observed in adult rats, but this sign disappeared within 15 – 30 min. All immature pups treated with 10 mg/kg showed serious tremors and choreoathetosis and died within 6 hr. Relevant pharmacokinetic parameters were calculated. The maximum plasma concentration of DLM in pups was  $3.30 \pm 0.95$  (mean ± SD) µg/mL, while that in adult rats was  $0.46 \pm 0.026$  µg/mL. The AUC<sub>06 hr</sub> of DLM in immature pups (12.30 mg·hr/L) was about 6.7-times greater than that in adult rats (1.85 mg·hr/L). Conversely, the AUC<sub>06 hr</sub> of PBA, a major DLM metabolite, in adult rats (4.00 mg·hr/L) was about 3.5-times greater than that in immature pups (1.14

mg·hr/L). These results indicate that the increased susceptibility of immature rats to acute DLM neurotoxicity is due to the higher systemic levels of DLM. The PBA data indicate that the immature rats' metabolic capacity is limited. Increased gastrointestinal absorption of DLM may also contribute to the higher blood levels of parent compound in this age-group. (Supported by EPA STAR Grant R830800)

**1248**

UPTAKE AND DISPOSITION OF INHALED METHANOL VAPORS IN HUMANS.

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Methanol is a widely used solvent and a potential fuel for motor vehicles. Human kinetic data of methanol are sparse. As a basis for biological exposure monitoring and risk assessment, we studied the inhalation toxicokinetics of methanol vapors in human volunteers. In addition sex differences and the suitability of bio-monitoring via saliva sampling were investigated. Four females and four males were exposed by inhalation to 0 (control exposure), 100, and 200 ppm methanol vapour for 2 h during light physical exercise (50 W) in an exposure chamber. Capillary blood, urine, saliva and exhaled air were sampled before, during, and after exposure and analyzed for methanol by head space gas chromatography. The exposures were performed according to the Helsinki declaration and after approval by the regional ethical committee. Methanol in blood increased from a background level of about 20 to 116 and 244  $\mu$ M at 0, 100 and 200 ppm, respectively. Saliva showed substantially higher concentration of methanol than blood the first several minutes after exposure. This difference disappeared within a few minutes, thereafter the levels in blood, urine and saliva closely followed one another. The concentration in all three media had returned to background levels later in the evening the same day. The half time of methanol in blood was about 110 minutes and the apparent total clearance 0.44 L/min. In blood, urine, saliva and exhaled air the AUC was about twice as high after exposure to 200 ppm of methanol compared to 100 ppm suggesting linear kinetics. No sex differences in the toxicokinetics of methanol could be detected. In conclusion the experimental results showed non-saturated metabolism of methanol and consistent patterns of methanol in blood, urine and saliva following a 2-h inhalation exposure.

**1249**

PERCUTANEOUS ABSORPTION OF WATER SOLUTION OF TRICHLOROETHYLENE IN VOLUNTEERS DURING PRE-STEADY AND STEADY STATE.

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To study dermal absorption of trichloroethylene (TRI) six volunteers (3 female and 3 male) were dermally exposed to 0.05% TRI water solution for 10, 20, 40, 90 and 120 min on the volar forearm over an area of 21 cm<sup>2</sup>. The internal exposure was assessed by measuring the concentration of TRI in exhaled air. An inhalation exposure with a known input rate and duration served as a reference dosage. The permeation rate courses were determined from exhaled air after both inhalation and dermal exposure using mathematical de-convolution. To determine permeability coefficient from non-steady state permeation data a mechanistic skin model of Kruse (2004) was used. The concentration of TRI in dosing solution decreased from 8 (10 min exposure) to 60 % (120 min exposure) revealing finite dose exposures. The average skin flux into the skin amounted to 0.0156, 0.0215, 0.0263, 0.0269 and 0.0323 mg cm<sup>-2</sup> h<sup>-1</sup> for 10, 20, 40, 90 and 120 min exposure durations, respectively. The respective maximal fluxes into the blood obtained from the slope of the cumulative permeation curve amounted to 0.0041, 0.0207, 0.0205, 0.0199 and 0.0351 mg cm<sup>-2</sup> h<sup>-1</sup>. The estimated K<sub>p</sub> amounted to 0.085 cm h<sup>-1</sup>. In contrast to theoretical considerations, the average skin fluxes after short exposures were smaller than those after longer exposures. This can be explained by relatively higher contribution of TRI evaporation from the skin after the end of exposure to the systemically absorbed amount. Incorporation of the loss of TRI from the skin due to evaporation into the skin model improved the goodness of the fit of the experimental data. The skin model used in the study showed to be feasible in estimation of steady state permeability from short term exposures and finite dose data. In addition, the model helps in the interpretation of experimental data of well described dermal permeation studies.

**1250**

INFLUENCE OF HEPATIC CLEARANCE ON THE TOXICITY OF THE TYPE I PYRETHROIDS BIFENTHRIN AND PERMETHRIN.

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Pyrethroids are a class of neurotoxic insecticides that are used in a variety of agricultural and household activities. Hepatic intrinsic clearance (CL<sub>int</sub>) of the Type I pyrethroids permethrin and bifenthrin may be a critical determinant of their toxic

effect. Rat LD<sub>50</sub>s reported in the literature for permethrin and bifenthrin are 1200 and 70 mg/kg, respectively. We determined *in vitro* metabolic parameters in adult male rat liver microsomes for permethrin and bifenthrin using a parent depletion approach. K<sub>m</sub> (3.82  $\mu$ M  $\pm$  0.07 and 5.41  $\mu$ M  $\pm$  7.26), V<sub>max</sub> (309.32  $\pm$  66.69 and 206.67 pmoles/min/mg protein  $\pm$  86.96) and hepatic CL<sub>int</sub> (9.7E-3 and 4.59E-3 L/hr/mg protein) were determined for the cis and trans isomers of permethrin, respectively. The *in vitro* parameters for the metabolism of bifenthrin were: K<sub>m</sub> = 34.75  $\mu$ M  $\pm$  41.94; V<sub>max</sub> = 845 pmoles/min/mg protein  $\pm$  479; hepatic CL<sub>int</sub> = 2.93E-3 L/hr/mg protein. The 3 to 4-fold difference in hepatic clearance rates between the permethrin isomers and bifenthrin do not account for their disparity in toxicity. This suggests that metabolism at other sites as well as pharmacodynamic effects are critical determinants of the toxicity of permethrin and bifenthrin. Hepatic clearance alone does not appear to fully explain the differences in the toxicity between bifenthrin and permethrin. (SJG supported by CT826513. This abstract does not represent USEPA policy.)

**1251**

METABOLISM OF DI(2-ETHYLHEXYL) PHTHALATE (DEHP) IN JUVENILE AND FETAL MARMOSET AND RAT.

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DEHP induces testicular lesion in juvenile rats, but not in marmosets (Kurata et al., SOT 2003). Since MEHP and its metabolites were suspected to be a cause of testicular lesions, we compared the metabolic profile of DEHP in juvenile and fetal marmoset with that of rats. STUDY-I: 14C-DEHP (100 mg/kg) was singly dosed to juveniles by gavage. Then the radioactivities in the plasma (2, 4, 8, 24hr after dosing), urine, feces (0 to 24hr), and tissues (24hr) were measured together with metabolite contents. The plasma radioactivity in rats was 20 to 100 times higher than that in marmosets. Three types of metabolites such as MEHP, conjugates of MEHP and MEHP-metabolites, or unconjugated MEHP-metabolites such as 5-Oxo, 5-OH, and 6 or 8-COOH MEHP were detected in rat plasma. However, unconjugated MEHP-metabolites were not detected in marmosets. About 60 % of the dose was excreted into the urine in rats. As for marmoset, although the bioavailability was presumed to be about 20 to 30%, the majority of the dose was excreted into the feces. As for rats, large amount of unconjugated MEHP-metabolites (about 35% of dose), which were the same kinds of plasma, were detected in the urine. However, as for marmosets, the majority was conjugated MEHP and its metabolites. Specific accumulation in the testes was noted neither in rats nor in marmosets, radioactivities in marmoset liver, kidney, or testes was much lower than those of rats. STUDY-II: we determined the tissue distribution (24hr) in fetuses when 14C-DEHP (100 mg/kg) was singly dosed to dams on gestation day of 130 for marmosets and day of 20 for rats. High radioactivity was observed in the digestive tract, liver, and kidney, and specific accumulation in the testis was noted in neither rats nor in marmosets. However, the radioactivity in rat testis was about 20-times higher than that in marmosets. Clear species differences in plasma and tissue radioactivity concentrations, and in the content of metabolites were demonstrated, and that might be the causes of species difference in testicular lesions.

**1252**

TRANSPLACENTAL TOXICOKINETICS OF 3'-AZIDO-3'-DEOXYTHYMIDINE (AZT) IN MICE.

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The anti-HIV drug 3'-azido-3'-deoxythymidine (AZT) has been demonstrated to be a perinatal carcinogen in mice, causing lung, liver, and mammary tumors in offspring. In these studies transplacental pharmacokinetics of AZT was determined in mice and both maternal and fetal exposures were estimated at doses that bridged effect and no effect doses in support of risk assessment. AZT was administered orally by gavage to pregnant CD-1 female mice at 50, 100, 200, and 300 mg/kg/day on GD 10-17 as 2 equal doses 6 hr apart. On the last day of dosing blood and fetuses from 3 mice per timepoint were sampled at 0.083, 0.17, 0.33, 0.5, 1, 2, 6.083, 6.17, 6.33, 6.5, 7, 8, 12, and 24 hours following the day's first dose. For determination of bioavailability and linearity of clearance, single IV doses of 50 and 100 mg/kg were administered to gravid females on GD 17. Plasma was collected at 0.083, 0.17, 0.33, 0.5, 1, 1.5, 2, 2.5, 3, 5, 7, 8, 12 and 24 hours post dose. Both parent compound and its metabolite AZT-glucuronide (GAZT) were determined in maternal plasma and whole fetuses using validated analytical methods. The plasma method was validated over the concentration range from 0.200 to 500  $\mu$ g AZT/mL plasma and 0.200 to 10.0  $\mu$ g GAZT/mL plasma. The method was validated from 0.1 to 100  $\mu$ g of AZT of pup tissue. The limit of detection (LOD) for the assays was estimated to be 0.04 and 0.014  $\mu$ g/g for fetus and dam plasma respectively. In the dams, plasma concentrations of AZT reached maximal levels within 15 minutes following oral doses, suggesting rapid absorption. Bioavailability was high in the dams. In fetal tissues, AZT concentrations reached maximal levels

at later times, suggesting that the placenta delayed absorption. Fetal maximal concentrations were generally lower than in dam plasma. Ratios of AZT exposure between fetus and dam plasma suggest that at doses of 50 mg/kg/day or higher fetal exposures were similar to that of dams. Overall the data suggests that in the mouse fetal AZT exposure mirrors that of the dam, though the profile differs.

### 1253 DISPOSITION OF BDE 99 AND BDE 153 IN FEMALE MICE.

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Polybrominated diphenylethers (PBDEs) represent a novel class of compounds typically used as flame retardants in electrical and household consumer goods. Recently, increased bioaccumulation of PBDE congeners has raised concern over the potential toxicity of these compounds in humans. Of particular interest are the congener profiles found in biota as they do not parallel profiles of commercial mixtures; a phenomenon that may be due to differential exposure and/or differences in metabolic capacity. Previous studies in our laboratory have shown major differences in excretion patterns of BDE 47 in rats and mice. In this study, the distribution and excretion of two other prominent BDE congeners were examined. Female C57/BL6 mice were given [<sup>14</sup>C] 2', 2', 4, 4'-pentaBDE (BDE 99) or [<sup>14</sup>C] 2', 4, 4', 5, 5'-hexaBDE (BDE 153) via intravenous administration (1mg/kg). The results indicate that BDEs 99 and 153 are not as rapidly excreted in the urine of mice as BDE 47. 21% of BDE 47 was excreted in the urine 24 hours following exposure, in contrast to 6% of BDE99 and <1% of BDE 153. A total of 42% BDE 47 was excreted in the urine over a five day period, versus 11% of BDE 99. Preliminary metabolite analyses reveal a majority of parent compound in the urine, a result analogous to BDE 47. Remaining BDE 99 and 153 were found primarily in adipose and other lipophilic tissues. Excretion and retention of PBDEs in mice appears to be dependent on the degree of bromination; the dominance of lower brominated congeners in biota despite the rapid excretion patterns demonstrated in mice raise questions as to the animal model that should be used in human health risk assessment. (This abstract does not reflect EPA policy. This work was partially funded by EPA NHEERL-DESE CT826513 and T32 ES07126).

### 1254 DEVELOPMENT OF AN *IN VITRO* BLOOD-BRAIN BARRIER MODEL FOR BRAIN DISPOSITION SCREENING OF PHARMACEUTICALS.

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Solute distribution between blood and brain is strictly regulated by the blood-brain barrier (BBB). While the BBB performs an important function in keeping unnecessary or harmful molecules from the brain, it poses a challenging problem in delivering therapeutics, including anticancer, antibiotic or antipsychotic drugs into the brain. Conversely, preventing potentially damaging molecules from overcoming the BBB is also an increasing problem, especially when combinations of therapeutics are encountered. Medical and pharmaceutical scientists therefore have a growing need for rapid, reliable *in vitro* models of the BBB for preclinical screening of pharmaceutical BBB transport properties. The current presentation describes initial results in development of an *in vitro* BBB model derived from bovine brain capillary endothelial cells (BCEC). Capillary vessels were first isolated from bovine brains. Individual BCECs were then released by further enzymatic digestion of the capillaries. BCEC thus obtained were cryopreserved. The isolation procedure produced a highly pure population of BCECs, as demonstrated by immunocytochemical staining for the endothelial cell marker von Willebrand factor. After recovery from cryopreservation, BCECs were cultured on microporous membrane culture inserts to produce the BBB model. Transmission electron microscopy and H&E stained light microscopy of the cultures show uniform endothelial cell monolayers with evidence of tight junction formation. Immunocytochemical staining also demonstrated uniform expression of the tight junction protein ZO-1 localized along the BCEC borders. Permeation of Lucifer yellow across the BBB culture was low, further demonstrating development of barrier function. Finally, Western blotting experiments were conducted to reveal the presence of the important BBB efflux transporter p-glycoprotein. These results show significant progress in development of a reliable *in vitro* BBB model that will be useful for preclinical screening of candidate pharmaceutical compounds. This work was supported by NCI Grant # R43 CA101703-02.

### 1255 PHARMACOKINETICS OF TAFA93, A NOVEL PRO-DRUG OF THE mTOR INHIBITOR RAPAMYCIN.

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**Introduction:** TAFA93, a novel mTOR inhibitor, is intended for use in the prevention of organ graft rejection and the treatment of autoimmune diseases. It was developed as an orally administered pro-drug of rapamycin that will maintain efficacy

while altering the pharmacokinetics (PK) in such a way as to decrease clinical adverse effects. It has been proposed that some drug related adverse effects are due to the rapid rise in whole blood rapamycin levels after dosing. TAFA93, upon hydrolysis to rapamycin, attenuates the rapid rise in rapamycin concentrations thereby potentially improving the safety profile. **Methods:** Pharmacokinetics were investigated in rats, dogs and primates. Hyperlipidemia was studied in a rat model. Efficacy was investigated in a rat heterotopic heart transplant model. **Results:** PK studies demonstrated that TAFA93 displayed a significantly different PK profile than rapamycin. Specifically, Cmax was blunted and Tmax was significantly increased. In the rat TAFA93 shifted the Tmax for rapamycin from approximately 0.5 hours to approximately 3 hours and reduced the Cmax for rapamycin by approximately 70% with only a 30% reduction in AUC. The altered PK in TAFA93 treated rats was associated with cholesterol levels that were significantly lower ( $p < 0.01$ ) than the levels measured in rapamycin treated animals. In addition, transplant data indicated that TAFA93 prolonged graft survival, compared to control at doses of 2.5 and 10 mg/kg/day, and was equally efficacious to rapamycin at 2.5 mg/kg/day despite a 1/3 decrease in AUC. Graft survival times of 41±4 and 39±4 days were obtained for TAFA93 and rapamycin respectively. **Conclusions:** TAFA93 is a novel pro-drug of rapamycin which in preclinical trials alters the PK of rapamycin in such a way as to decrease some of the known side effects while maintaining efficacy.

### 1256 RAT STRAIN DIFFERENCES IN ETHYLENE GLYCOL RENAL TOXICITY IS DRIVEN BY THE RENAL CLEARANCE OF OXALIC ACID.

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The kidney has been identified as a primary target organ in toxicity studies with ethylene glycol (EG), with rats being more sensitive than mice and males more sensitive than females. The male Wistar rat also appears to be more sensitive than male rats from other strains. Kidney toxicity results from a build up of the terminal metabolite, oxalic acid (OX), which can precipitate with calcium to form crystals. In this study, the renal clearance of OX as well as inulin (IN), a marker for glomerular filtration, was evaluated in young and old male Wistar rats and in rats that have been exposed for one year to 0, 50, 150 or 300 mg/kg/day EG via the diet. Age had a slight affect on the ability of male Wistar rats to clear OX, as the ratio of OX to IN clearance increased from ~0.6 to ~0.9. No effects were observed following chronic dietary exposures to ethylene glycol as the ratio of OX to IN clearance remained ~0.9. In all other rat strains and species studied (F344 rats, SD rats, dogs, sheep and humans), the clearance of OX was shown to be slightly higher than inulin (1.2-2.1), indicating the presence of an active transport process. In male Wistar rats, the net clearance of OX suggests that reabsorption processes are more important than active secretion, which likely contributes to the enhanced sensitivity of male Wistar rats. When OX and IN clearances are related to body weight, all species, with the exception of the male Wistar rat, have glomerular filtration and OX clearance rates that can be scaled allometrically. Since human risk assessments for renal toxicity may be driven by results from chronic studies in male Wistar rats, this quantitative assessment will be critical to determinations of human equivalent exposures and internal dose-response assessments. (Sponsored by the Ethylene Glycol Panel of the American Chemistry Council).

### 1257 DBDPO METABOLISM IN FISH AND MAMMALS: CONTRIBUTION TO LOWER BROMINATED DIPHENYL ETHERS.

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The flame retardant decabromodiphenyl oxide/ether (DBDPO) represents >80% of global polybrominated diphenyl ether (PBDE) use. PBDE typically detected in biota, tetra (BDE47), penta (BDE99, 100) and hexa (BDE153, 154), appear to originate from use of PentaBDE product. DBDPO metabolism has been questioned as a contributor; the evidence is reviewed. DBDPO is poorly absorbed. Its fish BCF<sub>water</sub> was < 50.<sup>1,2</sup> Trout absorbed 0.005% of 7.5 or 10 mg DBDPO/kg/d administered in food over 120 d.<sup>3</sup> DBDPO was not detected in carp fed treated food for 90 d (940 ng/fish/d); 0.4% uptake was estimated based on presumed metabolites.<sup>4</sup> PBDE typically reported in wild-caught fish were not detected. The test article purity was not stated by either, but one<sup>5</sup> used a former product whose only known composition was 77% DBDPO, ~23% nona- and octa-BDE, and concluded "no evidence of debromination to these congeners [e.g. BDE-47, 99 and 100] was found". Rats absorbed DBDPO to a limited extent (0.28–2%, oral dose) with 98% eliminated in feces as parent.<sup>5,6</sup> Production of metabolites was limited (2% @ 277ppm, diet) and thought to occur in the gut, not systemically. A rat study using a DBDPO formulation to enhance/maximize absorption/metabolite production reported trace amounts of 3 nonaBDE and perhaps OH-BDE.<sup>7</sup> The test article was 98% DBDPO; nonaBDE are typical impurities. Similar results were found

in a 2nd study with an absorption enhancing formulation.<sup>8</sup> The existing data indicate DBDPO metabolism does not contribute to the PBDE typically detected in biological samples. <sup>1</sup>Biodegradation and Bioaccumulation Data of Existing Chemicals. 1992. Ministry of International Trade&Industry. Chemicals Inspection&Testing Institute, Japan. <sup>2</sup>Hardy M. Chemosphere 2002 46:757-77. <sup>3</sup>Kierkegaard et al. Environment Sciences. Technol. 1999 33:1612-7. <sup>4</sup>Stapleton et al. Environ Sciences Technol. 2004 38:112-9. <sup>5</sup>N.T.P. Technical Report Series No.398. 1986. National Institutes of Health. Research Triangle Park, NC. <sup>6</sup>El Dareer et al. J Toxicol Environ Health 1987 22:405-415. <sup>7</sup>Morck et al. Drug Metabolism and Disposition 2003 31:900-7. <sup>8</sup>Sandholm et al. Xenobiotica 2003 33:1149-58.

**1258** BIO-PHYSICO CHEMICAL DETERMINANTS OF TIME-TO-STeady STATE FOR VOLATILE ORGANIC COMPOUNDS (VOCS).

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Human exposure to VOCs encountered in the environment is generally irregular and/or intermittent. However, steady state toxicokinetic (SST) conditions may be attained even under these circumstances, and often within shorter exposure durations for VOCs than many persistent organic pollutants. Therefore, it is relevant to characterize and optimize the biochemical and physical variables that control time-to-steady state for a given VOC. We used a physiologically-based toxicokinetic (PBT) human model constructed on an Excel spreadsheet and performed TK simulations. The end-point, time-to-steady state for arterial blood concentration (C<sub>ass</sub>), was evaluated for each VOC by varying the biochemical and physical variables. The biochemical and physical properties assessed in the model (blood: air partition coefficient (Pb), intrinsic clearance (Clint; V<sub>max</sub>/K<sub>m</sub>), and tissue: blood partition coefficient (Pt)) were varied from 1 to 10, 000. Pb was the major factor in attaining SST conditions. When all the variables (including Pb) were set at 1, 5.5 hours were required to reach SST conditions, whereas a Pb value of 1000 required 350 hours. Varying the Pt from 1 to 10, 000 gradually increased the biphasic character of the relationship, which is attributed to the redistribution of VOCs in different tissue compartments. Clint had a minimal effect on the time-to-steady state due to flow and Pt limitations in the liver compartment. The major single limiting factor to achieving time-to-steady state was alveolar absorption and elimination. We classified and validated several VOCs based on Pb. This classification can be useful for assessing internal dosimetry under various exposure conditions.

**1259** RELATIONSHIP BETWEEN CYP1A1 GENOTYPE AND BENZO(A)PYRENE (BP) HEMOGLOBIN (HB) ADDUCTS IN MATERNAL AND FETAL BLOOD.

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The cytochrome P450 isoform CYP1A1 has been widely studied because of its interactions with the PAH substrates such as BP. In addition to being a substrate for CYP1A1, BP is also a potent inducer of the enzyme. Because CYP1A1 plays such a pivotal role in the metabolism of BP, as well as several other PAH found in tobacco smoke, we deemed it justifiable to measure the levels of BP Hb adducts in cord blood samples with regards to CYP1A1 genetic polymorphisms of Msp I (T6235C) and Hinc II (A4889G). Matched maternal and fetal blood samples were obtained from smokers at delivery and stratified based on cotinine determinations. Subjects were divided into three groups based on CYP1A1 Msp I genotype: wild type (TT), heterozygous (TC), homozygous variant (CC). Subjects were divided into two groups based on CYP1A1 Hinc II genotype (AA) and heterozygous (AG). There were no homozygous variants for Hinc II. BP diol epoxide Hb adduct levels were highest in the cord blood samples with the CYP1A1 Msp I wild type (TT) genotype and lowest in the cord blood samples with the CYP1A1 Msp I homozygous variant (CC) genotype ( $\alpha = 0.05$ ,  $P < 0.39$ ). This was also the trend with BP-7, 8-oxide Hb adduct levels ( $\alpha = 0.05$ ,  $P < 0.61$ ) and BP-4, 5-oxide Hb adduct levels ( $\alpha = 0.05$ ,  $P < 0.13$ ). BP diol epoxide Hb adduct levels were slightly higher in cord blood samples with the CYP1A1 Hinc II heterozygous genotype than in the wild type ( $\alpha = 0.05$ ,  $P < 0.75$ ). Cord blood subjects with the CYP1A1 Hinc II wildtype genotype had higher BP-7, 8-oxide Hb adduct levels than those with the heterozygous genotype ( $\alpha = 0.05$ ,  $P < 0.21$ ). BP-4, 5-oxide Hb adduct levels were slightly higher in the cord subjects with the CYP1A1 Hinc II heterozygous genotype than in subjects with the wild type genotype ( $\alpha = 0.05$ ,  $P < 0.62$ ). These results suggest that polymorphisms of the CYP1A1 genotype may play a role in formation of hemoglobin adducts.

**1260**

ASSOCIATION BETWEEN GENETIC POLYMORPHISMS IN CYP1A1 AND CYP17, RACE, AND TAMOXIFEN SIDE EFFECTS IN BREAST CANCER PATIENTS.

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Despite the widely accepted use of tamoxifen to treat estrogen receptor positive (ER+) breast cancer patients, there are clear indications that this drug is not the optimal breast cancer treatment for all women. About 50% of women taking tamoxifen experience serious side effects. The nature of these side effects sometimes varies between African American women and Caucasian women. The reasons for the lack of optimal response to tamoxifen are unknown. Since genetic polymorphisms in metabolic enzymes are racially distributed and have been associated with suboptimal responses to some drug therapies, it is our hypothesis that racial disparities in tamoxifen-related side effects may be associated with genetic polymorphisms in selected cytochrome P450 enzymes (CYP1A1 and CYP17). We tested our hypothesis by conducting a cross-sectional study of 123 breast cancer patients (53 African Americans and 70 Caucasians). Each patient completed a questionnaire and provided a blood sample for evaluation of polymorphisms in CYP1A1 and CYP17 using polymerase chain reaction and enzyme digests. African American women had four times greater odds of having a CYP1A1 polymorphism compared with Caucasian women (odds ratio: 4.65; 95% confidence interval: 1.17 - 18.51). No differences were observed between the two ethnic groups for polymorphisms in CYP17 (odds ratio: 1.01; 95% confidence interval: 0.43 - 2.34). CYP1A1 polymorphisms were associated with an increased risk of one side effect from tamoxifen (migraines), but not with other side effects from the drug (nausea, migraines, depression, vaginal discharge, vaginal dryness, insomnia, or hot flashes). These data suggest that the risk of polymorphisms in CYP1A1 differs by race, and that the risk of at least one side effect from tamoxifen may be associated with genetic polymorphisms in CYP1A1. (Funded by University of Maryland, Other Tobacco-Related Disease Research Grant through the Maryland Cigarette Restitution Fund Program)

**1261**

STRUCTURAL HETEROGENEITY AT THE UDP-GLUCURONOSYLTRANSFERASE 1A LOCUS IN A KOREAN POPULATION.

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Glucuronidation catalyzed by UDP-glucuronosyltransferases (UGTs) is one of the most important mechanisms for a host defense against xenobiotics and endobiotics. UGT1A family is derived from a single gene locus (UGT1A) coding for nine functional proteins (UGT1A1, UGT1A3-UGT1A10) and three pseudogenes (UGT1A2, UGT1A11-UGT1A12). The N-terminal end of UGT1A isoforms is coded by distinct exon 1s located upstream of the common exons 2 through 5. Although genetic polymorphisms of several UGT1A isoforms have been reported separately, the haplotypes in all functional exons are not identified and little information is available regarding SNPs in Koreans. In the present study, genetic polymorphisms at all functional exons of UGT1A locus were analyzed by direct sequencing of genomic DNA in fifty healthy Korean subjects and their haplotypes were inferred from genotype data using an expectation-maximization algorithm. We identified forty-four SNPs at exon 1s and one SNP at exon 5 (1-39% of allelic frequencies). Eleven SNPs at exon 1 were novel, and the frequencies of known variants also showed significant differences between Koreans and other ethnic groups reported. For example, allelic frequencies of UGT1A7\*3 (N129K, R131K, and W208R) were 31.6% in Caucasian and 25.5% in Japanese, while it was 18.0% in Korean population. Haplotypes were estimated only for individuals with allelic frequency over 15%. Five major haplotypes that occur at frequency over 5% were identified, which together accounted for 78% of all haplotypes. Haplotype I, the reference sequence, was the most common (0.435), from which haplotype II (0.132) differed at positions in exon 1 of UGT1A4, UGT1A5, UGT1A7. These results suggest that genetic polymorphisms of UGT1A locus seem to be different between Korean and other ethnic populations.

**1262**

ROLE OF ALCOHOL DEHYDROGENASE GENOTYPE IN PREDICTING DEVELOPMENT OF FETAL ALCOHOL SYNDROME (FAS).

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Women who drink alcohol during pregnancy are at increased risk of delivering an infant with Fetal Alcohol Spectrum Disorders (FASD) or Fetal Alcohol Syndrome (FAS). However, not all women who drink while pregnant will have an affected

baby, suggesting that maternal or infant genes involved in alcohol metabolism play a role in disease susceptibility. Alcohol is principally metabolized to acetaldehyde via the enzyme Alcohol Dehydrogenase (ADH). Polymorphisms at ADH2 result in altered ADH kinetics therefore modulating individual metabolism of ethanol. Prior animal studies have linked metabolic changes in this locus to negative fetal outcomes in ethanol-exposed fetuses. Due to inconsistent human studies, this study was designed to further examine the ADH2 genotype of mother/infant pairs and determine the influence of genotype on risk of developing FAS/FASD. Peripheral blood samples from 69 mothers and cord blood from 60 infants have been collected, and the genomic DNA purified using a commercial kit. ADH2 genotype was determined using restriction fragment length polymorphism PCR (RFLP-PCR). Maternal ethanol use was estimated by AUDIT questionnaire and interview. Infant outcomes included weight, length, head circumference and gestational age at birth. In examining AUDIT scores, women expressing an ADH2\*3 allele drank less than ADH2\*1 homozygotes. In addition, ADH2\*1 homozygous mothers with the heaviest alcohol use gave birth to smaller infants with lower gestational ages at delivery. Infants requiring intensive care hospitalization postnatally were also seen predominantly in mothers who were ADH2\*1 homozygotes. To date, our data suggest a role for ADH genotype affecting maternal drinking patterns and infant outcomes. Showing ADH genotype to be a biomarker indicative of potential maternal alcohol use, as well as an accurate predictor of fetal outcome, is paramount to developing interventions for high-risk pregnancies.

## 1263 CHARACTERIZATION OF THE VAL108MET COMT POLYMORPHISM.

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A major detoxification pathway for the carcinogenic catechol estrogens is *O*-methylation by catechol-*O*-methyltransferase (COMT). The val108met polymorphism in the COMT gene has been positively associated with breast cancer and a number of neuropsychiatric conditions. Recently we found that human hepatocytes homozygous for the met108 allele (low activity genotype, COMT<sup>LL</sup>) had lower levels of cytosolic COMT protein and activity. In addition, cytosolic COMT activity levels correlated with cytosolic COMT protein levels. Thus, we hypothesized that the lower levels of COMT protein observed in cells with the COMT<sup>LL</sup> genotype may be due to an increased rate of degradation of the low activity COMT protein, COMT<sup>L</sup>. To investigate this hypothesis, COMT<sup>HH</sup> and COMT<sup>LL</sup> human breast cell lines were incubated with cycloheximide, to inhibit protein synthesis, for varying lengths of time before harvesting and preparing cytosolic fractions. Western blotting was performed to determine COMT protein half-life in each cell line. To date, our results indicate that the half-life of COMT protein in COMT<sup>HH</sup> and COMT<sup>LL</sup> cell lines is greater than 24 hours. In addition, we are investigating whether there is an association between the val108met COMT polymorphism and COMT mRNA levels. We hypothesized that the lower levels of cytosolic COMT protein in COMT<sup>LL</sup> cells may also be due to decreased levels of COMT mRNA. Northern blots to detect COMT mRNA were conducted on total RNA isolated from COMT<sup>HH</sup> and COMT<sup>LL</sup> human breast cell lines. Thus far, no clear trend or association between total COMT mRNA levels (normalized to actin mRNA levels) and the val108met COMT genotype has been observed. We are currently confirming the Northern blot results using the TaqMan™ real-time reverse transcription PCR assay. In addition we plan to confirm and extend our findings in cell lines by measuring COMT mRNA levels in cryopreserved COMT<sup>HH</sup> and COMT<sup>LL</sup> human hepatocytes. Support from R01CA77550 and T32ES07141

## 1264 POLYMORPHISM IN N-ACETYLTRANSFERASE 1 ALLELES NAT1\*10 AND NAT1\*14A IN BENZIDINE-EXPOSED WORKERS IN THE CHINESE DYESTUFF INDUSTRY: LACK OF ASSOCIATION WITH CYTOLOGICAL GRADING OF EXFOLIATED UROTHELIAL CELLS.

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N-Acetyltransferase 1 (NAT1) modifies bladder cancer risk in Caucasians formerly exposed to small amounts of aromatic amines in cigarette smoke. Thus, the present study was performed to investigate an association between NAT1\*10 and NAT1\*14A genotypes and bladder cancer risk in benzidine-exposed Chinese workers. Based on the cytological grading of exfoliated urothelial cells according to Papanicolaou ("Pap test"), members of our research cohort were stratified into dif-

ferent subgroups. An allele-specific PCR-based procedure was used to detect the polymorphism in polyadenylation signal at the locus of NAT1 T1088A. A nested-PCR-RFLP procedure was conducted to differentiate NAT1\*14A (T1088A, C1095A, and G560A) from NAT1\*10 (T1088A, C1095A). No significantly different frequencies of homozygous and heterozygous NAT1\*10 alleles were found among the subgroups with gradings according to Papanicolaou  $\leq$ II (18.3% and 40.2%, respect.), higher gradings according to Papanicolaou ( $>$ II; 28.0% and 34.1%, respect.), and with bladder cancer (26.3% and 34.2%, respect.). The present data show that NAT1\*10 neither displayed an association with an elevated grading of urothelial cells nor a clear impact on the risk for bladder cancer in benzidine-exposed Chinese workers.

## 1265 INDIRECT EVIDENCE FOR EXTRAGENIC AND/OR ENVIRONMENTAL INFLUENCE ON HUMAN NAT1 EXPRESSION: ANALYSIS OF PRIMARY HEPATOCYTES BY ALLELE-SPECIFIC QUANTITATIVE REAL-TIME RT-PCR.

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Arylamine N-acetyltransferase (NAT) isozymes play an important role in metabolizing environmental carcinogens and other xenobiotics through N-acetylation or O-acetylation reactions, thereby modifying individual cancer risk and individual drug response or toxicity. Two genetic loci, NAT1 and NAT2, exhibit single nucleotide polymorphisms that define genotype. We analyzed cryopreserved human hepatocytes for NAT1 genotype and acetylator phenotype. Genotyping individuals for known NAT1 polymorphisms did not predict their measured enzyme activity. To distinguish the possible involvement of cis-acting genetic factors versus environmental or genetic trans-acting factors in the regulation of hepatocyte NAT1 expression, we designed a real-time RT-PCR Taqman assay for quantitative measurement of allele-specific mRNA expression in NAT1\*4/\*10 heterozygotes. Genomic DNA from NAT1\*4/\*10 heterozygotes, with a defined allele ratio of 1, was used as a control for differences in allele-specific probe characteristics, PCR amplification and overall variability of the assay. NAT1\*4/\*4 and NAT1\*10/\*10 genomic DNAs were also mixed in different ratios and analyzed to create a standard curve. We established that NAT1\*4/NAT1\*10 ratio measurements outside of the range of 0.5 to 1.5 could be considered significantly different from 1.0 (equal expression of each allele). Analysis of the mRNA expression ratios from individual hepatocytes using the standard curve revealed that functionally significant cis-acting genetic factors do not appear to account for the discrepancy between NAT1 enzyme activity and NAT1 genotype in these hepatocytes. Although NAT1\*4/\*10 ratios ranged from 3.3 to 0.5, overall enzyme levels varied over 350-fold. These results suggest that environmental and/or genetic trans-acting factors influence the expression of NAT1 in human hepatocytes, and experiments have been designed to test this hypothesis. Partially supported by NCI grant CA34627 and NIEHS Training Grant T32-ES011564.

## 1266 FUNCTIONAL CHARACTERIZATION OF MOUSE N-ACETYLTRANSFERASES IN A NAT2 KNOCKOUT MODEL.

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Arylamine N-acetyltransferase (NAT) isozymes are linked to cancer susceptibility in multiple tissues. Three NAT isozymes have been identified in mice, with NAT1 and NAT2 being most abundant. Functional characterization of NAT1 and NAT2 was investigated in a Nat2 knockout (KO) model (Pharmacogenomics Journal 3: 169-177, 2003) and a Nat2 wild type (WT) strain for comparison. NAT1 and NAT2 catalytic activity, protein, and mRNA levels were determined in cytosols from liver, gut, pancreas, bladder, mammary, and prostate of both male and female adult mice. NAT2 and NAT1 catalytic activities were assessed using p-aminobenzoic acid (PABA) and isoniazid (INH), respectively. NAT1 and NAT2 protein was determined using NAT1- and NAT2-specific antisera. Nat1 and Nat2-specific mRNA levels were determined by qRT-PCR using Nat1- and Nat2-specific primers. PABA acetylating activity was undetectable in all KO mice in all tissues from both males and females, except for mammary tissue where very low levels of PABA acetylation were detected. In contrast, NAT2 catalytic activity was readily detected in all tissues from male and female WT mice. NAT1 catalytic activity levels were similar in KO and WT mice in all tissues. Nat1 and Nat2 protein expression levels were consistent with these results. However, no significant differences in Nat1- or Nat2-mRNA levels were observed between KO and WT mice, indicating that insertion of the LacZ ablation cassette eliminates NAT2 acetylating activity via disruption of the NAT2 protein, without significantly affecting transcription rates or transcript stability. Some gender effects were observed in the pancreas as both NAT1 and NAT2 catalytic activities were higher in males than females. In conclusion, NAT2 catalytic activity is substantially reduced in the KO mice. In contrast, NAT1 acetylation activity is detectable in all tissues tested and is not influenced by the Nat2 knockout. Partially supported by the Wellcome Trust, Summer Res. Scholars Prog. (University Louisville) & NCI grant CA34627.

MECHANISTIC STUDY OF THE A<sup>411</sup>T (L137F) GENETIC POLYMORPHISM IN HUMAN N-ACETYLTRANSFERASE 2.

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Human N-acetyltransferase 2 (NAT2) genetic polymorphisms may modify individual cancer susceptibility from carcinogen exposure. A<sup>411</sup>T (L137F) is a recently identified single nucleotide polymorphism (SNP) in the NAT2 coding region that coexists with three other SNPs T<sup>341</sup>C (I<sup>114</sup>T), C<sup>481</sup>T (silent) and A<sup>803</sup>G (K268R) in allele NAT2\*5I. cDNA cloning followed by transient expression in COS-1 cells showed that NAT2\*5I encoded an NAT2 isozyme with very low activity resulting from the combined effects of L137F and I114T. A<sup>411</sup>T reduced NAT2 immunoreactive protein to an undetectable level by western blot, whereas neither A<sup>411</sup>T alone nor in combination with other SNPs caused a change in mRNA level measured by real time RT-PCR. However, RNA distribution in polyosome profile indicated no change in translation initiation efficiency. Missense mutants displayed different effects on N-acetylation activity: (wild-type L137: 70.2±5.2; L137F: 1.34±0.03; L137W: non-detectable; L137I: 34.2±2.0; L137G: 0.52±0.04). To further test our hypothesis that A<sup>411</sup>T (L137F) destabilizes NAT2 and accelerates protein degradation induced by protein misfolding, various NAT2 alleles were cloned and recombinantly expressed in E.coli, which do not possess the ubiquitin-mediated degradation pathway. In contrast to mammalian cells, NAT2 possessing the A<sup>411</sup>T SNP recombinantly expressed in E.coli showed no reduction in immunoreactive NAT2 protein, though its catalytic activity was very low. These findings, suggest that the A<sup>411</sup>T(L137F) SNP confers slow acetylator phenotype resulting from enhanced ubiquitin-mediated degradation. Supported by a dissertation research grant from the Susan G. Komen Breast Cancer Foundation; NCI grant CA34627, and the Kentucky Lung Cancer Research Program.

PARAOXONASE POLYMORPHISM LEU-MET55 AND ITS RELATIONSHIP TO DIABETIC COMPLICATIONS IN PATIENTS WITH NON-INSULIN-DEPENDENT DIABETES MELLITUS.

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Serum paraoxonase (PON1) is an esterase that hydrolyses organophosphates such as paraoxon and sarin. On the other hand, this enzyme is located on HDL. It is proposed to have responsibility for the antioxidative activity of HDL and decreases the oxidative modification of LDL *in vitro*. The human PON1 gene contains two coding region polymorphisms leading to two different PON1 isoforms: one at position 192 and a second at position 55 [leucine (L) to methionine (M) substitution]. The polymorphisms affect the hydrolytic activity of the PON1 isoenzymes with respect to certain substrates, such as paraoxon and lipid peroxides. Several studies have shown that PON1 could be involved in the pathogenesis micro and macroangiopathy in diabetic late complications. A low PON1 activity in diabetes may contribute to this increased susceptibility by reducing its ability to retard LDL-oxidation. We investigated the effects of the PON1 L/M55 polymorphism on serum PON1 activity, glycaemic control and plasma lipoproteins in NIDDM patients with complications and without complications and in non-diabetic control subjects. Serum PON1 activity in the group with complications was significantly reduced by 23.5% than in the group with no complications and by 26.3% than in non-diabetic control group ( $P<0.05$ ). The distribution of PON1 activity for the PON1 L/M55 polymorphism was the same for three groups ; MM homozygotes have the lowest activity and LL homozygotes the highest, with LM heterozygotes having intermediate activity (LL > LM > MM). The frequency of PON1 M genotype for the group with complications was found higher compared with those of the group without complications and controls (0.37; 0.33; 0.33 respectively). In conclusion, PON1 M homozygosity and low serum PON1 activity in NIDDM patients with complications may be related to an increased tendency for lipid peroxidation (Supported by Research Fund of Ankara University).

GSTM1, GSTM3 AND SMOKING HABITS IN BLADDER CANCER CASES FROM TWO DIFFERENT INDUSTRIAL AREAS.

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Smoking is the most important risk factor of transitional cell carcinoma of the urinary bladder. Many, but not all publications report an association between the glutathione S-transferase M1 (GSTM1) negative genotype and smoking habits in

bladder cancer cases. Thus we investigated 210 bladder cancer cases from an area of the former chemical and rubber industry in Lutherstadt Wittenberg, 83 bladder cancer cases and 210 surgical controls without a history of malignancies from a hospital in Dortmund, located in a former area of coal, iron and steel industries. Genotyping for GSTM1 and GSTM3 was performed on nuclear DNA according to standard PCR/RFLP methods. Smoking habits were qualified by a standardized interview. Smokers and ex-smokers showed higher percentages in cases from Dortmund (43%/40%) and Lutherstadt Wittenberg (33%/45%) compared with controls (27%/35%). The percentage of GSTM1 negative cases was 63% in the entire bladder cancer patient group compared to 50% in the control group. The percentages of GSTM1 negative smokers in the two case groups were 67% and 67%, respectively, compared with 71% and 55% in non-smokers with bladder cancer. No relevant association between age at beginning of smoking, number of pack years and tumour classification or grading could be observed. The percentage of GSTM1 negative non-smoking controls was 50% and thus within the percentages commonly observed in healthy Caucasian populations. GSTM3 \*A/\*A was 76% in the entire group of bladder cancer cases and 74% in controls. No association between age at beginning of smoking, number of pack years and tumour classification or grading could be observed. Thus the elevated percentage of GSTM1 negative bladder cancer cases in the investigated industrial areas is, if at all, only in part due to smoking habits.

GST-T1, P53, AND CASPASE-8 POLYMORPHISMS AND COLON CANCER RISK.

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Glutathione-S-transferase T1 (GST-T1) detoxifies mutagenic compounds via conjugation with glutathione. p53 acts through various mechanisms to repair DNA damage and/or induce apoptosis. Caspase-8 plays a major contributing role in p53-mediated apoptosis. As such, polymorphisms in any of these genes could affect cancer risk. As no one has yet examined this, we were interested in whether there were interactions among these functional polymorphisms, independent of their main effects, that could alter colon cancer risk. We hypothesized that a C to G polymorphism 429 bp 3' of the stop codon in *GST-T1*, p53 Arg72Pro, and *caspase-8* Asp302His alone or in combination leads to an increased colon cancer risk. This hypothesis was tested in a case-control study of 830 subjects from the greater Baltimore area. Logistic regression analysis adjusted for age, race, and gender revealed an increased risk of colon cancer (OR=1.82, 95% CI: 1.19-2.80) in individuals homozygous for the *caspase-8* 302His allele carrying at least one G allele of *GST-T1* (vs. *GST-T1* C/C homozygotes). In contrast, a decreased colon cancer risk (OR=0.41, 95% CI: 0.19-0.89) was observed in individuals with at least one 302Asp *caspase-8* allele, who had at least one G allele of *GST-T1* (vs. *GST-T1* C/C homozygotes). Interestingly, the 72Pro allele of *p53* decreased cancer risk alone (OR (Pro/Arg vs. Arg/Arg)=0.90, 95% CI: 0.58-1.40; OR(Pro/Pro vs. Arg/Arg)=0.63, 95% CI: 0.39-1.00;  $P_{trend}=0.02$ ) and had a slight interaction with *GST-T1* ( $P_{interaction}=0.06$ ). There were no apparent interactions between p53 and caspase-8 polymorphisms. These results suggest that the effects of the *GST-T1* polymorphism on colon cancer risk vary according to one's *caspase-8* genotype but are not very dependent on *p53* genotype. We have also shown for the first time that the *p53* 72Arg allele is protective against colon cancer. These results should be cross-validated in other populations.

THE GLUTAMATE CYSTEINE LIGASE CATALYTIC SUBUNIT -129 C/T SINGLE NUCLEOTIDE POLYMORPHISM IS ASSOCIATED WITH THE LEVEL OF GAD65 AUTOANTIBODIES IN TYPE 1 DIABETES PATIENTS WITH A DELAYED AGE-AT-ONSET.

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GSH protects against reactive oxygen species (ROS) mediated cell injury in response to a variety of toxicants, and plays a role in the immune response and cytokine production. Type 1 diabetes mellitus (T1D) is an autoimmune disease involving auto-reactive T-cells and ROS, both of which are thought to lead to pancreatic beta-cell destruction. Peripheral blood GSH levels are depleted in many diseases including diabetes. In this investigation we hypothesized that GCLC expression may be a susceptibility factor for T1D. A polymorphic -129 C/T single nucleotide polymorphism (SNP) exists in the promoter region of the GCLC gene and the T allele is associated with lower GCL activity than the C allele. An association with the GCLC -129 C/T SNP and myocardial infarction has recently been reported in a Japanese population. The specific aim of this investigation was to determine the frequency of the GCLC -129 C/T polymorphism and its association

with T1D age-at-onset and T1D autoantibodies in T1D patients (n=761; ages 0-34 years) and in age-matched control subjects (n=551) from Sweden. There was no association between GCLC -129 C/T SNP and T1D age-at-onset. However, patients with an age-at-onset of 15-34 years who possess the GCLC -129 SNP T/T genotype and who are positive for glutamate decarboxylase autoantibodies (GAD65 Ab+) have a higher GAD65 Ab index than GAD65 Ab+ T1D patients with the C/C genotype (Kruskal-Wallis test p-value = 0.0405, Dunn's Multiple Comparison test, p-value < 0.05). Since the GCLC -129 SNP T/T genotype has been shown to result in lower GCL activity, these results suggest that lower GCL activity may be associated with increased GAD65 autoantibody levels in delayed age-at-onset T1D patients.

**1272 BIOLOGICAL CHARACTERIZATION OF THE MANGANESE SUPEROXIDE DISMUTASE (MNSOD) VAL-9ALA POLYMORPHISM.**

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A val-9ala polymorphism, found in the mitochondrial targeting signal of MnSOD, has been associated with a number of diseases, including breast cancer in several epidemiology studies. MnSOD functions in the mitochondrial matrix to dismutate superoxide to hydrogen peroxide. Previous studies have reported that the polymorphism may cause an increase in import of MnSOD to the mitochondrial matrix *in vitro*. Altered levels of MnSOD due to the polymorphism are hypothesized to disrupt the oxidative balance in the cell, making the cell more susceptible to oxidative stress. This study aimed to determine if the polymorphism is functional in breast epithelial cancer cell lines. We examined 4 breast epithelial cancer cell lines to evaluate MnSOD activity, protein, and mRNA expression levels. Two cell lines were genotyped as homozygous for the alanine allele, while two others were homozygous for the valine allele. Semi-quantitative western blots were used to evaluate MnSOD protein levels. Preliminary results suggest that 1 ala/ala cell line had significantly higher amounts of MnSOD protein versus the other 3 cell lines (p<0.001) when corrected for mitochondrial mass. MnSOD activity assays revealed that all 4 lines had very low levels of MnSOD activity and did not show any significant difference between the cell lines. MnSOD mRNA expression was analyzed using the TaqMan™ real-time reverse transcription PCR system. Preliminary results suggest that there is no difference in mRNA expression levels among the cell lines. We are currently adding 4 additional cell lines to our study to further investigate the functionality of this polymorphism. (Supported by NIH grants R01CA94747 and T32ES07141).

**1273 MOLECULAR ANALYSIS OF DIHYDROPYRIMIDINE DEHYDROGENASE AND THYMIDYLATE SYNTHASE GENE POLYMORPHISMS IN A TURKISH POPULATION.**

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Suzen HS, Yuce NN, Guvenc G, Duydu Y Ankara University, Faculty of Pharmacy, Department of Toxicology, Tandoğan, Ankara, Turkey. 5-Fluorouracil (5-FU) is a widely used chemotherapeutic agent to treat various malignancies. 5-FU is converted to a metabolite that inhibits thymidylate synthase (TS) which is a critical target in cancer chemotherapy. The detoxification of 5-FU *in vivo* is mainly due to cytosolic enzyme dihydropyrimidine dehydrogenase (DPD). Among other mutations in the DPYD gene, the most prominent one resulting in severe DPD deficiencies is G to A transition in 5-splice recognition site of intron 14 (IVS14+1G A). A genetic polymorphism that is characterized by a VNTR in the enhancer/promoter region of TS gene might also affect the outcome of 5-FU therapy. Together, the assessment of polymorphisms in genes that encode DPD and TS might be useful in selecting patients who are likely to tolerate and to respond adequately to 5-FU. The aim of this study was to identify IVS14+1G A and VNTR polymorphisms in a Turkish population and to make a comparison with the other populations. In this purpose, PCR-RFLP technique was used to identify frequency of the polymorphic DPYD and TS genes in 120 healthy unrelated individuals. Our preliminary results showed that no splice mutation in the DPYD gene was detected among 120 Turkish individuals. The frequencies of the allele TS (2R) and TS (3R) in Turkey were 0.415 and 0.585, respectively. Our results revealed that, compared with other populations, the molecular profile of polymorphism at DPYD gene loci is similar to many Asian and European populations. On the other hand Turkish population TS allele frequency data is very similar to Caucasian populations. Further studies in different populations will be contributed for prediction of the efficacy and toxicity of using 5-FU based chemotherapy. (Supported by Ankara University, Biotechnology Institute, Project No: 2001-K-120-240)

**1274 TWO POLYMORPHISMS IN THE DNA REPAIR GENE MGMT INCREASE HUMAN SENSITIVITY TO THE TOBACCO-SPECIFIC NITROSAMINE NNK.**

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Efficient DNA repair is critical for the protection of cells from carcinogenic agents present in tobacco smoke. Many polymorphisms in several DNA repair genes have been associated with increased risk of cancer. O6-Methylguanine-DNA-Methyltransferase (MGMT) is a direct reversal DNA-repair protein that plays an important role in protecting the cell from mutations resulting from exposure to alkylating mutagens. We propose that inherited polymorphisms in the coding region of MGMT, which result in amino acid substitutions, may significantly influence the level of smoking-induced genetic damage, a critical step in the cascade of events leading to cancer. We tested the hypothesis that the inheritance of the Leu84Phe or the Ile143Val polymorphism in MGMT is associated with increased genetic damage resulting from exposure to alkylating agents found in tobacco smoke. We used the mutagen sensitivity assay, with the tobacco-specific nitrosamine NNK as a model alkylating mutagen, to test this hypothesis. Lymphocytes obtained from healthy volunteers were exposed *in vitro* to NNK and the genotoxic response was measured by assessing the increase in chromosome aberration (CA) frequency. A significant increase in NNK-induced CA was observed in cells from individuals with the Phe allele compared to cells from individuals homozygous for the wild-type Leu allele. This was true especially for smokers, males and younger individuals. A significant increase in NNK-induced CA was observed for males or younger individuals with the Val allele compared to individuals homozygous for the Ile allele. These data suggest that the inheritance of the Phe allele and/or the Ile allele may affect the repair efficiency of genetic damage induced by NNK. It may also provide a partial mechanistic explanation for previously reported findings that indicate an association between MGMT polymorphism and increased risk of cancer. (Supported by an External Research Program grant from Philip Morris Inc. and by an NIEHS T32-07454 grant).

**1275 ASSOCIATIONS BETWEEN MERCURY, BDNF POLYMORPHISM, AND ATTENTIONAL ATTRIBUTES OF MOTOR FUNCTION.**

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We evaluated whether BDNF polymorphism (BDNF) is associated with attentional attributes of reduced motor function in mercury-exposed subjects. Behavioral scores, urine and buccal cell samples were collected for 194 male dentists (DDS) and 233 female dental assistants (DAs) who were exposed to mercury for 19 and 10 yrs. Spot urinary levels were respectively 3.32 (4.87) and 1.98 (2.29) ug/l. BDNF status was respectively 68% or 66% Wild Type, 26% and 30% single substitution, and 5% and 4% full mutation. Analyzing genders separately, multiple regression controlled for age, vocabulary, alcohol, medications, injury, repetitive trauma, and visual acuity. Performance on Digit Span, Finger Tap, and Hand Steadiness were adversely affected by joint exposure to mercury and BDNF in an additive manner in both groups. However, the dominant and non dominant factor for finger speed had no association with BDNF. But when we co-varied out the effect of both hands from Finger Tap Alternate, the resultant residual term, Finger Tap Alternate<sup>partial</sup> remained associated with BDNF. Further, the correlation between BDNF and the sequence of Hits and Contact Time for Hand Steadiness showed a significant decay in correlation from Hole 3 to Hole 9 (the largest to smallest hole) where only Hits and Time for Hole 3 were associated with the BDNF. [Hits 3-9: correlations were 0.27, 0.24, 0.03, 0.00, 0.02, 0.00, 0.01; Time 3-9: correlations were 0.45, 0.17, 0.06, 0.02, 0.04, 0.01, 0.01]. This observation is consistent for both groups. The results suggest that deficits in attention necessary to alternate finger taps or first learn to position a probe without touching the sides of a hole, is adversely affected by the polymorphism. Further, our methods are particularly useful when exposure levels approach that of the general population because identifying a vulnerable group with a BDNF polymorphism reveals the additive effect of a personal genetic factor with a more precise attentional deficit attributable to exposure to elemental mercury.

**1276 IDENTIFICATION OF A NOVEL VARIANT OF THE PREGNANE X RECEPTOR IN HEPATIC ADVERSE DRUG REACTION PATIENTS.**

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The incidence and economic impact of idiosyncratic drug-induced hepatotoxicity has been a longstanding concern for the pharmaceutical industry and regulatory bodies. Problems arise in the analysis of genetic variation in adverse drug reactions

(ADRs) due to the very low frequency of occurrence and thus the ability to acquire a significant patient cohort. In this study, access to an archive of 1900 clinical liver biopsies has enabled the identification of a cohort of 53 individuals displaying histological and biochemical evidence of ADRs. The Pregnen X Receptor (PXR) is a ligand-activated transcription factor known to regulate the transcription of a number of genes involved in hepatic xenobiotic metabolism and bile acid homeostasis. The PXR gene is a candidate for altered functionality in patients with ADRs, in particular drug-induced cholestasis. Sequence analysis of the PXR protein-coding region revealed the presence of a novel non-synonymous single nucleotide polymorphism within the ligand-binding domain. This resulted in a cysteine to arginine change at residue 301 (C301R), identified in 2 of the 53 ADR patients as a heterozygote. The variant has not been found in a cohort of over 300 non-ADR individuals of various ethnic origins. Electrophoretic Mobility Shift Assays using the CYP3A-DR3 response element show that the C301R variant binds to DNA with approximately the same affinity as the wild-type PXR. However, transfection studies using a CYP3A-DR3 reporter plasmid show that C301R has reduced basal transcriptional activity, and impaired responses to the PXR ligands rifampicin and hyperforin, compared to wild-type receptor. Since hepatic ADRs occur at such low frequency, and given that they appear to be multifactorial in their aetiology, it is possible that having enriched for a population of ADRs, one single genetic factor among a large number may have been identified. In the general population this may have such a low occurrence as to have remained undetected without such "enrichment".

**1277**

#### MEASURING CHOLINESTERASE ACTIVITY IN HUMAN SALIVA.

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Biomonitoring of organophosphorous and carbamate pesticides has focused primarily on the inhibition of blood cholinesterase. Blood biomonitoring, however, can be invasive, time-consuming, and costly, especially in young children and infants. Therefore, saliva biomonitoring has recently been explored as a practical and feasible alternative to blood. To determine whether an individual's salivary cholinesterase was consistent over time and which of two methods was more accurate and preferable, saliva samples were collected once per week for five consecutive weeks from 20 adults using a Salivette (cotton swab) and pipette. To measure cholinesterase activity, the radiometric method developed by Johnson and Russell (1975) was modified for human saliva by increasing tissue volume, substrate volume, incubation time, and incubation temperature. Using this method, cholinesterase was found to be present and measurable, with good repeatability (2.2% average difference between duplicate samples). Activity in pipette-collected samples ranged from 0 to 153.7 nmol hydrolyzed/min/ml saliva, while activity in Salivette-collected samples was slightly higher: 3.4 to 264.7 nmol hydrolyzed/min/ml saliva. The activity for some individuals was very consistent during the five weeks, whereas for others it was variable, and, in general, variability in activity for the two collection methods was comparable (mean coefficient of variation [CV] for pipette=34.8%; Salivette=35.6%). Cholinesterase levels from the two collection methods were significantly correlated ( $r=0.41$ ,  $n=100$ ,  $p<0.05$ , two tails). In terms of participant preference, the Salivette was preferred to the pipette method at the majority (86%) of visits. Results from this study demonstrate that (1) cholinesterase is measurable in saliva, (2) in some people (about 50% of our population) the activity is consistent from week to week, and (3) the collection methods yield comparable results, though participants prefer the Salivette. This is an abstract of a proposed presentation and does not reflect Agency policy.

**1278**

#### A CONVERSION FACTOR BETWEEN TWO CHOLINESTERASE ASSAYS AND ITS APPLICATION IN ESTABLISHING A NORMAL RANGE FOR HUMAN RBC AChE.

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The Cholinesterase (ChE) Reference Laboratory (CRL) at the US Army Center for Health Promotion and Preventive Medicine (USACHPPM) uses a modification of the delta pH method of Michel to annually analyze over 15,000 blood specimens from personnel that predominantly work at chemical agent storage sites and chemical demilitarization facilities. UC Davis and CRL are collaborating to derive a conversion factor between the delta pH and the colorimetric Ellman ChE assays. The factor is used to estimate a normal range of Ellman determined human blood acetylcholinesterase (AChE) levels based on CHPPM's data. Human red blood cells

(RBCs) from three volunteers were assayed at UCD by the delta pH and Ellman methods according to standard procedures. Both assays were carried out at 25 °C, and with optimal final substrate concentrations: 10 mM acetylcholine bromide for the delta pH assay and 1 mM acetylthiocholine iodide for the Ellman assay. RBCs were treated with diisopropyl fluorophosphate to generate a dose/response curve of inhibition. This yielded an approximate conversion factor: delta pH = 0.091 Ellman + 0.052 with an  $r^2$  of 0.96. This approach permits converting the extensive CRL database of baseline (presumably unexposed) delta pH AChE values into a normal range of Ellman AChE activities, assays used to evaluate occupational exposure to pesticides. The estimated normal 95% range of human AChE for the Ellman assay was 7.12 to 9.10  $\mu$ mol/min/mL with a mean value of  $8.23 \pm 0.62$  SD  $\mu$ mol/min/mL. This work was supported by the US Army Medical Research and Materiel Command under Grant Project Order DAMD17-01-1-0772, NIOSH (#CDC U07/CCU906162-06) and NIEHS (#ES05707).

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#### PESTICIDE EXPOSURE ASSESSMENT: CONCURRENT PASSIVE DOSIMETRY AND BIOLOGICAL MONITORING OF TRICLOPYR AND 2, 4-D EXPOSURES OF A BACKPACK APPLICATOR CREW.

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Strategies for pesticide exposure assessment represent different levels of quantitative certainty. This study concerned the consistency of exposure monitoring of workers using backpack sprayers. A crew of 8 applicators, a mixer/loader, and a supervisor applied two herbicides, triclopyr and 2, 4-D, as a tank mix for weed control in forestry. When coveralls were used as a passive dosimeter, the average residues recovered were  $2.50 \pm 1.55$  mg equiv/d for triclopyr and  $0.75 \pm 0.52$  mg equiv/d for 2, 4-D. When the herbicides were collected on cotton whole body dosimeters worn beneath the coveralls, measurements were reduced to  $0.22 \pm 0.23$  mg equiv/d and  $0.14 \pm 0.17$  mg equiv/d ( $n=5$ ) respectively. Cotton gloves (under nitrile gloves) and socks, and face/neck skin wipes were included in each case but they contributed less than 1% to the exposure estimate. Urine specimens (24 h) were analyzed for triclopyr and 2, 4-D, the biomarkers. The excreted doses of triclopyr and 2, 4-D were  $5.2 \pm 4.3$   $\mu$ g equiv/d and  $3.8 \pm 3.4$   $\mu$ g equiv/d respectively. Their corresponding excreted daily dosage was  $0.072 \pm 0.057$  and  $0.052 \pm 0.044$   $\mu$ g equiv/kg-d respectively. Based upon these results, the estimated clothing penetration was 11% for triclopyr and 20% for 2, 4-D. Their respective dermal absorption rates were 3.6%/d and 4.2%/d. For backpack application of triclopyr and 2, 4-D, worker dose based upon external pesticide deposition on coveralls was nearly 340-times and 180-times more than the dose determined by biomonitoring. Although external measures predicted excessive applicator exposures, use of backpack sprayers in a rugged forest terrain minimized releases to the forest environment and resulted in worker exposures far below toxic levels.

**1280**

#### ENVIRONMENTAL INDUCTION OF CYP1A-, CYP2M1- AND CYP2K1-LIKE PROTEINS IN TROPICAL FISH SPECIES BY PRODUCED FORMATION WATER ON THE NORTHWEST SHELF OF AUSTRALIA.

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Normal operation of oil well platforms results in the discharge of produced formation water (PFW). The expression of CYP1A, CYP2M1- and 2K1-like proteins was examined in Gold-Spotted Trevally (Carangoides fulvoguttatus) and Bar-Cheeked Coral Trout (Plectropomus maculatus) as possible biomarkers of PFW impact. The results of this pilot study indicated PFW contamination near the Harriet A platform may contribute to induction of CYP1A- and 2M1-like proteins in Trevally, while other contaminants associated with PFW may induce a CYP2K1-like protein. In a 2003 caged fish study, Stripey seaperch (Lutjanus carponotatus) were caught at a clean site, then distributed to three caging sites: A (near field), B (far field) and C (a non-impacted reference site). Fish were sampled at time (T) zero, T=3 and T=10 days. Significant increases of CYP1A, one CYP2K1- and two CYP2M1-like proteins were noted at Site A at T=10. For the other CYP2K1-like protein, a significant increase was observed at site A only at T=3, but not at T=10. Prevailing winds switched between day 6 and day 10 of sampling, moving the surface water due west, therefore exposing the fish to different components of PFW that may possibly induce this CYP2K1-like protein. These results indicate that CYP1A protein is sensitive to PFW exposure and may act as a good biomarker. Importantly, statistically significant environmental induction of both CYP2M1- and CYP2K1-like proteins in tropical fish due to PFW exposure has not previously been described and represents possible new biomarkers (other than CYP1A) of PFW fraction-specific contamination. (Supported by Apache Energy Party Ltd., Australian Institute of Marine Science and the Environmental Toxicology Research Program of University of Mississippi)

## ANALYSIS OF ENVIRONMENTAL POLLUTANTS IN BREAST MILK AND DNA DAMAGE IN BREAST-MILK CELLS.

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It has been hypothesized that increased genetic instability, resulting from exposure to environmental toxicants, may increase an individual's risk of developing breast cancer. It is important, therefore, to determine the extent to which exposure to environmental pollutants results in increased genetic instability. We have addressed this issue by examining both the levels of pollutants in breast milk, and DNA damage in breast-milk cells. Breast milk samples collected from twenty-one women living in the Springfield, MA and Albany, NY areas, were analyzed for levels of 101 polychlorinated biphenyl (PCB) congeners and 8 organochlorine pesticides/metabolites (Hexachlorobenzene, oxychlordane, pp'-DDE, op'-DDE, -Chlordane, trans-Nonachlor, Mirex, and cis-Nonachlor). Cells in the milk were separated by centrifugation within one hour of milk collection, and DNA damage was assessed using the single cell gel electrophoresis assay. Each woman completed a questionnaire providing information on her age, age of her nursing child, her cigarette smoking history and current exposure, and number of children she previously nursed. Mother's age ranged from 21 to 40 years, and child's age ranged from 3.5 to 67 weeks. PCBs and pesticides were detected in all 21 milk samples. Among women who had not previously nursed a child (n = 15), there were significant positive correlations between age of the woman and 1) total PCB level, 2) Hexachlorobenzene level, and 3) pp'-DDE level. None of these correlations were significant in women who had previously nursed a child (n = 6). All samples contained cells with detectable levels of DNA damage, but no significant correlations between levels of pollutants and DNA damage were observed.

## AMBIENT AND BIOLOGICAL MONITORING OF EXPOSURE TO AROMATIC AMINES IN THE RUBBER INDUSTRY.

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Diphenylguanidine and di-*o*-toluylguanidine are important vulcanization accelerators and are suspected to release aniline (A) and *o*-toluidine (*o*-T) during the manufacture process of automotive rubber parts. Both, A and *o*-T, are confirmed animal carcinogens. Recent studies also suggest at least a 6.5-fold increase in bladder cancer incidence of workers exposed to *o*-T [Ward *et al.* 1996, *J National Cancer Inst* 88: 1046; Markowitz and Levin 2004, *Occup Environ Health* 46: 154]. The purpose of this investigation was to determine exposure to A and *o*-T in 69 healthy workers of the rubber industry. For this purpose ambient monitoring by personal air sampling and biological monitoring of the internal dose (A and *o*-T in urine) and the effective dose (Hb adducts) was carried out by GC/MS analysis. Two known bladder carcinogens in humans, 4-aminodiphenyl (4-ADP) and 2-naphthylamine (2-NA) were included in the study. Urine and blood samples were collected post-shift and results were compared to those derived from 200 non-exposed individuals and those published in the cohorts of Ward *et al.* and Markowitz and Levin. A and *o*-T levels in the air were lower 0.11 ppm (TWA: 2 ppm). Urinary levels of A and *o*-T were higher compared to non-exposed individuals (A: 9.4 vs. 3.7 µg/L; *o*-T: 10.5 vs. 0.1 µg/L). Hb adducts were also higher in the exposed persons (A: 821 vs. 488 ng/L; *o*-T: 563 vs. 23 ng/L). No difference could be determined for Hb adduct levels of 4-ADP and 2-NA between exposed workers and controls. Hb adduct levels of *o*-T were only ~10-fold lower than those, which were reported in the cohort studies by Ward *et al.* and Markowitz and Levin. The results show that diphenylguanidine and di-*o*-toluylguanidine can release A and *o*-T during regular manufacturing processes of automotive rubber parts. The comparison between ambient and biological monitoring also shows that A and *o*-T are primarily taken up via the skin. Therefore, exposure is underestimated by ambient monitoring.

## SINGLE NUCLEOTIDE POLYMORPHISM AT EXON 4 IN THE MICROSOMAL EPOXIDE HYDROLASE GENE IS ASSOCIATED WITH INCREASED AFLATOXIN ALBUMIN ADDUCTS IN THE BLOOD OF HUMANS IN GHANA.

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Hepatocellular carcinoma (HCC) is a multifactorial disease with various host and environmental factors involved in its etiology. Of these, aflatoxin exposure has been established as an important risk factor in the development of HCC and the pres-

ence of aflatoxin-albumin adducts in the blood serves as a valuable indicator of human exposure. In this research, the relationship between a variety of different HCC host factors and the incidence of aflatoxin-albumin adduct levels was studied in a Ghanaian population at risk for HCC. These factors included age, sex, HBV and HCV status and genetic polymorphisms in both microsomal epoxide hydrolase (mEH) and glutathione S-transferases (GSTs). Blood samples were analyzed for aflatoxin-albumin adducts, HBV and HCV status. GSTM1 and GSTT1 deletion polymorphisms and mEH exon 3 and exon 4 single nucleotide polymorphisms (SNP) were determined from urine samples. In univariate analysis, age, HBV and HCV status, GSTT1 and mEH exon 3 genotypes were not associated with aflatoxin-albumin adduct levels. However, mean adduct levels were significantly higher in both females and individuals typed heterozygous for mEH exon 4 (vs. wild types). Stratification analysis also showed that gender along with mEH exon 4 genotype and HBV status had a significant effect on adduct levels. Both females typed HBsAg+ and males with mEH exon 4 heterozygote genotypes showed significantly higher adduct levels as compared to the HBsAg- and wild types, respectively. Understanding the relationships between these host factors and the variability in aflatoxin-adduct levels may help in identifying susceptible populations and targeting aflatoxin-specific interventions for humans at high risk for HCC and chronic liver diseases (NIEHS P42 ES04917, USAID LAG-G-00-96-90013-00).

## CORRELATION OF MATERNAL HEMOGLOBIN (HB) 4-AMINOBIPHENYL ADDUCT LEVELS WITH RESPECT TO COTININE LEVELS AND MATERNAL GENOTYPES.

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Of all the avoidable risks for adverse pregnancy outcomes, cigarette smoking is the most important and obvious. Cigarette smoke affects the primary user, the mother, but also, the fragile fetus. It has been estimated that between fifteen and thirty percent of pregnant women in the United States smoke during pregnancy. Furthermore, Kentucky regularly ranks among the states with the highest percentage of pregnant smokers with twenty-three percent, a close second to West Virginia while Utah ranks the lowest at approximately fourteen percent. These extremes may be attributable to cultural and agricultural differences between the populations of these states. In the current study, we correlated the level of hemoglobin adducts to 4-aminobiphenyl found in tobacco smoke. Maternal hemoglobin adduct levels were measured in subjects based on cotinine concentrations and specific genotypes of interest. Maternal subjects with cotinine levels  $\geq$  50 ng/ml who possessed a NAT1\*10 allele had higher 4-aminobiphenyl hemoglobin adduct levels than subjects with the same cotinine levels who did not possess a NAT1\*10 allele ( $\alpha = 0.05$ ,  $P < 0.13$ ). Maternal subject with cotinine levels  $\geq$  50 ng/ml who were NAT2 slow acetylators had slightly higher 4-aminobiphenyl hemoglobin adduct levels than subjects with the same cotinine levels who were NAT2 rapid or intermediate acetylators ( $\alpha = 0.05$ ,  $P < 0.52$ ). Maternal subject with cotinine levels  $< 5$  ng/ml who were NAT2 slow acetylators had slightly lower 4-aminobiphenyl hemoglobin adduct levels than subjects with the same cotinine levels who were NAT2 rapid or intermediate acetylators ( $\alpha = 0.05$ ,  $P < 0.30$ ). These data suggest that the genetic polymorphisms of N-acetyltransferase may contribute to the variations in hemoglobin adduct levels seen in smokers, especially among the aromatic amines.

## CORRELATION OF MATERNAL AND FETAL HEMOGLOBIN (HB) ADDUCTS IN SMOKERS WITH RESPECT TO GENOTYPE.

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Hb adducts have been suggested as biochemical monitors of carcinogen exposure and measures of genotoxic risk. Studies have demonstrated that carcinogenic compounds, especially those present in cigarette smoke bind covalently to Hb. To further elucidate the relationship between maternal and fetal Hb adducts as a measure of tobacco smoke exposure, matched maternal/cord blood samples were obtained and 4-aminobiphenyl and benzo(a)pyrene adducts characterized. The results were graphed and linear regressions were performed for each of the adducts determined. Correlations between Hb adducts of paired maternal and fetal samples were assessed and Pearson's correlation coefficient (*r*) was used to determine the tightness of the data. In order to account for deviations from the regression line, individual data points were examined with regard to the genotypes of the maternal and fetal samples. A positive correlation was found to exist among both maternal and fetal samples for both 4-aminobiphenyl (*r* = .536,  $\alpha = 0.05$ ,  $P < 0.0001$ ) and benzo(a)pyrene (*r* = .346,  $\alpha = 0.05$ ,  $P < 0.0001$ ). Based on the inferred NAT1, NAT2, GSTM1 and GSTT1 genotypes for each of the maternal/fetal data points, no trend in hemoglobin adduct levels could be established. Genotypes appeared to

be distributed evenly across the scatter plot. NAT2 slow acetylators were slightly more common in pairs who had low levels of 4-aminobiphenyl hemoglobin adducts for both maternal and fetal samples. In addition, no trend in genotype was observed when considering CYP1A1 Msp I, CYP1A1 Hinc II, GSTM1 or GSTT1 polymorphisms, suggesting that either additional genotype polymorphisms or combinations of genotype may play an important role in the overall level of tobacco smoke carcinogen Hb adducts

## 1286

### LEVELS OF POLYCYCLIC AROMATIC HYDROCARBONS IN AMNIOTIC FLUID SAMPLES FROM SMOKERS AND NONSMOKERS.

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Previous studies from this laboratory have focused on the characterization of blood protein adducts formed *in utero* as a result of maternal smoking during pregnancy. These biological samples, obtained during the third trimester of pregnancy, at delivery, have clearly shown a correlation between maternal smoking and exposure of the fetus to tobacco smoke carcinogens, including 4-aminobiphenyl and benzo(a)pyrene. In the present study, we examined exposure of the fetus during the first trimester of development to various environmental carcinogens, particularly those found in tobacco smoke. Amniotic fluid samples were obtained from women undergoing routine amniocentesis at between 16 and 20 weeks gestational age. Amniotic fluid, produced by the fetal lungs and kidneys, is an important part of pregnancy and fetal development and this fluid surrounds the fetus throughout pregnancy. In these studies, samples of amniotic fluid were obtained from non-smokers as well as 0.5 pk/da smokers through >2pk/da smokers. Amniotic fluid samples were extracted and analyzed by HPLC and GC/MS for the presence of polycyclic aromatic hydrocarbons (PAHs). Amniotic fluid levels of PAHs were found in almost all samples analyzed. However, there was a clear correlation between levels of maternal smoking and PAHs in the amniotic fluid. 1-hydroxypyrene levels ranged from  $1.54 \pm 0.12 \mu\text{g/L}$  in nonsmokers to  $11.72 \pm 0.67 \mu\text{g/L}$  in women smoking >2 pk/da, indicating approximately a 10X increase over non-smokers. Similar results were found with more widely established carcinogens, including hydroxylated benzo(a)pyrene derivatives, which ranged from  $1.41 \pm 0.13 \mu\text{g/L}$  in nonsmokers to  $11.56 \pm 0.59 \mu\text{g/L}$  in >2pk/da smokers. These results indicate that exposure to harmful environmental carcinogens can occur during early gestational periods and may place the fetus at a risk of genotoxic as well as teratogenic events.

## 1287

### INVESTIGATIONS OF VARIATION IN URINARY PROFILES BETWEEN SMOKERS AND NON-SMOKERS USING METABONOMIC (NMR SPECTROSCOPIC) URINALYSIS.

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Development and evaluation of potentially reduced exposure tobacco products (PREPs) requires knowledge of smoke constituent uptake in smokers. Since smoke is a complex mixture, altering product specifications often results in conflicting yield and uptake estimates. Alternatives to developing and measuring countless constituent-specific biomarkers are needed. Metabonomics has the potential to provide this holistic approach for PREP assessment; however, initial exploratory research is required to demonstrate the potential. In this study, the hypothesis was tested that the metabolic profiles of smokers differ from those of non-smokers, and these differences can be mapped and quantified by metabonomic (NMR spectroscopic) urinalysis. Urine samples (24-h) were collected from smokers (N=24) and non-smokers (N=9) of mixed gender and varying ages on a controlled low mutagenicity diet. Samples were analyzed using 600 MHz  $^1\text{H}$  NMR spectroscopy. The resulting NMR spectra were interpreted using pattern recognition methods to characterize metabonomic alterations between the two groups. An initial principal component analysis (PCA) showed that several sources of variation existed in the data other than smoking. This resulted in the two sets of data overlapping in a plot of the first two components. However, when a supervised approach (PLS-DA) was applied to the data, the resulting plot showed that the majority of smokers could be separated from non-smokers. A number of urine samples contained a series of unknown compounds, mostly from smokers, but also in a small number of samples from non-smokers. These compounds were unrelated to nicotine and its major metabolites. No correlations were noted between NMR spectral data and demographic data, but the NMR data correlated with Ames urinary mutagenicity assays (both TA98 and YG1024;  $R^2=0.6$ ). Interestingly, no correlations were noted with tobacco-selective biomarker data (major nicotine and NNK metabolites). Reducing the conflicting sources of variation (as seen by PCA) may improve the predictive quality of the NMR spectral data.

## 1288

### RACE/ETHNICITY, INCOME AND PERCHLOROETHYLENE (PERC) EXPOSURES AMONG ADULT AND CHILD RESIDENTS OF BUILDINGS WITH DRY CLEANERS.

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Residential indoor air levels of perc were documented in 65 apartments in 24 New York City multifamily buildings where dry cleaners using perc were operating. Alveolar breath and blood perc level for adult (20-55 yrs old) - child (5-14 yrs old) pairs residing in each sampled apartment were also determined. Mean indoor air perc levels were significantly higher in buildings located in minority (population < 48.9% White) ( $72 \mu\text{g/m}^3$ ) or low-income (>23.6% households below poverty threshold) ( $230 \mu\text{g/m}^3$ ) census block groups than in buildings located in non-minority (18  $\mu\text{g/m}^3$ ) or higher income (23  $\mu\text{g/m}^3$ ) census block groups. Mean indoor air perc levels were also significantly higher in households identifying themselves as minority (African-American or Hispanic alone or in combination with any other race) (82  $\mu\text{g/m}^3$ ) than in households identifying themselves as non-minority (White) (16  $\mu\text{g/m}^3$ ) and significantly higher in low-income households (annual income <\$30,000) (106  $\mu\text{g/m}^3$ ) compared to higher income households (annual income >\$60,000) (18  $\mu\text{g/m}^3$ ). Individual adult and child blood and breath perc levels were significantly correlated with indoor air perc levels ( $R^2$  range 0.55-0.68). Not surprisingly then, blood perc levels in minority (1.07-1.96 ng/mL) or low income (1.17-2.16 ng/mL) residents were significantly higher than in non-minority (0.54 ng/mL) or low income residents (0.50-0.51 ng/mL). Alveolar breath perc levels were also higher in minority or low-income residents than in non-minority or higher income residents but not significantly so. These findings suggest that minority and low income residents of multifamily buildings with co-located dry cleaners constitute a subpopulation in New York City with exposure to perc greater than non-minority and higher income residents of buildings with co-located dry cleaners. Funded through USEPA STAR Grant R827446010.

## 1289

### HEALTH EVALUATION OF MARINE FISHES FROM JOHNSTON ATOLL NATIONAL WILDLIFE REFUGE, A FORMER MILITARY INSTALLATION.

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The JANWR was recognized as an important seabird refuge in 1926. Over decades, defense activities in the area have resulted in contamination with radioisotopes, pesticides, dioxins, metals, PCBs, and PAHs. At the request of the USFWS, the USGS evaluated the extent of contamination and fish health at 3 areas on Johnston Island (JI) and a reference site. The assessment was designed to determine if adverse effects were present in fishes from contaminated sites, not to determine causality. Dioxins and PCBs were measured in sediments along pre-defined transects; while fish collected along these transects were examined through gross, histological (blood, liver, kidney, spleen, gonad), and biochemical (EROD) metrics. Dioxins ( $\leq 40\text{pg TCDD-equiv/g dry wt.}$ ) and PCBs ( $\leq 20-10, 600\text{ng tPCB/g dry wt.}$ ) in the near-shore sediments off JI were an order of magnitude or 300-fold greater, respectively, than concentrations in reference sediments collected off another island at the atoll. *Chaetodon auriga*, *Acanthurus triostegus*, *Mulloidichthys flavolineatus*, *Parupeneus multifasciatus*, and *Gymnothorax javanicus* dominated the 233 fish examined. Fish from JI tended to have larger livers, smaller spleens, and a higher incidence and severity of lesions. Gross lesions included: fin erosions and deformities, eye cataracts, epidermal papilloma, head and pigmentation abnormalities. Microscopic lesions in tissues of fish from JI were moderate, yet generally greater in severity and incidence than lesions in fish from the reference site. Anomalies identified included: granulomas, macrophage aggregates, reduced fat/glycogen, inflammation, and necrosis. Micronuclei were up to 3-fold higher in fish from some JI sites. EROD was generally elevated in JI fish, but results were variable due to inter-species differences and numbers of individuals tested. Overall, results of the health assessment indicated that fish collected off JI were in poorer health than those from the reference site.

## 1290

### EXPOSURE ASSESSMENT IN CONTAMINATED ENVIRONMENTS.

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Humans are frequently exposed to complex mixtures of polycyclic aromatic hydrocarbons and other compounds. These include exposures due to chemically contaminated, or Superfund, sites, as well as exposures to combustion by-products.

Human populations in contact with these chemicals may experience adverse health effects if the exposures are of a sufficient dose and duration. However, limited information is available to understand the relationship between environmental concentrations and absorbed dose. Studies have been conducted at a contaminated and control site in Azerbaijan to investigate the relationship between environmental concentrations and biomarkers of exposure. Data have been collected to measure environmental concentrations, and biomarkers of exposure in human populations. The biomarkers being investigated include blood plasma concentrations of PAHs, and DNA adduct formation in lymphocytes. The results indicate that concentrations of total polycyclic aromatic hydrocarbons (tPAHs) range from 2, 300 ng/m<sup>2</sup> for a window swipe to 40, 400 ng/m<sup>2</sup> for a floor swipe sample. The model carcinogen, benzo(a)pyrene, was detected in house dust at concentrations ranging from 178 ng/m<sup>2</sup> to 483 ng/m<sup>2</sup>. Analysis of human blood plasma of all exposed individuals for PAH residues found mean total PAH levels of 303 ng/ml blood and mean carcinogenic PAH levels of 5.6 ng/ml blood. DNA adducts were significantly higher in the total exposed population than in the total control population. However adducts in lymphocytes from men in the control region were comparable to those in men from the exposed region. Overall, the results indicate there is a correlation between PAHs in environmental samples and PAH adducts in an exposed population.

## 1291 INTEGRATION OF BIOMONITORING DATA INTO THE RISK ASSESSMENT PROCESS.

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Improved biomonitoring (BM) technologies are being used to measure very low levels of environmental chemicals in the tissues of adults, children and developing fetuses. These measurements can show that people have been exposed to chemicals and that these chemicals have been absorbed into the body. However, linkages to exposure on the one hand and linkages to human health on the other are not always clear. The ILSI Biomonitoring workshop explored the process and information needed to put BM information into perspective by associating these data with exposure and potential health outcomes. Key questions associated with the use of BM data were discussed, these included How can BM data be used more widely?, What other data is needed to use BM data in risk assessment?, How might BM data be used inappropriately? Case studies of arsenic, phthalates, perfluorooctane sulfonic acid, polycyclic aromatic hydrocarbons and organophosphates were conducted to evaluate BM data for different classes of chemicals against these questions. The case-studies identified specific scientific issues and developed perspective on broader scientific questions. An overarching need is for the development of consensus criteria similar to the Bradford-Hill criteria that are commonly applied to epidemiology studies for application and interpretation of BM data. In general it was determined that in many cases there is an absence of human toxicokinetic data that are essential when evaluating human exposure relative to animal toxicology data. Furthermore, PBPK and exposure models should be developed along with gaining a clearer understanding of dose metrics are needed. In addition, there is a lack of information on how to identify appropriate biomarkers that reflect low level exposure at or near the threshold for effect. There is also a need to carefully examine the risk assessment paradigm in the context of BM data and to develop a risk communication strategy.

## 1292 A FLEXIBLE APPROACH FOR EVALUATING FIXED RATIO MIXTURES OF FULL AND PARTIAL AGONISTS FOR MIXTURES OF MANY CHEMICALS.

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Detecting interaction in chemical mixtures can be complicated by differences in the shapes of the dose-response curves of the individual components (e.g. mixtures of full and partial agonists with differing response maxima). We present an analysis scheme where flexible single chemical dose-response curves are combined in an 'additivity model' with prediction for the mixture based on a constraint of additivity. Iterative algorithms are used to estimate mean responses for this model at observed mixture combinations using only single chemical parameters. A 'full model' is also fit to the data with additional mixture parameters included. A likelihood-ratio test is used to test the hypothesis of additivity by comparing the full and additivity model predictions in the likelihood functions. When interaction is detected, an interaction threshold model may be estimated along the mixture ray. This model has two components: an implicit additivity region and an explicit part that describes the departure from additivity; the interaction threshold is the boundary between the two regions. The methods are illustrated with resulting data from a study of a mixture of 18 PHAHs in female Long Evans rats exposed by oral gavage for four

consecutive days with serum thyroxine (T4) as the response variable. The mixing ratio was selected based on the ratios found in breast milk, fish and other sources of human exposure. The methods demonstrated three plateau levels for the maximum effects of the single chemicals and allowed for dose threshold effects for each single chemical and the fixed-ratio mixture. The likelihood-ratio test of additivity was rejected due to evidence of greater than additive interaction in the high dose region. Estimation of the interaction threshold within the observed experimental region suggested evidence of additivity in the low dose region. (This abstract does not reflect USEPA policy.)

## 1293 A BAYESIAN NETWORK MODEL TO PREDICT HAZARD POTENCY FOR SKIN SENSITISATION.

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When assessing the skin sensitisation potency of a chemical, a risk assessor is often faced with information from a range of tests and/or predictive models. Under these circumstances the implicit approach is to take a weight of evidence approach, where the value assigned to each piece of evidence depends on its type and quality. In this present work, we have built a Bayesian Belief Network model to make this process explicit. The model provides a framework for assessing the relative contribution of different sources and for quantifying uncertainty, and ultimately facilitates prediction for new substances with limited data. It combines test results from Buehler, Guinea Pig Maximisation (GPMT), Human Repeat Insult Patch (HRIP), Human Maximisation (HMT) and Local Lymph Node (LLNA) tests and also incorporates Quantitative Structure Activity Relationship (QSAR) predictions for sensitisation and penetration. The methodology combines prior knowledge provided by experts, with evidence on the relationships between tests that is learnt from data. The output of the model is a prediction of the concentration that will give sensitisation at a defined level (typically 5% and 1%) in an HRIP, with a measure of uncertainty. The model has been built on a training set of 30 chemicals chosen to cover a range of chemical classes and sensitisation potencies, and then has been tested on a further nine. Initial results are promising; within the training set there is good correlation between model predictions and HRIP estimates from data, and the model predicts well for the additional evaluation set.

## 1294 TOXICITY-RELATED MOLECULAR PARAMETERS CALCULATED FOR ALDEHYDES AND KETONES FOUND IN DIESEL EXHAUST.

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Quantitative structure activity relationship (QSAR) studies can help elucidate toxicological mechanisms of complex mixtures, e.g., vehicle emissions. Some of the aldehydes and ketones found in vehicle emissions have been identified as irritants, mutagens and carcinogens. Several are listed as Hazardous Air Pollutants regulated by the 1990 Clean Air Act. Environmental sources of these carbonyls are numerous with automobile and truck emissions a major source in outdoor air. Diesel engine exhaust (DE) is a complex mixture of gases, vapors, liquid aerosol and particulate matter. This mixture contains combustion products such as carbon (soot), nitrogen, water, carbon monoxide, nitrogen dioxide, sulfur dioxide, polycyclic aromatic hydrocarbons and aldehydes. Historically, the soot fraction of DE has received the most attention as its compositional fraction can vary from 60%-80% of the total depending on the fuel, engine types and load. More recently, gas and vapor phase constituents, e.g., aldehydes and ketones, have been considered. Toward understanding this complex mixture, 75 DE carbonyls have been identified from the literature. A number of molecular parameters related to biological activity have been calculated for these 75 carbonyls, including calculated base ten logarithm of the octanol-water partition coefficient (Clog P), molecular volume (MgVol), calculated molar refractivity (CMR) and number of valence electrons (NVE). These calculations suggest that DE carbonyls tend to be hydrophobic, with 48 of the 75 compounds (64%) being either hydrophobic or strongly hydrophobic (0.5 < Clog P >2), 8% being weakly hydrophobic (0 < Clog P < 0.5) and only 28% being hydrophilic (Clog P <0). [This abstract may not represent official EPA policy.]

## 1295 THREE-DIMENSIONAL ANATOMICAL RECONSTRUCTION OF THE UPPER RESPIRATORY TRACT OF B6C3F1 MICE.

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The influence of the anatomy of the upper respiratory tract (URT) on airflow distribution and ventilation rate is a critical factor determining uptake of inhaled agents and interspecies differences in lesion location. Computational fluid dynamics (CFD) models based on three-dimensional anatomical reconstructions of the

URT have been used in rats, monkeys and humans to improve the accuracy of the descriptions of uptake of inhaled gases and particles for use in risk assessment. Because most bioassays are conducted in both rats and mice and since the mouse serves as the species on which many genomic platforms are based, a CFD model of the URT of the B6C3F1 mouse was undertaken. The methods used to develop the specimens for other CFD models were modified to reduce artifacts and improve the fidelity of the *in vivo* architecture. A male B6C3F1 mouse was killed without exsanguination. The head was fixed for 2 hr in Bouin's fluid rather than formalin to minimize shrinkage. The nasal airways of the specimen were filled with cryo-embedding medium that was saturated with Xerox copy toner to enhance the contrast between the airways and tissue structures and to provide support during sectioning. The head was embedded nose tip down and sectioned in frozen carboxymethylcellulose rather than paraffin to eliminate distortion and facilitate realignment. High quality digital photography was used to capture images of the block face. Images (412 total) were collected at sectioning intervals ranging from 10 to 50 $\mu$ . The three-dimensional image processing software, Mimics (Materialise, Ann Arbor, MI), was used to convert the series of digital photographs to a three-dimensional reconstruction using operator controlled thresholding criteria. This reconstruction will form the basis for a CFD model of airflow and inhaled material transport in the mouse nasal passages and is expected to improve the accuracy of uptake estimates for this important species, facilitating quantitative interspecies comparative risk assessment.

## 1296

### A PHARMACOKINETIC-PHARMACODYNAMIC MODEL FOR GENE-REGULATED PROSTATE MAINTENANCE: COMPARING THE EFFECTS OF CASTRATION WITH ANTIANDROGEN EXPOSURE IN THE RAT.

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Antiandrogens affect prostate maintenance in two ways. Androgen antagonists, such as the fungicide vinclozolin, act as competitive ligands for the androgen receptor (AR). Enzyme inhibitors, such as the therapeutic drug Finasteride, inhibit the enzyme 5 $\alpha$ -reductase (5 $\alpha$ R) from metabolizing testosterone (T) to the more potent dihydrotestosterone (DHT). Each mode of action results in decreased DHT levels and hence decreased prostate size. Castration leads to nearly total removal of T and DHT from the body, resulting in acute, significant prostate regression. A model describing the pharmacokinetics (PK) of T, DHT, luteinizing hormone (LH), and antiandrogens was linked to a pharmacodynamic (PD) model describing the androgen-controlled gene regulation of the prostate in the adult male rat. The resulting PK-PD model is able to simulate the prostatic effects of castration and antiandrogen exposure. The model describes the metabolism of T to DHT by 5 $\alpha$ R with feedback loops for the positive regulation of T synthesis by LH and negative regulation of LH by T and DHT. The gene-regulated processes involved in prostate maintenance, including cell proliferation, apoptosis, fluid production, and 5 $\alpha$ R activity, are each controlled in the model by the occupancy of a single gene by androgen-AR dimerized complexes. The model accurately captures the dynamics of the prostate after castration vs. daily doses of Finasteride, compared with experimental data. Daily dosing of Finasteride results in significantly decreased levels of DHT and raised levels of T due to the feedback loop. The result is approximately 60 percent prostate regression after 21 days compared to over 90 percent regression due to castration. This modeling effort provides a framework for extension from the adult to the more sensitive pubertal developmental period by including growth and changes in hormone synthesis and metabolism. (Funded by EPA/UNC Training Agreement CT827206. This abstract does not reflect EPA policy.)

## 1297

### PHYSIOLOGICALLY BASED PHARMACOKINETIC/PHARMACODYNAMIC MODELING FOR THE N-METHYL CARBAMATE PESTICIDE CARBARYL: INSIGHT INTO MECHANISM AND RISK ASSESSMENT.

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A physiologically based whole body pharmacokinetic/pharmacodynamic (PBPK/PD) model was developed for the N-methyl carbamate pesticide carbaryl (1-naphthyl N-methylcarbamate) as well as its major metabolites naphthol and hydroxylated carbaryl in rats and humans. The model consists of liver, blood, brain, fat, lumped slowly perfused and rapidly perfused compartments, with blood circulation linking the compartments for compound distribution. Intravenous (IV), oral, and dermal exposures as well as fecal and urinary elimination were described. The model simulates inhibition of acetylcholinesterase (AChE) in blood and brain based on tissue dosimetry. Tissue concentration and AChE inhibition data from

Sprague-Dawley rats, as well as fecal and urinary elimination data, were used for model calibration. Model simulations were consistent with experimental data and highlighted that the key pharmacokinetic characteristics of carbaryl are quick absorption, quick metabolism, and quick excretion. In the case of oral gavage, about 85-90% of a dose goes into urine through blood circulation while the half-life of compounds in circulation is less than 0.75 hour. PBPK modeling indicated that whole body pharmacokinetic model has the advantage for such a case in gaining insight to pharmacokinetic reality over those models which only consider concentration-time profiles in blood or specific tissues. The model was scaled up from rats to human toddlers and used to predict AChE inhibition associated with lawn care application of carbaryl. The predicted level of AChE inhibition in toddlers was about 1%, a level that is not clinically detectable. This predicted low level of AChE inhibition reflects its rapid clearance from target tissues and its reversible interaction with AChE.

## 1298

### USE OF EXPOSURE RELATED DOSE ESTIMATING MODEL (ERDEM) FOR ASSESSMENT OF AGGREGATE EXPOSURE OF INFANTS AND CHILDREN TO N-METHYL CARBAMATE INSECTICIDES.

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A physiologically based pharmacokinetic (PBPK) model was developed within the Exposure Related Dose Estimating Model (ERDEM) framework to investigate selected exposure inputs related to recognized exposure scenarios of infants and children to N-methyl carbamate pesticides as specified under the Food Quality Protection Act (FQPA). Conservative assumptions underlying residential exposure and cumulative risk were examined as inputs for particular exposure scenarios using residential dermal transfer coefficients, ambient air concentrations, and dietary intake. Physiological, pharmacokinetic and pharmacodynamic parameters describing the fate and effects of carbaryl, aldicarb, and propoxur in rats were scaled to establish the model structure for exposure to humans. Adjustments were made for differences in metabolism and physiology between children and adults. Michaelis-Menten kinetics were used to describe metabolism, where the chemical species compete for the catalytic enzymes. Bimolecular rate constants,  $k_i$  (pM-1 hr-1), were used to describe inhibition of acetylcholinesterase by the parent compounds, where the effects are assumed to be additive. The simulation results over a day reveal the effects of residential exposure on cholinesterase activity, and highlight the scenarios and biological pathways where chemical interactions are important. The interactions are a cause for differences in risk between cumulative and single-chemical exposures. Although this work was reviewed by EPA and approved for publication, it may not necessarily reflect official Agency policy.

## 1299

### SPATIAL/TEMPORAL MODELING OF CYTOCHROME P450-MEDIATED PHOSPHOROTHIONATE INSECTICIDE METABOLISM IN THE RAT LIVER ACINUS.

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In order to be effective anticholinesterases, the phosphorothionate insecticides (e.g., parathion) containing a P=S group, require CYP-mediated bioactivation (desulfuration) to their oxon metabolites which contain a P=O group (e.g., paraoxon). The spatial patterns of parathion desulfuration in the rat liver acinus were investigated by measuring the cellular content of CYP mRNA and performing mathematical modeling of the transport of the insecticide and its metabolite, as well as the rate of formation of the oxon. Adult male rats were treated i.p. with 80 mg/kg phenobarbital (PB) to induce CYP 2B1/2. Laser capture microdissection was used to determine the regional distribution of mRNA for CYP 2B1/2 in livers of control and treated rats. Real time PCR was used to quantify mRNA, and indicated higher levels of CYP 2B1/2 mRNA from the PB treated rats. The levels of CYP-mediated desulfuration were determined in hepatic microsomes by monitoring the amount of paraoxon (the active metabolite) produced by paraoxon's ability to inhibit an exogenous source of acetylcholinesterase. The spatial dimensions of the hepatic sinusoids were determined microscopically. The data were used to create predictive mathematical models for the spatial metabolism of insecticides through the hepatic acinus. The modeling involves partial differential equations using estimates of diffusion rates for the insecticide and its oxon through cell membranes, sinusoidal blood flow rates, P450 content and P450 rates of desulfuration. The desulfuration modeling is the initial model to more realistically predict the spatial/temporal metabolism of phosphorothionates. (Supported by NIH P20 RR 017661)

**1300****BIOTRANS: A NEW TOOL FOR PREDICTING THE METABOLISM OF CHEMICAL MIXTURES.**

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Metabolism of a single xenobiotic may produce one or more reactive species via complex pathways. Metabolism of a mixture of compounds is more complicated, as metabolites shared among the pathways can be formed, as well as species not seen using each constituent chemical alone. Experimental studies of chemical mixtures to determine toxic intermediates and understand the interconnected metabolic pathways can be resource prohibitive; hence, novel, alternative approaches are needed. Here we describe the structure and use of a computer-assisted modeling tool for "predictive metabolomics" that we call BioTRaNS (Biochemical Tool for Reaction Network Simulation). This tool simulates metabolism *in silico* by using "virtual enzymes" (each comprising reaction rules and binding feasibility predictors) and "virtual agents" (that perform non-enzymatic reactions) to mediate the transformation of "virtual molecules". The simulation generates a rich, time-dependent inventory of metabolites interlinked by specific reactions, thus forming a biochemical reaction network. As a test case, we selected a mixture of four VOCs: trichloroethylene, perchloroethylene, methyl chloroform, and chloroform. These VOCs are common groundwater contaminants, and each can exert harmful health effects. Using BioTRaNS to create a system containing a number of Phase I and Phase II virtual enzymes, as well as appropriate virtual agents, we were able to predict the metabolites of the four pollutants, describe their metabolic pathways, and identify metabolites in common and intermediates expected to be unstable or reactive, including epoxides, acid chlorides, ketenes, and radicals. We were also able to predict and highlight species based on mechanism-based rules, such as cytochrome P450 heme-oxo-substrate complexes. Our work represents the first steps in using this technology to describe and understand the interwoven and complex metabolic pathways of chemical mixtures. (This study was supported, in part, by NIEHS grants K25 ES11146 and T32 ES 07321.)

**1301****INTER-INDIVIDUAL VARIABILITY IN BENZENE METABOLITES.**

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The deleterious effects of benzene exposure are thought to be caused by the production of cytotoxic and genotoxic metabolites. Since metabolism varies among individuals, we investigated interindividual variability in the production of benzene metabolites among 116 workers exposed to benzene in China (mean exposure = 4.8 ppm; 10th-90th percentiles 0.40-10 ppm). Each worker had repeated measurements of both personal airborne exposure to benzene and toluene and urinary benzene metabolites (tt-muconic acid, S-phenylmercapturic acid, phenol, catechol, and hydroquinone), as well as of unmetabolized benzene in urine. We used mixed effects statistical models that incorporated random slope and intercept terms for each worker to evaluate interindividual variability. In this context, the random slope explains the subject-specific relationship between each metabolite (or unmetabolized benzene) and benzene exposure, while the random intercept relates to the background metabolite level for each subject (due to the presence of non-benzene sources of the metabolites, particularly the phenolic compounds). Significant interindividual variability was detected for each benzene metabolite and for unmetabolized benzene in urine. Fixed effects were included in the models to investigate the influence of potential covariates on benzene metabolite levels, notably co-exposure to toluene, lifestyle factors, and smoking. Several of these covariates were significantly associated with the production of benzene metabolites. \* Supported by grant P42ES05948 from NIEHS/NIH.

**1302****USING OBSERVED VARIABILITY AS A CRITERION FOR CELLULAR KINETICS MODELS AFTER FURAN EXPOSURE.**

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Potential carcinogens are frequently examined by investigating their effect on the balance between the replication and apoptosis rates of cells. Observations on cell replication (by BrdU labeling) cover the entire period of BrdU exposure (usually several days), while observations on apoptosis are restricted to the brief period (sev-

eral hours) that apoptotic cells are identifiable; thus the data are difficult to compare directly. Additionally it is difficult to take into account the effect of the experimental treatment on the length of the time interval of apoptotic identifiability. Dynamic models linking cellular replication and apoptosis may be stochastic (linked birth and death models) or statistical (differential equation system with covariance structure determined by the data). The data from a furan exposure experiment (Fransson-Steen, R. et al.(1997)) are re-analyzed to address the above concerns. Four groups of mice were exposed to 4 levels of furan (0, 4, 8 and 15 mg/kg) for 3 weeks. BrdU was supplied for the last 7 days. Using a derived expression for the variability of the fraction of unlabeled cells, the statistical model was found to be more appropriate for this data. Hypothesis tests showed a significant increase in cell replication and apoptotic rates at the highest dose level as well as an increase in the apoptotic duration at the highest 2 dose levels.

**1303****QUANTITATIVE MODELS OF BYSTANDER EFFECTS FROM IONIZING RADIATION IN NON-TARGETED CELLS.**

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Major revisions of our understanding of the mechanisms of how ionizing radiation causes effects such as cytotoxicity and mutations have occurred in the last decade. This is due to accumulating evidence that these effects, called bystander effects, can occur in cells distant from those being irradiated (see J.B. Little, *Oncogene*, 2003). Observations made in many types of systems suggest these effects are due to secreted factors, although no specific factor has been identified. The secreted factors have been shown to be transmissible through both gap junctions and extra cellular media. Secreted transmissible factors through gap junctions can cause cytotoxicity and mutations, whereas factors in extra cellular appear to only cause cytotoxicity. In this work we integrate many of these observations in quantitative models of the secreted factors to better understand how the secreted factors may operate and how to better extrapolate *in vitro* observations to *in vivo* systems. We examine a number of possible assumptions that are consistent with the observations. One type of model used for gap junctions treats each cell as a separate compartment connected by first order rates in a two dimensional geometry, and within cells the secreted factor is deactivated at a constant rate. Exposure to ionizing radiation is simulated as an initial amount of the factor being deposited in the central compartment. Variations on this model describe the deactivation of the secreted factor as a saturable mechanism. Other types of models describe transmission across cells as involving a variable time delay. These models are extrapolated to three dimensional geometries to describe tissues. The models are shown to potentially provide a method for classifying diverse *in vitro* systems in terms of the rate of transmission of secreted factors, and how to estimate these rates from mutation rates or cytotoxicity fractions. We also suggest the types of experiments that might be performed to distinguish between the types of proposed models. This work was supported by a research grant from the Department of Energy Low-Dose Program.

**1304****A MECHANISTIC MODEL OF LIFETIME CANCER RISK FOR INHALATION EXPOSURES TO REACTIVE GASES.**

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Quantitative cancer dose-response analyses typically estimate lifetime risk using adult human parameter values (i.e., 70 kg, 70 years, 20 m<sup>3</sup> of air/day). Despite recent regulatory emphasis, children's risk is often relegated to a qualitative discussion of intrahuman variability or use of an uncertainty factor. Mechanistic models of dosimetry and cancer using age-specific parameters can increase the accuracy of lifetime risk estimates. Previously, a clonal growth (CG) model predicted cancer risks from inhaled formaldehyde (HCHO) based in part on estimates of (1) regional HCHO flux to respiratory tissues and (2) numbers of cells at risk. In this model, the number of cells at risk was estimated for the adult and assumed to vary in proportion to age-specific body weight. The CG model used regional HCHO fluxes from adult computational fluid dynamics (CFD) and typical-path models of the nasal passages and lung, respectively, and adult ventilation rates and activity patterns throughout life. Here, age-specific flux predictions and childhood ventilation rates and activity patterns are used to test the hypothesis that these factors affect risk estimates using HCHO as representative reactive gas. Age-specific HCHO fluxes were estimated for the human respiratory tract using a CFD model of HCHO uptake in the nasal passages and a single-path model of HCHO uptake in the lower respiratory tract isotropically scaled to estimates of nasal and lung volumes, respectively, in children aged 3 months and 1, 5, 10, and 15 years. Surface areas for each lung generation and 20 levels of flux in the nasal passages were estimated for each age group and used to calculate age-specific numbers of respiratory cells at risk. These values are input sequentially by age group to the CG model to develop lifetime cancer risk estimates. This information helps determine the importance of incorporating age-specific parameters during childhood in risk estimates for lifetime exposures to inhaled reactive gases.

## PRELIMINARY DEVELOPMENT OF A MECHANISTIC THYROID HORMONE MODEL.

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An existing model for free iodide (I<sup>-</sup>) kinetics in the adult rat was expanded into a pharmacodynamic thyroid hormone feedback model. Portions of Kohn *et al.*'s model were incorporated to account for processes such as hormone production, storage and secretion in the thyroid, deiodination of thyroxine (T<sub>4</sub>) to triiodothyronine (T<sub>3</sub>) to less active forms, and the feedback mechanisms between free hormone levels, TSH and sodium iodide symporter (NIS) up-regulation and down-regulation. The previously developed iodide model successfully described iodide kinetics and the inhibition of iodide uptake at the thyroid NIS by a competitive inhibitor, perchlorate (ClO<sub>4</sub><sup>-</sup>), but did not predict subsequent blood hormone perturbations. The new model uses first order hormone production rates from the thyroid and M-M kinetics to describe deiodination of T<sub>4</sub> by Type I deiodinase in the kidney and liver, Type II T<sub>4</sub> 5'deiodinase in other lumped tissues, and T<sub>3</sub> deiodination by Type III deiodinase and its inhibition by free T<sub>4</sub>. Transport of thyroid hormones bound to thyroxin-binding prealbumin and albumin are described as flow limited. While the preliminary model structure and parameters established here are intended to describe normal thyroid conditions and early upregulation, future modifications of parameter values may allow hormone predictions based on a disrupted thyroid status. Ultimately, the improved rat model may be extrapolated to a human PBPD model and validated against published human data.

## BIOLOGICALLY-BASED MODELING OF THE SPONTANEOUS TRANSFORMATION OF HUMAN KERATINOCTYES, RHEK-1.

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Neoplastic transformation of an immortalized human keratinocyte cell line, RHEK-1, by various carcinogens and chemical mixtures has been investigated in our laboratory and elsewhere. This human cell line, though useful for studying multi-stage carcinogenesis, exhibits certain tendency for spontaneous transformation, particularly after multiple passages. In the present study, computational models of cellular growth and the step-wise process of spontaneous malignant transformation are developed for the RHEK-1 cell line. Cellular growth and time-course acquisition of transformation-associated characteristics such as anchorage independent growth (AIG) in methylcellulose and tumor formation in immunocompromised mice were incorporated in the model. Normal cell growth is described logically with a carrying capacity estimated by saturation density analysis. Conceptually, three compartments are included in the transformation model: normal RHEK-1 cells which can die, divide, or mutate, AIG positive cells which can die, divide, or further mutate, and finally, malignant cells capable of forming tumors in nude mice. Distinct division and death rates, estimated from experimental data, are used for cells in each compartment. Furthermore, cell growth and cell death is assumed to be deterministic, while mutation is assumed to be a stochastic process. Accurate model simulations of RHEK-1 cells under negative control experimental conditions formulate a basis for chemically-induced neoplastic transformation. Thus, further development includes the integration of extensive time-course cell and molecular biology data from chemically treated keratinocytes to establish the carcinogenic potential of chemicals (polycyclic aromatic hydrocarbons) and chemical mixtures (marine diesel fuel and Jet A). (This study is supported, in part, by ATSDR Cooperative Agreement #U61/ATU881475 and NIEHS Training Grant 1 T32 ES 07321).

## EVALUATION OF MOUSE MODELS FOR ASSESSING THE ALLERGENIC POTENTIAL OF PROTEINS.

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Animal models may prove useful in evaluating the allergenic potential of novel proteins in the context of biotechnology. However, no standardized and validated model currently exists. The objective was to evaluate the utility of mouse models to differentiate between allergens and putative non-allergens. Human allergenic proteins (peanut Ara h 1 and Ara h 2, cows' milk  $\beta$ -lactoglobulin [B-LG]) and putative non-allergenic proteins (spinach RUBISCO and soy lipoxygenase [SLG]) were pu-

rified to > 90%. Each of four labs used the intraperitoneal (ip) route of exposure with the BALB/c, BDF-1, C3H/HeJ, A/J, or C57/Bl6 strains. Other variables included the presence or absence of adjuvant, the number and time between ip injections, protein doses, and the use of homologous or heterologous PCA to assess IgE. One lab also evaluated clinical signs. All labs demonstrated significant PCA responses to RUBISCO in the absence of adjuvant. In two labs, purified Ara h 2 provided poor IgE PCA titers, and the relative response via PCA to RUBISCO was significantly greater than Ara h 2. In another lab, a much higher clinical score was seen with SLG than B-LG. The nature and magnitude of the responses varied between different mouse strains. In general, it was not possible to differentiate between the allergens and putative non-allergens in any lab using the strains and endpoints evaluated. The presence of endotoxin contamination in the putative non-allergenic proteins may, in part, explain the relatively large PCA responses observed with the putative non-allergenic proteins. In conclusion, no ip mouse model evaluated could differentiate between the purified allergens and putative non-allergens. In conclusion, further work will be necessary before mouse models could be utilized to evaluate the potential allergenicity of proteins.

## VALIDATION OF FUMONISIN BIOMARKERS IN F344 RATS.

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Fumonisins are ubiquitous contaminants of cereal grains around the world. Fumonisin B1 (FB1) is a carcinogen and a strong tumor promoter in animal models. Dietary exposure to FB1 has etiologically been linked to human neural tube defect and elevated esophageal and liver cancers in certain areas of the world. Disruption of sphingolipids metabolism and metabolites of sphingolipids, especially ratio of sphinganine (Sa) and sphingosine (So) have been proposed as potential biomarkers for FB1 exposure. To validate FB1 biomarkers for studying human diseases risks, male F344 rats were administrated p.o. with either a single-dose of 0, 10, and 25 mg FB1/kg body weight (b.w.), or multiple-doses of 0, 1.0 or 2.5 mg FB1/kg /day b.w. for 5 weeks. The time course of FB1 induced metabolic alteration of sphingolipids was assessed in serum and urine samples collected from the treated rats at different time interval by a robust HPLC-fluorescence detection with D-erythrosphingosine (C20) as the internal standard. In the single-dose study, urinary Sa/So ratio reaches the maximum in day 8 (30.11 ± 19.40) for 25 mg FB1/kg group and in day 6 (11.48 ± 6.54) for 10 mg FB1/kg group, whereas serum Sa/So ratio shows a similar pattern for the two treatment groups and reaches the maximum around day 3 with the Sa/So ratio of 0.79 ± 0.01 for 10 mg/kg and 1.02 ± 0.05 for 25 mg/kg, respectively. In the multiple-doses study, serum and urinary Sa/So ratio reach the maximum more gradually, and kept at that level more lastingly than those observed in the single-dose study. The dose-response relationship between treatment of FB1 and FB1 metabolites in feces and Sa/So ratio in urine and serum was further examined. (Supported by the NIH grant CA94683)

## SHORT-TERM SAFETY EVALUATION OF NOVASIL IN HUMANS.

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Novasil clay (NS) provides significant protection from the adverse effects of aflatoxins (AFs) in multiple animal species by decreasing bioavailability from the GI tract. It is postulated that NS clay can be safely added to human diets to diminish exposure and health risks from AF contaminated food. To determine the safety and tolerance of NS in humans and establish dosimetry protocols for long-term efficacy studies, a randomized and double-blinded phase I clinical trial was conducted. Volunteers (20 - 45 yr in age), were clinically screened for confirmation of their health status. Fifty subjects (23 males and 27 females) were randomly divided into two groups: the low-dose group received 6 capsules containing 1.5 g/day, and the high-dose group received 6 capsules containing 3.0 g/day for a period of 2 wk. NS capsules were manufactured in same color and size and were distributed to each participant 3 times a day at designated sites, where follow-up was taken to record any side effects and complaints. Blood and urine samples were collected before and after the study for laboratory analysis. All participants completed the trial and compliance was 99.1%. Mild GI effects were reported in some participants. Symptoms

included abdominal pain (6%, 3/50), bloating (4%, 2/50), constipation (2%, 1/50), diarrhea (2%, 1/50), and flatulence (8%, 4/50). No statistical significance was found between the two groups for these adverse effects ( $P>0.25$ ). No significant differences were shown in hematology, liver and kidney function, electrolytes, vitamin A & E, and minerals in either group. These results demonstrate relative safety of NS clay in human subjects and will serve as a basis for long-term human trials in populations at high risk for aflatoxicosis (Supported by USAID LAG-G-00-96-90013-00).

### 1310

#### COMPARISON OF FORMALIN FIXED AND FROZEN TISSUES FOR SPHINGANINE AND SPHINGOSINE DETERMINATION FOLLOWING FUMONISIN B<sub>1</sub> EXPOSURE IN SWINE.

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Fumonisin B<sub>1</sub> (FB1), a mycotoxin produced by *Fusarium verticillioides*, is a contaminant of corn and causes lethal pulmonary edema in swine and leukoencephalomalacia in horses. Since FB1 inhibits sphingolipid metabolism, sphinganine (Sa) and sphingosine (So) can be used as biomarkers of exposure. Serum and fresh tissues (stored at -80°C) are used experimentally to determine Sa and So concentrations following FB1 exposure. However, formalin fixed tissues are more routinely available for diagnostic purposes and would provide multipurpose, low cost, long term preservation for research studies. To determine whether formalin fixed tissues could be used, we quantified Sa and So concentration by reverse-phase high-performance liquid chromatography (HPLC) in kidney, liver, and lung obtained 3 months previously from FB1-treated and control pigs, and compared the results for fresh frozen and formalin fixed tissues. Formalin fixed tissues had lower Sa and So than the corresponding fresh frozen tissues. The Sa:So ratio in formalin fixed tissues was higher because the loss for So was greater than for Sa. The correlation coefficients between fresh frozen and formalin fixed tissues was high ( $>0.95$ ). These results indicate that formalin fixed liver, kidney and lung can be used to determine alterations in Sa and So concentrations, with the Sa:So ratio being the most useful. This information should allow retrospective determination of FB1 as a cause of toxicity in spontaneous diseases. Supported by C-FAR (02E-082-4VR) and the International Life Sciences Institute.

### 1311

#### INFLUENCE OF THE SOY ISOFLAVONES GENISTEIN AND DAIDZEIN ON THE EXPRESSION OF 17 $\beta$ -ESTRADIOL-METABOLIZING ENZYMES IN CULTURED MCF-7 BUS127 CELLS.

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The activation of 17 $\beta$ -estradiol (E2) to the catechol estrogens 2-hydroxyestradiol (2-HO-E2) and 4-hydroxyestradiol (4-HO-E2) constitutes an important risk factor in hormonal carcinogenesis. 4-HO-E2 exhibits a stronger genotoxic potential than 2-HO-E2. In human breast tissue, 2-HO-E2 is mainly formed by cytochrome P450 (CYP) 1A1, and 4-HO-E2 is formed by CYP1B1. The catechol estrogens are detoxified by catechol-O-methyltransferase (COMT). Since genistein (GEN) und daidzein (DAI) are believed to exhibit a protective function on hormonal carcinogenesis, we examined the influence of E2, GEN und DAI alone and in combination on the expression of COMT, CYP1A1, and CYP1B1 in cultured MCF-7 BUS127 cells by reverse transcription/competitive polymerase chain reaction. CYP1A1 enzyme activity was determined by measurement of the conversion of ethoxresorufin to the fluorescent dye resorufin. In addition, estrogen-induced cell proliferation was determined by means of the E-screen. E2, GEN, and DAI inhibited the expression of COMT. The maximal effect (reduction by 70% of the original relative mRNA level) was obtained with 100 pM E2, 1 microM GEN, and 1 microM DAI, respectively. The same concentrations also induced the most pronounced cell proliferation in the E-screen. The effects of E2, GEN und DAI were additive up to the maximal effect and could be suppressed by the estrogen receptor antagonist ICI 182, 780. The expression of CYP1B1 was only slightly modulated by E2 and the isoflavones, whereas the expression of CYP1A1 was inhibited by both E2 and the soy isoflavones. CYP1A1 mRNA levels correlated with enzyme activity. These studies indicate that GEN und DAI stimulate the formation of the more genotoxic catechol metabolite of E2 but inhibit the detoxification of both catechol metabolites in estrogen-responsive tumor cells. Supported by Deutsche Forschungsgemeinschaft (Grant Me 574/21-1).

### 1312

#### MODIFIED METHOD FOR EXTRACTION AND HPLC ANALYSIS OF SPHINGANINE AND SPHINGOSINE FROM BODY FLUIDS, CELLS AND TISSUES.

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Free sphinganine (Sa) and sphingosine (So) are important biomarkers for fumonisin B<sub>1</sub> exposure. The method for analyzing tissue Sa and So levels was developed by Merrill et al., 1988 (*Anal Biochem* 171:373). Yoo et al., 1996 (*Toxicol in Vitro* 10:77), proposed a more economical method with simplified steps and improved recoveries, as well as allowing the entire extraction to be performed in 1.5 mL microcentrifuge tubes. This method worked well for cells and rodent kidney, but was not recommended for rodent liver and tissues from other species. Norred et al., 1996 (*Toxicol in Vitro* 10:349), proposed dual cycles of extraction for rat liver and kidney. In this study, we reevaluated the methods developed by Yoo and Norred for analyzing Sa and So in a variety of body fluids, cells and tissues. Using Yoo's extraction protocol and modified HPLC conditions, we successfully quantified Sa and So in cells (HepG2 hepatocytes, PK15 renal cells, A10 smooth muscle cells, H9C2[2-1] cardiomyocytes), serum and urine from pigs and calves, as well as fresh frozen swine tissues (liver, kidney, lung and heart). The modified method also worked well for formalin fixed swine liver, kidney and lung but not heart, due to unexplained peaks in the chromatogram interfering with interpretation of Sa and So. Modifications in HPLC conditions included a lower % of potassium phosphate buffer in the mobile phase (8%), a lower flow rate (1 mL/min) and a lower excitation wavelength (230 nm). Norred's method did not work well for our samples. Our modified method provides a relatively rapid and economical method for quantifying Sa and So in a variety of body fluids, cells and tissues. Supported by International Life Sciences Institute.

### 1313

#### EFFECT OF AFLATOXIN B1 ON DRY GRIND ETHANOL PROCESS.

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Aflatoxins, like all mycotoxins, are toxic fungal metabolites which can have adverse health effects on animals and human beings. Aflatoxins are a major concern for the dry grind corn processing industry as it is believed that aflatoxins affect yeast and reduce its efficacy in producing ethanol. In the present study, aflatoxin B1 (100, 200, 350 or 775 ppb) was added to mycotoxin free (aflatoxin B1, B2, G1, G2; fumonisin B1, B2; ochratoxin A, zearalenone and deoxynivalenol) corn and laboratory scale fermentations were conducted. Fermentation, using *Saccharomyces cerevisiae*, was carried out at 30°C for 72 hr with continuous agitation. Mean ethanol concentrations in the fermenter ranged from 14.01 to 14.51 % (v/v) at 60 hr for all treatments. No effect of aflatoxin B1 was observed on fermentation rates or on final ethanol concentrations. In the dry grind ethanol process, 55% of aflatoxin B1 was detected in wet grains and 45% in thin stillage (supernatant). Aflatoxin B1, up to 775 ppb, had no effect on ethanol.

### 1314

#### SERUM AND URINE SPHINGANINE ARE USEFUL BIOMARKERS OF FUMONISIN B<sub>1</sub> EXPOSURE IN SWINE DUE TO SLOW SYSTEMIC CLEARANCE.

G. Tyagi<sup>1</sup>, S. Hsiao<sup>1</sup>, J. L. Marlatt<sup>2</sup>, M. E. Tumbleson<sup>3</sup>, P. D. Constable<sup>2</sup>, R. M. Eppley<sup>4</sup> and W. M. Haschek<sup>1</sup>. <sup>1</sup>*Veterinary Pathobiology, University of Illinois at Urbana-Champaign, Urbana, IL*, <sup>2</sup>*Veterinary Clinical Medicine, University of Illinois at Urbana-Champaign, Urbana, IL*, <sup>3</sup>*Agricultural and Biological Engineering, University of Illinois at Urbana-Champaign, Urbana, IL* and <sup>4</sup>*CFSAN, USFDA, Laurel, MD*.

We have shown that fumonisin B<sub>1</sub> (FB1) toxicity in swine manifests as cardiogenic pulmonary edema. The mechanism of FB1 toxicity is believed to be inhibition of sphingolipid metabolism, resulting in altered sphinganine (Sa) and sphingosine (So) concentrations. In swine, alterations in Sa and So are found in serum and urine, as well as tissues such as heart, liver, lung and kidney. This study characterized the clearance of Sa and So from blood and urine in pigs in order to determine the usefulness of Sa and So as biomarkers of FB1 exposure. Male castrated pigs (15 kg body wt) were given 1 mg FB1/kg or saline (controls) IV at 0, 24, and 48 hr. Blood and urine were collected from FB1 pigs (n = 4 to 8) and from controls (n = 4) over 144 hr. The serum and urine Sa concentration as well as the serum Sa:So ratio increased in FB1 treated pigs, peaking at 96 hr in serum and 120 hr in urine.

Sa and So concentrations decreased after this time but were still elevated at 144 hr in both serum and urine. The serum and urine So concentrations were also increased, though to a much lesser extent. These results indicate that intracellular sphingolipids continued to be released following termination of FB1 exposure. Therefore, serum and urine Sa and the Sa:So ratio are useful biomarkers of recent FB1 exposure. *Supported by International Life Sciences Institute.*

**1315**

CLINOPATHOLOGY AND TISSUE SPHINGOLIPID CLEARANCE AFTER FUMONISIN B<sub>1</sub> EXPOSURE OF SWINE.

J. L. Marlatt<sup>1</sup>, G. Tyagi<sup>2</sup>, S. Hsiao<sup>2</sup>, M. E. Tumbleson<sup>3</sup>, R. M. Eppley<sup>4</sup>, P. D. Constable<sup>1</sup> and W. M. Haschek<sup>2</sup>. <sup>1</sup>*Veterinary Clinical Medicine, University of Illinois at Urbana-Champaign, Urbana, IL*, <sup>2</sup>*Veterinary Pathobiology, University of Illinois at Urbana-Champaign, Urbana, IL*, <sup>3</sup>*Agricultural and Biological Engineering, University of Illinois at Urbana-Champaign, Urbana, IL* and <sup>4</sup>*CFSAN, USFDA, Laurel, MD.*

Fumonisin B<sub>1</sub> (FB1) inhibits sphingolipid metabolism which is believed to be the mechanism of FB1 toxicity. In pigs, we have shown that FB1 causes cardiotoxicity that results in pulmonary edema and increases sphinganine (Sa) and, often, sphingosine (So) concentrations in serum and urine, as well as heart, liver, lung and kidney. Therefore, Sa and So are considered biomarkers of FB1 exposure. This study characterized the clinopathology and tissue sphingolipid clearance in pigs after FB1 exposure to determine the usefulness of tissue Sa and So as biomarkers. Male castrated pigs (15 kg body wt) were given 1 mg FB1/kg or saline (controls) IV at 0, 24, and 48 hr. FB1 pigs were euthanized at 72 (Group A, n = 4) or 144 hr (Group B n = 4), and controls (Group C, n = 4) at 144 hr. Liver specific serum biochemistry and urinalysis (creatinine, protein and specific gravity) were determined at 0, 72 and 144 hr, and were not significantly different from controls though the values in the FB1 groups were often higher. Two FB1 treated pigs developed respiratory distress with one dying due to pulmonary edema. The Sa and So concentrations and Sa:So ratio in liver, kidney, lung and heart from Groups A and B pigs were significantly increased compared to controls ( $p < 0.05$ ), but there was no difference in values in treated pigs at 72 and 144 hr. These results suggest that accumulated intracellular sphingolipids do not rapidly leave the cell and/or that sphingolipid metabolism continues to be inhibited after cessation of FB1 treatment. Therefore, tissue Sa and So concentration are a useful biomarker for recent FB1 exposure. *Supported by International Life Sciences Institute.*

**1316**

SENSITIVE SCREENING BIOASSAY FOR DEOXYNIVALENOL DETECTION IN FOOD SAMPLES.

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A bioassay was used to screen for deoxynivalenol (DON) in the range of common contamination in a variety of food samples. Six corn and five wheat foods were acquired from local commercial sources and one naturally contaminated wheat (research) sample was tested. Parallel samples were prepared from extraction of 25 g food samples with acetonitrile: water (84:16), cleanup with an alumina: charcoal SPE column, and evaporation under nitrogen over warm water. One aliquot of each sample was dissolved in complete RPMI-1640 media supplemented with 0.03% ethanol and 1.7% DMSO. Samples were plated to 96-well low-evaporation plates in triplicate and serially diluted 1:1. DON standard controls and blanks were included on each plate. Human K-562 erythroleukemia cells were added at a density of  $2.5 \times 10^4$  cells/well. Plates were incubated for 48 hours at 37° C, 5% CO<sub>2</sub>. Cell proliferation was assessed by the MTS dye reduction assay. A second aliquot of each sample was dissolved in 20% aqueous methanol for HPLC/UV analysis of DON. 3-Acetyl DON and 15-Acetyl DON were not detected in any samples. The experiment was replicated three times. DON was detected in one corn and two wheat samples by both methods. The positive corn sample had DON levels of 368 and 464 ng/g food; the commercial positive wheat sample had 255 and 152 ng/g food; the research wheat sample had DON levels 3893 and 2735 ng/g for HPLC and bioassay methods respectively. The IC50 for DON was 389 ng/ml media and samples negative for DON by HPLC had bioassay responses as percent of controls  $\geq 85\%$ . The correlation between the two methods was 0.87 for the DON-contaminated samples. This sensitive bioassay detected DON in a variety of food matrixes within the range of contamination that could be present in the human food supply.

**1317**

INTEGRATION OF FDA DATABASES TO DEVELOP METHODOLOGIES FOR PREDICTIVE TOXICOLOGY.

M. Cheeseman and M. Twaroski. *CFSAN/OFAS, USFDA, College Park, MD.*

Chemically induced toxicity continues to be one of the major concerns of pharmaceutical, agricultural, and other chemical industries. Toxicology disciplines relevant in these industries are extremely diverse, involving mammalian, environmental, and

ecological systems. Many of these endpoints required by regulatory agencies at FDA and EPA involve costly and lengthy animal testing. These long term studies may, for example, utilize as many as 160 rodents or 32 dogs. The valuable information can be used to: 1) build predictive models based on structure activity relationships; 2) reduce the time required for evaluations; 3) reduce the number of animal studies. In this regard, USFDA has been actively collaborating with the public toxicity database standardization effort, ToxML, to create a set of controlled vocabulary to represent toxicity data. Using the templates based on RedBook, groups in FDA initiated construction of endpoint-specific databases according to the ToxML standard. These databases will greatly assist our reviewers in their evaluations. In addition, non-proprietary portions will be made available so that high quality data from FDA can contribute to the success of predictive toxicology.

**1318**

MATERNAL EXPOSURE TO GENISTEIN DURING PREGNANCY SUPPRESSIVELY EFFECTS ON TUMOR INITIATION INDUCED BY 7, 12-DIMETHYLBENZ[A]ANTHRACENE IN STEROIDOGENIC ORGANS OF F1 OFFSPRING.

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Recently, much attention has been focused on the protective effects of isoflavones against tumor. However, little is known about an indirect effect of isoflavones on the F1 offspring via maternal exposure during pregnancy. In this study, we examined whether maternal exposure to genistein, a soy isoflavone, during pregnancy was effective to prevent tumor initiation induced by 7, 12-dimethylbenz[a]anthracene (DMBA) on the reproductive organs of the F1 offspring. Twenty-five pregnant ICR mice were used. Genistein dissolved in corn oil (0.1, 1 and 10 mg/kg/day) was subcutaneously injected every day from gestation day 14 to day of birth. On 8 weeks old of the F1 offspring, DMBA (100 mg/kg, i.p.) was injected and after 16 hours of the injection, testis, prostate, ovary and uterus were removed from the F1 offspring, and subjected to single-cell gel electrophoresis assay to examine indirect suppressive effect of genistein on the tumor initiation induced by DMBA. There was no significant difference in the organ weight between control and genistein-treated groups. DNA damage was clearly shown in all of the reproductive organs in non-genistein treated group. In genistein treated group, no suppressive effect for DNA damage was found in prostate and uterus, however, significant suppressive effect was observed in testis and ovary. Generation of fetal reproductive organs has been known to begin gestation day 14 in mice, suggesting a possibility that fatal testis and ovary, which are steroidogenic organs, are affected by genistein under maternal exposure in this study. Since cytochrome P450 (CYP) 1B1 is constitutively expressed in steroidogenic organs and induced by polycyclic aromatic hydrocarbons such as DMBA, induction of CYP1B1 may be suppressed by genistein. To elucidate the underlying mechanism, we further examined induction of CYP1B1 mRNA by semi-quantitative RT-PCR and quantified serum estrogen level. We discuss this possibility based on these results.

**1319**

EFFECTS OF N-6 AND N-3 POLYUNSATURATED FATTY ACIDS ON COLORECTAL CARCINOGENESIS.

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*In vivo* studies have demonstrated that high fat fish oil (HFFO) diets with high levels of n-3 polyunsaturated fatty acids (PUFAs) can inhibit the formation of chemically-induced colon tumors compared to high fat corn oil (HFCO) diets which are rich in n-6 PUFAs. Our studies show that HFFO diets also protect against the initiation of aberrant crypt foci in F344 rats compared to HFCO diets. Furthermore eicosapentaenoic acid (EPA) also inhibited the proliferation of human colon adenocarcinoma Caco-2 cells compared to linoleic acid (LA). The mechanism responsible for the inhibitory effects of n-3 PUFAs on colorectal tumors may partly be related to inhibition of PGE<sub>2</sub> synthesis from arachidonic acid (AA). Plasma levels of PGE<sub>2</sub> were indeed lower in HFFO fed rats compared to HFCO fed rats. However, reductions in PGE<sub>2</sub> synthesis by EPA compared to AA in Caco-2 cells did not lead to differential effects on cell proliferation, which suggests that PGE<sub>2</sub> is not directly involved in regulation of cell proliferation in colon cancer cells by n-6 and n-3 PUFAs. Our results suggest that lipid peroxidation-induced oxidative stress might be an important mechanism by which n-3 PUFAs possess anticarcinogenic effects. This is supported by the fact that HFFO diets with a high amount of EPA increased the amount of lipid peroxidation in F344 rats compared to HFCO diets with a high amount of LA. Levels of malondialdehyde, which is an end product of lipid peroxidation were also increased after incubation of Caco-2 cells with EPA. Furthermore, transcription of genes involved in oxidative stress is increased in HFFO fed rats, whereas addition of antioxidants diminishes the anticancer effects of n-3 PUFAs in Caco-2 cells, which also suggests that oxidation of n-3 PUFAs underlies their anticancer effects. Overall, the results might imply that n-3 PUFAs

protect against colon carcinogenesis via increased oxidative stress that ultimately leads to inhibition of cell proliferation. Diets enriched with high levels of n-3 PUFAs may thus have beneficial colon cancer inhibiting effects, which may be reduced by high levels of dietary antioxidants

## 1320 BIOMARKERS OF OXIDATIVE STRESS: NEW PROSPECTS AND APPROACHES.

**D. C. Liebler.** *Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN.*

Oxidative stress plays a key role in the development of cardiovascular disease, neurodegenerative diseases, and cancer. Many toxic chemicals associated with increased risk of these diseases also induce oxidative stress. Assessment of oxidative stress in clinical studies has been hampered by a dearth of robust, sensitive, and well-validated biomarkers. This symposium will address new opportunities to develop oxidative stress biomarkers for application to human clinical studies. Dr. Maria Kadiiska (NIEHS) will discuss the Biomarkers of Oxidative Stress Study (BOSS), which evaluated several different noninvasive or minimally invasive chemical markers of carbon tetrachloride- or ozone-induced oxidative stress in rats. Dr. Stanley Hazen (Cleveland Clinic Foundation) will describe the development and validation of multiplexed LC-MS-MS assays for specific lipid and protein oxidation products in human serum and the application of these markers to assess oxidative stress endpoints as risk factors for cardiovascular disease. Dr. Ian Blair (University of Pennsylvania) will present recent developments in the identification and LC-MS-MS-based analysis of products of oxidative DNA damage and DNA adducts derived from electrophilic lipid oxidation products. Dr. Lawrence Marnett (Vanderbilt University) will discuss recent findings that DNA adducts derived from electrophilic products of lipid oxidation undergo metabolism to a series of products that can be analyzed as potential urinary biomarkers of oxidative stress. Dr. Daniel Liebler (Vanderbilt University) will discuss the identification of albumin adducts formed by reactive products of lipid oxidation and the potential application of these adducts as serum biomarkers of oxidative stress *in vivo*.

## 1321 PRODUCTS OF OXIDATION AS MEASURABLE INDICATORS OF OXIDATIVE STRESS: VALIDATION OF BIOMARKERS FROM RODENT CCL4 AND OZONE EXPOSURE.

**M. Kadiiska.** *Environmental Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, NC.* Sponsor: D. Liebler.

Oxidation products of lipids, proteins and DNA in plasma and urine of rats were measured as part of a comprehensive, multilaboratory validation study searching for non-invasive biomarkers of oxidative stress. The goal of the study was to find the most sensitive, selective and specific markers of oxidative stress that are applicable to different oxidative insults and stored specimens. Investigators from 24 labs worldwide are participating in this study. The focus of this presentation will be on the findings from measurement of oxidative stress in experimental animal models of CCl<sub>4</sub> poisoning and ozone exposure. The time and dose-dependent effects of CCl<sub>4</sub> and ozone exposure on concentrations of lipid hydroperoxides, TBARS, malondialdehyde (MDA) and isoprostanes were investigated with different techniques. In addition, measures of oxidation products of proteins (protein carbonyls, methionine sulfoxidation, tyrosine oxidation products) and DNA (strand breaks, 8-OHdG, M1G) were carried out as well. The pattern of oxidative stress biomarkers seen in these two exposures will offer insight into the specificity and sensitivity of the markers and will provide evidence that a given product of oxidation may be a marker for some type of oxidative stress but not others.

## 1322 MOLECULAR MECHANISMS OF OXIDANT STRESS IN AT-RISK SUBJECTS UNDERGOING REVASCULARIZATION.

**S. Hazen.** *Department of Molecular Medicine, Cleveland Clinic Foundation, Cleveland, OH.* Sponsor: D. Liebler.

Our group has successfully developed and applied LC/MS/MS assays that permit multiplexed stable isotope dilution quantitative analyses of multiple molecular markers of distinct protein and lipid oxidation products in clinical specimens. This approach permits comprehensive metabolic profiling of clinical and biological specimens, and is equally applicable to clinical and Mechanism-based investigations. For example, in recent clinical studies we have shown that nitrotyrosine, a protein oxidation product of nitric oxide (NO)-generated oxidants, predicts atherosclerotic risks and burden better than current diagnostic laboratory tests employed in clinical practice (Shishehbor et al, (2003) JAMA). Through simultaneous analyses of ni-

trotyrosine and other specific oxidation products of distinct oxidant generating pathways, such as chlorotyrosine for myeloperoxidase (MPO)-catalyzed oxidation, dityrosine for tyrosyl radical-mediated oxidative crosslinking, and ortho-tyrosine for hydroxyl radical-like oxidant damage to proteins, complementary studies have demonstrated the potential clinical utility of select oxidative stress biomarkers as targets for therapy and response to lipid lowering (statins) (Shishehbor et al, (2003) Circulation).

## 1323 NOVEL BIOMARKERS OF OXIDATIVE STRESS-INDUCED DNA DAMAGE.

**I. Blair.** *Department of Pharmacology, University of Pennsylvania, Philadelphia, PA.* Sponsor: D. Liebler.

A major consequence of oxidative stress is increased lipid peroxidation resulting from reactive oxygen species or from the action of oxygenases such as cyclooxygenase or lipoxygenase. Depletion of intracellular reducing equivalents during oxidative stress provides the opportunity for lipid hydroperoxides to undergo homolytic decomposition to a series of bifunctional aldehydic electrophiles. For example, we have shown that omega-6 polyunsaturated fatty acids can decompose to 4-hydroperoxy-2-nonenal, 4-oxo-2-nonenal, 4-hydroxy-2-nonenal, 4, 5-epoxy-2-decenal, and 9, 11-dodecadienoic acid. Each of these bifunctional electrophiles is able to interact with DNA to induce specific lesions that are potential biomarkers of oxidative stress. We have recently identified carboxylate-containing DNA-adducts as specific biomarkers for up-regulation of the cyclooxygenase (COX) or lipoxygenase (LOX) pathways. This presentation will describe the application of stable isotope dilution LC-MS-MS to develop sensitive assays for specific products of DNA damage through these oxidative stress mechanisms.

## 1324 URINARY DNA ADDUCTS AND THEIR METABOLITES AS BIOMARKERS OF OXIDATIVE STRESS.

**L. Marnett.** *Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN.*

Oxidizing agents, nitrosating agents, or halogenating agents react directly with DNA to form modified bases or induce strand breaks. In addition, aldehyde, ketone, or epoxide products of lipid, protein, or DNA oxidation react with DNA bases to form stable adducts. These adducts may remain in genomic DNA, allowing quantification in tissue extracts, or they may be removed by repair pathways and excreted allowing quantification in urine. Several products of direct or indirect oxidative damage to DNA have been detected in genomic DNA from human subjects, but attempts to develop biomarkers based on these products have met with uneven success. For large-scale biomarker screening, a urine-based assay is ideal. Yet detection of a biomarker in urine requires that it be excreted intact without prior metabolism. Our recent studies demonstrate that the primary adduct formed from the reaction of malondialdehyde with DNA, M1dG, is metabolized prior to excretion in the rat. This finding indicates that in order to adequately evaluate the usefulness of M1dG as a biomarker *in vivo*, both the parent compound and its metabolite(s) must be considered. Furthermore, these results suggest the likelihood that other DNA adducts undergo a similar metabolic fate. This presentation will address both the *in vivo* analysis of urinary M1dG and the potential metabolism of other DNA adducts as biomarkers for oxidative stress-induced DNA damage.

## 1325 PROTEIN ADDUCTS AS OXIDATIVE STRESS BIOMARKERS.

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Reactive oxidants modify essentially all biomolecules and nonradical electrophiles produced as byproducts of lipid oxidation also react with proteins and DNA. In contrast to DNA modifications, relatively little is known about protein modifications by reactive endogenous electrophiles associated with oxidative stress. Several examples of protein modifications by electrophilic products of lipid oxidation are known (e.g., modification of apolipoproteinB-100 (ApoB-100) in LDL) and are considered critical steps in disease pathogenesis. However, the reactions of many electrophilic products of lipid peroxidation with proteins have not been explored. New proteomics approaches based on tandem mass spectrometry (MS-MS) now enable the high-throughput characterization of protein adducts, mapping of modification sites and quantitation of adducts. We have characterized adducts formed by reactive electrophilic products of lipid oxidation with human serum albumin and

other proteins and have mapped the adducts at the level of amino acid sequence. This presentation will describe the identification of oxidative stress-derived protein modifications and their application as biomarkers of oxidative stress *in vivo*.

#### **1326** GENETIC BASIS FOR SUSCEPTIBILITY TO METALS: METABOLISM AND MOLECULAR TARGETS.

**E. K. Silbergeld.** *Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD.*

There are substantial individual differences in human susceptibilities to many toxicants, including metals. Understanding these differences in susceptibility is fundamental to identifying factors important in assessing and managing health risks associated with metals such as lead, cadmium, mercury, and arsenic. Genetic factors may play a significant role in the variable human response to metal exposures. The goal of this symposium is to present and discuss information from both epidemiology and basic research that is relevant to refining our knowledge of metal toxicity and the relationships between metal exposures and human diseases.

#### **1327** DO GENETIC POLYMORPHISMS MODIFY THE KINETICS OR TOXICITY OF LEAD? INSIGHTS FROM EPIDEMIOLOGY.

**B. S. Schwartz.** *Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD.*

Lead is widely distributed in the body. With its affinity for sulfhydryl and carboxyl groups, it binds to proteins in blood and tissues, including many polymorphic gene products. We evaluated interactions between lead dose and 7 polymorphic genes in 3 large scale epidemiologic studies involving 3, 186 subjects. In studies of former organolead workers (N=1, 109), current and former Korean lead workers (N=937), and Baltimore residents (N=1, 140), we measured lead in blood by AAS and in bone by <sup>109</sup>Cd K-shell X-ray fluorescence. Genotyping was completed for 9 SNPs including those in  $\delta$ -aminolevulinic acid dehydratase (ALAD; G177C), apolipoprotein E (ApoE; C393T and C407T), and vitamin D receptor (VDR; C47T [BsmI] and T1202C [FokI]). Health outcomes included neurobehavioral assessment and blood pressure. Linear regression was used to examine associations of genotypes with dose biomarkers and health outcomes, and interactions of lead dose and genotypes in associations with health outcomes. ALAD genotype was consistently associated with blood lead but not with bone lead in the Korea study. VDR-BsmI genotype was associated with blood lead, DMSA-lead, and tibia lead in the organolead and Korea studies. This genotype was also associated with blood pressure and cognitive function, and modified relations of blood lead with cognitive function in Baltimore residents. ApoE genotype was associated with tibia lead and modified its relations with cognitive function in the organolead study. Our results demonstrate that genetic polymorphisms, especially for ALAD and VDR, modify the kinetics of lead, as evidenced by differences in lead biomarkers by genotype. In contrast, the polymorphisms were inconsistently found to modify relations of lead dose with health outcomes. By comparing and contrasting associations of genotypes with lead dose measures, and of the lead-gene interactions in their associations with health effects, hypotheses can be generated regarding the mechanistic role of the gene products, that is, whether these modify the toxicokinetics or toxicodynamics of lead.

#### **1328** USE OF GENETICALLY ALTERED ANIMALS TO DETERMINE THE ROLE OF CADMIUM IN METALLOTHIONEIN TOXICITY.

**C. D. Klaassen.** *Pharmacology, U Kansas Med. Ctr, Kansas City, KS.*

The role of metallothionein (MT) in Cd toxicology has been studied using MT-I transgenic (MT-TG) and MT-I and II knock-out (MT-null) mice. We have shown that (1) MT-TG and MT-null mice appear to be normal except for altered tissue MT levels; (2) MT does not appear to affect Cd absorption from the gastrointestinal tract, and tissue Cd distribution, but MT plays an important role in the elimination and tissue retention of Cd; (3) MT protects against acute inorganic Cd-induced lethality and hepatotoxicity, and the mechanism of protection appears to be sequestration of Cd in the cytosol, thus reducing the amount of Cd in critical organelles; (4) MT modulates Cd-induced expression of protooncogene (c-jun) and tumor suppressor gene (p53) in mouse liver; (5) MT protects against Cd-induced nephrotoxicity, but does not appear to be a major factor in modulating acute CdMT-induced renal injury and Zn-induced protection; (6) MT protects against chronic Cd-induced hematotoxicity, ameliorates Cd-induced elevation of serum TNF- $\alpha$  and IL-1 $\alpha$  levels, and splenomegaly; (7) MT protects against chronic Cd-induced hepatotoxicity, as evidenced by decreased hepatic inflammation, apoptosis,

and granulomas; and (8) MT-null mice are more susceptible than controls to chronic Cd osteotoxicity. These studies using MT-TG and MT-null mice have been excellent tools in advancing our understanding of the role of MT in Cd toxicology, and suggest that MT plays a major role in protection from Cd toxicity.

#### **1329** ARSENIC METABOLISM: KNOCKOUT MICE AND POLYMORPHISM STUDIES.

**H. V. Aposhian, R. A. Zakharyan, U. K. Chowdhury, M. D. Avram, M. L. Wollenberg, A. Hernandez and M. M. Aposhian.** *Molecular and Cellular Biology, University of Arizona, Tucson, AZ.*

There are at present questions about how inorganic arsenic metabolism is regulated and why is there variability in the metabolic processing of inorganic arsenic in humans as judged by the concentration of arsenic species excreted in the urine. A critical enzyme in this process is MMA(V) reductase, which reduces arsenate, MMA(V), and DMA(V) to more toxic metabolites. The enzyme has an absolute requirement for GSH. The MMA(V) reductase protein is identical to the most recently discovered glutathione S-transferase omega. Our laboratory has given sodium arsenite to MMA(V) reductase knockout mice and to wild mice. Although exon 3 of the MMA(V) reductase gene has been removed, the livers of wild mice still have 20% of the MMA(V) reductase activity of the knockout mice indicating that another protein may have some supplemental MMA(V) reductase activity. No such studies are available for arsenic methyltransferases. The genetics of these mammalian enzymes is lacking. Polymorphism studies are becoming available to help to decipher and correlate changes in the genes responsible for arsenic metabolism and urinary arsenic species. These studies also will be reviewed. (Supported by the Superfund Basic Research Program NIEHS Grant No. ES-04940 from the National Institute of Environmental Health Sciences).

#### **1330** MERCURY-INDUCED AUTOIMMUNITY IN GENETICALLY SUSCEPTIBLE MICE.

**M. Monestier.** *Microbiology & Immunology, Temple University School of Medicine, Philadelphia, PA.* Sponsor: E. Silbergeld.

The incidence of human autoimmune diseases is increased following exposure to various chemical or pollutants. Only a few animal models are available to help us understand the mechanisms of chemically-induced autoimmunity. In susceptible mice, subtoxic doses of mercury elicit a complex autoimmune syndrome with production of highly specific IgG antinucleolar antibodies, a polyclonal increase in serum IgG1 and IgE, and tissue lesions that include a glomerulonephritis and vasculitis. Susceptibility to mercury is conferred by various genetic factors. The production of autoantibodies is under the control of major histocompatibility complex class II molecules (with H2<sup>s</sup> mice being especially susceptible), whereas undetermined polygenic factors control the polyclonal response to mercury. My laboratory has focused on the mechanisms, such as cytokine production and costimulatory molecules expression, which regulate mercury-induced autoimmunity. Our most recent work indicates that resting dendritic cells and CD25+CD4+ regulatory T cells cooperate in maintaining tolerance to mercury. If dendritic cells are activated or if regulatory T cells are depleted, tolerance to mercury is lost even in mice that are normally resistant. Because dendritic cells are best activated by microbial components, these experiments indicate that infectious agents and chemical challenges can conspire to trigger autoimmune manifestations in previously tolerant individuals.

#### **1331** MOLECULAR MECHANISMS OF LEAD-INDUCED CARDIOVASCULAR DISEASES.

**E. K. Silbergeld.** *Environmental Health Sciences, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD.*

There is compelling epidemiological evidence associating lead exposures with increased risks of cardiovascular diseases, including hypertension, stroke, and peripheral arterial disease. We have reported that relatively small increases in blood lead [PbB], on the order of 2 $\mu$ g/dL, increases risks of both prevalent disease and mortality in adult men and women. For peripheral arterial disease, odds of disease were increased 3-5 fold as PbB increased from 1.4 to 2.8  $\mu$ g/dL. These effects are observed at Pb exposures in the range of 1-5  $\mu$ g/dL PbB, well below those associated with altered renal function. The epidemiological studies indicate no biological interactions between Pb and serum cholesterol or calcium. To understand mechanisms by which Pb affects the cardiovascular system, we have examined possible interactions between Pb and polymorphisms in genes that encode proteins known to be involved in both lead metabolism and regulation of vascular function. These candidate genes include endothelial nitric oxide synthase (eNOS), superoxide dismutase (SOD), vitamin D receptor (VDR), and aminolevulinic acid dehydratase (ALAD). These studies utilized a population of lead workers in Korea, who have been followed from 1997 to 2002 under protocols approved by Johns Hopkins, University of Maryland, and the Soonchunhyang University School of Medicine. There was a

significant relationship between both blood Pb and bone Pb levels and increased systolic blood pressure. The G<sup>894</sup>-T<sup>894</sup> polymorphism in eNOS was not associated with either blood pressure or an effect modification of the Pb:blood pressure relationship. However, this eNOS polymorphism did modify the distribution of Pb into bone. These results indicate that gene:environment interactions may be highly complex, involving both toxicokinetics and potential target proteins.

### 1332 PESTICIDE NEUROTOXICITY AT THE INTERSECTION OF EPIDEMIOLOGY AND TOXICOLOGY.

W. K. Boyes<sup>1</sup> and F. Kamel<sup>2</sup>. <sup>1</sup>EPA, Research Triangle Park, NC and <sup>2</sup>NIEHS, Research Triangle Park, NC.

Neurotoxicity of high-level pesticide exposure in adults is well-documented, but less is known about the consequences of chronic exposure to moderate dose levels. Results from human epidemiology studies and animal toxicology studies are not always consistent. Recent human studies suggest that moderate pesticide exposure may be associated with increased risk of an array of neurologic symptoms, particularly affective outcomes including depression and suicide. Laboratory animal studies do not address comparable outcomes, and typically have not identified subtle neurological impairments that persist beyond the exposure period, despite higher dose levels than are typically experienced by humans. Are these apparent species differences real? If so, are they related to pharmacokinetic or pharmacodynamic factors? Alternatively, are differences related to the types of outcomes assessed, duration of exposures, combined exposures to multiple agents, or to other factors? Understanding and predicting human health risks from pesticide exposure requires complex integration of the differing data provided by human and animal studies.

### 1333 NEUROTOXICITY OF CHRONIC PESTICIDE EXPOSURE IN LICENSED PESTICIDE APPLICATORS IN THE AGRICULTURAL HEALTH STUDY (AHS).

F. Kamel<sup>1</sup>, L. S. Engel<sup>2</sup>, B. C. Gladen<sup>1</sup>, J. A. Hoppin<sup>1</sup>, M. R. Alavanja<sup>3</sup> and D. P. Sandler<sup>1</sup>. <sup>1</sup>NIEHS, NIH, DHHS, Research Triangle Park, NC, <sup>2</sup>Memorial Sloan-Kettering Cancer Center, New York, NY and <sup>3</sup>NCI, NIH, DHHS, Rockville, MD. Sponsor: W. Boyes.

Little is known about the neurological consequences of chronic exposure to moderate pesticide levels in the absence of poisoning. We explored this issue in a cross-sectional analysis of data from the AHS, a cohort of licensed pesticide applicators enrolled in 1993-97 in Iowa and North Carolina. At enrollment, ~19,000 applicators reported lifetime pesticide exposure and frequency of 23 neurologic symptoms reflecting cognitive, motor, sensory and nonspecific dysfunction experienced during the prior year. A summary measure indicating greater symptom number (10+ vs <10 symptoms) was associated with lifetime use of any insecticide, showing a significant dose-response trend; adjusted relative risk (RR, 95% confidence interval) for the highest exposure category was 2.5 (2.1-3.0). Weaker relationships were seen for other pesticides - in the highest exposure category, RR was 1.4 (1.2-1.7) for fumigants, 1.3 (1.0-1.7) for herbicides, and 1.2 (1.1-1.4) for fungicides. Among chemical classes of insecticides, the strongest relationships were for organophosphates [RR=2.2 (1.9-2.6)] and organochlorines [RR=2.0 (1.8-2.2)]. Events involving high pesticide exposure were associated with greater symptom number [RR=3.0 (2.7-3.5)], as were physician diagnoses of pesticide poisoning [RR=2.5 (2.0-3.1)]. Increased symptom number was associated with insecticide use even when applicators who had experienced a pesticide poisoning or high-exposure event were excluded from the analysis. Insecticide and fumigant use were also associated with increased prevalence of individual symptoms as well as groups of symptoms defined a priori and reflecting affect, cognition, systemic effects, peripheral neuropathy, motor dysfunction, or vision. These results suggest that, even in the absence of pesticide poisoning, an increase in self-reported neurologic symptoms is associated with lifetime use of pesticides, particularly organophosphate and organochlorine insecticides.

### 1334 ORGANOPHOSPHATE EXPOSURE, DEPRESSION AND SUICIDE: MATCHING EPIDEMIOLOGICAL DATA TO MODELS BASED ON ANIMAL STUDIES AND CASE SERIES.

L. London<sup>1</sup>, A. Flisher<sup>2</sup>, V. Major<sup>3</sup>, H. Kromhout<sup>4</sup> and D. Mergler<sup>5</sup>. <sup>1</sup>School of Public Health and Family Medicine, University of Cape Town, Observatory, South Africa, <sup>2</sup>Department of Psychiatry and Mental Health, University of Cape Town, Observatory, South Africa, <sup>3</sup>Department of Health Sciences, Peninsula Technicon, Cape Town, South Africa, <sup>4</sup>Institute for Risk Assessment Sciences, Utrecht University, Utrecht, Netherlands and <sup>5</sup>CINBIOSE, University of Quebec a Montreal, Montreal, QC, Canada. Sponsor: W. Boyes.

The role of organophosphates as agents used in suicide in the developing world is well-documented. However, the potential role of exposure to OPs as a causal factor in suicide has not been explored to date. Animal data have identified the impact of

OP exposure on serotonin, which is known to be an important neurotransmitter implicated in depression amongst humans, and in increasing levels of impulsivity, itself a significant factor in suicide attempts. Ecological studies and case series data provide additional circumstantial evidence to support the hypothesis that humans exposed to OPs may be at increased risk for affective disorders and suicide, although evidence from epidemiological studies is less consistent. Data will be presented from a cross-sectional study conducted in 2002 amongst 813 farm workers in the rural Western Cape Province of South Africa, which examined a range of standardised neuropsychological and psychiatric outcomes in relation to a history of OP exposure. These included the General Health Questionnaire (GHQ), the Beck Depression Inventory (BDI), the Brief Symptom Inventory (BSI), the Refined 12 - Item Four-Factor Measurement Model of the Aggression Questionnaire, the Barrat Impulsiveness Scale, and suicidal ideation. Preliminary analyses indicate important differences in affective outcomes by gender, farm type and spray activities. These differences will be elaborated in presentation, outlining their relevance to better understanding the potential hazards associated with organophosphate pesticides.

### 1335 ANIMAL MODELS OF CHRONIC PESTICIDE TOXICITY.

V. C. Moser, P. J. Bushnell, R. C. MacPhail, D. W. Herr, R. S. Marshall, D. L. Hunter and W. K. Boyes. NTD/NHEERL/ORD, USEPA, Research Triangle Park, NC.

Despite the wealth of literature on acute neurotoxicological effects of organophosphorus (OP) pesticides in laboratory animals, there are relatively few reports of long-term exposure. Reports in the literature describing  $\pm$ chronic $\pm$  exposures to pesticides are, in fact, as short as 7 days and rarely as long as 4 months. Furthermore, routes of administration range from subcutaneous to dietary. Doses used in many of the studies produce signs of acute toxicity and/or significant brain or blood cholinesterase (ChE) inhibition. In contrast, human exposures are often prolonged and without obvious toxic effects. In rats, few neurobehavioral effects have been reported following exposures of 3-4 month duration. Decreased motor activity has been reported occasionally, and one study reported a transient improvement in learning at low doses. Shorter-term exposures, however, appear to produce various neuromotor and cognitive effects; some of these effects were observed at doses producing considerable ChE inhibition. On the other hand, subcutaneous exposure to a few OPs produced cognitive changes even after recovery of enzyme activity. The comparability of these findings to human exposures is limited due to the dose, length, and route of exposures. Data will be presented from a 1-year study of chlorpyrifos, given via the feed with and without intermittent high-dose challenges. Chronic chlorpyrifos produced some neurobehavioral changes, altered the rats $\pm$  response to acute challenges, and caused a mild but persistent cognitive deficit. These subtle changes in behavior may correspond to the relatively vague changes in psychomotor and cognitive function reported in humans. Overall, however, the generally available data are insufficient to assess the ability of chronic animal studies to predict the outcomes in OP-exposed humans. This is an abstract of a proposed presentation and does not reflect EPA policy.

### 1336 DEVELOPMENT OF PHYSIOLOGICALLY BASED PHARMACOKINETIC AND PHARMACODYNAMIC MODELS TO DETERMINE DOSIMETRY, DYNAMIC RESPONSE, AND TO ASSESS RISK FOLLOWING EXPOSURE TO ORGANOPHOSPHORUS INSECTICIDES.

C. Timchalk and T. S. Poet. Pacific Northwest National Laboratory, Richland, WA.

There is an ongoing need to develop approaches for quantitatively assessing risk associated with exposures to a broad-range of organophosphorus insecticides and to determine the potential implications of these exposures to human health. These insecticides share common metabolic activation/detoxification pathways and a common mechanism of neurotoxicity associated with excessive cholinergic stimulation, due to the inhibition of acetylcholinesterase in nerve tissues. Efforts are underway to develop state-of-the-art physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) modeling approaches to assess dosimetry and biological response following exposure to single organophosphorus insecticides or more complex mixtures. A PBPK/PD model has been developed for the organophosphorus insecticides chlorpyrifos and diazinon utilizing available data from rats and humans. These models have been extended to incorporate age- and polymorphism-dependent changes and have been successfully used to quantitatively determine the dose-dependent impact of insecticide exposure. Secondly, a binary PBPK/PD model has been developed to quantitatively assess dosimetry and response due to mixed exposures. This binary model facilitates understanding the mixture interactions and the potential for additivity, synergism or antagonism from multiple insecticide exposures. These results demonstrate that PBPK/PD modeling can be used to

quantitatively assess exposure and biological response to classes of insecticides involving multiple routes of exposure (i.e. skin, ingestion, inhalation) that are routinely encountered in the environment. In summary, it is envisioned that once fully validated, these modeling approaches will be very useful for assessing exposure and health risk as a component of a broader biomonitoring or epidemiology assessment strategy for a wide range of potentially exposed individuals. Supported by: CDC/NIOSH 1 R01 OH03629-01A2; EPA-STAR R828608.

 **1337** PESTICIDE NEUROTOXICITY IN ADULTS: IMPLICATIONS FOR PESTICIDE SAFETY TESTING AND PUBLIC HEALTH.

W. K. Boyes, *EPA, Research Triangle Park, NC.*

Pesticide safety evaluations depend on the best available scientific information, including data from animal toxicity and human epidemiological studies. Several recent epidemiological studies indicate that chronic pesticide exposure increases the risk of neurologic symptoms and affective disorders. Animal studies, in contrast, do not investigate comparable outcomes and generally have not demonstrated analogous chronic impairments. There are several factors to consider when evaluating the comparability of epidemiological and animal neurotoxicity studies. Regarding the epidemiological literature, issues include co-exposure to other agents, concurrent, chronic or previous exposures, use of appropriate controls, potential confounding factors, methods of exposure assessment, and subjective or objective evaluation of neurological status. In epidemiological studies, it is difficult to attribute health outcomes to specific exposures, and dose levels are difficult to quantify. Animal experiments must be evaluated regarding factors such as dose level and duration, procedures used to assess neurological or behavioral status, and appropriateness of the animal model to human neurotoxicity. Factors which may explain apparent differences between animal and human studies include: animal neurological status is evaluated with different procedures than those used in humans; animal studies may involve shorter exposure durations and higher dose levels; most animal studies evaluate a single pesticide ingredient whereas humans are typically exposed to many agents in product formulations. Since neither type of study can adequately address all issues, an appropriate blend of scientific information is necessary to evaluate potential human risks of chronic pesticide exposure. [This is an abstract of a proposed presentation and does not reflect EPA policy]

 **1338** CURRENT REGULATORY AND SCIENTIFIC VIEWS REGARDING CHEMICAL HAZARDS TO CHILDREN.

D. R. Juberg<sup>1</sup> and D. J. Paustenbach<sup>2</sup>. <sup>1</sup>*Regulatory Laboratories, Dow AgroSciences, Indianapolis, IN* and <sup>2</sup>*ChemRisk, San Francisco, CA.*

The evaluation of the health of children, specifically the estimated risk due to environmental chemical exposures, continues to receive increasing regulatory attention. It has resulted in decision-making that has scientific, policy, and public health impacts. At the center of this discussion is whether children are uniquely susceptible and whether current regulatory approaches are protective of children. This workshop will discuss ongoing initiatives by the EPA and CDC aimed at characterizing children's exposures and evaluating biomonitoring data. Other parameters that are influential in predicting susceptibility, namely, pharmacokinetics and pharmacodynamics, will also be addressed. The use of uncertainty factors in setting environmental criteria and estimating safe doses will be discussed and a case study from the VCCEP program will be presented. A view from the medical community, frequently the first responders to questions and concerns over children's health, will be offered. The scientific questions about whether children are significantly more susceptible to toxicants and the current regulatory response to this concern (e.g., FQPA, testing requirements, basic research, cancer risk assessment guidelines) are the focus of this workshop. An anticipated outcome is the identification of those areas of research that will give the toxicology community the opportunity to be a central figure in properly addressing this important public health question.

 **1339** OVERVIEW OF USEPA RESEARCH ACTIVITIES AIMED AT CHARACTERIZING CHILDREN'S EXPOSURES.

E. A. Cohen Hubal, *USEPA, Research Triangle Park, NC.* Sponsor: D. Juberg.

Given the potential vulnerability of children to the effects of environmental exposures, understanding the relationship between children's health outcomes and environmental exposures is an important research need to reduce uncertainty in risk assessment. Over the past 8 years, significant research activities have been initiated at the USEPA to increase understanding of children's vulnerabilities and to better characterize children's exposures to chemical stressors in the environment. Research efforts include development of models, methods, and data to quantitatively de-

scribe ways that children are exposed to environmental stressors. Current and recently completed studies include large field studies to measure children's exposures to chemicals in their homes and daycare centers as well as targeted studies to better understand the determinants of exposure. CTEPP, a study of 260 preschool-age children, has recently been completed and data are being analyzed to identify important exposure factors and pathways. A longitudinal children's study of 60 infants and toddlers is being implemented over the next couple of years to assess exposures to current-use pesticides, phthalates, and BFRs. As a result of these and other Agency initiatives, important data are being collected and assessment approaches are being developed and used to improve the scientific basis of exposure assessments for children. Despite this significant progress, there are many important gaps associated with how to effectively measure and characterize exposure for health studies and risk assessment. In this presentation, ongoing and recent USEPA initiatives aimed at evaluating children's exposures and health risks will be discussed, including issues associated with characterizing cumulative risks from exposures to multiple environmental stressors. The information and data obtained from these efforts will help identify the most important exposures for children and enable decision to prioritize environmental health related activities. This work has been funded wholly or in part by the USEPA. It has been subjected to Agency review and approved for publication.

 **1340** USING CDC BIOMONITORING DATA FOR ASSESSING CHILDRENS' EXPOSURES TO ENVITONMENTAL CHEMICALS.

L. L. Needham, *NCEH, CDC, Atlanta, GA.* Sponsor: P. Williams.

It has been well documented that children on a kilogram basis are more highly exposed to a variety of environmental chemicals than are adults. In addition, because their body functions are still developing, the impact of these exposures may be greater; thus, they are more susceptible to these exposures than are adults. These early life exposures can not only potentially affect the child near the time of the exposure but in some instances can lead to adverse health outcomes later in life. Thus, it is important to monitor exposures occurring especially *in utero*, infant, toddler, and early childhood life stages. CDC has utilized samples collected from its National Health and Nutrition Examination Survey (NHANES) to estimate background levels for 116 chemicals in the U. S. general population, based on race/ethnicity, gender and age. The biggest limitation on the NHANES was age, specifically the younger ages. For most of the analytes, measurements were made in urine samples only in those aged 6 years and older and in serum samples only in those aged 12 years and older. Exceptions to these age ranges were lead, cadmium, mercury, and cotinine measurements. To obtain more data on exposures occurring early in life, our laboratory has collaborated extensively with other groups to determine levels of environmental chemicals in the younger populations and in some instances we relate these levels to adverse health outcomes. Specific examples will include organophosphorus pesticides, phthalates, and perfluorinated chemicals.

 **1341** EVALUATION OF CHILDHOOD EXPOSURES TO INDUSTRIAL CHEMICALS THROUGH VCCEP.

P. R. Williams, *ChemRisk, Boulder, CO.* Sponsor: D. Paustenbach.

The Voluntary Children's Chemical Evaluation Program (VCCEP) is designed to provide data to enable the public and risk assessors to better understand or evaluate the potential health risks to children associated with certain chemical exposures. Specifically, in response to a request from the USEPA, chemical manufacturers have voluntarily begun to collect and analyze data for 23 different chemicals in the first tier of a pilot of this program. These chemicals were selected for initial evaluation because they have been found to be present as contaminants in either human tissues or fluids (e.g., adipose tissue, blood, breath, breast milk, urine); food and water children may eat and drink; or air children may breathe (including residential or school air). A key question of VCCEP is whether the potential hazards, exposures, and risks to children have been adequately characterized, or whether additional data are needed to evaluate children's unique risks. In this presentation, the tiered testing protocol of VCCEP's pilot program will be discussed, as well as the key findings from the first set of chemicals that have undergone peer consultation. These include: acetone, decabromodiphenylether, methyl ethyl ketone, pentabromodiphenyl ether, octabromodiphenyl ether, and vinylidenechloride. The status of other chemicals currently being evaluated under VCCEP will also be discussed (e.g., benzene, ethylbenzene, xylene, n-dodecane, undecane, decane). Particular focus will be given to the lessons learned and data limitations associated with VCCEP, as well as recommendations for areas of improvement in future evaluations.



### 1342 EVALUATION AND PREDICTION OF DOSIMETRY IN CHILDREN: EMPIRICAL AND MECHANISTIC APPROACHES.

A. M. Jarabek. USEPA Visiting Scientist, CIIT Centers for Health Research, Research Triangle Park, NC.

Susceptibility in children to the adverse health effects of hazardous chemicals is a function of exposure, pharmacokinetics (PK), and pharmacodynamics (PD). This presentation will focus on consideration of the potential susceptibility of various life stages due to differences from adults in PK and PD parameters. Current approaches for dosimetry adjustment and response analysis will be described. These approaches range from empirical descriptions of dose-response at various levels of observation (e.g., at the population versus tissue levels) in different age groups to mechanistically motivated models that attempt to account for mode of action by explicitly incorporating anatomical and physiological determinants of PK and PD on an age-specific basis. Semi-empirical analyses of specific mechanistic parameters (e.g., scaling of metabolic clearance rates) have also been explored to address differences due to life stage. Examples of ingested compounds and inhaled particles or gases will be used to illustrate the range of approaches. Predictions based on empirical versus mechanistic approaches for various dose metrics and response outcomes will be compared, including predictions of lifetime cancer risk constructed with distinct age groups and activity patterns compared to adult daily average simulations. Because of the many challenges in ensuring proper protection of children's health, it is important that the range of approaches for dosimetry and response analysis be described in a context that provides an appreciation of the uncertainty versus accuracy afforded by the empirical versus mechanistic descriptions. Characterization of the context provides a basis for the proper application of data-derived uncertainty factors in health risk assessment and can facilitate an interface with exposure models. The emphasis on empirical versus mechanistic approaches provides a framework that can be used to construct characterization of this context for these critical interfaces. The presentation also readily reveals research needs for various PK and PD parameters.



### 1343 DIFFERENTIAL SENSITIVITY OF CHILDREN AND ADULTS TO CHEMICAL TOXICITY AND THE USE OF UNCERTAINTY FACTORS IN REGULATING RISKS.

G. Charnley<sup>3</sup>, M. L. Dourson<sup>1</sup> and R. Scheuplein<sup>2</sup>. <sup>1</sup>*Toxicology Excellence for Risk Assessment (TERA), Cincinnati, OH*, <sup>2</sup>*Keller and Heckman, Stafford, VA* and <sup>3</sup>*HealthRisk Strategies, Washington, WA*.

We investigated the current uses and level of protectiveness of uncertainty factors in determining safe doses for chemical exposures. We found that when experimental animal studies are available in teenage-to-adult animals but not in younger animals, the division of the lowest teenage-to-adult animal NOAEL by an Uncertainty Factor for Database Deficiency (UFD) of 3 or 10 accounts for between ~92% and 98% of the possible occurrences of lower younger-animal NOAELs. Drawing conclusions about the adequacy of an Uncertainty Factor for within Human Variability (UFH) is more challenging, though, based on specific comparisons for newborns, infants, children, adults and those with severe disease, where the population protected is between 60% and 100%. For example, with severe disease, the population protected is closer to 60%; in larger populations that include sensitive individuals a percentage closer to 100% of the population (including children) is protected by using either a 10-fold uncertainty factor for human variability or by using a 3.16-fold factor each for toxicokinetic and toxicodynamic variability. We conclude that an additional factor to limit environmental chemical exposures is unlikely to provide significantly greater protection to children over 6 months of age when compared to the current method. The same conclusion might not always hold true for children younger than 6 months of age, however, in the absence of adequate developmental or systemic toxicity testing.



### 1344 AN EVALUATION OF ENVIRONMENTAL HEALTH TRENDS AMONG CHILDREN BASED ON MEDICAL AND SCIENTIFIC EVIDENCE.

D. A. Goldstein. A2NE, Monsanto Company, St. Louis, MO. Sponsor: P. Williams.

Identification of high priority childrens environmental health issues currently relies heavily upon recognition of broad trends in childrens health outcomes. Widely held beliefs regarding trends in childrens health, frequently described as epidemics of childhood cancer or autism, are often unsubstantiated by or sometimes at odds with existing scientific evidence. In this presentation, a systematic review of the literature regarding child health trends is discussed in order to identify validated trends in child health. For example, although clear evidence exists for increases in childhood asthma in recent years, only suggestive evidence exists for some other allergic disorders (allergic rhinitis, sinusitis). Changes in population adjusted child

cancer rates indicate small alterations in the frequency of major childhood cancers, possible trends for certain rare tumors, and increases in brain tumor diagnosis rates resulting from improvements in diagnostic technology. There is no convincing evidence available regarding trends in neuro-developmental disorders, including autism, ADHD, and learning disabilities. While data document a declining age of puberty in females, evidence for other changes in reproductive function demonstrates wide temporo-spatial variation, but no clearly defined global trends. Some caution is warranted, however, when relying on trends to identify key areas of environmental contribution to disease. Accurate trend data is contingent upon stable diagnostic criteria and disease recognition over time and place in addition to adequate sampling methodologies. Further, while changes in disease rate over time are unlikely to change for purely genetic conditions, environmental contributions to disease burden do not necessarily result in observable trends in the general population. Broad trends may miss large, but static, contributions to disease, or may ignore susceptible sub-populations or locally important environmental factors.



### 1345 TOXICOLOGIC EVALUATION OF INHALED VACCINES.

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Mucosal and systemic immunity stimulated by aerosolized vaccines have been recognized as effective pathways for preventative immunizations and therapies for pathogens and diseases ranging from flu (e.g., FluMist) to measles (World Health Organization) to asthma. Likewise, in the face of an ever-present risk of aerosol delivery of chemical and biological agents, inhaled vaccines make sense by stimulating immunity at the portal of pathogen/ chemical entry. However, several toxicological hurdles exist for those challenged with developing or regulating vaccines, especially those designed for administration to the respiratory tract. General toxicological assessment as are required for all vaccine subtypes are necessary as well as special considerations including safety pharmacology. Adjuvant type and possible transport to the brain via the olfactory pathway are of concern as well. This symposium will bring together experts in the field of inhaled therapeutics and vaccine development, to give insight into the required and perceived toxicology of aerosolized vaccines.



### 1346 AIRWAY DRUG DELIVERY OPTIONS FOR INHALED BIOLOGICS AND VACCINES.

C. Leach. *Preclinical Development, Lovelace Respiratory Research Institute, Albuquerque, NM*.

There is a growing interest among academic, commercial, and government entities in developing vaccines for delivery to the respiratory tract both to the nasal and pulmonary tissues. Over 50 of the currently approved vaccines target respiratory diseases yet only one product actually utilizes the respiratory epithelia as its initial delivery site. Respiratory delivery offers the obvious advantages of being needle-free and eliciting immunity at the port of entry for the pathogen. Thus there may be additional mucosal immunity in addition to humoral immunity, and at lower total doses. There are many ways of achieving drug delivery to the respiratory tract each of which may offer advantages specific to the respective vaccine. Nebulizers offer the advantage of being readily available and easy to use in clinical settings. However they are expensive, time consuming, not portable and not practical in developing nations. In addition, some vaccines may not stand up to the high shear forces generated by some nebulizers. Metered dose inhalers (MDIs) are inexpensive, portable, and provide a semi-sterile, light and moisture-free environment. However MDIs may not be able to deliver high doses of vaccines where required. Dry powder inhalers are perhaps the best option since technology exists whereby low density, respirable, high drug load particles can be generated for use in simple, inexpensive, and portable devices. Devices and/or particles sizes can be adjusted to provide optimum delivery to either the nasal or deep respiratory tissue. Other technology such as piezoelectric aerosol technology may also prove useful for respiratory tract delivery of vaccines. Because of the novelty of aerosolized vaccine delivery, there will most likely be heavy dependence on knowledge of existing aerosol drug technologies and understanding such as insulin, antibiotics and others to lead developers in the proper direction to provide safe, effective, inexpensive and easy to use inhaled vaccine products.



### 1347 IMMUNOGENICITY AND SAFETY TESTING OF VACCINES: A REGULATORY PERSPECTIVE ON GENERAL REQUIREMENTS AND SPECIAL ISSUES OF THE RESPIRATORY TRACT.

K. L. Hastings. *Office of New Drugs, CDER, USFDA, Rockville, MD*.

Nonclinical safety assessment of inhaled therapeutics is a standard component of drug development. Usually, this involves what are generally referred to as "bridging toxicology" studies to determine the potential adverse effects of drugs that were previously administered by the oral or parenteral route. As part of this assessment,

studies to determine the ability of an inhaled drug to induce respiratory hypersensitivity reactions are usually conducted, although the methodologies used are of questionable utility. Safety assessment of vaccines to be administered by inhalation presents several unique issues. Although it is obvious that an immune response should be induced, distinguishing between pharmacodynamic and adverse immunologic reactions can be very difficult. As with other forms of hypersensitivity, it is likely that individual genetic makeup puts individuals at risk for immunologically-mediated adverse reactions, and it is unlikely that nonclinical methods would be useful for risk assessment. However, inhalation toxicology methods are available which could be useful in hazard identification. Adaptations of these methods to assessing the safety of inhaled vaccine products will be discussed.

 **1348** UNIQUE ISSUES ASSOCIATED WITH TOXICOLOGY ASSESSMENT OF VACCINES FOR BIOWARFARE AGENTS.

R. House. *Dynport Vaccine Company, Frederick, MD.*

Recent geopolitical events have resulted in an increased urgency in the development and licensure of vaccines designed to protect against bioterrorist/biowarfare agents. However, development of such vaccines presents the researcher and regulator with multiple scientific and practical challenges. These challenges can be complicated even further when live vaccines are considered. Some of these challenges include choice of the appropriate animal model(s) for toxicology testing and how they relate to the model(s) used for demonstration of efficacy, potential difficulties in locating facilities to perform the work (primarily related to ability to handle certain biological agents and GLP compliance), and physiochemical and biochemical/microbiological characterization. This presentation will discuss the development strategies associated with preventative vaccines for protection against select agent pathogens, with special emphasis on preclinical considerations for inhaled live vaccines.

 **1349** WHO SPONSORED PRECLINICAL TOXICITY TESTS FOR INHALED MEASLES VACCINE.

M. Papania. *Centers for Disease Control and Prevention, Atlanta, GA.* Sponsor: M. Reed.

The goal of the WHO's Measles Aerosol Project is to license at least one method for respiratory delivery of currently licensed measles vaccines. Preclinical toxicity testing is an important component of the regulatory process for licensing the vaccine/device combination. WHO solicited proposals for the study, which was conducted by the Centre International de Toxicologie in cynomolgus macaques. There were 4 groups of 6 male and 6 female, measles-negative animals per group. A control and study group were used for each of two nebulizers studied. One nebulizer delivered particles  $<5\text{ }\mu\text{m}$  and the other  $>10\text{ }\mu\text{m}$ . The test article was lyophilized measles vaccine with measured potency. The control article was vaccine excipients. Two 5x human doses of vaccine or placebo were given, on day 1 and day 21. Exposure estimates were based on breathing rate, lung volumes and aerosol  $\text{J}$ . Three animals per sex per group were sacrificed on day 7 and 42. Endpoints measured were: A) Vital signs and clinical observations: before, during, and 60 min post aerosol exposure (emphasis on respiratory symptoms). B) Serum chemistry, hematology (with coag), and urinalysis C) Serum serology at 7, 14, 21, 28 and 42 days. D) Daily observations: of health, activities, behavior, food and water consumption, etc with special attention to any neurological effects and to observations of the inside of the nose and mouth for local effects. Ophthalmologic observations were also made. E) Gross pathology, lung lavage, organ weight, ophthalmology, and histopathology (emphasis on localized virus staining, cribiform plate, olfactory bulb, and mouth, nasal and throat passages). The study was completed in 2004. Results are pending at the time of this abstract but will be included in the presentation.

 **1350** NONCLINICAL SAFETY EVALUATION OF TLR4 AGONISTS ADMINISTERED BY NASAL SPRAY.

C. M. Lynch. *Nonclinical Development, Corixa Corporation, Seattle, WA.* Sponsor: M. Reed.

Over the past few years, it has become clear that most vaccine adjuvants and immunomodulatory agents act on various members of the Toll-like receptor family. Toll-like receptors recognize structural components of pathogens and, through this interaction, activate the immune system. The first microbial product discovered to be a Toll-like receptor agonist was lipopolysaccharide (LPS), a membrane component of Gram-negative bacteria, which activates Toll-like receptor 4 (TLR4). Although LPS is a potent immunomodulatory agent, its medicinal use is limited by

its extreme toxicity. We have developed a series of natural and synthetic TLR4 agonists that retain many of the immunomodulatory properties of LPS, but have greatly reduced the toxicity. These TLR4 agonists have potent immunomodulatory effects when administered to the airway mucosa. They can induce both nonspecific innate resistance to respiratory pathogens and specific adaptive immunity when used as mucosal adjuvants. These compounds can be potentially used to protect against respiratory infections caused by bacteria and viruses and to modulate immune responses to allergens associated with the development of atopic disorders. We conducted a number of toxicity studies in rodents and dogs to assess the safety and potential toxicity after intranasal administration of TLR4 agonists to support clinical trials in humans. This presentation will discuss the factors affecting successful delivery to the nasal cavity such as, selection of the appropriate animal model, anatomical features of the nasal cavity, administration techniques and delivery devices. Special considerations for toxicity assessment of products administered via the intranasal route, specifically, the potential for retrograde transport along the olfactory nerves to the brain will be discussed. Efforts to expose the olfactory epithelium will be described. The IND-enabling nonclinical safety studies of TLR4 agonists will be presented.

 **1351** TOXICOLOGICAL RESEARCH AND TESTING: BEST PRACTICES AND OPPORTUNITIES FOR LABORATORY ANIMAL REFINEMENT, REDUCTION, AND REPLACEMENT.

S. M. Lasley<sup>1</sup> and W. S. Stokes<sup>2</sup>. <sup>1</sup>*Department of Pharmacology, University of Illinois College of Medicine, Peoria, IL* and <sup>2</sup>*NICEATM, NIEHS/NIH/DHHS, Research Triangle Park, NC.*

In the performance of toxicology studies, whether for purposes of product safety testing or identifying mechanisms of toxicant action, it is becoming increasingly important to adopt practices and approaches that refine, reduce, and replace the numbers of laboratory animals utilized. Incorporation of best practices into studies will help ensure that animals are used in the most humane and judicious manner consistent with successful attainment of the research or testing objectives. Adoption of these practices is of timely importance because of continually increasing regulatory oversight of animal care and use, and thus consideration of these issues from different viewpoints is of broad interest to toxicologists. Best practices for regulatory testing involves careful consideration and appropriate incorporation of *in vitro* methods, humane endpoints, and tiered testing strategies (Stokes). Current best practices for housing and providing environmental enrichment for study animals should be consistently utilized, and factors considered that might potentially influence study outcomes (Brown). GLP requirements for pre-clinical safety studies are important factors to address, and optimal animal welfare practices consistent with compliance must be ensured (McCormack). Application of toxicogenomics to pre-clinical safety studies involving animals is an emerging concern, and potential opportunities for these methodologies to refine, reduce, and replace animal use are being developed (Schechman). Finally, updating of European Union animal welfare laws continue to evolve, and their potential impact on harmonization of animal care programs and toxicological research is a relevant concern to multinational companies (Donovan).

 **1352** BEST PRACTICES FOR USING HUMANE ENDPOINTS AND TIERED TESTING STRATEGIES TO REFINE, REDUCE, AND REPLACE ANIMAL USE IN TOXICOLOGICAL RESEARCH AND TESTING.

W. S. Stokes. *NICEATM, NIEHS/NIH/DHHS, Research Triangle Park, NC.*

Animal care and use regulations, guidelines, and policies require consideration of alternatives prior to the use of animals for research and testing. Toxicology studies using animals should therefore incorporate methods and approaches consistent with study objectives that minimize the use of animals and that reduce or avoid potential pain and distress. Several new and recently revised regulatory testing guidelines for acute local and systemic toxicity now recommend the use of humane endpoints and tiered testing strategies. The guidelines allow for hazard identification to be accomplished in some situations with reduced or no animal use. This approach involves an initial review of all available relevant information and data on the test and related substances. If further information is needed, this is generated in a step-wise manner and may include determination of physical/chemical properties, structure-activity relationship evaluations, and *in vitro* studies. A weight-of-evidence evaluation is made at each stage to determine if there is sufficient information for determination of hazard or if additional data is needed. If animal studies are deemed necessary, the use of sequential testing can often reduce the total number required, especially for those chemicals causing severe effects. The establishment of humane endpoints prior to the initiation of animal studies can also provide a basis for appropriate interventions to reduce the severity and/or duration of pain and distress. This involves anticipating possible toxic effects and establishing appropriate criteria that can serve as the basis for ending a procedure before or when pain and

distress become apparent. Appropriate consideration and incorporation of best practices in toxicology studies can be expected to refine, reduce, and/or replace animal use while supporting the attainment of study objectives

 **1353** BEST PRACTICES FOR ENVIRONMENTAL ENRICHMENT AND HOUSING FOR LABORATORY ANIMALS USED IN TOXICOLOGICAL RESEARCH AND TESTING.

M. J. Brown. *Charles River Laboratories, East Thetford, VT.* Sponsor: S. Lasley.

Environmental enrichment (EE) is defined as any measure which promotes expression of natural, species specific behaviors and a decrease in, if not the disappearance of, abnormal behaviors. The importance of environmental enrichment for laboratory animals is recognized in national regulations and guidelines such as the Animal Welfare Act and Guide for the Care and Use of Laboratory Animals, as well as international regulations such as ETS123. However, in animal testing, there has been ever increasing efforts to standardize animal use over the years through control of genetics, hygiene, feeding and housing. EE is viewed by some as potentially decreasing this standardization and thus increasing variability, increasing the numbers of animals which must be used and invalidating the historical database. Concerns are confounded by the numerous published reports with conflicting conclusions - enrichment does, or does not affect body weights; food consumption; organ weights; urine and plasma corticosterone; behavioral tests; endocrine activations; aggressiveness, and the list goes on. The effect of the stress of a non-enriched environment on study results has also been discussed. Science has been a continually evolving field. Standards of housing and handling laboratory animals have continued to improve as we learn more about topics such as laboratory animal disease and housing needs. We are continuing to learn about animal needs as more research is done into the effects of environment. Many toxicology labs have embraced the concept of EE. All those involved in toxicological testing must use this new knowledge to strive to improve animal well being in a manner that is consistent with testing objectives. The diverse types of studies, differing objectives, different regulatory agencies and chemical and toxicological properties of the test substance will all affect the type and extent of EE which can be incorporated into studies. Scientists, veterinarians, IACUCs and regulatory agencies should be working together to enhance animal well being through the use of EE whenever possible.

 **1354** BALANCING ANIMAL WELFARE AND GLP COMPLIANCE IN NONCLINICAL LABORATORY SAFETY STUDIES.

J. F. McCormack. *Office of Regulatory Affairs, US Food and Drug Administration, Rockville, MD.* Sponsor: W. Stokes.

Persons conducting nonclinical laboratory studies frequently face circumstances they perceive as necessitating either a compromise in animal care or compliance with FDA GLP regulations. This presentation will address the objectives of the GLP regulations and the use of the GLP quality management system in balancing adequate veterinary care with the need to acquire critical product safety information. The roles of testing facility management, the study director, and the attending veterinarian and their cooperative interaction in resolving conflicts will be emphasized. Study directors and attending veterinarians often view their respective responsibilities under FDA GLP regulations and animal care regulations as incompatible. The presentation will discuss how a thorough understanding of the GLP regulations can support the compatibility of these responsibilities. The presentation will also discuss the role of testing facility management in establishing procedures and an organizational structure to minimize conflict and ensure the quality and integrity of studies conducted in their facility. A discussion of common misperceptions about GLP requirements related to animal care and use will be conducted. Topics related to facility design, standard operating procedures and protocols, treatment of study animals, record retention, and FDA inspections will be covered. The presentation will also address the objectives of and activities occurring under the interagency agreement among NIH, USFDA, and USDA on animal care and use in nonclinical laboratories. The discussion will cover the responsibilities of the respective regulatory authorities and the type of information they share and how they interact under the Memorandum of Understanding.

 **1355** APPLICATION OF TOXICOGENOMICS TO PRE-CLINICAL SAFETY TESTING: VALIDATION CONSIDERATIONS FOR POTENTIAL LABORATORY ANIMAL REFINEMENT, REDUCTION, AND REPLACEMENT.

L. M. Schechtmann. *FDA/NCTR, Rockville, MD.*

Toxicogenomics offers promise for numerous key areas of research/testing, e.g. identification of early more predictive biomarkers of toxicity, new approaches for human, animal, and environmental safety/hazard/risk assessment, and characteriza-

tion of the biological activity and targets of chemicals/drugs. The technology could also potentially stimulate development of a new generation of alternative predictive testing and screening methods that could ultimately influence reduction, refinement and replacement (3Rs) of animals used for such purposes. Regulatory acceptance of the technology for decision-making purposes will necessitate standardization, validation, and demonstration of biological relevance. Efforts are already underway (e.g. ECVAM-ICCVAM/NICEATM collaboration) to begin to lay groundwork for the eventual standardization and validation of toxicogenomic-based technologies that would render them reproducible and reliable for informed regulatory decision-making. It is recognized that such an evaluation may involve considerable complexity, in that both the technology itself and any predictive alternative test methods derived from it will warrant validation. Thus, a strategy involving parallel and interactive processes of technological advancement and validation considerations will help guide the evolution of, establish confidence in, and promote adoption of this promising research/regulatory tool. Efforts directed at regulatory implementation have been in progress at FDA, e.g. draft guidances on (a) submission of pharmacogenomic data and, and (b) tests/devices that employ multiplex and microarray-based technologies. Initiatives such as these will form the foundation for the appropriate utilization of both the toxicogenomics technology and test methods based on toxicogenomics and will pave the way forward for the use of alternative (e.g. *in vitro*-based) toxicogenomics methods with potential regulatory utility and that are responsive to the 3Rs.

 **1356** REVISIONS TO EUROPEAN ANIMAL WELFARE LEGISLATION: IMPACT ON ANIMAL CARE AND TOXICOLOGY STUDIES.

J. C. Donovan. *BioResources, Wyeth Research, Collegeville, PA.* Sponsor: S. Lasley.

Conventional wisdom holds that the evolution of animal welfare legislation in Europe portends what is likely to follow in the US. Accordingly, animal researchers have for some years now been monitoring the development of new European animal welfare regulations. Since 1997 when the Council of Europe decided to revise ETS 123, the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, a lengthy and complex process ensued that will eventually lead to a new animal welfare Directive, or law, in the European Union. As the Council of Europe neared completion of revising ETS 123 in 2003, the European Union recognized a need to revise Council Directive 86/609, the European Directive on the Approximation of Laws, Regulations and Administrative Provisions Regarding the Protection of Animals Used for Experimental and other Scientific Purposes. In 2003 the European Commission appointed a Technical Expert Working Group (TEWG) to provide scientific input on the promulgation of new legislation. The TEWG reports were published in mid-2004 and will be used as the basis for a first draft. The reports address the scope of the legislation, how experimentation should be authorized, the function of ethical review committees and pain categorization. Following consultation with stakeholders, including animal welfare groups, the Commission will present a final draft to the European Parliament and Council of Ministers for legislative action. This presentation will review the possible implications of the TEWG reports and ETS 123 for US-based animal research institutions, with an emphasis on toxicology studies. Specific areas of concern include: restrictions on non-human primate usage; enrichment requirements; cage size modifications; protocol authorization procedures; other administrative burdens; and, escalation of animal testing costs. A status report on the progress of the evolving legislation will be provided and, if a draft of the regulations is available, a review and analysis of the document.

**1357** THE ROLE OF THE COOH-TERMINAL TRANSACTIVATION DOMAIN OF THE MOUSE AH RECEPTOR IN LIGAND-DEPENDENT AND INDEPENDENT DEGRADATION.

R. S. Pollenz and J. Popat. *Biology, University of South Florida, Tampa, FL.*

The AHR receptor (AHR) is a ligand activated transcription factor that is a member of the bHLH/PAS family of proteins. Following ligand activation, the AHR is capable of forming a heterodimer with the ARNT protein and binding to DNA to regulate specific genes. Several studies have implicated the degradation of the AHR via the 26S proteasome as one of the mechanism that may attenuate receptor action. In addition, it has been suggested that the COOH-terminal domain of the AHR may be necessary for ligand-mediated degradation. This implies that amino acids within the COOH-terminal region may be modified for degradation, or that induction of specific genes may be required for degradation. To address these questions, two truncated AHRs were produced. A stop codon was placed at nucleotide #1501 to generate an AHR with 500 amino acids (AHR500) or at nucleotide 1294 to generate an AHR with 431 amino acids (AHR431). The truncated AHRs were ligated into retroviral expression vectors and used to generate stable cell lines. Treatment of stable cell lines with TCDD for 0-6 hrs showed that the time course

and magnitude of AHR degradation was not impaired in AHR500 or AHR431 when compared to wild type AHR. Geldanamycin (GA) treatment also resulted in similar levels of degradation in all AHRs. Importantly, ligand activation of AHR431 did not result in detectable levels of CYP1A1, and AHR500 induced reduced levels when compared to wild type AHR. Interestingly, the subcellular localization of the AHR431 was predominantly nuclear when compared to AHR500, and immunoprecipitation studies showed that AHR431 was associated with reduced levels of hsp90 and did not contain XAP2. These studies suggest that the COOH-terminal 374 amino acids of the AHR that include the transactivation domain, is not required for ligand-dependent or independent degradation. This implies that AHR degradation does not require genes induced by the AHR-ARNT heterodimer, and that degradation can occur efficiently when the AHR is nuclear. Supported by ES 10991.

**1358**

KINETIC STUDIES OF ARYL HYDROCARBON RECEPTOR ACTIVATION BY INDOLO[3, 2-B]CARBAZOLES, INDIGOS AND 2, 3, 7, 8-TETRACHLORODIBENZO-P-DIOXIN IN HUMAN HEPG2 CELLS.

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The arylhydrocarbon receptor (AhR) is considered to be an unusually promiscuous receptor and several candidate endogenous ligands have been suggested. These include indoles as well as fatty acid derived molecules. Among these, the naturally occurring ligand indirubin has been claimed to be most efficient, inducing transcription of cytochrome P4501A1 (CYP1A1) in human HepG2 cells at a concentration of 1pM (Adachi et al., *Toxicology Sciences* 80, 2004, 161-9). Other investigators have reported lower affinity of indirubin as measured by competitive binding to rat and mouse liver cytosolic receptors and in assays of AhR activation in a reporter cell line derived from the human hepatoma HepG2 cell line (Rannug et al., *Mutat Res* 282, 1992, 219-25; Guengerich et al., *Arch Biochem Biophys* 423, 2004, 309-16). In this study, we examined the ligand dependent activation of indigo, indirubin, indolo[3, 2-b]carbazole (ICZ), 6-formyl-indolo[3, 2-b]carbazole (FICZ) and 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) using stably transfected HepG2 cells that express luciferase under the control of one xenobiotic response element from the human CYP1A1 gene. Sub-confluent cells were treated with the compounds dissolved in DMSO, at a final DMSO concentration of 0.1 %. Special care was taken to protect cell culture media from light to avoid formation of FICZ from tryptophan present in the medium. Time-course and dose-response studies were conducted and it was observed that indirubin, ICZ and FICZ were the most potent activators, causing a half maximal induction of luciferase activity at concentrations that were approximately 20-fold lower than those of TCDD. The maximum response was obtained at short incubation times (3-6 h) and this may explain the discrepancies between the earlier reports from studies with HepG2 cells. Therefore we conclude that the indolo[3, 2-b]carbazoles and indirubin are more potent activators of the human AhR than TCDD. (Supported by USPHS Grant ES10337)

**1359**

DRE BINDING IS NOT REQUIRED FOR REPRESSION OF AHR SIGNALING BY AHRR.

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TCDD causes a variety of effects including toxicity, alterations in the cell cycle, and developmental defects, and the aryl hydrocarbon receptor (AHR) is required to mediate many of these effects. Once ligand activated, the AHR translocates to the nucleus where it forms a transcriptionally active AHR-ARNT heterodimer that binds to dioxin-response elements (DREs), causing changes in gene expression. The AHR repressor (AHRR) is a TCDD-inducible, AHR-regulated protein that can inhibit AHR action, forming a negative feedback loop with the AHR. It has been proposed that the AHRR acts as a repressor through competition with AHR for binding to ARNT and binding to DREs. We previously cloned a zebrafish AHRR and demonstrated that competition for ARNT2b was not the primary mechanism of repression. To investigate the role of DRE binding in the mechanism of AHRR repression, a point mutation was made at tyrosine 9 of zebrafish AHRR. Previous research has shown that a single mutation at the homologous residue of AHR disrupts DRE binding. Gel shift assays confirmed that DRE binding of the mutant AHRR was disrupted. In transient transfection assays in COS-7 cells, both wild-type and mutant AHRRs were able to repress the AHR2-dependent induction of a luciferase reporter (pGudLuc6.1), although repression by the mutant AHRR was slightly attenuated. Co-transfection of increasing amounts of zebrafish ARNT2b failed to reverse repression caused by either the wild-type or mutant AHRR. These

data indicate that the AHRR can act as a repressor independent of DRE binding and in the presence of excess ARNT. Therefore, additional mechanisms of repression must exist. [NIH ES06272 and NIH ES07831]

**1360**

ERK KINASE ACTIVITY CONTRIBUTES TO AH RECEPTOR TARGETED PROTEIN DEGRADATION AND TRANSCRIPTIONAL POTENTIAL.

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Along with the mechanics of ligand binding and nuclear translocation, evidence exists that intracellular signaling pathways play an important role in Ah receptor function. Ah receptor ligands such as 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) are known to activate mitogen activated protein kinases (MAPK), such as the Erk kinases. In this study, we focused on the elucidation of the potential effects of Erk kinases on Ah receptor function. Cotreatment of hepalc1c7 cells with TCDD and Erk kinase inhibitors PD98059, U0126 or SL327 synergized TCDD enhanced nuclear accumulation of the Ah receptor, but also led to a reduced capacity to complement induction of Cyp1a1. This is explained by potential modifications through phosphorylation of the carboxyl region of the receptor, leading to diminished transactivation potential. Coincidentally, inhibition of Erk kinase leads to the induction of cellular Ah receptor levels by altering the steady-state concentrations through delayed degradation of the receptor. Erk kinase activity is directly linked to Ah receptor stabilization as demonstrated by reduction in total Ah receptor levels following the overexpression of constitutively active MEK1. Since overexpression of MEK1 enhanced TCDD initiated transactivation potential of the receptor, the transactivation region could be a target for Erk kinases. Immunoprecipitation experiments of the Ah receptor indicate that Erk kinase activity is directly associated with the receptor. Since the carboxyl region of the receptor is responsible for targeted ubiquitination in addition to controlling transactivation potential, these results indicate that Erk kinase activity promotes ligand initiated transcriptional activation while targeting the Ah receptor for degradation. (Supported by USPHS Grant ES10337)

**1361**

INVOLVEMENT OF KINASE SIGNALING PATHWAYS IN AH-RECEPTOR MEDIATED GENE EXPRESSION IN RAT LIVER CELLS.

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Agonists of aryl hydrocarbon (Ah) receptor such as TCDD and certain PCBs induce the expression of genes containing DREs. Cross-talk with other receptor and non-receptor signaling pathways can alter such gene expression. To identify the interactions of Ah-receptor signaling with other signaling cascades a rat liver cell line WB-F344 that expresses Ah-receptor and ARNT protein was stably transfected with the plasmid containing green fluorescent protein (GFP) gene with four DREs. One clone that expressed high levels of GFP in response to TCDD (0.1 nM) or 3, 3, '4, 4', 5-pentachlorobiphenyl (PeCB, 2.0  $\mu$ M) exposure was used to examine Ah-receptor interactions with other signaling pathways. Cultures of the clone were treated with known pharmacological inhibitors of various signaling pathways prior to exposure to TCDD or (PeCB). GFP expression was examined two days after treatment. Pretreatment of the cultures with a PCB congener 2, 2', 4, 4'-tetrachlorobiphenyl (TCB, 20  $\mu$ M) that activated the mitogen activated protein kinases (MAPK) ERKs inhibited Ah-receptor mediated expression of GFP by more than 90%. Pretreatment of cells with a pharmacological inhibitor of ERK U-0126 (10.0  $\mu$ M) or a protein kinase C inhibitor bis indolylmaleimide (BIS, 5.0  $\mu$ M) did not have significant inhibitory effect on GFP expression. In contrast compound 56, a highly specific inhibitor of EGF receptor tyrosine kinase activity completely abrogated GFP expression. A src tyrosine kinase inhibitor PP2 (10.0  $\mu$ M) partially inhibited GFP expression by 50-60%. Taken together these results suggest that interactions of Ah-receptor signaling with other cell signaling pathways may alter Ah-receptor mediated gene expression and potential biological effects of Ah receptor agonists (supported by NIEHS/Superfund grant ES-04911 and Philip Morris External Research Program).

**1362**

ACTIVATION OF THE ARYL HYDROCARBON RECEPTOR ALTERS IN VIVO HEPATOCYTE PROLIFERATION.

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Increasing evidence links the aryl hydrocarbon receptor (AhR) to cell cycle regulation. For instance, AhR-defective mouse hepatoma cells exhibit a prolonged transition through G1 phase. Similarly, treatment with the persistent AhR agonist 2, 3, 7,

8-tetrachlorodibenzo-p-dioxin (TCDD) stalls proliferation in hepatoma cells and primary hepatocyte cultures, presumably by disrupting the normal physiological processes of the AhR, yet the mechanism is unclear. The goal of this study was to determine how the AhR regulates *in vivo* hepatocyte proliferation induced either by partial hepatectomy (PH) or by treatment with the tumor-promoting mitogen 1,4-bis[3, 5-dichloro-pyridyloxy]benzene (TCPOBOP). Whereas liver regeneration following PH depends on the production of a cascade of growth factors that culminate in proliferation, TCPOBOP-induced proliferation depends on activation of the constitutive androstane receptor (CAR) and occurs independently of PH-induced stimuli. To test how AhR activation affects proliferation, mice were gavaged with TCDD (10-20  $\mu$ g/kg) or peanut oil one day prior to 70% PH or TCPOBOP treatment (3 mg/kg, gavage). Mice were sacrificed 18-60 hours later, following a 2-hr pulse with bromodeoxyuridine (BrdU), and liver was fixed for histological analysis or homogenized for western blotting. Whereas exposure to TCDD resulted in a 5-fold decrease in the number of BrdU+ hepatocytes 36 hours after PH, it increased proliferation in TCPOBOP-treated mice. AhR levels were upregulated in hepatocytes following either PH or TCPOBOP treatment, but were not altered by TCDD. Interestingly, exposure to TCDD had not effect on cyclin D1 levels, but consistently increased cyclin E levels in both model systems; however, the ramifications of this are unclear. In summary, these findings indicate that the AhR may play a role in regulating hepatocyte proliferation induced by two separate stimuli comprising distinct pathways.

### 1363

#### TEMPORAL AND DOSE-DEPENDENT HEPATIC GENE EXPRESSION PATTERNS IN MICE PROVIDE NEW INSIGHTS INTO TCDD-MEDIATED HEPATOTOXICITY.

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Many, if not all, of the toxic effects of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) are due to sustained, inappropriate changes in gene expression mediated via the activation of the aryl hydrocarbon receptor (AhR). Despite years of research the exact mechanisms responsible for its toxic effects remain unknown. In an effort to further characterize the mechanisms of TCDD-mediated toxicity, comprehensive temporal and dose response microarray analyses were performed on hepatic tissue from immature ovariectomized C57BL/6 mice treated with TCDD. For temporal analysis, mice were gavaged with 30  $\mu$ g/kg of TCDD or vehicle and sacrificed after 2, 4, 8, 12, 18, 24, 72 or 168 hours. Dose response mice were gavaged with 0, 0.001, 0.01, 0.1, 1, 10, 100 or 300  $\mu$ g/kg of TCDD and sacrificed after 24 hours. Hepatic gene expression profiles were monitored using custom cDNA microarrays containing 13, 361 cDNA clones. 443 and 315 features exhibited a significant change at one or more doses or time points, respectively, as determined using an empirical Bayes approach. Functional gene annotation extracted from public databases associated gene expression profiles with physiological processes such as oxidative stress and metabolism, differentiation, apoptosis, gluconeogenesis, and fatty acid uptake and metabolism. Complementary H&E examination, Oil Red O staining, clinical chemistry (i.e. ALT, TG, FFA, cholesterol) and high resolution gas chromatography/mass spectrometry assessment of hepatic TCDD levels were also performed in order to phenotypically anchor changes in gene expression to toxicity. Collectively, the data support a proposed mechanism for TCDD mediated hepatotoxicity including fatty liver which involves mobilization of peripheral fat and increased uptake of fatty acids potentially as an alternative energy source due to inhibition of hepatic gluconeogenesis. Supported by grant ES11271

### 1364

#### EXPRESSION OF DOMINANT NEGATIVE N-CADHERIN RESULTS IN AHR-DEPENDENT GENE INDUCTION IN HEPA 1C1C7 WILD-TYPE CELLS.

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We have established that suspension-mediated disruption of all cell adhesion leads to the AhR-dependent induction of the prototypical XRE-regulated target gene, CYP1A1, in Hepa 1c1c7 wild-type cells. Increases in steady state CYP1A1 mRNA following a cadherin-disrupting shift from high to low calcium medium indicated that adherens junctions, mediators of cell-cell adhesion, may play a role in the suspension-induced AhR/ARNT signaling response. Our hypothesis is that disruption of adherens junctions leads to an increase in  $\beta$ -catenin, a cadherin-binding intracellular adherens junction protein, which promotes the AhR-dependent induction of CYP1A1. In order to further study the mechanism by which suspension induces AhR/ARNT signal transduction, a doxycycline-inducible dominant negative N-cadherin (dn-N-cad) protein was used to specifically disrupt cadherin-mediated cell-cell adhesion in adherent Hepa 1c1c7 wild-type cells. Maximal induction of

the dn-N-cad protein occurred 24-48 hours post treatment with doxycycline and was clone dependent. As predicted, expression of the dn-N-cad transgene decreased endogenous cadherin levels and resulted in increased  $\beta$ -catenin levels as early as 24 hours post treatment. Increases in mRNA levels of CYP1A1 were observed at 32, 48 and 56 hours following treatment with doxycycline. These studies directly demonstrate that in adherent Hepa 1c1c7 wild-type cells disruption of N-cadherin-containing junctions results in the endogenous upregulation of CYP1A1 expression. Our findings suggest a role for  $\beta$ -catenin in endogenous regulation of AhR/ARNT signaling. These data support the overall hypothesis that AhR/ARNT signaling may be endogenously regulated by cell-cell interactions *in vitro*.

### 1365

#### ER $\alpha$ IS A LIGAND-DEPENDENT MODULATOR OF AHR TRANSCRIPTION: INTERPLAY BETWEEN THE TWO RECEPTOR SYSTEMS.

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Using chromatin immunoprecipitation (ChIP) we have demonstrated the TCDD-mediated recruitment of ER $\alpha$  to AhR target genes, and transient transfection experiments in HuH7 liver cells showed that ER $\alpha$  increased AhR/ARNT complex (AhRC) activity. In this study we used real-time PCR analysis of endogenous CYP1A1 levels to confirm the enhancing effect of ER $\alpha$  on AhR activity, and re-ChIP analysis further demonstrated the presence of ER $\alpha$  in the active AhRC. No oscillatory cycling was apparent; however the use of  $\alpha$ -amanitin pretreatment was effective in dissecting the differential recruitment kinetics between the enhancer and tata-box regions of the CYP1A1 promoter. We also examined the ability of different AhR and ER ligands to influence the recruitment of ER $\alpha$ , and the AhRC to pS2 and CYP1A1 using ChIP followed by real-time PCR. As expected, TCDD induced the recruitment of ER $\alpha$  to the CYP1A1 promoter, but not to the pS2 promoter. Surprisingly, DIM, ICZ, and  $\beta$ -naphthoflavone, induced the recruitment of ER $\alpha$  to both the CYP1A1 and the pS2 promoters, whereas  $\alpha$ -naphthoflavone only induced the recruitment of ER $\alpha$  to pS2 and not CYP1A1. This suggests that diverse AhR ligands can differentially influence ER $\alpha$  activity. Phytoestrogens genistein and coumestrol, as well as 4-t-octylphenyl and BPA caused a similar time-dependent recruitment of ER $\alpha$  to pS2; however preferential recruitment of the p160 coactivators was evident. For instance, E2 caused the recruitment of all three p160 family members to pS2, whereas 4-t-octylphenol preferentially recruited TIF2 as opposed to AIB1 or SRC-1. In cotreatment with TCDD, all estrogenic compounds increased the recruitment of ER $\alpha$  to CYP1A1. Collectively, these data implicate ER $\alpha$  as a modulator of AhR signaling cascades, that different AhR ligands can differential influence ER $\alpha$ , supporting a new mechanism of AhR-ER $\alpha$  cross-talk. In addition, these data also suggest that different ligands can influence the coregulator complex composition during transcription activity.

### 1366

#### THERAPEUTIC CONCENTRATIONS OF HALOPERIDOL DO NOT DIRECTLY AFFECT OVARIAN FUNCTION AND OOCYTE QUALITY AS TESTED IN MOUSE OVARIAN FOLLICLE CULTURE MODEL.

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Patient plasma concentrations of the neuroleptic drug haloperidol used in antipsychotic and behaviour therapies range between 5  $\eta$ M and 100  $\eta$ M. Several in-vivo animal studies suggested that haloperidol can influence the gonadotrophin and prolactin release and can have an impact on reproduction. It is not clear whether haloperidol can provoke cellular down-stream effects via receptor binding in the ovary, which would create additional disturbances for female fertility. The in-vitro mouse ovarian follicle bioassay was used to investigate the direct effects of haloperidol on the ovarian function and on oocyte quality. Preantral mouse ovarian follicles were cultured individually during 12 days in continuous presence of haloperidol (1, 10, 50, 100, 1000  $\eta$ M). A total of 420 follicles were used in 6 replicates. *In vitro* ovulation was induced by hCG and mature oocytes were harvested for morphological analysis and grading of meiotic maturation. Haloperidol exposed follicle cultures were evaluated for follicle differentiation, oocyte maturation and steroid hormone secretion pattern. Up to a dose of 100  $\eta$ M haloperidol had no effects on follicle survival and differentiation (antrum formation), oocyte maturation or steroid production. But at a dose of 1000  $\eta$ M haloperidol oocyte maturation capacity was significantly decreased: only 64 $\pm$ 17% of the oocytes extruded a polar body, compared to 97 $\pm$ 5% in the control group. Also 17  $\beta$ -estradiol secretion was decreased compared to the control group ( $P=0.05$  in t-test). The mouse in-vitro follicle bioassay reveals that haloperidol doses within the human therapeutic dose window do not alter folliculogenesis, oocyte quality and steroidogenesis by a direct interaction with the ovarian follicle. Doses of haloperidol over 100  $\eta$ M interfere with oocyte maturation perhaps via cellular receptor-mediated pathways in the ovary that bind the compound with a low affinity.

BCL-2 FAMILY MEMBERS PROTECT AGAINST  
METHOXYCHLOR-INDUCED TOXICITY OF MOUSE  
OVARIAN ANTRAL FOLLICLES *IN VITRO*.

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The organochlorine pesticide methoxychlor (MXC) is a reproductive toxicant that destroys antral follicles in the mammalian ovary. In response to *in vivo* MXC exposure, mouse antral follicles show increased atresia and altered apoptotic protein expression. Moreover, mice overexpressing the anti-apoptotic protein Bcl-2 (Bcl-2+), and mice deficient in the pro-apoptotic protein Bax (BaxKO) are protected from *in vivo* MXC-induced atresia. Likewise, studies using an *in vitro* culture model indicate that MXC inhibits antral follicle growth and increases atresia over controls. This current study tested the hypothesis that isolated antral follicles from Bcl-2+ and BaxKO mice are protected from MXC-induced atresia and growth inhibition, and that our *in vitro* culture model mimics *in vivo* MXC exposure. Mouse antral follicles were isolated and exposed *in vitro* to vehicle (DMSO) or MXC (1-100 µg/ml) for 96hrs. Follicle diameters were measured at 24hr intervals to assess growth in response to MXC. After 96hrs, follicles were processed for histological analysis of atresia. MXC (10 and 100 µg/ml) significantly inhibited growth of wild type (WT) antral follicles *in vitro* compared to controls at 72 and 96hrs (n=25 follicles/treatment; p≤0.03). In contrast, MXC did not inhibit growth of Bcl-2+ and BaxKO follicles until 96hrs (n=28 follicles/treatment; Bcl-2+ p≤0.004; BaxKO p≤0.001). MXC also increased atresia of small and large WT antral follicles over controls (n=6-11 follicles/treatment; p≤0.03), but did not increase atresia of large Bcl-2+ and BaxKO antral follicles over control levels. These data suggest that Bcl-2 overexpression or Bax deletion provides protection from MXC-induced antral follicle toxicity. These data also indicate that this *in vitro* culture model mimics our findings *in vivo*, supporting the use of this method to evaluate ovarian follicle toxicity. (Supported by NIH HD38955, T32 ES07263-13, T32 HD07170 and a Colgate-Palmolive Postdoctoral Fellowship)

REVERSAL OF BENZO-A-PYRENE EFFECTS ON  
OXYTOCIN-INDUCED  $Ca^{2+}$  OSCILLATIONS IN  
HUMAN MYOMETRIAL PHM1-41 CELLS.

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Benzo-a-pyrene (BaP) is one of the polycyclic aromatic hydrocarbons that exist as major environmental pollutants. In the present study, we have examined the effect of this carcinogen/mutagen upon human myometrial  $Ca^{2+}$  signaling. Exposure of PHM1-41 cells to BaP did not alter basal calcium levels of these cells or the IP3-releasable  $Ca^{2+}$  pool. However, BaP significantly decreased the oxytocin-induced  $Ca^{2+}$  transient as well as the frequency of oxytocin-induced oscillations. To determine the source of these alterations, a variety of pharmacologic agents such as the endoplasmic reticulum Ca-ATPase inhibitor thapsigargin, an activator and inhibitor of protein kinase C, a K<sup>+</sup> channels blocker, and tyrosine kinase inhibitors and activators were used. Our data revealed that the store operated channels are significantly affected by BaP treatment. The inhibition of myometrial smooth muscle oscillations induced by BaP seems to be mediated, at least in part, via activation of  $Ca^{2+}$  activated K<sup>+</sup> channels, because partial inhibition was eliminated by the K<sup>+</sup> channel blocker tetraethylammonium chloride. The non-specific tyrosine kinase inhibitor genistein and the epidermal growth factor receptor tyrosine kinase inhibitor AG1478 markedly reduced the oxytocin induced  $Ca^{2+}$  oscillations in control cells but not in BaP treated cells. The effect of these inhibitors on the tyrosine kinase pathway in control myometrial cells is similar to the BaP effect suggesting that BaP inhibits tyrosine kinase activity in PHM1-41 cells. The use of FBS or charcoal/dextran treated FBS (1.25%) prior to oxytocin, restimulated the  $Ca^{2+}$  oscillations induced by oxytocin in BaP treated cells to a level similar to control cells. These data suggest that the action of BaP effect on  $Ca^{2+}$  oscillations in myometrial cells can be reversed.

EFFECTS OF ATRAZINE AND AN ATRAZINE  
METABOLITE MIXTURE ON DIFFERENTIATED  
MAMMARY EPITHELIAL CELL MILK PROTEIN  
PRODUCTION IN CULTURE.

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Previous studies have shown that prenatal exposure to the commonly used herbicide, atrazine (ATR) can have adverse effects on mammary gland development and function in female rat offspring. To evaluate the effects of ATR on differentiated mammary epithelia, HC11 cells were tested for a dose response to atrazine or an atrazine metabolite mixture (EBM), containing 25% ATR. Cells were grown to

confluence on plastic in growth medium, then induced to differentiate (produce milk proteins) by adding prolactin, dexamethasone, and insulin (lactation media). The cells were simultaneously dosed with 0, 16, 64, or 250 µM ATR or 0.17, 17.4, or 1740 ng/ml EBM. Cells not induced with lactation media and grown concomitantly were the negative control. Following 4 days of differentiation (induces caseins), media were collected and concentrated, and attached cells were lysed in a solution containing protease inhibitors. Western blot analyses were performed for actin, prolactin and caseins using 50 µg total protein per sample. Actin (40 kDa) was detected at similar levels in all cell treatments and was absent in media samples. Prolactin (28 kDa) was detected in all treatment groups in similar amounts, but was absent in negative controls. Cellular prolactin levels were unchanged by treatment (24 kDa band slightly reduced in negative control). Caseins were detected with the antibody, and the use of lactation medium induced a 5- and more than 20-fold induction of caseins of about 28 and 21 kDa, respectively, over that in negative controls. EBM caused a dose dependent decrease in 21 kDa casein (72, 43, and 1% of control, respectively), whereas only the highest dose of EBM affected the 28 kDa species (36% of control). ATR alone had no effect on the 28 kDa casein, but decreased the 21 kDa species 78, 77, and 68% of control, respectively. Studies to discern the effects of these compounds on gene expression of milk proteins are ongoing. (This abstract is of a proposed presentation and does not reflect EPA policy)

NEONATAL EXPOSURE TO THE PHYTOESTROGEN  
GENISTEIN ALTERS REPRODUCTION IN FEMALE CD-  
1 MICE.

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Developmental exposure to estrogenic substances causes adverse effects on the developing reproductive tract. Genistein (Gen), the primary phytoestrogen in soy products, was investigated for potential adverse effects on the developing female reproductive system. Outbred CD-1 mice were treated by subcu. injection on neonatal days 1-5 with Gen 0, 0.5, 5 or 50 mg/kg/day in corn oil. Mice were weaned at 21 days of age and observed daily for vaginal opening (n=16 mice per group). Vaginal opening occurred earlier in Gen 0.5 and 5 mice than controls (3/16 and 2/16, respectively). However, the highest dose of genistein showed a marked delay in vaginal opening with only 6% open at 30 days of age compared to 40% in controls. At 2 mo., daily vaginal smears showed that Gen altered estrus cycle length as well as caused a dose related increase in severity of abnormal cycles. When Gen mice were bred to control males, the 0.5 and 5 group had normal fertility at 2 and 4 mo. of age with similar numbers of mice delivering live pups and similar litter sizes as controls. However, by 6 mo. of age, there was a sharp decrease in the percent of mice delivering live pups in both Gen 0.5 and 5 doses (60% and 40%) compared to controls (100%). Mice treated with the highest dose of Gen did not deliver any live pups at any age examined. To study the infertility at the high dose, mice were bred and uteri collected at various gestational ages to assess pregnancy loss. There were less Gen 50 mice showing signs of pregnancy on day 6, 8 or 10 (62%, 37% and 45%) compared to controls (89%, 95% and 100%). Further, Gen mice that were pregnant had fewer embryos (<50%) than their control counterparts. Reabsorptions were commonly seen in Gen 50 mice. In summary, mice treated neonatally with genistein exhibit altered vaginal opening, abnormal estrus cycles, early reproductive senescence and subfertility/infertility at doses of Gen that are environmentally relevant.

ESTROGEN-INDUCED PATTERN OF GENE  
EXPRESSION AT HIGH AND LOW DOSES IN FETAL  
MALE RAT TESTIS.

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Exposure of developing fetal rodents to some estrogenic compounds at relatively high doses induces abnormalities in the male reproductive system and causes a predisposition to abnormal function during adulthood. We hypothesize these latent developmental effects are preceded by immediate changes in the fetal gene expression. High-density oligonucleotide arrays were used to determine the transcriptional program elicited by transplacental exposure to 17 $\alpha$ -ethynodiol (EE), genistein (Ges), or bisphenol A (BPA), in the developing rat testis and epididymis on GD 20, following maternal exposure on GD 11-20. Dosages were: 0.001-10 µg EE/kg/day, 0.001-100 mg Ges/kg/day and 0.002-400 mg BPA/kg/day, none of which induced detectable morphological changes in the developing reproductive system. The gene expression profile of testis and epididymis was modified by exposure to these estrogen receptor (ER) agonists in a consistent way. The highest dosage of each compound produced a significant change in expression level of 141, 46 and 67 genes (out of 8740), respectively. Dose-dependent analysis of the transcript profile revealed that the expression of 56 genes is significantly modified by

EE, while only 28 genes and 15 genes are dose responsive to Ges or BPA, respectively. Global comparison of the effect of EE, Ges or BPA exposure, at the three highest doses, indicated that the expression of 52 genes change in the same direction, although at a different magnitude. The dose-response curve for gene expression was monotonic, with both the magnitude of change and number of genes significantly changed decreasing with decreasing dose. Further, the gene expression changes induced show clear evidence of a variety of cellular pathways affected by estrogen exposure, some of which may have an impact in both gonadal differentiation and subsequent reproductive function. These results indicate that gene expression data are diagnostic of mode of action and can be used to elucidate dose-response characteristics at low dose.

**1372** LOW DOSE CELLULAR RESPONSES IN THE FETAL RAT TESTIS *IN UTERO* EXPOSED TO DI (N-BUTYL) PHTHALATE.

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Di(n-butyl)phthalate (DBP) is used in personal care products and as plasticizer to soften plastics. Adult male rats *in utero* exposed to DBP at 250-500 mg/kg/day dose-levels have malformations of the reproductive tract and testicular atrophy. These dose-levels also cause development of multinucleated gonocytes (MNG), inhibit cell proliferation, alter formation of seminiferous tubules, and disrupt contacts between Sertoli and germ cells in the fetal rat testis. The goal of the study was to determine if exposure *in utero* to low doses of DBP result in these cellular responses in the fetal testis. Timed-pregnant Sprague-Dawley rats were treated with 0.1, 1, 10, 30, 50, 100, and 500 mg/kg/day DBP by oral gavage on gestation days 12 to 20, and euthanized on day 21. Two hours prior to euthanasia, dams were i.p. injected with 50 mg/kg BrdU. Fetal testes were fixed in situ in modified Davidson's fixative, dissected, and embedded in paraffin. Cellular responses were assessed using H&E- or immunostained with P-cadherin tissue slides. The effect of DBP treatment on the size, total cell number, and cordial cross-section number was significant at 50 mg/kg/day dose-level. Although there was a trend indicating that the 50 mg/kg/day dose-level increases the incidence of MNG, statistical significance was achieved only at the 100 mg/kg/day dose-level. Consistent with this trend, fetal rat testes exposed to 30 and 50 mg/kg/day DBP had focal disruption of Sertoli-germ cell contacts as indicated by P-cadherin immunostaining. Our data demonstrate that in the rat, adverse cellular responses in the fetal testis can be detected at lower doses compared to those causing gross pathological changes. The lowest dose that significantly altered size, total cell number, and cordial cross-section number also decreased the concentration of testicular testosterone in the fetal rat testes *in utero* exposed to DBP. Whether reduction in testicular testosterone following *in utero* exposure to DBP is linked with decreased cell proliferation, disruption of Sertoli-germ cell interaction, and increased occurrence of MNG remains to be determined.

**1373** IDENTIFYING CANDIDATES FOR THE CDM GENE, WHICH CONFFERS RESISTANCE TO CADMIUM-INDUCED TESTICULAR DAMAGE IN MICE.

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Cadmium is an industrial and environmental toxin that damages the testes, prostate, placenta, kidneys, liver, lungs, and pancreas in humans and other mammals. Strain specificity for cadmium-induced testicular damage in mice is due to a single recessive gene, named cdm. Cdm/cdm mice are afforded complete resistance to cadmium-induced testicular damage, while both wildtype and heterozygous mice are susceptible. Of 44 mouse strains, 14 including C57BL/6J are cdm/cdm, and 30 including DBA/2J are wildtype. The cdm gene has been previously mapped to a region between microsatellite markers D3Mit110 and D3Mit255, a 4.7 Mb region on mouse chromosome 3, using C57BL/6J x DBA/2J (BxD) recombinant inbred strains (Dalton et al., 2000). The present study used 1, 850 F2 mice from a BxD F1 cross to narrow this region to 1.8 Mb. Thirty-two mice with a recombination between two cdm flanking markers (D3Nds2 and D3Mit291) which are 4.3 Mb apart were injected with 6 mg/kg CdCl<sub>2</sub> and phenotyped for testicular damage. Additional polymorphic markers were designed to refine the region to 1.8 Mb. RT-PCR has been run on the 30 candidate genes in this region. Preliminary data indicate expression differences in an oxidoreductase, a ubiquitin-conjugating enzyme, Nfcb1, and two predicted but not yet annotated genes. Real time PCR and protein inhibitor experiments are currently underway for these and other candidate genes in the region. Damage to spermatogenesis, Sertoli cells, and endothelial tissue has been quantified via histopathology. The function of the cdm gene has not yet been identified but it is conceivable that elucidation of the cdm pathway will provide insight into the mechanisms for human cadmium toxicity in reproductive and other organs.

**1374** PERIPUBERTAL DEHP EXPOSURE INHIBITS ANDROGEN-DEPENDENT DEVELOPMENT IN SPRAGUE-DAWLEY RATS.

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The plasticizer Di (2-ethylhexyl) phthalate (DEHP) may present reproductive risk by demasculinizing prenatal and juvenile males. The current study was designed to assess DEHP effects throughout pubertal development in Sprague-Dawley rats. Males were dosed with DEHP at 0, 100, 300 and 900 mg/kg-bw/day from PND 23. A "peri-pubertal" group was dosed until mid puberty (euthanized on PND 43/44), and a "post-pubertal" group was dosed throughout puberty (euthanized on PND 63/64). At the time of first necropsy, where half of the male study population was euthanized, there was a dose dependent reduction in the number of males showing complete preputial separation. Females dosed daily from PND 23 with either corn oil or 900 mg/kg-bw/day DEHP showed no differences in time to vaginal opening. In males, weights of androgen dependent tissues (testis, epididymis, ventral prostate, seminal vesicle, Cowper's glands and levator ani + bulbocavernosus muscles were reduced at high doses in peri and post-pubertal groups. Serum testosterone (T) levels and adrenal gland weights showed dose dependent reductions in the peri-pubertal group, but were not significantly affected in the post-pubertal group. *Ex-vivo* testis T production was reduced (with and without gonadotropin stimulation) in a dose-dependent manner in peri and post-pubertal groups. On a per weight basis (T/mg of testis), there was a dose dependent increase in *ex-vivo* testis T production in the post-pubertal group. These findings support the hypothesis that DEHP treatment around early puberty decreases Leydig cell T output, whereas prolonged treatment throughout puberty increases Leydig cell T output. These data do not support the hypothesis that DEHP exposure promotes precocial pubertal development by elevating serum T. This is an abstract of a proposed presentation and does not necessarily reflect EPA policy.

**1375** GENE ONTOLOGY MAPPING OF EARLY TRANSCRIPTIONAL RESPONSES ASSOCIATED WITH HEPATOCYTE PROLIFERATION IN THE LIVER OF DIETHYLHEXYLPHthalATE-EXPOSED MICE.

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A number of industrial chemicals cause liver tumours in rodents without damaging DNA directly. Previous studies have suggested that these chemicals induce carcinogenesis by controlling cell growth, proliferation and apoptosis. Assessment of the likely risk to humans posed by nongenotoxic carcinogens will be facilitated by identification of the molecular pathways through which these compounds control cell proliferation. We have investigated these pathways using global gene expression profiling and bioinformatic analyses. Mice were dosed with the model rodent nongenotoxic carcinogen and peroxisome proliferator (PP) diethylhexylphthalate (DEHP) to induce liver growth and peroxisome proliferation within 3 days. Gene expression levels at 2, 8, 24 and 72h were measured using Affymetrix GeneChips and 1, 786 DEHP-responsive genes were identified by ANOVA. Gene Ontology (GO) analysis tools were used to identify the predominant Biological Processes and Cellular Components associated with early DEHP-responsive genes. Consistent with previous studies of DEHP-induced liver cell growth and proliferation, our GO analysis of transcript profiling data revealed a striking overrepresentation of genes involved in acyl-CoA and fatty acid metabolism together with genes associated with the peroxisomal cellular component. Furthermore, this GO analysis revealed gene expression changes associated with a variety of biological functions, including in amino acid metabolism, hemostasis, complement activation, the endoplasmic reticulum overload response and circadian rhythm. Together, these data reveal potential new pathways of PP action and may shed new light on the mechanisms by which nongenotoxic carcinogens control hepatocyte growth and proliferation. Furthermore, our data demonstrate that GO mapping is a powerful approach for elucidating modes of toxicity based on Toxicogenomic data.

**1376** COMPARATIVE ANALYSIS OF GENE NETWORKS AT MULTIPLE DOSES AND TIME POINTS IN LIVERS OF RATS EXPOSED TO ACETAMINOPHEN.

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As a part of systems toxicology, gene interaction network analysis using microarray data has been developed to determine systematic changes of gene expression after chemical exposure. We have developed two algorithms for analyzing gene-expression networks, both based on Bayesian network structures: the TAO-Gen

(Theoretical Algorithm for identifying Optimal Gene interaction networks) algorithms for identifying networks (1) and a statistical algorithm for quantifying network connections (2). In this paper, these methods were further applied to the gene network profiling of a rat liver exposed to acetaminophen (APAP) to compare differentially expressed genes at multiple doses of exposure chemicals and multiple time points after their exposure. APAP is well known for its use in pain relievers and fever reducers. The maximum daily dose is 4 g (66-77 mg/kg b.w.) for adults. Hepatotoxicity of APAP has been considered to be involved in apoptosis and oxidative stress. Therefore, 17 genes related to apoptosis and oxidative stress were chosen based on ontology to determine gene-interaction networks. The behavior of networks that are made up of the 17 genes selected was comparatively analyzed at multiple doses (from a dose equivalent to the human maximum daily dose to a dose 3 times higher) and time points. The results showed that the network structure at the lower doses can be separated from the high dose structure, suggesting that this analysis can detect different gene interactions at lower doses.

### 1377 A SIGNATURE GENE NETWORK APPROACH TO TOXICITY.

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The different physiological responses to toxicant challenge, both protective and pathological, reflect differential activity of the underlying cellular pathways. Although gene expression profiling has been used to classify distinct cellular states, it is still problematic to identify mechanisms of toxicity from these profiles. To address this limitation, we have developed a gene network-based approach that supports identification of the genes and pathways mediating cellular toxicity. Gene expression microarray data is used to identify interacting, differentially expressed genes that are assembled into toxicant-“signature” gene networks. First, using a yeast experimental model, we demonstrate that all of the cellular responses to hydrogen peroxide exposure can be captured by microarray profiling to construct a phenotypic response network for oxidative stress. Next, using data from published mammalian studies, we demonstrate that MetaCoreTM, a computational platform for pathway and gene network analysis, can be used to construct a gene network that identifies the underlying cellular pathways and processes of chemical-induced toxicity. As an example, gene expression microarray data for benzene-induced hematotoxicity has been used to construct static signaling maps that reveal both oxidative stress and of p53 inhibition of apoptosis. To further illustrate our approach, gene network maps have been constructed that distinguish exposure to two hepatotoxins, furan and acetaminophen (APAP), that cause different liver pathologies. For example, the furan-induced network connects up-regulated genes of cell proliferation and cell cycle progression with down-regulated genes for nuclear receptors and several P450s and their transcriptional regulators. In contrast, the regulatory network for APAP includes the energy producing pathways of cellular glycolysis and mitochondrial -hydroxylation. These examples demonstrate the power of signature gene networks to reveal mechanisms of toxicity and represents a novel method for accessing complex tissue-level processes.

### 1378 A GENETIC NETWORK APPROACH TO COMPARATIVE TOXICOGENOMICS AND RISK ASSESSMENT: THE OXIDATIVE STRESS RESPONSE.

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Although the precise mechanisms of chemically induced cellular toxicity are often unknown, they are presumed to be associated with diagnostic changes in gene expression of the proteins that mediate the toxicant response. Comparison of gene expression profiling data from different species reveals a high degree of evolutionary conservation of the functional modules of the underlying genetic network. We are exploiting this conservation to develop a yeast computational model for the highly conserved phenotypic responses to oxidative stress and to use this model to identify cellular patterns of gene expression that will be predictive for the underlying mechanisms of toxicity. As a proof of concept for our approach, we have modeled the gene expression profiles for both protective and adverse oxidative stress responses that are induced by hydrogen peroxide in yeast, and demonstrate that this computational model can identify genetic pathways that are conserved among yeast, animal models, and the human. We have separately visualized the genetic networks of the mitochondrial versus the cytoplasmic responses to hydrogen peroxide-induced stress and demonstrate an intimate coupling of the pathways for energy production and protein biosynthesis between these two cellular compartments. Loss of mitochondrial function is an early adverse consequence of exposure of yeast cells to hydrogen peroxide. Ortholog mapping of conserved genes identifies oxidative phosphorylation as a key process in the mitochondrial genetic response network that is

also a source of cellular free radical damage in response to oxidant challenge. Using this key pathway as the seed to visualize the human mitochondrial proteome network has allowed us to identify key signaling nodes (genetic hubs) in the mitochondrial response. Thus, our genetic network analysis enables comparative toxicogenomics and the construction of predictive models for human risk assessment.

### 1379 PREDICTION OF HEPATOCARCINOGENICITY OF CHEMICALS USING THE GENE EXPRESSION IN THE 28-DAY REPEAT-DOSE TOXICITY STUDY.

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We developed a prediction method for hepatocarcinogenicity of chemicals using the microarray techniques. Male Fischer 344 rats aged 5-week old were treated by daily oral gavages with hepatocarcinogenic and non-hepatocarcinogenic compounds at two dose levels for up to 28 days. The gene expression profiles of the livers were analyzed using the in-house microarray (NEDO-ToxArray) and GeneChip (RGU34A, Affymetrix) on Day-1, 3, 7, 14 and 28 after treatment. Several statistical methods were applied to select the characteristic genes that showed a significant increase or decrease in the gene expression levels between the hepatocarcinogens and the non-hepatocarcinogens. Unsupervised and supervised methods were then adopted to verify the validity of this approach for gene selection. First, in cluster analysis with the selected gene sets, the chemicals were classified into two groups of hepatocarcinogens and non-hepatocarcinogens. Next, in Support Vector Machine with the selected gene sets, prediction of hepatocarcinogens showed good values at high dose on Day-14 and Day-28. In this approach, the selected gene sets were found to be effective to predict hepatocarcinogenicity of the chemicals. This study is supported by NEDO (New Energy and Industrial Technology Development Organization).

### 1380 TOXICOGENOMICS FOCUSING ON THE HEMOPOIETIC STEM CELL TOXICOLOGY.

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The application of toxicogenomics to hematotoxicology should eventually focus on the alteration of the expression profilings of the hematopoietic stem cell/progenitor compartment. Since the hematopoietic system consists of a mixture of heterogeneous cells from the view of not only functionally different cellular lineages, but also different stages in differentiation, an unfractionated blood sample may give different gene expression profiles, and the microarray data from the net tissue may not always provide an efficient and predictable outcome. Specific attention should be paid to the preparation and interpretation of microarray data. As case studies, hematotoxic gene expression profilings after a single dose of 300 cGy irradiation and repeated 300 ppm benzene inhalation, 6 hours a day, 5 days a week, for 2 weeks were introduced as an inductive approach of toxicogenomics, and then, a couple of plausible genes were selected with respect to the “stemness profiling”. In the WT mice, up-regulation of p53 gene was not appeared in 2 weeks after benzene intermittent exposure, but weakly detected in a month after 300 cGy irradiation. Up-regulation of cyclinG1, down stream of p53, was observed after both, benzene inhalation and 300 cGy irradiation, implying that the up-regulation of cyclinG1 should be a relevant reflect to the prolonged DNA damage. When benzene is exposed, down-regulation of caspase 12 and up-regulation of cyclin B1 is seen only when p53 is knocked out (these gene changes may be hidden by p53 gene regulation in the case of WT mice). In the case of benzene exposure, participation of the same gene repertoire is observed even in the WT mice one month after 300cGy irradiation, suggesting the result may be reflect of a possible p53 dysfunction in these irradiated mice.

### 1381 QC METRICS AND THRESHOLDS FOR ASSESSING THE OVERALL QUALITY OF A MICROARRAY STUDY.

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The regulatory review of microarray-based pharmacogenomic data submissions is challenged by the complexity of the DNA microarray experimental process, the use of multiple platforms available in the market, and the lack of standardization across

this process. It is difficult to standardize individual microarray steps for regulatory quality control (QC) purposes, but we believe that platform-independent QC metrics and thresholds for assessing the overall quality of a microarray study can and should be established. Cross-lab/platform comparability is required for the transformation of DNA microarray technology from a research tool to clinical practice, and depends on the availability of such platform-independent QC metrics and thresholds. We have re-analyzed several published data sets and have identified broad differences in data quality. Intra-lab/platform quality control problems were evident for studies that claimed a lack of cross-lab/platform comparability. We have demonstrated problems in data quality as well as the lack of QC metrics and thresholds that objectively assess the overall quality of a microarray study. This process was shown to require independent assessments of the quality of the microarray and the study. It also required appropriate approaches to establish QC metrics and thresholds. Three fundamental questions were addressed: (1) QC Metrics (parameters): How should the quality of a microarray study be assessed? (2) QC Thresholds (cutoffs): Is the quality of a microarray study acceptable? (3) Approaches: What should be done to establish microarray QC metrics and thresholds?

### 1382

#### PROTOCOLS FOR THE ASSURANCE OF MICROARRAY DATA QUALITY AND PROCESS CONTROL.

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Microarrays represent a powerful technology that provides the ability to simultaneously measure the expression of thousands of genes. However, it is a multi-step process with numerous potential sources of variation that can compromise data analysis and interpretation if left uncontrolled, necessitating the development of quality control protocols to ensure assay consistency and high data quality. In response to emerging standards, such as the Minimum Information About a Microarray Experiment (MIAME) standard, tools are required to ascertain the quality and reproducibility of results within and across studies. To this end, a comprehensive quality control protocol for microarrays was developed using cDNA microarrays from *in vivo* and *in vitro* dose-response and time-course studies. The protocol combines: 1) diagnostic plots monitoring the degree of feature saturation, global feature and background intensities, and feature misalignments with 2) plots monitoring the intensity distributions within arrays with 3) a support vector machine (SVM) model. This work has been supported by NIH grants ES11271, ES12245 and Superfund P42 ES04911. Support for LDB and JEE was provided by T32 ES07255 and NCI grant R25 CA92049, respectively. TRZ is partially supported by the Michigan Agriculture Experiment Station.

### 1383

#### DBZACH: A COMPREHENSIVE TOXICOGENOMIC INFORMATION MANAGEMENT AND KNOWLEDGE DISCOVERY SYSTEM.

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dbZach (<http://dbzach.fst.msu.edu>), a Minimum Information About a Microarray Experiment -Toxicology (MIAME/Toxicology) supportive toxicogenomic relational database, uses Java-based data mining and visualization tools to facilitate toxicogenomic data quality assurance and control, and knowledge discovery. Currently, dbZach contains subsystems for the management of in-life and *in vitro* sample annotation, pathology, and toxicity data, cDNA clones and annotation, microarray images, raw and normalized data, and quantitative real-time PCR results. Knowledge from cDNA and Affymetrix microarray experiments are anchored to data from complementary studies (e.g. pathology, clinical chemistry) to facilitate mechanistic understanding of toxicological responses. By managing multi-species data, and leveraging orthologous mappings through the integration of data from Ensembl, direct cross-species comparisons are made. dbZach also streamlines microarray data submittal to repositories (e.g., Gene Expression Omnibus and ArrayExpress) by formatting data in Microarray Gene Expression (MAGE) Markup Language files (MAGE-ML). Future developments for dbZach include management of biological pathway information, and integrated quality control tools. The dbZach System will be made source-available for local implementation as an inde-

pendently-operated laboratory information management and knowledge discovery system. Supported by NIH grants \*T32 ES07255 ES 04911-12, ES 011271 and ES 011777.

### 1384

#### LEVAMISOLE RESIDUES IN CHICKEN TISSUES AND EGGS.

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Levamisole is currently being used to treat capillaria infection in chickens even though there is no published withdrawal information available for levamisole in chickens. The tissue residue withdrawal of levamisole in chickens was studied in thirty-two healthy broiler breeders at the age of thirty-two weeks (peak of egg production). Levamisole residues in chicken tissues, eggs and plasma were determined by high performance liquid chromatography with ultraviolet (HPLC-UV) detection method at wavelength 225 nm. The highest level of residue and longest withdrawal time after oral administration of 40 mg/kg levamisole to chickens was in the liver. On day 3 the level of levamisole were undetectable in the plasma. On day 9 levamisole residue level in eggs was 0.096 ug/g and on day 18 it was 0.06 ug/g or less in all the analyzed chicken tissues, those levels were lower than the recommended Maximum Residue Limit (MRL). The withdrawal time for levamisole in chickens is longer than for other species tested. This is due in part to the larger dose of levamisole that is recommended in chickens. In conclusion from this research, levamisole needs 9 days for eggs to be under the MRL and 18 withdrawal days before slaughter of medicated birds for their tissues to be safe for human consumption.

### 1385

#### FUMONISIN B-GLUCOSE REACTION PRODUCTS ARE LESS TOXIC WHEN FED TO SWINE.

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Reduction of liver toxicity by fumonisin B1 (FB1)-glucose reaction products has been demonstrated in swine via intraperitoneal delivery. The goal of this study was to evaluate this detoxification strategy using a dietary approach. Eighteen 3-week-old pigs divided in 3 treatments (6 pigs/group) were exposed for 15-d to diets containing either 528  $\mu$ mol total FB/kg (FB), FB-glucose products at 528  $\mu$ mol total FB/kg diet containing 23% unreacted FB (122  $\mu$ mol total FB/kg of diet) (FB-G) or 0  $\mu$ mol total FB/kg diet. Data was analyzed by a repeated measurements analysis at  $\alpha=5\%$ . On d 15, aspartate aminotransferase was highest in FB (576 IU/L  $\pm$  83.2) as compared to FB-G (180.7  $\pm$  51.1) and controls (120.2  $\pm$  18.7), which were not different. Levels of  $\gamma$ -glutamyl transferase were highest in FB pigs (250.5 IU/L  $\pm$  48), followed by FB-G (99.3  $\pm$  23.3) and controls (47.3  $\pm$  10.1), which were different. Total bilirubin was highest in FB treated pigs (3.31 mg/dL  $\pm$  1.2), followed by FB-G (0.41  $\pm$  0.3) and control (0.11  $\pm$  0.02) group, which were similar. Serum Sa/So ratio in FB pigs (1.78  $\pm$  0.2) was higher than in FB-G (1.14  $\pm$  0.2) and control (0.5  $\pm$  0.08) pigs, which were different. Liver Sa/So ratio in FB pigs (0.15  $\pm$  0.05) was higher than in controls (0.03  $\pm$  0.009) but similar to FB-G (0.11  $\pm$  0.04). Weight gain in FB pigs (1.75 kg  $\pm$  0.6) was significantly lower than FB-G (5.1  $\pm$  0.5) and controls (4.17  $\pm$  0.6), which correlated with feed intake reduction in FB pigs. At histopathology, FB pigs showed generalized liver necrosis with cellular and nuclear variability and disorganization of tissue. Liver and kidneys in the FB-G and control groups appeared normal. Results suggest that dietary FB-glucose products could substantially protect swine in instances of widespread FB grain contamination.

### 1386

#### THE NATIONAL RESIDUE PROGRAM AS A FOOD SAFETY TOOL.

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The National Residue Program (NRP) is a multi-component analytical sampling effort designed to monitor chemical residues in domestic and imported meat, poultry, and egg products. The NRP is administered by the Food Safety and Inspection Service (FSIS), a public health regulatory agency of the USDA. FSIS, USFDA, and EPA are the key developers of the NRP. They work cooperatively with other organizations to achieve mutual food safety objectives with respect to residues. FDA and EPA have statutory authority for setting tolerances and action levels for chemical residues and for enforcing compliance with those tolerances. This authority is

granted under the FFDCA(FDA) and the FIFRA (EPA). FSIS protects consumers by testing meat, poultry, and egg products for chemical residues and preventing adulterated products from entering the food supply. This testing also allows FDA and EPA to enforce compliance with the tolerances and action levels that they set. In 2003, the FSIS Domestic Monitoring Plan sampled and tested 12 compound classes of drugs and pesticides, comprising approximately 59 residues. Of the 26, 214 Domestic Monitoring Plan samples analyzed, 85 residue violations were found in 85 animals. The residue violations consisted of 36 antibiotics, 20 zeranol, 14 sulfonamides, nine avermectins/milbemycins, four chlorinated hydrocarbon/chlorinated organophosphate, three arsenicals, and two flunixin. No residue violations were found in the testing for arsenicals, chloramphenicol, ractopamine, beta-agonists, melengestrol acetate, and diethylstilbestrol. The most common cause of violations with approved drugs in livestock, poultry, and egg products is a failure to allow an adequate withdrawal time. FSIS sampling focuses on analyzing kidney and liver tissues, since most FDA limits are established for these tissues

**1387 ASSESSMENT OF METALS CONCENTRATIONS IN SALMONBERRIES AND SOURDOCK COLLECTED NEAR A MINING TRANSPORT ROAD IN NORTHWEST ALASKA.**

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Historical transport of ore concentrate over a 50-mile haul road from the Red Dog lead/zinc mine in northwest Alaska to a seaport has been associated with elevated lead levels on moss growing near the road, and with concerns regarding subsistence foods. Initial studies of berries and sourdock, which are part of the local subsistence diet, suggested little impact from mine related activities but results were inconclusive. As part of a comprehensive assessment of potential impacts to human health and the environment associated with metals released during overland transport of ore concentrate, salmonberries and sourdock were evaluated for their potential contribution to dietary metals exposure during subsistence use. In summer 2004, salmonberry and sourdock samples were collected from three traditional harvesting locations, at increasing distance from the port facilities: Site A (1.5 miles NW), Site B (12 miles NW), and Site C (17 miles NW). Washed and unwashed samples were analyzed for lead and cadmium, two metals that had previously been identified as chemicals of concern for the site. Metals concentrations were compared between sites, and to samples collected in 2001 from Site A and from a reference location. Site A salmonberry lead was lower in 2004 than in 2001 ( $p \leq 0.001$ ). Sites B and C berry lead were similar to each other, but slightly lower than in Site A ( $p \leq 0.001$ ). Nevertheless, 2004 berry lead concentrations from all sites were similar to reference conditions. 2004 cadmium concentrations were similar at Sites A, B, and C, and were similar to 2001 Site A and reference concentrations. Sourdock lead and cadmium concentrations were similar at Sites A, B, and C, and in 2001 Site A samples. However, while sourdock lead concentrations at each of the three sites were similar to reference conditions, cadmium concentrations were lower than reference concentrations at all three sites ( $p \leq 0.01$ ). These results suggest that no limitations on subsistence harvest are necessary at any of the sites studied.

**1388 LACK OF EFFECTS OF BETA-CAROTENE DERIVED FROM BLAKESLEA TRISPORA, A NATURAL FOOD COLOR, IN A THIRTEEN-WEEK ORAL TOXICITY STUDY IN F344 RATS.**

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Beta-Carotene derived from Blakeslea trispora is considered equivalent to chemically synthesized beta-carotene. A group Acceptable Daily Intake (ADI) for both synthetic beta-carotene and beta-carotene derived from Blakeslea trispora has been set at up to 5 mg/kg bw (JECFA, 2002). However, safety assessment data for beta-carotene derived from Blakeslea trispora are limited, and therefore the present sub-chronic oral toxicity study was conducted in rats. When groups of 10 male and 10 female F344 animals were fed beta-carotene at dietary levels of 0, 0.2, 1.0 and 5.0 % for 13 weeks, there were no treatment-related adverse effects on body weight, food and water consumption, urinalysis, ophthalmology, hematology and serum biochemistry findings and organ weight data. On clinical observation, red feces were observed in both sexes of the 0.2%, 1.0% and 5.0% group animals and necropsy revealed all rats of the treated groups to have reddish discoloration of the contents of the gastro-intestinal tract, due to pigmentation and thus lacking toxicological significance. On histopathological examination, sporadic spontaneous lesions known occur in this strain of rats were the only findings, with no specific relation to the test compound. Thus, the No-Observed-Adverse-Effect-Level

(NOAEL) was judged to be a dietary level of 5.0% (3127 mg/kg body weight/day for males, 3362 mg/kg body weight/day for females) of beta-carotene derived from Blakeslea trispora under the present experimental conditions.

**1389 DEPLETION OF T CELLS BY MONOCLONAL ANTIBODIES AGAINST MTHY-1.2 ANTIGEN REDUCES FUMONISIN B<sub>1</sub> TOXICITY IN MICE.**

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Fumonisin B<sub>1</sub> is a mycotoxin produced by *Fusarium verticillioides* that is frequently associated with corn. It produces species-specific and organ-specific toxicity, including equine leukoencephalomalacia, porcine pulmonary edema, and hepatic or renal damage in most animal species. Fumonisin B<sub>1</sub> perturbs sphingolipid metabolism by inhibiting ceramide synthase. Our previous studies indicated that fumonisin B<sub>1</sub> caused localized activation of cytokines secreted by macrophages and other cell types, which modulate fumonisin B<sub>1</sub>-induced hepatic apoptosis in male mice. In the current study, male athymic nude (nu/nu) mice and their wild type counterparts (nu/+), with and without depletion of T cells, were treated subcutaneously with 2.25 mg/kg/day of fumonisin B<sub>1</sub> for 5 days and sampled 1 day after the last injection. Depletion of T cells was achieved by intravenous injection of 50 µg monoclonal antibody against Thy-1.2 surface antigen of mature peripheral T lymphocytes, 24 h before first fumonisin B<sub>1</sub> treatment. The fumonisin B<sub>1</sub>-induced elevation in activities of circulating liver enzymes were not different between nu/nu and nu/+ mice, while depletion of T cells in nu/+ mice significantly prevented fumonisin B<sub>1</sub>-mediated liver toxicity, confirmed by analyses of circulating liver enzymes and apoptotic hepatocytes. Since all the fumonisin B<sub>1</sub>-treated groups had similar extent of hepatic sphinganine accumulation; the reduced toxicity of fumonisin B<sub>1</sub> in T cell depleted mice was not due to its effect on sphinganine accumulation. Expression of fumonisin B<sub>1</sub>-induced tumor necrosis factor  $\alpha$  and interleukin-12 was observed in nu/nu and nu/+ mice but not in T cell depleted nu/+ mice. This study suggested that T cells and corresponding proinflammatory cytokines have an important role in mediating fumonisin B<sub>1</sub>-induced hepatic toxicity. (Supported in part by USPHS ES 09403)

**1390 SAFETY EVALUATION OF A NEW ANTI-STALING AMYLASE ENZYME FOR BAKERY APPLICATIONS.**

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This anti-staling product is a maltotetraose-forming amylase enzyme produced with a recombinant strain of *B. subtilis*. The enzyme was protein-engineered to increase thermostability and exo-specificity needed for bakery applications. Toxicology studies designed to demonstrate the product is safe for its intended uses were performed following the guidelines recommended by Pariza and Johnson (2001). Acutely, anti-staling amylase is non toxic by ingestion. No genotoxicity was observed in a bacterial reverse mutation assay at dose levels up to 5240 µg total protein/plate and an *in vitro* human lymphocyte chromosomal aberration test up to moderately toxic concentrations, both with and without metabolic activation. In a 90-day oral toxicity study in Sprague-Dawley rats, daily gavage of anti-staling amylase up to and including a dose level of 314.1 mg total protein/kg/day (509.4 mg TOS/kg/day; 17.5 mg enzyme protein/kg/day) did not result in any biological or statistical differences in body weight, feed consumption, hematology, clinical chemistry, organ weights, and clinical observations. In the open field testing, decreases in ambulatory movements were noted in treated females but were still within the historical control ranges collected at the testing facility. There were no histopathologic changes noted in treated animals. The NOAEL was established at 17.5 mg enzyme protein/kg/day. The average consumption of flour from bakery products is 3.25 g/kg bw/day and the typical dosage of anti-staling amylase is 0.5 to 4.0 mg enzyme protein/kg flour. The estimated daily intake of anti-staling amylase is thus 0.0016 to 0.0130 mg/kg bw. The NOAEL of 17.5 mg enzyme protein/kg/day would offer more than a 1000 X margin of safety. Collectively, the historic safety profile of the production organism *B. subtilis* and the results of the toxicology studies attest to the safety of this anti-staling amylase for bakery applications.

**1391 TIME- AND DOSE-DEPENDENT CHANGES IN SPHINGOID BASE 1 PHOSPHATES IN TISSUES FROM RATS FED DIETS CONTAINING FUMONISINS.**

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Fumonisins (FB) are mycotoxins found in corn. They are inhibitors of ceramide synthase (CS), a key enzyme in de novo sphingolipid biosynthesis. Inhibition of CS results in a marked increase in free sphinganine (Sa), an intermediate in de novo

biosynthesis, in rat liver and kidney; the primary target organs in FB fed rats. There is a close correlation between elevation in Sa and the extent and severity of toxicity in rodent liver and kidney. Sa is toxic to cultured cells and is believed to contribute to the liver and kidney toxicity of FB, however, inhibition of CS also decreases ceramide formation and potentially, increases levels of Sa-1-phosphate (Sa-1-P) and sphingosine (So)-1-phosphate (So-1-P). The present study was conducted to quantify the time- and dose dependent changes in Sa, So, Sa-1-P and So-1-P in kidney and liver of male Sprague-Dawley rats fed diets containing 0, 5 ppm and 50 ppm FB1 for 10 days. These tissues were microscopically examined for increased apoptosis and other FB-induced toxicity. At 50 ppm FB1, renal toxicity was first evident on day 3 and was maximal on day five, whereas, Sa and Sa-1-P were first elevated at day 1 and maximally elevated at day 5 to similar levels. So-1-P was first elevated on day 3 and maximally elevated on day 5, however, the level of elevation was 50 fold less than Sa-1-P. Neither So-1-P nor Sa-1-P was detected in control kidney. At 5 ppm FB1, renal toxicity was first evident on day 5 and was more severe on day 10, whereas, Sa was first elevated on day 1 and increased throughout the 10 day study. Sa-1-P and So-1-P were first elevated at days 3 and 5, respectively, and were further increased on day 10. Compared to kidney, liver toxicity was much more subtle, with an obvious but moderate effect only being seen in the high dose group on days 5 and 10 and was preceded by increases in Sa on day 3. There was no So-1-P or Sa-1-P detected in liver from control or FB1-fed animals. The high level of Sa-1-P in kidney from FB-treated rats suggests its involvement in FB-induced renal toxicity.

### 1392 TOXICOLOGY STUDIES ON ALLANBLACKIA SEED OIL.

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Allanblackia seed oil is derived from the seed of the Allanblackia tree (*Allanblackia stuhlmanni*) which grows in the tropical rain forest of Africa. The oil has a unique fatty acid composition, as it is composed predominately of stearic acid and oleic acid. This fatty acid profile and the arrangement of the fatty acids on the triglyceride backbone make the oil highly suitable for use in foods such as margarine. The oil has not been consumed to any significant extent outside Africa and has very little food use in the countries where Allanblackia trees grow such as Ghana and Tanzania. A programme of toxicology studies was conducted to confirm that Allanblackia seed oil is safe for use in food. Allanblackia seed oil was tested in two *in vitro* mutagenicity assays, an Ames test and mouse lymphoma assay, and a 90-day repeat dose oral toxicity rat study. Allanblackia seed oil was non-genotoxic in both *in vitro* assays. In the 90-day study Allanblackia seed oil was administered to Wistar (Cr: (WI) WU BR) rats at a dose level of 20% in a synthetic diet (AIN-93G). A high fat control diet containing 20% shea oleine was also tested in the study alongside a standard control diet with 7% soya oil. Minor changes in haematology parameters, clinical chemistry parameters and organ weights were observed in the Allanblackia seed oil group compared with the standard control. None of the organ weight changes were accompanied by histopathological alterations. These changes also occurred to a similar or lesser extent in the shea oleine control group indicating that they were associated with a high fat diet rather an adverse effect of Allanblackia seed oil. These data demonstrate that Allanblackia seed oil is safe for use in food.

### 1393 SAFETY AND EFFICACY POTENTIAL OF A NOVEL ANTHOCYANIN-RICH MULTIPLE BERRY EXTRACT IN *IN VIVO* MODELS.

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Edible berry extracts rich in anthocyanins possess a broad spectrum of therapeutic and anti-carcinogenic properties. Anthocyanins repair and protect genomic DNA integrity. Six berry extracts (wild blueberry, bilberry, cranberry, elderberry, raspberry seeds, and strawberry) were studied for antioxidant efficacy, cytotoxic potential, cellular uptake, and anti-angiogenic properties. Recently we evaluated various combinations of edible berry extracts and developed a synergistic formula, OptiBerry, which exhibited high ORAC value, low cytotoxicity, and superior anti-angiogenic properties compared to the other combinations tested. This study was focused to determine the broad spectrum safety and antioxidant efficacy of OptiBerry. We investigated the acute oral, acute dermal, primary dermal irritation and primary eye irritation toxicity of OptiBerry. Acute oral LD<sub>50</sub> of OptiBerry was found to be greater than 5, 000 mg/kg in both male and female Sprague-Dawley rats. Acute dermal LD<sub>50</sub> of OptiBerry was found to be >2, 000 mg/kg. No changes in body weight or adverse effects were observed following necropsy. In New Zealand albino rabbits, primary skin and primary eye irritation tests were also conducted. OptiBerry was classified as slightly irritating to the skin and minimally irritating to the eye. We assessed the antioxidant potential of OptiBerry in rats and mice using EPR imaging for whole-body redox status and glutathione levels by

HPLC. This study employed a clinically relevant hyperbaric oxygen (HBO) exposure system to study the antioxidant properties of OptiBerry. OptiBerry feeding (8 weeks) significantly prevented HBO-induced GSH oxidation in the lung and liver of vitamin E deficient Sprague-Dawley rats. Furthermore, OptiBerry fed mice (2 weeks), when exposed to HBO, demonstrated significant protection against HBO-induced oxidation of mice compared to the unfed controls by EPR imaging. Taken together, these results indicate that OptiBerry is safe and possesses antioxidant properties in addition to being anti-angiogenic.

### 1394

#### A 90-DAY DIETARY TOXICITY STUDY OF HEATED DAG OIL AND HEATED TG OIL IN RATS.

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This study evaluated the possible toxic effects of heated DAG oil (diacylglycerol oil) and heated TG oil (Triacylglycerol oil) when given in the diet at levels up to 5.5% (55, 000 ppm) for 90 days to rats. DAG oil is a cooking oil which contains >80% diglycerides, <20% triglycerides and <5% monoglycerides. The heated oils were prepared by taking DAG oil and TG oil separately and batch frying potato slices in deep fryers at 180 degrees Celsius for 8 hours per day on three consecutive days. The treated oils were then stored frozen until use on study. A special diet was prepared so that all of the dietary fat could be provided by heated DAG oil or unheated DAG oil at varied concentrations. Dietary concentrations (% heated DAG oil/% unheated DAG oil) of 0%/5.5% (unheated DAG oil control), 1.0%/4.5%, 2.75%/2.75%, and 5.5%/0% were presented daily, seven days per week, for a minimum of 90 days. Two additional groups received the special diet with 5.5% of unheated TG oil or 5.5% of heated TG oil. The TG oil contained >85% triglycerides, <10% diglycerides and <5% monoglycerides. The fatty acid composition for DAG oil and TG oil was closely matched. All groups consisted of 10 male and 10 female Cr:CD(SD)IGS BR rats and were fed ad libitum. The clinical condition of the animals, body weights, body weight gains, food consumption, ophthalmic examinations, functional observational battery and motor activity, clinical pathology evaluations, organ weights, gross and histopathologic findings were unaffected by either heated DAG oil or heated TG oil compared to the unheated oils or when the results in the two 5.5% heated oil groups were compared. Feeding diets prepared with the heated DAG oil or heated TG oil resulted in no obvious toxic effects. Thus, the no-observed-effect levels (NOELs) for dietary exposure of heated DAG oil or heated TG oil were 5.5% (55, 000 ppm), the highest levels tested, under the conditions of this study.

### 1395

#### ACCUMULATION OF SPHINGOID BASES AND SPHINGOID BASE 1-PHOSPHATES: A POSSIBLE MECHANISM FOR FUSARIUM VERTICILLIOIDES CORN-SEEDLING DISEASE.

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Sphingolipids are important structural components of membranes involved in signaling pathways that regulate cell growth and death. Fumonisins (FB) are water soluble mycotoxins produced by *F. verticillioides*, which is parasitic to corn. FBs are inhibitors of ceramide synthase (CS), a key enzyme in sphingolipid biosynthesis. Inhibition of CS causes an increase in sphinganine and sphingosine (phytosphingosine in plants), as well as their respective 1-phosphates, which is used as a biomarker for FB activity. While FB is not known to cause plant disease in the field, it is found in the ear, roots, and stalks of corn. The objectives of this study were to determine the dose- and time-dependent effects of FB1 in soils on i) root development and ii) disruption of sphingolipid metabolism in roots. Sterilized corn seeds were germinated and planted in sterile potting soil (10/pot) and watered with solutions of FB1 (0, 1, 5, 10, and 20 µg/ml). The seedlings were dosed with FB1 on days 2, 4, and 6 after planting, followed by ddH<sub>2</sub>O as needed until harvest. The experiment was split into two time groups (3 reps per dose), the first group was harvested on day 8 and the second group was harvested on day 21. There was a dose dependent reduction in root mass in the 21 day time group at 5, 10, and 20 µg/ml FB1, while there was a slight increase in root growth at 1 µg/ml FB1. FB1 was detected in the soils as well as the roots, and the levels were closely correlated with the FB1 dosage. There was a dose dependent elevation in sphingoid bases and their 1-phosphates in the root tissues from both time groups. These results show that under laboratory conditions FB1 in soils can i) affect root growth and alter seedling performance, ii) FB1 in soil can be taken up by roots, iii) FB1 in soils can cause marked dose-dependent elevation in sphingoid bases and their 1-phosphates that precede reduced root growth.

DEVELOPMENTAL TOXICITY OF DIETS CONTAINING FUMONISIN B<sub>1</sub> TO LM/BC AND CD1 MICE: A COMPARATIVE STUDY.

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Fumonisin mycotoxins are found in corn and corn-based food. Their health effects in humans are unknown, however, it has been suggested that they might be a risk factor for neural tube defects (NTDs) in populations consuming contaminated corn as a dietary staple. Fumonisin B<sub>1</sub> (FB<sub>1</sub>) was not teratogenic when given orally to pregnant CD1 mice during gestation days (GD) 7-15 whereas intraperitoneal injection of  $\geq 5$  mg/kg BW FB<sub>1</sub> to pregnant LM/Bc mice on GD 7.5-8.5 caused NTDs. To compare NTD susceptibility in these strains, female LM/Bc (n=8/group) and CD1 mice (n=10/group) were fed diets containing 0 (control), 50 or 150 ppm FB<sub>1</sub> (provided by *F. verticillioides* culture material) beginning 5 weeks before mating. Pregnant females and their litters (mating produced  $\geq 5$  litters/strain-group) were examined for gross malformations, especially NTDs in the fetuses, by necropsy after GD16. The high-dose diet was maternally toxic to both strains as established by microscopic examination of the maternal livers. Within each strain, no unequivocal effects on mating, fertility, maternal weight, and litter size were noted. Neither fetotoxicity nor NTDs were found in control or low-dose litters of either strain. One of five (20%) LM/Bc high-dose litters was positive for NTDs (1 of 10 fetuses was affected). In contrast to the LM/Bc strain, no NTDs were found in the high-dose CD1 litters but fetotoxicity was found in two of the litters (n=9) from this group; the incidence of dead fetuses therein ranged from 40 to 64%. These results suggest that (a) the dietary NOEL for NTDs in LM/Bc mice is  $> 50$  ppm FB<sub>1</sub>; (b) NTDs develop at maternally toxic doses of FB<sub>1</sub>; (c) strain-dependent differences in sensitivity to fetotoxicity and NTDs exist; and (d) studies are needed to define the NTD dose-response and the physiological factors underlying NTD development in FB<sub>1</sub>-fed mice.

## A 90-DAY DIETARY STUDY ON CARRAGEENAN WITH EMPHASIS ON THE GASTROINTESTINAL TRACT.

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Carrageenan is a food additive, approved globally for food use. Recent publications have alleged carrageenan as a carcinogen. In response, a GLP subchronic dietary toxicity study in rats was conducted on commercial kappa carrageenan, molecular weight distribution at 4.6% below 50k Daltons. The molecular weight was tested by triple detection (right angle/low angle light scattering, viscometer & refractometer). Groups of Fischer 344 rats (20/sex/group) received control or treated diets at levels of 0, 2.5, and 5.0% for 90 days. Homogeneity, stability and verification of dose level in test diets were determined weekly. Clinical examinations were done daily. Individual food consumption/body weight measurements were made weekly. Ophthalmic exam was conducted prior to and at the end of treatment. Hematology/serum chemistry and urinalysis evaluations were done at necropsy, as were organ weight determinations for adrenals, brain, heart, kidneys, liver, ovaries, spleen, testes, and thyroid with parathyroids. Full histopathological evaluation of organs was conducted on the control and 5.0% groups. Special microscopic examinations of hematoxylin-eosin stained cross sections of paraffin-embedded rolled colon were conducted in control and high-dose animals. Clinical signs were limited to soft feces in high dose animals and to a lesser extent in low dose animals. There were no treatment-related effects on body weights or hematology and urinalysis parameters. Lower serum albumin and cholesterol levels in high dose males were not considered to be biologically significant. There were no treatment-related effects on organ weights or ophthalmic, macroscopic, or microscopic findings. The gastro-intestinal tract appeared normal in detailed histopathology. The NOAEL is 5% in the diet (3394 and 3867 mg/kg/day in males and females, respectively).

## TANNIC ACID IS A PROOXIDANT AND INDUCES CYCLOOXYGENASE-2 IN HUMAN COLON CELLS.

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Tannic acid (penta-*m*-digalloyl glucose) is a common dietary constituent present in a variety of foods, beverages and medicinal products. It is generally thought to be an antioxidant and in some studies has been shown to exhibit antimutagenic and anti-carcinogenic properties. At low levels in the diet, tannic acid reduces spontaneous

hepatomas in C3H mice and has been proposed as a chemopreventative agent for colon cancer. In the present studies we demonstrate that tannic acid also possess prooxidant activity which may be cytotoxic for colon cells. We found that, in the presence of transition metals, tannic acid (10-100  $\mu$ M) generates hydroxyl radicals as measured by the hydroxylation of terephthalate, an aromatic trapping agent. Hydroxyl radical production by tannic acid was pH dependent and optimal at pH 7.4. Using plasmid DNA unwinding assays, hydroxyl radical production by tannic acid was found to be sufficient to cause single and double strand DNA breaks. In human colon CX-1 cells, we found that tannic acid readily induced lipid peroxidation. This was associated with induction of cyclooxygenases-2 (COX-2) mRNA, an important gene product known to regulate inflammatory mediator production in the colon. Inhibition of tannic acid-induced production of reactive oxygen intermediates by DMSO (0.5%), a hydroxyl radical trap, was found to suppress plasmid DNA strand breaks and inhibit lipid peroxidation and expression of COX-2 in CX-1 cells. Taken together, our data suggest that dietary tannic acid has prooxidant activity that can damage DNA and cause production of cytotoxic inflammatory mediators via production of hydroxyl radicals. Production of reactive oxygen intermediates by tannic acid and subsequent cellular injury may contribute to the development of colon cancer. Supported by NIH grants ES05022, ES007148, CA093798 and CA100994.

## GENOTOXICITY STUDIES ON DIETARY DIACYLGLYCEROL (DAG) OIL.

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Dietary Diacylglycerol (DAG) oil is an edible oil enriched in DAG (more than 80%). A recent investigation indicated that DAG oil or its components may have beneficial effects on the prevention and management of obesity. We evaluated the genotoxic potential of DAG oil using standard genotoxicity tests. Bacterial reverse mutation assay (Ames test), the chromosomal aberration assay in cultured Chinese hamster lung cells (CHL/IU), and a bone marrow micronucleus assay in ICR CD mice were employed in the present study. In addition we have tested the possibility that genotoxic substances may be formed during cooking, heated DAG oil was prepared by batch frying potato slices in the oil at 180°C for 8 hr/day for 3 consecutive days. Therefore, genotoxicity tests were also performed on heated DAG oil. Results obtained did not show any genotoxic effect on either unheated DAG oil or heated DAG oil. We conclude that there are no safety concerns on the genotoxicity of DAG oil under the conditions for normal use.

## ISOTHERMAL MODELS FOR THE PREDICTION OF NUTRIENT INTERACTIONS WITH MYCOTOXIN SORBENTS.

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A variety of sorbent materials are frequently added to animal feeds to bind and reduce the bioavailability of mycotoxins in the gastrointestinal tract. However, the safety of many of these products has not been established. Importantly, sorbents that are nonselective may interact with nutrients, minerals, and other feedborne chemicals to pose significant hidden risks. The purpose of this study was to develop a rapid screening method to assess dietary sorbent interactions with selected nutrients. A variety of clay-based materials and activated carbon, were tested for their ability to sorb vitamin A and riboflavin from aqueous solution. Equilibrium adsorption isotherms were run for each sorbent using a modification of methods previously established. Vitamin A and riboflavin were analyzed with a UV-VIS spectrophotometer at 292 nm and at 445 nm, respectively. The maximum capacities ( $Q_{\max}$ ) and affinity constants ( $K_d$ ) at 25°C for the sorption of each vitamin were determined with a computer fitted Langmuir equation. An organophilic montmorillonite clay and an activated carbon strongly sorbed vitamin A with  $Q_{\max}$  values of 4.00 mol/kg and 0.552 mol/kg, respectively. Affinity constants ( $K_d$ ) for these sorbents were determined to be  $1.94 \times 10^4$  and  $4.15 \times 10^5$ , respectively. Sodium montmorillonite and carbon also exhibited significant binding of riboflavin ( $Q_{\max} = 0.193$  mol/kg;  $K_d = 1.95 \times 10^5$  and  $Q_{\max} = 0.267$  mol/kg;  $K_d = 1.48 \times 10^6$ , respectively). Other sorbents including NovaSil clay showed negligible interaction with either vitamin. In previous studies, NovaSil has been shown to bind aflatoxins with high affinity *in vivo*. These experiments confirm the selectivity of this clay and suggest that isothermal analysis can be utilized to predict interactions of dietary sorbents with important nutrients. Further studies will assess the ability of isothermal modeling to delineate interactions of sorbents with other important feed additives (NIEHS P42 ES04917, USAID LAG-G-00-96-90013-00).

**1401**

EFFECTS OF A LOW-CARBOHYDRATE DIET ON MARKERS OF OXIDATIVE STRESS IN TISSUES FROM DIABETIC AND NON-DIABETIC RATS.

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Increased oxidative stress in diabetes is partly attributed to the generation of reactive molecules from elevated blood glucose levels, with consequent development of secondary complications. Because a low-carbohydrate (low-carb) diet improves glycemic parameters, this study examined whether a low-carb diet (carbohydrates 5.5%, fats 68% by calories) confers beneficial effects on the oxidative status of the heart, kidney and liver in diabetes. Male and female normal and streptozotocin-induced diabetic rats were fed standard chow (carbohydrates 63%, fats 11%, by calories) or low-carb diet for 30 days. Elevated glucose, HbA1c, and alanine and aspartate aminotransferases in diabetic animals were reduced or normalized by the low-carb diet. Diabetes increased cardiac activities of glutathione peroxidase and catalase relative to controls. The low-carb diet normalized cardiac glutathione peroxidase in diabetic animals, and reduced catalase in the female rats. In kidney, glutathione peroxidase activity was increased in diabetic animals relative to non-diabetic controls and was not corrected by the diet. Low-carb fed diabetic rats had increased and decreased activities of renal glutathione reductase and SOD relative to diabetic animals on the control diet. No significant differences were observed between normal controls and normal low-carb diet rats except for increased renal glutathione peroxidase activity in the male rats. In liver, diabetes was associated with a decrease in catalase activity and glutathione levels and an increase in glutathione peroxidase and  $\gamma$ -glutamyltranspeptidase activities. Decreased hepatic glutathione peroxidase activity and MDA content were noted in diet-treated diabetic rats. No diet-induced changes in superoxide dismutase were noted in any tissue. Overall, the low-carb diet helped stabilize hyperglycemia and did not produce overtly negative effects in tissues of normal or diabetic rats.

**1402**

CHRONIC TOXICOLOGICAL EVALUATION OF NOVASIL CLAY IN THE DIET OF SPRAGUE-DAWLEY RATS.

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NovaSil (NS) clay, a common anti-caking agent in animal feeds, has been reported to diminish bioavailability and adverse effects of aflatoxins in short-term studies. The potential toxicity of long-term dietary exposure to NS has not been determined. Five to six week old male and female Sprague-Dawley rats were fed rations containing 0, 0.25, 0.5, 1.0, or 2.0% (w/w) levels of NS for 28 weeks. NS showed negligible levels of dioxin and furan contaminants. Octachlorodibenzo-p-dioxin (2.34 ng/kg) was the only contaminant found above the limit of detection (LOD = 1.02 ng/kg). Total feed consumption, cumulative feed consumption, body weight, total body weight gain, feed conversion efficiency, and relative organ weights were unaffected in either sex at the doses tested. At necropsy, there were no abnormalities in tissues of interest (liver, kidneys, lungs, heart, brain, spleen, tibia, uteri and ovaries). Analysis of whole blood showed no significant differences in hematological parameters between NS-fed rats and controls with the exception of males consuming 0.25% NS (higher mean corpuscular hemoglobin). Red and white blood cells appeared normal and morphologically similar. Serum biochemical analysis was generally normal but indicated that Ca was slightly increased in 0.5% and 2.0% NS-treated females. Serum and hepatic vitamin A and vitamin E were unchanged in all groups except females consuming 1.0% NS and showing a slight increase in serum vitamin A. Serum Fe and Zn levels were unchanged except in males consuming 1.0% NS which showed increased Fe. However, the difference observed did not demonstrate dose-dependency. Results suggest that dietary inclusion of NS at levels as great as 2.0% (w/w) does not result in overt toxicity. These findings (as well as others) support the use of NS clay dietary interventions for human populations at high risk for aflatoxicosis (NIEHS P42 ES04917, USAID LAG-G-00-96-90013-00).

**1403**

ACRYLAMIDE, HEAVY METAL COMBINATION TOXICITY AND GLUTATHIONE DEPLETION.

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Acrylamide (AA) has recently been detected as contamination in heated food products. In higher concentrations it is neurotoxic and clastogenic and has been classified as a possible human carcinogen. Although it has low toxicity in cell cultures of hepatic, renal and intestinal cells its toxicity is increased by addition of heavy met-

als like Hg or Cd, but not As. Since AA as well as its metabolite glycinamide are partly detoxified by glutathione coupling, combination toxicity may be related to additive glutathione depletion. In order to clarify the mechanism of AA toxicity singly and in combination we measured glutathione content and depletion of glutathione by L-buthionine sulfoximide (1 mM BSO) and determined cytotoxicity in parallel by neutral red uptake and LDH release. In renal LLC-MK2 and intestinal I-407 cells treatment of cells by BSO for 24 hours and additional AA treatment with 0, 1  $\mu$ g/ml for another 24 hours cytotoxicity was not detectable, in accordance with the lack of AA toxicity in control cells. In hepatic Hep G2 cells LDH release was increased and neutral red uptake decreased in parallel at the highest AA concentrations tested (>0.06  $\mu$ g/ml); control Hep G2 cells were not affected by AA up to 100  $\mu$ g/ml. Compared to control cells addition of AA did slightly decreased glutathione content by 25% in either of these cell lines. Glutathione depletion has been shown to increase cytotoxicity of all three heavy metals in cells and *in vivo*. Therefore, data will be presented on cellular glutathione changes by heavy metals, effect of glutathione depletion on heavy metal and AA cytotoxicity and the relationship of combination toxicity to alterations in glutathione changes.

**1404**

SAFETY ASSESSMENT OF LYSINE MAIZE.

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Maize grain used in animal diets is deficient in the essential amino acid lysine. A new maize variety has been developed for animal feed that has increased levels of lysine. Although this variety will be identity preserved for use in poultry and swine feed, it might inadvertently enter the human food chain. Therefore, the human food safety assessment of lysine maize (LM) grain was undertaken, including analysis of the introduced protein (*Corynebacterium* dihydrodipicolinate synthase - cDHDPS). cDHDPS has the same function as the DHDPS protein present in maize, but is not feedback inhibited by lysine. Bioinformatic analysis revealed that the protein is not structurally or functionally related to known protein toxins or allergens, and the function of this enzyme raise no safety concerns. cDHDPS is readily degraded *in vitro* in simulated gastric fluid. cDHDPS did not produce adverse effects in mice when given as a single dosage of 800 mg/kg BW, which provides a large safety margin compared to potential human dietary exposures. The high dose acute mouse test is appropriate for assessing protein safety since most known protein toxins act through acute mechanisms. Compositional analysis of the grain was also undertaken to assess the level of increased lysine content and to characterize any changes in lysine-related metabolites. The composition of LM was comparable to that of conventional maize varieties, with the exception of increased lysine and its catabolites, saccharopine and alpha-amino adipate. There is a history of safe consumption of these catabolites as they are present in common foods. Dietary exposure to lysine catabolites from ingestion of common foods exceeds exposures from inadvertent human food use of LM. In addition, grain from LM, along with the control and several conventional maize varieties, was also fed to rats for 90 days at levels up to 33% in the diet. There were no adverse effects in rats fed LM grain, and the rat dietary exposure considerably exceeded potential human exposure, should the grain inadvertently enter the human food chain. The weight of evidence supports the safety of LM for farm animal and human consumption.

**1405**

EVALUATION OF THE SUBCHRONIC TOXICITY OF 1507XNK603 TRANSGENIC MAIZE.

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Three groups of adult male and female rats (12/sex/group) were fed diets formulated with a transgenic maize line (1507xNK603) containing genes that express the Cry1F, CP4 EPSPS, and PAT proteins, for approximately 90 days. The grain received 2 in-field applications of glufosinate and/or glyphosate herbicides. Four additional groups were fed diets formulated with a near isoline, non-transgenic hybrid maize line, or one of three non-transgenic, commercial hybrid maize lines. The Cry1F protein provides insecticidal activity against lepidopteran pests. The CP4 EPSPS and PAT proteins provide tolerance to herbicides containing glyphosate and glufosinate-ammonium, respectively. All grains and diets were analyzed for nutrients, fiber/energy, amino acids, minerals, vitamins, and contaminants and found to be comparable. Grain and diets were also analyzed for transgenic genes and/or proteins (presence and activity). No diet-related differences were observed among groups fed the different diets, with respect to body weight and nutritional parameters, clinical signs, ophthalmology, neurobehavioral assessments, clinical pathology, organ weights, and gross or microscopic pathology. Under the conditions of this study, diets containing a transgenic strain of maize, irrespective of herbicide treatment, produced no adverse effects in rats, compared to diets containing a non-transgenic, near isoline strain of maize or a non-transgenic, commercial strain of maize.

**1406**

INDUCTION OF COLON TUMORS IN C57BL/6J MICE FED MEIQX, IQ OR PHIP FOLLOWED BY DEXTRAN SULFATE SODIUM TREATMENT.

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MeIQx, a heterocyclic amine, has been shown to induce tumors in several organs including the liver, but consistently failed to induce intestinal tumors in rodents whereas the other heterocyclic amines IQ and PhIP exert colon tumorigenicity in rats. Recently, however, we found that dietary MeIQx induces genotoxicity in the colon as well as the liver of 2 different types of reporter gene-transgenic mice at subcarcinogenic doses such as 300 ppm. In the present study, in order to clarify a possibility whether MeIQx can induce colon tumors, C57BL/6J mice that have been used for both transgenic models were fed MeIQx, IQ or PhIP at a dose of 300 ppm for 12 weeks, and thereafter twice received 1-week treatment with dextran sulfate sodium (DSS) apart 2 weeks. After twenty weeks, colon tumors including adenocarcinomas were found at incidences of 26%, 24% and 45% in the groups receiving MeIQx, IQ and PhIP, respectively, which were significantly ( $P<0.05$  or 0.01) different from the DSS alone value (0%). On the other hand, MeIQx did not significantly induce any tumors in C57BL/6J mice or gpt delta mice even when fed at 300 ppm for 78 weeks, suggesting that the treatment of MeIQx alone was not sufficient to promote colon tumors. Thus our results clearly indicate that in addition to IQ and PhIP, MeIQx can induce colon tumors in mice under a colon tumor-promotional condition.

**1407**

INHIBITION OF AFB<sub>1</sub> ACTIVATION AND AFB<sub>1</sub>-DNA ADDUCT FORMATION BY DIETARY BUTYLATED HYDROXYTOLUENE IN TURKEYS.

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Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is potent hepatotoxin and a common contaminant of foods and feeds. Poultry are extremely susceptible to this mycotoxin, a condition we have shown to be associated with a combination of efficient hepatic cytochrome P450 (CYP)-mediated activation and deficient glutathione S-transferase (GST)-mediated detoxification. Previously, we showed that dietary butylated hydroxytoluene (BHT) protects against nearly all clinical signs of aflatoxicosis in turkeys. To determine the mechanism of chemoprevention, we examined the effect of BHT on CYP-mediated activation and GST-mediated detoxification of AFB<sub>1</sub>. Our data indicates that BHT appears to act primarily by inhibition of AFB<sub>1</sub> activation, rather than by induction of protective GSTs. Liver microsomal activation of AFB1 to AFBO was inhibited by BHT by apparent classic Michaelis competitive kinetics ( $K_i = 5.38$  mM). Likewise, dietary BHT significantly inhibited hepatic microsomal AFB<sub>1</sub> activation in addition to related CYP indicator activities. Dietary BHT also significantly reduced AFB<sub>1</sub> bioavailability as well as hepatic AFB<sub>1</sub>-DNA adduct formation at several time points compared to control birds after oral administration of [<sup>3</sup>H]-AFB<sub>1</sub>. Despite induction of hepatic GST indicator activities, we found no measurable effect toward specific AFB<sub>1</sub> detoxification by GST. These data support our hypothesis that the observed *in vivo* chemoprevention by BHT is due to inhibition of hepatic CYP-mediated AFB<sub>1</sub> activation to reactive toxic intermediate(s). (Supported in part by USDA-National Research Initiative grants 97-3081 and 02-35204-12294)

**1408**

SAFETY ASSESSMENT AND RISK/BENEFIT ANALYSIS OF THE USE OF AZODICARBONAMIDE IN BABY FOOD JAR CLOSURE TECHNOLOGY: PUTTING TRACE LEVELS OF SEMICARBAZIDE EXPOSURE INTO PERSPECTIVE.

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Trace levels of semicarbazide (SEM) discovered in bottled foods (especially baby foods) led to action by the European Food Safety Authority (EFSA) to eliminate possible exposure to this hydrazine compound. This would be via a ban in 2005 in Europe of the jar sealing technology known as Press On-Twist Off ("PT") closures. Azodicarbonamide, which is used in PT closures for the formation of a hermetic seal, partially degrades with the heat of processing to form SEM. The potential risks of SEM have been evaluated in the context of the benefits of the PT technology. SEM shows limited genotoxicity *in vitro* that is largely prevented by S9. Negative results were found *in vivo* in DNA alkaline elution assays and in UDS assays. This is in contrast to the genotoxic hydrazines that also have been shown to cause tumors. Carcinogenicity studies of SEM are of limited quality and show a questionable weak effect in mice and no effect in the rat at high doses, which are not relevant

to human exposure at trace levels. IARC has assigned SEM as Group 3, "not classifiable as to its carcinogenicity to humans". Based on exaggerated estimates of exposure to infants consuming baby foods compared to a NOAEL in developmental toxicity studies, the margin of safety is more than 21,000. Since the risk of an adverse effect is negligible, it is clear that any theoretical risk is outweighed by the benefits of continuing use of the PT closure to ensure both the microbial integrity and availability of commercial baby foods as a valuable source of infant nutrition.

**1409**

INHIBITORY EFFECTS OF PLATYCODON GRANDIFLORUM ON 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE (NNK)-INDUCED LUNG TUMORIGENESIS IN A/J MICE.

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The present study was undertaken to estimate the effect of aqueous extract from the roots of *Platycodon grandiflorum* A. DC, Changil (CK) on 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung tumorigenesis in A/J mice. Mice were given an intraperitoneal injection of NNK to induce pulmonary neoplasms. They also received CK as a drinking water as night for 23 weeks, starting 1 week after the NNK injection. Treatments with CK reduced the incidence of lung tumors. CK treatment lowered the multiplicity of lung neoplasms with statistical significance. Immunohistochemically, CK reduced proliferating cell nuclear antigen (PCNA)-positive index in the lung tumors without affecting PCNA index in hyperplastic alveolar cell lesions. These results suggest that CK inhibits the development of lung tumors in mice treated with NNK, due to the suppression of initiation stage.

**1410**

FOUR WEEKS EXPOSURE TO A NOVEL NUTRITIONAL MIXTURE CONTAINING A SERIES OF POLYPHENOLIC PHYTOCHEMICALS ANTAGONIZES ACETAMINOPHEN-INDUCED HEPATOTOXICITY *IN VIVO*.

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From a disease-prevention perspective, recent progress in phytochemical and nutraceutical research clearly suggests 'benefits outweigh the risk' pattern. Powerful antioxidant properties have been the most acclaimed mechanism of action for these entities. Surprisingly, however, no studies have attempted to evaluate the combined antitoxic potential of a phytochemical-nutraceutical mixture (PNM) in *in vivo* models. Therefore, this study was designed to investigate whether pre-exposure to a unique PNM has the ability to impede mechanistic events involved in acetaminophen (AP)-induced hepatotoxicity. Besides several vitamins and minerals, key phytochemical components of PNM were: citrus flavonoids, red wine polyphenols, Garcinia, Gymnema, Ginkgo, Ephedra sinica, Camelia sinensis, Silybum, Guarana, Eluthero, Allium sativum and Ocimum basilicum extracts. To assess PNM's anti-toxic properties, groups of animals (ICR mice, 3 months old) received either a control diet or PNM containing diet for 4 weeks. On day-28, half of each group received saline i.p., while the other half of each group of mice received AP 400 mg/kg i.p. All the animals were sacrificed 24 hours after AP exposure. Serum and liver samples were analyzed. AP caused massive liver injury (ALT U/L: Control- 26±2; AP: 12880±1320) and oxidative stress (%Lipid peroxidation, MDA: Con-100%; AP:268%) coupled with genomic DNA fragmentation (Control-100%; AP:288%). PNM-exposure significantly reduced animal mortality and all the AP-induced events (In PNM+AP: ALT- 1397±122 U/L; MDA-125%; DNA frag-122%) including apoptotic and necrotic cell deaths. Collectively, our investigation suggests that PNM-exposure can serve as a much more powerful blend in antagonizing toxicants rather than phytochemical or nutraceutical alone, and PNM may potentially shield vital target organs from toxicities caused by intentional or accidental exposures to structurally and functionally diverse drug and chemical entities.

**1411**

*IN VITRO* EFFICACY OF PLANT-EXTRACTS USED FOR ALTERNATIVE TREATMENT OF SNAKEBITES IN NIGERIA.

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Snakebites are a significant cause of death in Nigeria. Many citizens come in contact with deadly snakes on a daily basis. Due to high cost of conventional antivenom many victims patronize native doctors who use decoctions from plant ex-

tracts. This study describes the *in vitro* protective effects of a variety of plant extracts used for treating snakebite victims. Active ingredients were extracted from plant aqueous homogenates with 50% methanol. The extracts were tested for protective activity against the venoms of *Echis ocellatus* (*EO*), *Bitis arietans* and *Naja naja nigricollis* (*NN*) that are either neurotoxic, haemotoxic or myotoxic. Protective effects of the extracts against corresponding toxic activities of the venoms were tested in the chick biventer cervicis (cbc) muscle preparation, the egg embryo anti-haemorrhagic assay and cytotoxicity assay in skeletal muscle cells ( $C_2C_{12}$ ). Extracts that showed protective effects against some or all of the toxic effects of the venoms were further fractionated by bioassay-guided chromatographic separation methods to obtain active fractions or pure compounds. Five plant extracts showed protective activities against the toxic effects of the snake venoms. *Parkia biglobosa* (*PB*) stem bark extract for instance, significantly ( $p \leq 0.001$ ) protected the cbc muscle preparation from *NN* venom-induced inhibition of neurally evoked twitches when added to the bath 3–5 min before or after the venom. It reduced the loss of agonist responses to acetylcholine, carbachol and KCl, which are normally inhibited by *NN* venom. The extract (75–300  $\mu$ g/ml) significantly ( $p \leq 0.05$ ) protected  $C_2C_{12}$  cells against the cytotoxicity of *NN* and *EO* venoms. Egg embryos exposed to lethal concentrations of *EO* venom for  $\geq 12$  h were protected by *PB* extracts. The extract (5–10  $\mu$ g/1.5 ml) completely blocked the haemorrhagic activity of the venom. Plant extracts thus offer an alternative to antivenom therapy.

#### 1412

#### IMMUNOMODULATING AND ANTIOXIDANT EFFECTS OF MEXICAN STRAINS OF LENTINUS LEPIDEUS, GANODERMA APPLANATUM AND ARMILLARIA TABESCENS.

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A number of bioactive molecules (polysaccharides), including antitumor substances, have been identified in many Basidiomycetes mushrooms species growing in Asia. Those substances produce their antitumor effects by activating different immune responses in the host. In spite of the great number of mushrooms species growing in Mexican forests most of them have scarcely been investigated. In this study we evaluated the antitumoral, immunomodulating and antioxidant activity of aqueous extracts prepared from *Lentinus lepideus*(L.l), *Ganoderma applanatum*(G.a) and *Armillaria tabescens*(A.t) collected in Nuevo Leon, Mexico and cultivated *in vitro*. Aqueous extracts were administered p.o. at 20 mg/Kg to Balb/c mice and immunomodulating activity was evaluated according to the technique of Cunningham. Chang liver cells and HepG2 cells were exposed to the extracts (0.24 to 500 mg/mL) for 72 h and selective cytotoxicity was evaluated by the MTT test. The antioxidant effect was evaluated by the dichlorofluorescein diacetate (DCFDA) fluorescent probe in Chang liver cells. No significant difference between the CT50 of neoplastic and benign cells was found with any of the extracts. L.l and A.t increased the immune response by 84% and 55% respectively. L.l, G.a and A.t produced an inhibition of 75%, 65% and 49% of the xantine oxidase activity. Considering the results obtained in this study fractioning of the extracts of L.l, G.a and A.t will be carried out in order to know which fraction has the active principles.

#### 1413

#### NUCLEAR TRANSLOCATION OF NF-KB/REL COMPONENT BY PCSC22 ISOLATED FROM PORIA COCOS SCLEROTIUM.

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The sclerotium of *Poria cocos* Wolf, which grows on the roots of pine trees, has long been used as a sedative and diuretic (Chang and But, 1987). The accumulating data revealed that certain ingredients of the sclerotium of *Poria cocos* showed anti-tumor activities (Kanayama, 1986). Although the mechanism of anti-tumor activity is not known, the polysaccharides may potentiate the host defense mechanism through the activation of immune system. In the present study we show that PCSC22, a polysaccharide isolated from the sclerotium of *Poria cocos* with one percent sodium carbonate, significantly induces nitric oxide (NO) production and inducible NO synthase (iNOS) transcription. To further investigate the mechanism responsible for the induction of iNOS gene expression, we investigated the effect of PCSC22 on the activation of NF-kB/Rel, whose binding site was located in the promoter of iNOS gene. Immunohistochemical staining of p65 and p50 showed that PCSC22 produced strong induction of NF-kB/Rel nuclear translocation. Electrophoretic mobility shift assay (EMSA) and reporter gene assay further confirmed the activation of NF-kB/Rel by PCSC22. In conclusion, we demonstrate that PCSC stimulates macrophages to express iNOS gene through the activation of NF-kB/Rel.

#### 1414

#### SAFETY AND TOXICOLOGICAL EVALUATION OF A NOVEL NIACIN-BOUND CHROMIUM.

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Chromium is an essential trace element required for normal protein, fat and carbohydrate metabolism. Niacin-bound chromium (NBC) is a unique form of bioavailable chromium that promotes a healthy lipid profile. This study was carried out to determine the safety of NBC. Acute oral, acute dermal, primary dermal irritation and primary eye irritation toxicity of NBC was evaluated. Ames bacterial reverse mutation assay, mouse lymphoma test and a dose-dependent 90-day subchronic toxicity was also conducted. In safety studies, the acute oral LD<sub>50</sub> of NBC was greater than 5,000 mg/kg in male and female Sprague-Dawley rats. No changes in body weight or adverse effects were observed following necropsy. Acute dermal LD<sub>50</sub> of NBC was greater 2,000 mg/kg. NBC was classified as slightly irritating in primary skin irritation testing in New Zealand Albino rabbits. Primary eye irritation test was conducted with NBC on rabbits. NBC was non-irritating to the eye. NBC did not induce mutagenic effects in bacterial reverse mutation test in five *S. typhimurium* strains (TA1535, TA98, TA100, TA97a and TA102), either with or without metabolic activation. Similarly, NBC didn't induce mutagenic effects in the mammalian cell gene mutation test in L5178Y mouse lymphoma cells TK (+/-), either with or without metabolic activation. A subchronic oral toxicity study was conducted at doses of 5, 50 and 125 ppm NBC for 90 consecutive days. No significant changes in selected organ weights individually and as percentages of body and brain weights were observed at 90 days of treatment. NBC supplementation did not cause changes in hepatic lipid peroxidation and DNA fragmentation after 30, 60 or 90 days of treatment. Hematology, clinical chemistry and histopathological evaluations revealed no abnormal findings. Taken together, the above results demonstrate the safety of NBC.

#### 1415

#### INHIBITORY EFFECTS OF KAHWEOL AND CAFESTOL ON CARRAGEENAN-INDUCED INFLAMMATION IN MOUSE AIR POUCH MODEL.

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Recent studies have shown the chemoprotective effects of kahweol and cafestol, which are coffee-specific diterpenes. This study investigated the effects of kahweol and cafestol on the inflammatory response induced by carrageenan in the mice. Air pouches were induced subcutaneously on the backs of mice and injected with carrageenan. The mice were treated with vehicle, kahweol, or cafestol at a dose of 5–10  $\mu$ g/kg one hour before carrageenan challenge. After carrageenan challenge (48 h), the air pouches were removed and analyzed. The volume, protein amounts, and cell count in the exudation obtained from the kahweol, or cafestol-treated mice were significantly reduced compared to those from vehicle-treated animals. The contents of prostaglandin E2, TNF-alpha and the mRNA for cyclooxygenase-2 were also suppressed in these animals. The histological examination displayed the suppression of the inflammatory response in the pouch tissues from kahweol, or cafestol-treated mice. Histological examination showed suppression of the inflammatory response in the pouch tissue from kahweol, or cafestol-treated mice.

#### 1416

#### UP-REGULATION OF CYCLOOXYGENASE-2 EXPRESSION BY CHICKEN OVALBUMIN IN MURINE MACROPHAGES.

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Chicken ovalbumin is one of the major egg white allergens that cause IgE-mediated food hypersensitivity. In the present study, we investigated the effects of ovalbumin on the cyclooxygenase-2 (COX-2) gene expression in the mouse macrophage cell line RAW 264.7. Ovalbumin significantly increased the prostaglandin E2 production and the expression of COX-2 mRNA and COX-2 protein in a dose-dependent manner. To investigate the significant cis-acting regions which COX-2 promoter, transient transfection experiments were carried out using reporter vectors harboring deleted COX-2 promoters. The transcriptional factor binding sites for activator protein 1 (AP-1) and NF-kB between -574 and -51 could be important for the induction of COX-2 by ovalbumin. The results of these studies suggest that induction of transcriptional activation of COX-2 by ovalbumin might be mediated through the AP-1 and NF-kB activation.

ANTIOXIDATIVE EFFECTS OF *PAECILOMYCES TENUIPES* ON CARBON TETRACHLORIDE-INDUCED HEPATOTOXICITY IN PRIMARY CULTURES OF ADULT RAT HEPATOCYTES AND *IN VIVO*.

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*Paecilomyces tenuipes* (PT), which is one of the cordyceps species belonging to *Ascomycetes*, has been used for medicinal purposes due to its wide physiological activities. The protective effects of PT against carbon tetrachloride (CCl<sub>4</sub>)-induced toxicity in adult rat hepatocytes were investigated in the present studies. After the monolayer was obtained by culturing the hepatocytes for 4 hrs, the medium was changed with fresh medium. Then PT extracts at 1, 2 and 5 mg/ml and/or CCl<sub>4</sub> at 4 mM were treated directly to the culture medium for 24 hrs. PT extracts protected the levels of glutathione, oxidized glutathione, glutathione peroxidase and catalase from reduction by CCl<sub>4</sub>. For *in vivo* studies, Sprague-Dawley rats were administered with PT extracts at 0.25, 0.5 and 1.0 g/kg p.o. once daily for 7 consecutive days. Thirty minutes after the final administration with PT, the rats were treated i.p. with CCl<sub>4</sub> at 0.5 ml/kg. PT extracts also protected the levels of glutathione, oxidized glutathione, glutathione peroxidase and catalase from reduction by CCl<sub>4</sub>. These results indicated that PT extracts might have hepatoprotective activities against CCl<sub>4</sub> by the protection of hepatic sulphydryl depletion.

NATURAL PRODUCTS DISCOVERY THROUGH DEVELOPMENT THE CHALLENGES AND THE OPPORTUNITIES.

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The WHO estimates that approximately 80% of the world population relies primarily on traditional medicines as sources for their primary health care. Over 100 chemicals that are considered to be important drugs that are either currently in use or have been widely used in one or more countries in the world have been derived from a little under 100 plants. Approximately 75% of these substances were discovered as a direct result of studies focused on the isolation of active substances from plants used in traditional medicine. Drug development historically has relied only on a small number of molecular prototypes to produce new medicines. Only approximately 250 discrete structural prototypes have been used up to 1995 and most of these chemical platforms have been derived from natural sources. While recombinant proteins and peptides are gaining market share, low molecular weight compounds still remain the predominant choice for therapeutic intervention. The overwhelming concern today in the pharmaceutical industry is to improve the ability to quickly find new drugs. This will only be successfully accomplished if the procedures for drug target elucidation and lead compound identification and optimization are themselves optimized. However, the ability to accelerate the identification of pertinent lead compounds will only be achieved with the implementation of new ideas to generate varieties of structurally diverse test samples. Natural products are the most potent sources of new drug leads. This is because the degree of chemical diversity found in natural products is broader than that from any other source. However, natural products present challenging problems in the management of their discovery programs as well as the management of their development programs. Of particular note are the issues of purification, synthesis, identification and formulation. Lessons learned from prior problems and failures in development are presented. The use of totally new strategies for discovery and solutions to the problems of development of natural products need to be considered and are presented.

PRO-APOPTOTIC EFFECTS OF THE TROPICAL GINGER COMPOUND, 1'-ACETOXYCHAVICOL ACETATE, ON HUMAN BREAST CANCER CELLS *IN VITRO*.

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The tropical ginger compound, 1'-acetoxychavicol acetate (ACA) possesses cancer chemopreventive properties in several models (oral mucosa, skin, colon) but its effects on breast cancer have not been evaluated. To determine whether ACA may also be effective at blocking mammary carcinogenesis, preliminary studies were performed in human breast cancer derived MCF-7 and MDA-MB-231 cells. The effects of ACA on cell viability were assessed using 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonylphenyl)-2H-tetrazolium (MTS) and Trypan Blue exclusion analysis. ACA significantly decreased cell viability in a time-and dose-dependent manner using both assays, with effective concentrations  $\leq 10 \mu\text{M}$ . Morphological examination of cells through light microscopy, as well as, 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) staining, suggested ACA induced apoptosis. To further investigate its pro-apoptotic effects, Annexin V/Alexa Fluor 88 staining was visualized using Flow Cytometry. At 6 h and 24 h after treatment with ACA (10-50  $\mu\text{M}$ ), there was a significant dose-dependent increase in the percentage of cells undergoing late apoptosis in both cell lines. We hypothesized that

ACA may have inhibitory effects on ornithine decarboxylase (ODC), a putative oncogene upregulated in most tumors, that metabolizes polyamines that are essential for cell growth. ACA (10-50  $\mu\text{M}$ , 6 h) blocked ODC activity by 90-99% and 84-85% in MCF-7 and MDA-MB-231 cells, respectively. These results suggest ACA may have potential chemoprotective effects against the later stages of breast cancer. This research was sponsored by a Grant-in-Aid (Intramural Award).

NOTOGINSENG ATTENUATES LPS-INDUCED PRO-INFLAMMATORY MEDIATORS IN ANTIGEN PRESENTING CELLS.

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The USFDA does not regulate herbal or dietary supplements as drugs although many may have associated therapeutic effects and/or toxicities. In this regard, the immunomodulatory effects of *Panax notoginseng* on cultured dendritic cells (DC2.4) and macrophages (RAW 264.7) were investigated. Notoginseng inhibited the production of LPS-induced TNF $\alpha$  by the phagocytic cells in a concentration-dependent manner as measured by ELISA. TNF $\alpha$  production by both the dendritic cells and macrophages was inhibited in samples treated with notoginseng 24 hours prior to and concurrent with LPS addition, but not in samples treated 8 hours after LPS stimulation. Also, COX-2 and IL-1 $\beta$  mRNA levels in RAW264.7 cells stimulated with LPS were inhibited by notoginseng. The effects of notoginseng on expression of the accessory molecules CD40, CD14, CD86 and TLR4 on phagocytic cells stimulated with LPS were analyzed by flow cytometry. CD86 expression was decreased while CD14 expression was unaffected following exposure to 50  $\mu\text{g}/\text{ml}$  notoginseng. There was also a trend towards a decrease in CD40 expression and an increase in TLR4 expression in both the macrophages and dendritic cells. The immunomodulatory effects of Rb1 and Rg1 ginsenosides, the purported bioactive components of our notoginseng extract, were investigated. Both Rb1 and Rg1 inhibited TNF $\alpha$  levels in the RAW264.7 cells following LPS stimulation, whereas only Rg1 inhibited TNF $\alpha$  production by DC2.4 cells. However, neither purified ginsenoside compound inhibited LPS-induced TNF $\alpha$  production to the same extent as the whole notoginseng extract suggesting that additional, as of yet unidentified, compounds probably contribute to the overall bioactivity of this herbal extract. Collectively, these results indicate that notoginseng inhibits the activation of RAW264.7 and DC2.4 cells and demonstrate that notoginseng possesses anti-inflammatory properties *in vitro*. This work was supported by a grant from NSF-EPSCoR (EPS0091995).  $\alpha\beta\mu$

EFFECTS OF MURINE CYP1A1 IN MOUSE HEPATOMA HEPA-1C1C7 CELLS BY CATECHIN AND 3', 4', 3, 5, 7-O-METHYLATED CATECHIN.

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Green tea catechin is known to exert chemopreventive effects in many cancer models. In the present study, we investigated the effect of catechin and 3', 4', 3, 5, 7-O-methylated catechin on 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxine (TCDD)-inducible P450 1A1 gene expression in mouse hepatoma Hepa-1c1c7 cells. 7-ethoxresorufin O-deethylase (EROD) activity in the Hepa-1c1c7 cells was significantly increased by 3', 4', 3, 5, 7-O-methylated catechin but not catechin. TCDD-induced cytochrome CYP1A1-specific EROD activity was markedly reduced in the concomitant treatment of TCDD and 3', 4', 3, 5, 7-O-methylated catechin but not catechin. TCDD-induced CYP1A1 mRNA level was also markedly suppressed in the concomitant treatment of TCDD and 3', 4', 3, 5, 7-O-methylated catechin but not catechin. A transient transfection assay using dioxin-response element (DRE)-linked luciferase and electrophoretic mobility shift assay revealed that 3', 4', 3, 5, 7-O-methylated catechin reduced transformation of the aryl hydrocarbons (Ah) receptor to a form capable of specifically binding to the DRE sequence in the promoter of the CYP1A1 gene. These results suggest the down regulation of the CYP1A1 gene expression by 3', 4', 3, 5, 7-O-methylated catechin in Hepa-1c1c7 cells might be antagonism of the DRE binding potential of nuclear Ah receptor.

SUPPRESSION OF MURINE CYP1A1 IN MOUSE HEPATOMA HEPA-1C1C7 CELLS BY FORMONONETIN.

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Isoflavone phytoestrogen, formononetin, is 4-O-methyl derivatives of daidzein. In the present study, we investigated the effect of formononetin on 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxine (TCDD)-inducible P450 1A1 gene expression in mouse hepatoma Hepa-1c1c7 cells. TCDD-induced cytochrome CYP1A1-specific

7-ethoxyresorufin O-deethylase (EROD) activity was markedly reduced in the concomitant treatment of TCDD and formononetin in a dose dependent manner. TCDD-induced CYP1A1 mRNA level was also markedly suppressed in the concomitant treatment of TCDD and formononetin. A transient transfection assay using dioxin-response element (DRE)-linked luciferase and electrophoretic mobility shift assay revealed that formononetin reduced transformation of the aryl hydrocarbons (Ah) receptor to a form capable of specifically binding to the DRE sequence in the promoter of the CYP1A1 gene. These results suggest the down regulation of the CYP1A1 gene expression by formononetin in Hepa-1c1c7 cells might be antagonism of the DRE binding potential of nuclear Ah receptor.

#### 1423 CAFFEIC ACID PHENETHYL ESTER SUPPRESSED IN B16F10 MELANOMA CELL METASTASIS

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Metastasis of cancer cells to distant sites is one of the major deciding factors in cancer outcome. In fact, prognosis of cancer is mainly determined by the invasiveness of the tumor and its ability to metastasize. In this study, we assayed the preventive and therapeutic effects of Caffeic acid phenethyl ester (CAPE) derived from honey-bee propolis on the experimental lung metastasis induced by B16F10 melanoma cells in C56BL6 mice. CAPE clearly inhibited both B16F10 melanoma cell adhesions to the extracellular matrix proteins as well as invasion through Matrigel-coated filter. In addition, CAPE significantly inhibited the proliferation of B16F10 melanoma cells in a dose-dependent manner. In B16F10 melanoma lung metastasis experiment, CAPE showed remarkable inhibitory effects on the lung tumor colonization in a dose-dependent manner. These results demonstrate that anti-metastatic activity of CAPE resulted from blocking of proliferation and invasion of the melanoma cells. Taken together, the results of our study provide evidence that CAPE possess an anti-metastatic activity.

#### 1424 SUPPRESSIVE EFFECT OF GAMMA-TOCOPHEROL ON PROSTATE CANCER DEVELOPMENT IN PB/SV40 T ANTIGEN TRANSGENIC RATS.

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Vitamin E is the general name for a family of eight different compounds which is one of the most researched in medicine.  $\gamma$ -Tocopherol is the major form of vitamin E, but has drawn little attention compared with  $\alpha$ -tocopherol, the predominant form of vitamin E in tissues and the primary form in supplements. Epidemiological studies have indicated that plasma concentrations of  $\gamma$ -tocopherol are inversely associated with the risk of prostate cancer. In addition, recent studies have indicated that  $\gamma$ -tocopherol exerts inhibitory effects on growth of prostate cancer cell lines. However, *in vivo* studies of effects of  $\gamma$ -tocopherol on prostate carcinogenesis have not been reported. The aim of the present study was to evaluate the effects of  $\gamma$ -tocopherol on prostate cancer development using the probasin(PB)/simian virus 40(SV40) T antigen transgenic rat established in our laboratory. Five week old male PB/SV40 T antigen transgenic rats were given  $\alpha$ - (50mg/kg diet) or  $\gamma$ -tocopherol (50, 100mg/kg diet) in vitamin E free diet for 10 weeks. There were no treatment-related changes with reference to body or relative prostate weights, as well as food consumption. Furthermore, there were no significant differences in the incidences of prostate adenocarcinomas in any of the prostate lobes. However, quantitative analysis of proliferative lesions revealed a significant suppressive effect of  $\gamma$ -tocopherol in the ventral ( $P<0.05$ ) and dorsal lobes ( $P<0.01$ ), with dose-dependence. The results thus indicate that  $\gamma$ -tocopherol may suppress rat prostate carcinogenesis. Investigations of potential mechanisms of the observed anticancer effects are now on-going.

#### 1425 SUBCHRONIC GAVAGE TOXICITY STUDY OF GINKGO BILOBA EXTRACT IN FISCHER 344 RATS AND B6C3F<sub>1</sub> MICE.

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There is limited information pertaining to the toxicity of the widely used dietary supplement *Ginkgo biloba* extract (GBE). The purpose of this study was to evaluate the toxicity of GBE in 0.5 percent aqueous methylcellulose administered to male and female Fischer 344 (F344) rats and B6C3F<sub>1</sub> mice. Rats were administered 0, 62.5, 125, 250, 500, and 1000 mg/kg GBE (at 5 mL/kg) and mice were administered 0, 125, 250, 500, 1000 and 2000 mg/kg GBE (at 10 mL/kg) by the gavage route for up to 14 weeks. All treated rats were similar to controls in overt behavior and in general health and appearance. Weekly and terminal mean body weights of GBE treated rats were similar to control. Slight, but statistically significant increases in mean total protein and albumin concentrations were observed in male rats at all

dosages. Group mean liver and liver-to-body weights were statistically significantly increased in all dosage groups. Histopathological observations included centrilobular and midzonal hepatocellular hypertrophy in GBE treated rats of both sexes and midzonal hepatocellular vacuolization in treated males. Thyroid hyperplasia was observed in all GBE treated rats. Weekly and terminal mean body weights of GBE treated mice were similar to control. Ruffled fur was observed in 1000 and 2000 mg/kg male mice between weeks 7 and 8 and weeks 5 and 9, respectively. There were no alterations in measured hematologic parameters. Increased mean liver and liver-to-body weights were observed at all male and female mouse dosages. Decreased mean kidney and kidney-to-body weights were observed in 2000 mg/kg male mice. Organ weight changes were correlated with histopathological findings. Hepatocellular hypertrophy was noted in all male and female treatment groups and decreased renal cytoplasmic vacuolization was noted in male mice at  $\geq 500$  mg/kg. In conclusion GBE treatment to F344 rats resulted in hepatic and thyroid effects while hepatic and renal effects were observed in B6C3F<sub>1</sub> mice. This work was supported by NIEHS Contract No. N01-ES-35502

#### 1426 INHIBITORY EFFECT OF KAHWEOL AND CAFESTOL ON TGF-BETA-INDUCED FIBROSIS IN HEPATIC STELLATE CELLS.

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Activation of hepatic stellate cells and increasing of collagen content have been identified as a critical step in hepatic fibrogenesis and are regulated by several factors including cytokines and oxidative stress. In this study, we investigated the effects of kahweol and its dehydro derivative, cafestol, two diterpenes that are present in considerable quantities in coffee beans, on hepatic fibrosis in hepatic stellate cells. We report that kahweol and cafestol decreases TGF-beta-stimulated collagen I/III mRNA expression. The mRNA expressions of TGF-beta-induced collagen type I/III were measured by RT-PCR. As the results, kahweol and cafestol inhibited collagen I/III mRNA expression in a dose dependent manner. Furthermore, the effects of kahweol and cafestol on expression of alpha-smooth muscle actin (a-SMA) and collagen type I/III, were evaluated utilizing immunocytochemistry. Kahweol and cafestol reduced expression of a-SMA and collagen type I/III proteins compared with TGF-beta-induced hepatic stellate cells. Moreover, kahweol and cafestol decreased TGF-beta-induced proliferation of hepatic stellate cells. These results suggested that the effects of kahweol and cafestol on the hepatic fibrosis in stellate cells may, at least in part, be due to its ability to reduce proliferation and collagen synthesis.

#### 1427 ANTI-ANGIOGENETIC EFFECTS OF CAFESTOL AND KAHWEOL ON HT-1080 FIBROSARCOMA CELLS.

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Cafestol (CA) and its dehydro derivative, Kahweol (KA), are two diterpenes that are present in considerable quantities in coffee beans, as well as in the final, unfiltered beverage e.g. in Turkish or Scandinavian style coffees. They have been shown to possess both adverse and chemoprotective properties. In the present study, we identified the effect of CA and KA on angiogenesis. A cytotoxicity assay of CA and KA in HT-1080 fibrosarcoma cell showed the decrease effect in cell viability by a dose-dependent manner but no significant influence on the growth of human microvesSEL endothelial cell-1 (HMEC-1). CA and KA inhibited the vascular endothelial growth factor (VEGF)-induced tube formation of HMEC-1 in dose-dependent manner. VEGF production from HT-1080 cells was also inhibited by treatment with CA and KA. CA and KA inhibited both VEGF-induced vessel formations in the C57BL/6 mouse matrigel plug assay. Taken together, the results of our study provide evidence that CA and KA possess an anti-angiogenic potential that is a new insights and previously unrecognized biologic activity.

#### 1428 GREEN TEA INFUSION PREVENTS DUAL PROMOTING EFFECTS OF PENTACHLOROPHENOL, AN ENVIRONMENTAL POLLUTANT, ON HEPATO- AND CHOLANGIO-CARCINOGENESIS OF MICE INDUCED BY DIETHYLNITROSAMINE.

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In order to explore a possibility of custom drinking of green tea infusion being efficacious to reduce the carcinogenic risk induced by environmental exposure to pentachlorophenol (PCP), we examined the effects of green tea infusion on the dual

promoting action to hepato- and cholangio- carcinogenesis in mice exposed to DEN. In the first experiment, groups of 15 male mice were initially treated with diethylnitrosamine (DEN) at a dose of 20 ppm in the drinking water for the first 8 weeks followed a 4 week recovery interval by PCP at concentrations of 0 (basal diet), 300 and 600 ppm in the diet for 23 weeks. Further groups of animals were treated with DEN and PCP in the same manner as above and 2 % green tea infusion (GT) instead of the drinking water was given 2 weeks before and during PCP exposure. PCP exposure at the high dose promoted DEN-induced hepatocarcinogenesis, and also progressed cystic hyperplasias of intrahepatic bile ducts to cholangiocellular tumors. Co-administration of GT was able to prevent the increases of incidences and multiplicities of DEN-induced hepatocellular tumors due to PCP exposure and also arrest the progression of cystic hyperplasias to cholangiocellular tumors by PCP treatment. In the second experiment, co-treatment with GT in the drinking water from 1 week before 300 or 600 ppm PCP treatment in the diet to the end of the experiment at week 3 in B6C3F1 male mice was able to inhibit increases of serum ALT activities, 8-oxodeoxyguanosine (8-oxodG) levels in liver DNA and labeling indices (LIs) of bromodeoxyuridine (BrdU) in hepatocytes and intrahepatic biliary epithelia induced by PCP. These findings suggest a possibility of usual intake of green tea reducing the carcinogenic risk caused by an environmental pollutant, PCP which might involve oxidative stress in its carcinogenesis.

#### 1429 TWO-GENERATION REPRODUCTION STUDY OF TEAVIGO™ (EPIGALLOCATECHIN GALLATE) IN RATS.

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Epigallocatechin gallate (EGCG) is a principal polyphenolic compound of green tea and has received wide attention for its potential anti-cancer and cardioprotective effects. Although several *in vivo* toxicity studies have been reported, the effects of EGCG on reproduction and fertility are not clearly documented. Sprague-Dawley rats were administered 0, 1200, 3600 or 12000 ppm Teavigo™ (91% EGCG) in their diet for two consecutive generations, commencing 10 weeks prior to F<sub>0</sub> mating and continuing through to the maturation and reproductive evaluation of the F<sub>1</sub> generation. Although the highest dosed F<sub>0</sub> rats showed signs of reduced body weight and food consumption there was no influence on their mating, fertility or survival. However, a slight increase in the post-natal mortality of their offspring was observed. Also, a slight delay in sexual maturation of the F<sub>1</sub> animals receiving 12000 ppm was noted, which is likely due to the reduced growth rate of these rats. No obvious evidence of toxicity effects on reproduction, or reduced fertility was observed among adult animals dosed at 3600 ppm and histology showed no abnormal findings. A slight increase in pup mortality in the 3600 ppm group was attributed to the increased food consumption and increased EGCG intake by nursing dams. Also, weanling pups had EGCG plasma levels higher than in the adults. Teavigo™ was found to have a NOAEL of 3600 ppm for general toxicity, fecundity and fertility in adult rats. A NOAEL of 1200 ppm is posited for effects on growth and development of nursing offspring.

#### 1430 SUPPRESSION OF LIPOPOLYSACCHARIDE-ACTIVATED CYCLOOXYGENASE-2 EXPRESSION BY BIOCHANIN A IN MURINE MACROPHAGE RAW 264.7 CELLS.

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Biochanin A is an isoflavonoid phytoestrogen found in soybean and possesses anti-tumorigenic, anti-oxidant, anti-microbial properties. In the present study, we investigated the effects of biochanin A on the cyclooxygenase-2 (COX-2) gene expression, which plays a crucial role in many physiological and pathological processes in macrophages. Treatment with biochanin A significantly decreased the production of LPS-induced prostaglandin E2 and the expression of COX-2 mRNA in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages in a dose-dependent manner. Transient transfection experiments also showed that LPS-induced increase in COX-2 promoter activities was suppressed by biochanin A. This study suggests that modulation of COX-2 expression by biochanin A may be important in the prevention of inflammation.

#### 1431 RUTAECARPINE IS AN AGONIST OF THE ARYL HYDROCARBON RECEPTOR.

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Rutaecarpine is a major quinazolinocarboline alkaloidal component of *Evodia rutaecarpa* Bentham (Rutaceae). Rutaecarpine has been characterized to the effects of cytochrome P450 s (CYPs) metabolism *in vivo* and reported as a potent inhibitor of

CYP1A2 in both mouse and human liver microsomes. In the present study, we investigated the effect of rutaecarpine on CYP1A1 gene expression in mouse hepatoma Hepa-1c1c7 cells. Cultured mouse hepatoma Hepa-1c1c7 cells were treated with rutaecarpine to assess the role of rutaecarpine on CYP1A1 expression. CYP1A1-specific 7-ethoxyresorufin O-deethylase (EROD) activity in the Hepa-1c1c7 cells was significantly increased by rutaecarpine. Furthermore, rutaecarpine caused an increase in the level of CYP1A1 mRNA, indicating that it may be an agonist of the aryl hydrocarbon receptor (AhR). A transient transfection assay using dioxin-response element-linked luciferase and electrophoretic mobility shift assay revealed that rutaecarpine increased transcription of a reporter vector containing the CYP1A1 promoter. These results suggest that rutaecarpine might be an agonist of the AhR in Hepa-1c1c7 cells.

#### 1432 EXPRESSION PROFILING OF ESTROGEN RESPONSIVE GENES FOR PHYTOESTROGENS BY DNA MICROARRAY ASSAY.

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Phytoestrogens have attracted significant attention because of their ability to modulate the action of estrogen, which can be used for anti-cancer agents, hormone therapeutic agents, dietary additives and supplements, and antimicrobial agents. However, the gene networks and molecular pathways by which phytoestrogens regulate these events are only partially understood. Understanding more about the gene function networks and signal transduction pathways initiated or mediated by these compounds, we here examined phytoestrogens such as isoflavones, flavones, flavonols, and coumestrol, naringenin and phloretins by using a DNA microarray assay. Of the 172 estrogen responsive genes, expression profiles of phytoestrogens were compared using correlation coefficients, or R values, after a correlation analysis by linear regression. While R values indicate the similarity of the response by the genes, we also examined the genes by cluster analysis and by their specificity to phytoestrogens (specific to isoflavones, flavonols, flavones and all except with ipriflavone) or gene functions. Several genes were selected from p53-related genes, Akt2-related genes, MAPK-related genes, Ras superfamily genes and AP-1 family and related genes. We further examined the extracts from two local crops of soy beans such as Kuro-daizu or Mochi-daizu by comparing the gene expression profiles with those of a natural estrogen or phytoestrogens as a first step in utilizing the expression profiles for various applications. The approach described here would be useful for analyzing plant extracts for various purposes including screening new estrogenic compounds, examining food processing methods, a quality control of food products and understanding the effects of phytoestrogens. Supported by the Preventing Public Pollution from the Ministry of the Environment of Japan.

#### 1433 MICROARRAY ANALYSIS OF T-2 TOXIN-INDUCED APOPTOTIC LESIONS OF THE LIVER, PLACENTA AND FETAL LIVER IN PREGNANT RATS.

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T-2 toxin is a trichothecene mycotoxin produced by *Fusarium* spp. It is known that T-2 toxin induces apoptotic lesions in many tissues. It is also reported that pregnant mice treated with T-2 toxin exhibited fetal death and fetotoxicity in addition to maternal toxicity, and apoptosis was also observed in these systems. However, the mechanism of T-2 toxin-induced toxicity in pregnant rats is unknown. The purpose of the present study is to examine the T-2 toxin-induced gene expression changes in pregnant rats. Pregnant rats on day 13 of gestation were treated orally with 2 mg/kg of T-2 toxin and sacrificed at 1, 3, 6, 9, 12 and 24 hours after the treatment. Histopathologically, the number of apoptotic cells was increased in the dams liver, placenta and fetal liver after the treatment. To examine the gene expression profiles, we performed GeneChip microarray analysis in these tissues at 3 selected time points based on the results of TUNEL-staining. As the results, the increased expressions of oxidative stress- and apoptosis-related genes were detected in the dams liver, placenta and fetal liver from pregnant rats treated with T-2 toxin. The decreased expressions of lipid metabolism- and drug-metabolizing enzyme-related genes were also detected in these tissues. As regarding apoptosis, the results suggested that MAPK pathway might be involved in the mechanisms of T-2 toxin-induced apoptosis. In addition, increased expression of *c-jun* gene was consistently observed in these tissues. Our results suggest that the mechanism of T-2 toxin-induced toxicity in pregnant rats is due to oxidative stress followed by the activation of MAPK pathway, and finally apoptosis may be induced. *c-jun* gene may play an important role in T-2 toxin-induced apoptosis.

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FUMONISIN B<sub>1</sub> ACTIVATES SERINE PALMITOYLTRANSFERASE AND SPHINGOSINE KINASE, ENZYMES IMPORTANT IN SPHINGOLIPID METABOLISM, IN MOUSE LIVER.

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Fumonisins B<sub>1</sub> (FB<sub>1</sub>) is a mycotoxin produced by *Fusarium verticillioides*, a common fungus in corn and other crops. FB<sub>1</sub> causes equine leukoencephalomalacia, porcine pulmonary edema, cancer in liver of female mice and in kidney and liver of male rats. It is hepatotoxic and nephrotoxic in most animal species. The primary biochemical effect of FB<sub>1</sub> is inhibition of ceramide synthase leading to the accumulation of free sphingoid bases (sphinganine and sphingosine), sphingoid base metabolites, and depletion of more complex sphingolipids. The treatment of FB<sub>1</sub> increases the sphinganine and sphingosine levels in liver of female mice. The hepatopathy is closely correlated with the disruption of sphingolipid metabolism. In this study, we investigated the effects of FB<sub>1</sub> on serine palmitoyltransferase (SPT), the enzyme synthesizing 3-ketosphinganine in *de novo* biosynthetic pathway of sphingolipids, and sphingosine kinase (SPHK), the enzyme converting free sphingoid bases to their respective phosphates. Female BALB/c mice were subcutaneously injected with 2.25 mg FB<sub>1</sub>/kg daily for 5 days. One day after the last treatment, the expression of SPT1 and SPT2 was increased by 4.7- and 5.3-fold, respectively, in liver. The activity of SPT was increased in liver from FB<sub>1</sub>-treated mice. FB<sub>1</sub> dramatically increased the expression of hepatic SPHK to 25-fold. These increases were accompanied by induction of inducible nitric oxide synthase (iNOS) in liver, suggesting that the production of enzymes was mediated via NF<sub>κ</sub>B activation. It appears that the accumulation of free sphingoid bases and their metabolites will be influenced by activation of the above enzymes and may amplify the toxic response to FB<sub>1</sub>. (Supported in part by USPHS ES 09403)

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IN VITRO STUDIES CONFIRM SYNERGISTIC EFFECTS OF FUMONISIN B1 AND OCHRATOXIN A IN VIVO.

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Contamination of food and feeds by mycotoxins is a major problem of human and animals health concern which is also extremely detrimental to economy. Mycotoxins producing moulds may produce a diversity of toxins such as aflatoxins, ochratoxins, trichothecenes, zearalenone, fumonisins, tremorgenic toxins and ergot alkaloids. Indeed, more than one mycotoxin are found usually in the same contaminated commodities, rising the problem of multi-toxicosis in which the respective metabolites are also involved. The risk assessment for humans potentially exposed to multi-mycotoxins suffers very much from the lack of adequate food consumption data. Furthermore, for a given mycotoxin synergism and antagonism with other mycotoxins found in the same food commodities are not taken into account. The case of combination of ochratoxin A (OTA) and fumonisins B1 (FB1) has been addressed in the present paper with the purpose of predicting the *in vivo* toxicity using a simple *in vitro* test, i.e. neutral red uptake, in three different cell-lines, C6 glioma cells, Caco-2 cells and Vero cells. Using the following equation [1], *in vivo* toxicity (LD<sub>50</sub>) is in adequation with *in vitro* data, (IC<sub>50</sub> values) for both toxins as well as for the combination of 10 microM OTA and variable concentrations of FB1 (10 to 50 microM). Thus such a simple *in vitro* test may help predicting *in vivo* toxicity of combinations of mycotoxins naturally occurring in foodstuffs. Reference [1] Spielmann, H., Genschow, E., Liebsch, M., Halle, W., Determination of the starting dose for acute oral toxicity (LD<sub>50</sub>) testing in the up and down procedure (UDP) from cytotoxicity data. ATLA 27; 957/966, 1999.

1436

CYTOKINE GENE EXPRESSION IN RAT MICROGLIA EXPOSED TO THE MARINE TOXIN DOMOIC ACID.

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The molecular pathology of Amnesic Shellfish Poisoning, one of the shellfish poisoning syndromes in the United States caused by the marine glutamate analog domoic acid (DOM), is incompletely understood. Our working hypothesis is that DOM may activate rat neonatal microglia to cause generation of cytokines, and toxicity to the nervous system. We have reported that *in vitro* treatment of microglia with DOM leads to TNF- $\alpha$  protein release (Mayer et al. *BioMedCentral Pharmacology* 1:7-19, 2001). The purpose of our study was to investigate cytokine gene expression in 4-24 hour DOM [1mM]-treated microglia using a cytokine-specific cDNA array (SuperArray Inc., Bethesda, MD). Through side-by-side hybridization with cDNA probes prepared from mRNA from control or 4, 8, 16 and 24 hour DOM-treated microglia the expression of 18 cytokine genes was determined. Control microglia expressed 4 genes constitutively *in vitro*: interleukin (IL)-

1 $\beta$ , IL-2, IL-16 and TGF  $\beta$ 1. Over a 24-hour observation period, DOM increased expression of IL-2, IL-16 and TGF  $\beta$ 1 by 201, 333 and 220 % of control expression, respectively. Concomitantly, DOM decreased expression of IL-1 $\beta$ . Our current data demonstrates that DOM can affect microglia cytokine gene expression *in vitro* in a time-dependent manner. Furthermore these data provide additional experimental evidence to support our working hypothesis (Mayer A. et al. *The Toxicologist* 78 (S-1): 162, 2004). Supported by R15 ES12654-01 from NIEHS, NIH to AMSM.

1437

COMPARISON OF WEEKLY EXPOSURES TO ANATOXIN-A AND NICOTINE ON THE MOTOR ACTIVITY OF RATS.

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Anatoxin-a is a nicotinic cholinergic agonist that is produced by several genera of cyanobacteria, and has been implicated in several poisoning episodes of wildlife, livestock, domestic animals and people. Previous research on nicotine has obtained tolerance and sensitization to its effects on motor activity even when it was administered at weekly intervals. We therefore compared the effects of weekly anatoxin-a and nicotine on the motor activity of rats. Adult male Long Evans rats were tested daily (M-F) in a photocell device, that recorded both horizontal and vertical activity, during 30-min sessions. Anatoxin-a and nicotine were given s.c. once a week for four weeks, just prior to a test session. Anatoxin-a was given as the (+) isomer and as the racemate. Dose ranges for each compound were: (+)-anatoxin-a, 75-225  $\mu$ g/kg; (+/-)-anatoxin-a, 200-950  $\mu$ g/kg; and (-)-nicotine, 300-1800  $\mu$ g/kg. Each experiment also included a saline-vehicle control group. Nicotine initially decreased both horizontal and vertical activity. Tolerance developed to nicotine's effects with weekly administration. Both forms of anatoxin-a also initially decreased horizontal and vertical activity. In contrast to nicotine, however, the effects of either form of anatoxin-a did not change with weekly administration. Thus, anatoxin-a and nicotine can be distinguished by their effects on motor activity with weekly treatment. This is an abstract of a proposed presentation; the information does not necessarily reflect Agency policy.

1438

NICOTINE EFFECTS ON THE ACTIVITY OF MICE EXPOSED PRENATALLY TO THE NICOTINIC AGONIST ANATOXIN-A.

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Several studies in the literature have shown that exposure of mice and rats to nicotine early in development alters its effects when the rodents are subsequently challenged with nicotine. Anatoxin-a is a nicotinic agonist produced by several genera of cyanobacteria, and has caused numerous deaths of wildlife, livestock and domestic animals world-wide. We therefore determined the effect of nicotine on the motor activity of adult mice that had been exposed prenatally to anatoxin-a. Pregnant CD-1 mice received either saline vehicle or one of two doses of (+/-)-anatoxin-a (125, 200  $\mu$ g/kg), i.p., on GD 13-17. As adults (8 months), control mice of both genders were used to determine the effect of nicotine (0, 0.1, 0.3, 1.0 or 3.0 mg/kg, s.c.) on motor activity measured for 30-min in a photocell device. Under these conditions, nicotine produced dose-related decreases in both horizontal and vertical activity, with an ED<sub>50</sub> estimated to be 0.65 mg/kg. Next, additional control mice and mice exposed prenatally to anatoxin-a received the nicotine ED<sub>50</sub> and saline vehicle, in a counterbalanced fashion, with one week separating treatments. Nicotine decreased both horizontal and vertical activity in all mice, regardless of prenatal anatoxin-a treatment. Thus, no enduring effects of prenatal anatoxin-a were obtained in adult mice following nicotine challenge. This is an abstract of a proposed presentation; the information does not necessarily reflect Agency policy.

1439

HEAVY METAL EXPOSURE FROM HERBAL SUPPLEMENTS IN NIGERIA.

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The uses of herbal products are not regulated in Nigeria and many low-income countries and are freely available to everyone. The safety of these herbal medicines is poorly understood. This study characterizes the content of cadmium, copper, iron, nickel, selenium, zinc, lead and mercury in a random sample of Nigerian traditional products. Ready-to-use herbal products were purchased from the open market and digested using HNO<sub>3</sub>. The heavy metal content of the digested filtrate

was determined by atomic absorption/flame emission spectrometry, Uni-Cam Model 929. Mercury content was determined by the flameless Atomic Absorption Spectrometry using the cold vapor phase. Reference blanks and spiked samples used for quality control were within  $\pm 5\%$ . The result showed that 100% of the samples contained elevated amounts of heavy metals. The estimated daily intake (range; threshold g/day) from consumption of these products was - cadmium (0.6-4.8; 0.06), copper (15.3-97.5; 12), iron (13.7-123.8; >1), nickel (2.5-78; >0.1), selenium (6.3-123.8; >0.2), zinc (0.8-27.5; >20), and lead (0-27; 0.5). Mercury was detected in one sample (0.06; >0.05). These data alert us to the possibility of heavy metal toxicity from herbal products in Nigerian. The public health hazards from ingestion of herbal medicines should be identified and disclosed by in-depth risk assessment studies.

#### 1440

#### ADDITIVE EFFECTS OF FUMONISIN B1 AND DEOXYNIVALENOL IN THE HUMAN INTESTINAL CELL LINE, CACO2.

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Fumonisin B1 (FB1) and vomitoxin, Deoxynivalenol (DON) are mycotoxins produced by Fusarium species, namely *F. Verticilloides* (formerly *F. Moniliforme*) and *F. graminearum* and *F. Culmorum* respectively. These mycotoxins jointly occur in maize. Therefore combinations of these toxins have been tested in the human intestinal cell line, Caco2 for general cytotoxicity i.e. Neutral red and MTT tests and protein synthesis measured by 3H-Leucin incorporation. Data show that both decrease cell viability by 37% at 10 microM for FB1 and by 19 to 48% at 4 to 20 microM for DON respectively. Both inhibit protein synthesis by 45% and 61% for FB1 and DON at 10 microM respectively. Interestingly the combination of these two toxins leads to additive effects and not to synergistic effects as previously reported for the combination of FB1 and ochratoxin A (OTA). Reference: Creppy, E.E., Chiarappa, P., Baudrimont I., Borracci, P., Moukha, S., Carratu, M.-R. Synergistic effects of fumonisin B1 and ochratoxin A: are *in vitro* cytotoxicity data predictive of *in vivo* acute toxicity? *Toxicology*. 201; 115/23, 2004.

#### 1441

#### POLYNUCLEAR AROMATIC HYDROCARBONS (PAHS) FROM BUTADIENE SOOT ARE CONCENTRATED WITHIN LIPID RESERVOIRS IN HUMAN RESPIRATORY EPITHELIAL CELLS.

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Butadiene soot (BDS), a polynuclear aromatic hydrocarbon (PAH)-rich, metals-poor particulate mixture generated from the incomplete combustion of 1, 3-butadiene, a high volume petrochemical, is an environmental pollutant and a potential respiratory toxicant. The majority of freshly generated BDS is <2.5 microns in diameter (PM2.5). Actively growing, non-confluent BEAS-2B cells, a human broncho-epithelial cell line, exposed to BDS generated in our laboratory, displayed a PAH-associated, time-dependent, cytosolic, punctate blue fluorescence following excitation at 365 nm. Cell proliferation was suppressed during exposure to BDS (24-72 hr). Thorough washing of the culture dishes to remove BDS allowed proliferation to resume. The BDS (PAH)-associated cytosolic, punctate fluorescence exhibited low levels of co-localization with a lysosomal marker (Lyso-Tracker Red) and an autophagic vacuole marker (monodansylcadaverine). Exposure of BDS-treated cells to an endosomal compartment marker (dextran-tetramethylrhodamine) revealed no discernable co-localization. These results suggest that the cytosolic punctate fluorescence develops neither from a purely endocytic process nor through a predominantly lysosomal association. Cells stained with a fluorescent cholesterol ester analog (cholesterol BODIPY C11), however, exhibited pronounced co-localization of cytosolic lipid-staining and BDS-associated fluorescent vesicles, strongly suggesting that PAHs initially adsorbed onto BDS particles are concentrated within BEAS-2B cells in lipid vesicles. Combined with previous studies of the physico-chemical properties of BDS, this evidence supports the hypothesis that BDS damages respiratory epithelial cells through a process that includes retention of PAHs within cellular lipid storage units. (Supported by Louisiana Governors Biotechnology Initiative).

#### 1442

#### ACTIVATION OF INFLAMMATORY MEDIATORS AND POTENTIAL ROLE OF AIR POLLUTION PARTICULATES AND AH-RECEPTOR LIGANDS IN FOAM CELL FORMATION.

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Epidemiological data and animal experiments have indicated that exposure to air pollution particulates (PM) as well as Ah-receptor (AhR) ligands such as 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) can lead to cardiovascular toxicity and atherosclerosis.

Monocytes/macrophages are known to play a key role in atherosclerosis by releasing proinflammatory cytokines and the formation of foam cells. Foam cells are lipid-laden macrophages residing in the vessel wall and furthering the local inflammatory response. Our hypothesis is that PM, especially their organic components, exert an inflammatory response leading to the formation of macrophage-derived foam cells. Here, we investigated the effect of TCDD, used as prototype of AhR ligands, and Urban Dust Particulate Matter (UDP; SRM#1649a) including the organic extracts of UDP (OE-UDP), and stripped UDP (sUDP) on formation of foam cells. Our findings show that, like oxidized low density lipoprotein (oxLDL), TCDD as well as UDP promote the formation of atherosgenic foam cells derived from U937 macrophages, verified by increased lipid/cholesterol accumulation and extensive formation of blebs on the cell surface, which are hallmarks of foam cells. Screening the expression pattern of the most important mediators of the inflammatory response involved in atherosclerosis and foam cell formation demonstrated a time- and dose-dependent increase of cyclooxygenase-2 (COX-2), interleukin-1 $\beta$  (IL-1 $\beta$ ), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in U937 macrophages treated with TCDD or UDP. Changes of these inflammatory mediators were followed by significantly elevated levels of matrix degrading metalloproteinases (MMP)-1, MMP-3, MMP-12 and MMP-13. Increased expression of MMPs was associated with enhanced cell migration of U937 macrophages indicating the biological activity of the MMPs. These findings clearly indicate that UDP and AhR ligands, like TCDD, stimulate differentiation of U937 macrophages into potentially plaque forming foam cells.

#### 1443

#### PULMONARY EXPOSURE TO RESIDUAL OIL FLY ASH (ROFA) IMPAIRS SYSTEMIC MICROVASCULAR ENDOTHELIUM-DEPENDENT DILATION.

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Epidemiology studies link elevated ambient particulate matter (PM) with increased cardiovascular mortality and morbidity. However, the mechanisms involved are unclear. The present study investigated whether pulmonary exposure to PM could alter systemic microvascular function. Rats were exposed to ROFA (0.1, 0.25, 1, or 2 mg/rat), TiO<sub>2</sub> (0.25 mg/rat) or saline by intratracheal instillation. At 24 hr post-exposure, bronchoalveolar lavage (BAL) was performed to monitor markers of pulmonary damage and inflammation. Another set of rats was prepared for histopathological analysis. A third set of rats was used for *in vivo* microscopic analysis of the arterioles of the exteriorized right spinotrapezius muscle, leaving innervation and feed vessels intact. BAL markers of pulmonary damage and inflammation were elevated at 1 and 2 mg/rat ROFA, but not after 0.1 and 0.25 mg/rat ROFA or 0.25 mg/rat TiO<sub>2</sub>. Histopathology indicated the presence of slight focal inflammation at all PM exposures. *In vivo* microscopic analysis indicated that ROFA did not affect resting systemic microvascular diameter or tone. However, ROFA significantly inhibited vasodilation in response to luminaly injected Ca<sup>2+</sup> ionophore (A23187), with complete inhibition at  $\geq 0.25$  mg/rat ROFA and 65% inhibition at 0.1 mg/rat. The effect of 0.25 mg/rat TiO<sub>2</sub> on A23187-induced vasodilation was similar to 0.25 mg/rat ROFA. Pulmonary PM exposure did not affect the responsiveness of arterial smooth muscle to a nitric oxide generator indicating an effect on endothelial cells. In addition, ROFA (2 mg/rat) resulted in systemic inflammation as evidenced by: (1) adherence of PMN to the systemic arterioles, (2) increased FMLP-induced chemiluminescence from isolated blood PMN, and (3) increased dihydroethidium oxidation at the arteriolar wall. These results indicate that pulmonary exposure to ROFA causes systemic inflammation, which compromises the influence of nitric oxide on endothelium-dependent vasodilation.

#### 1444

#### DETECTION AND MOLECULAR ANALYSIS OF PARTICULATE AIR POLLUTION-INDUCED CARDIOPULMONARY OXIDATIVE STRESS USING A TRANSGENIC MOUSE MODEL AND EMERGING TECHNOLOGIES.

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Identification of particle characteristics and biological mechanism(s) responsible for the adverse pulmonary and cardiovascular responses associated with particulate air pollution exposure remains a critical research activity. We have employed an oxidative stress sensitive and bioluminescent reporter transgenic mouse model, hemeoxygenase-1 (HO-1) luciferase, in order to identify *in situ* induction of oxidative stress within lung and heart tissues following pulmonary aspiration (50  $\mu$ g/50  $\mu$ l/mouse) of a suspension of residual oil fly ash (ROFA) particles. HO-1 induction in lung and heart tissue protein extracts was measured by chemiluminescence at various times following exposure. Pulmonary and cardiac chemiluminescence increased at

2h ( $p<0.001$ ) and 24h ( $p<0.05$ ) post-exposure, respectively. Increased chemiluminescence in lung tissue protein extracts correlated with elevated levels of luciferase protein. Bioluminescent staining of lung and heart frozen tissue sections demonstrated localized areas of oxidative stress. RNA was recovered from these regions of interest following laser capture microdissection. Gene expression profiling by microarray analysis of recovered RNA samples is currently ongoing. These results demonstrate the ability of air pollution particles derived from oil combustion to induce local and systemic oxidative stress which in turn may result in acute organ injury as well as potentially contribute to disease processes that have oxidative stress as a common pathological etiology. Funding: EPA/NCSU Training Agreement CT826512 to E. Roberts. (This abstract does not reflect EPA policy)

#### 1445 CONCENTRATED AMBIENT AIR POLLUTION CREATES OXIDATIVE STRESS IN CNS MICROGLIA

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Particulate matter (PM) is known to affect extrapulmonary targets. Nanometer size PM particles carry free radical activity on their surface and damage target tissues through oxidative stress (OS). Recently, we've reported a 29% reduction of CNS neurons (i.e., n. compacta of the substantia nigra) in a transgenically modified mouse strain (Apo E<sup>-/-</sup>) exposed for 5 mo to concentrated northeastern regional background ambient particles (CAPs) (Veronesi et al., Inhalation Toxicology in press). Since these neurons are highly sensitive to OS mediated damage, the response of microglia (the brain macrophage) to the same CAPs was examined. Immortalized, mouse microglia (BV2) were exposed to high and low potency CAPs (as determined by NF- $\kappa$ B activity) and endpoints, indicative of oxidative stress mediated inflammation, were collected. ATP levels, assayed with Luciferase-based chemiluminescence, were significantly reduced after 5 min exposure to  $>250$   $\mu$ g/ml mitochondrial depolarization after 30 min exposure. Significant increases in TNF  $\alpha$ , IL6 and IL1  $\beta$  were shown after 6 hr exposure. Glutathione and non-protein sulphydryl levels, markers of OS were also significantly increased at 25-100  $\mu$ g/ml concentrations. Cells, exposed for 4 hrs to 75  $\mu$ g/ml of the high or low potency CAPs, were analyzed for genomic expressions, using an Affymetrix microarray. Bioinformatics indicated highly significant changes in genes associated with cell cycling, apoptosis, adhesion molecules, NF- $\kappa$ B, Toll receptors, Superoxide dismutase, proinflammatory cytokines, oncogenes, TNF receptors and others. Taken together, these data indicate that CNS microglia respond to concentrated ambient PM with changes suggestive of OS mediated inflammation. (This abstract has been reviewed by the USEPA, NHEERL and does not necessarily reflect its policy).

#### 1446 EFFECTS OF PARTICULATE MATTER ON THE PULMONARY AND VASCULAR SYSTEM: TIME COURSE IN SPONTANEOUSLY HYPERTENSIVE RATS.

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Within the scope of two multi center projects, "Health effects of particles from motor engine exhaust and ambient pollution" (HEPMAP) and "Chemical and biological characterization of ambient air coarse, fine, and ultrafine particles for human health risk assessment in Europe" (PAMCHAR), the present study aimed to optimize the design for PM toxicity screening studies in terms of dose and time between a single intratracheal exposure and the determination of the biological responses in compromised rats. PM was sampled at a road tunnel (RTD) using a high volume cascade impactor. Spontaneously hypertensive rats were exposed to EHC-93 or different doses of RTD. Autopsy was performed at 4, 24 or 48 hrs post-exposure. The neutrophil numbers in bronchoalveolar lavage fluid increased tremendously after exposure to relatively high RTD concentrations (10-15 fold) or EHC-93 (25 fold). Furthermore, PM exposure affected blood coagulation since plasma fibrinogen levels were increased significantly (1.2 fold). Pulmonary inflammation, oxidative stress, and changes in blood coagulation factors as well as circulating blood cell populations were observed within the range of 3 to 10 mg PM/kg body weight without the presence of significant pulmonary injury. At a lower dose only some inflammatory effects can be detected, which will probably be too small to discriminate between PM samples, and potential confounding effects were observed with the highest dose. In addition to dose, 24 hrs post-exposure seemed to be the appropriate time to assess relative toxic potency of PM since the majority of the health effects were observed one day after PM exposure compared to the other times examined. Aforementioned considerations provide a good foundation for the design of PM toxicity screening studies.

#### 1447

#### ENDOTHELIAL INJURY IN PARTICULATE MATTER (PM)-INDUCED CARDIOVASCULAR INJURY: KINETIC ANALYSIS OF GENE EXPRESSION PROFILES.

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Numerous epidemiological studies established positive associations between ambient fine PM and cardiovascular morbidity and mortality. The biological basis for these adverse health effects is yet to be elucidated. Cardiovascular toxicity of fine PM and its toxic constituents may be direct or indirect. Vascular endothelial cells have been implicated in both direct and indirect cardiovascular effects of PM. To understand the role of endothelial injury and dysfunction in PM cardiovascular toxicity, we have initiated *in vitro* studies using primary human vascular endothelial cells. Acute exposure (25min) to a very low concentration (1mg/ml) of a model emission source PM, residual oil fly ash (ROFA) indicated differential gene expression profile for genes representing various functional groups such as cytokines, growth factors, vascular tone regulators, adhesion molecules, transporter proteins and voltage gated signaling mediators. Molecular observations observed at this early time point predicted alterations in plasma membrane structural and functional properties. Cytological observations at 8h post exposure indicated a role for these genes in the endothelial injury. To further understand the role of continuous exposure of endothelial cells to the same concentration of ROFA, we carried out exposure time kinetic studies at 25 min, 1, 3, 8, 12 and 24h. Gene expression profiles analyzed for ~20, 000 genes using human Affymetrix gene chips (H133A) at these time points clearly show time-dependent differential gene expression profiles consistent with progressive endothelial injury and dysfunction. The results of this study may provide insight to understand the role of the endothelium and the molecular basis for PM cardiovascular toxicity. It also may provide a biological basis for the discrepancies observed on the onset of acute myocardial events in cardiac patients associated with that ambient PM exposures. (This abstract does not reflect USEPA policy).

#### 1448

#### AIRBORNE PARTICULATE MATTER UPREGULATES INFLAMMATORY MARKERS IN THE MOUSE BRAIN: IMPLICATIONS FOR NEURODEGENERATION.

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Neurodegenerative diseases are multifactorial. Senescence is a common predisposing factor. However, genetic mutations and environmental factors have also been linked to the onset and progression of these disorders. While the pathological hallmarks of different age-related neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis are unique, certain underlying processes appear to be generally enhanced. These include oxidative and inflammatory events. Concentrated particulate matter present in polluted air enhances proinflammatory markers in the brain. The activated levels of the immune related transcription factor NF- $\kappa$ B were increased. Furthermore, the concentration of the cytokines IL-1 $\alpha$  and TNF- $\alpha$  were elevated. These effects were not due to the presence of endotoxin in the particulate matter. It is possible that this environmental exposure can trigger an innate-immune response in the CNS and by doing so aggravate already existing age-related adverse events. Transgenic models of neurodegenerative disorders can be an essential tool in determining whether environmental exposures do indeed contribute to and exacerbate pathological lesions associated with age-related disorders.

#### 1449

#### SOLUBLE NICKEL ASSOCIATED WITH RESIDUAL OIL FLY ASH INCREASES SUSCEPTIBILITY TO PULMONARY INFECTION IN RATS.

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Soluble metals of residual oil fly ash (ROFA), an air pollutant from the combustion of fossil fuel, may be associated with lung infection and morbidity in susceptible populations. The present objective was to determine which soluble metal in ROFA was associated with decreased clearance of bacteria from the lungs of rats. At day 0, rats were intratracheally instilled (IT) with soluble NiCl<sub>2</sub> (55.7 $\mu$ g), FeSO<sub>4</sub> (37.2 $\mu$ g), AlSO<sub>4</sub> (46.6 $\mu$ g), ZnCl<sub>2</sub> (8.69 $\mu$ g), or a mixture of the metals in quantities present in a 2.0 mg dose of ROFA as determined by elemental analysis. On day 3, rats were infected with an IT dose of  $5 \times 10^4$  *Listeria monocytogenes* and euthanized on days 6, 8 and 10. The left lungs were homogenized to assess bacterial load, and bronchoalveolar lavage (BAL) was performed on the right lungs to measure lung injury and inflammation. Cells from lymph nodes were analyzed to determine phenotype. On day 6, rats exposed to Ni or the metal mixture had an increased bacterial lung burden as compared to all groups, and rats exposed to Zn had a higher bacterial load when compared to rats exposed to Al, Fe, or saline control. Lactate

dehydrogenase and albumin levels in BAL fluid were 3 times greater in the Ni and mixed metal groups on day 6 as compared to controls. Treatment with Ni and the metal mixture resulted in a 3 and 2 fold increase, respectively, in the number of cells in the BAL at all time points. The number of T cells in the BAL of the Ni and the mixed metal groups was decreased on day 6 and the number of natural killer cells was decreased in the Ni group at all time points as compared to controls. The ratio of CD4<sup>+</sup> to CD8<sup>+</sup> T cells in the BAL and in the lymph nodes was higher in the Ni and mixed metal groups on day 6. In summary, rats treated with soluble Ni, alone or in a mixture with soluble Fe, Zn, and Al, showed increased lung injury, decreased bacterial clearance, and altered lymphocyte profiles at early time points post-infection. Thus, soluble Ni may play an important role in the increase in susceptibility to infection after ROFA exposure.

**1450**

MEMBRANE PERMEABILITY AS A DETERMINANT OF ZN<sup>2+</sup>-INDUCED SIGNALING IN HUMAN AIRWAY EPITHELIAL CELLS.

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Zinc (Zn<sup>2+</sup>) is an essential micronutrient but also a common metallic constituent of ambient air particulate matter (PM) that may play a role in PM-induced adverse health effects. *In vivo* and *in vitro* studies have shown that Zn<sup>2+</sup> exposure induces inflammatory responses in the airways. We have previously shown that exposure to Zn<sup>2+</sup> results in activation of multiple intracellular signaling intermediates, including the epidermal growth factor receptor (EGFR), in a variety of cell types. Based on this variability, we hypothesized that cellular permeability is a factor limiting the magnitude of signal transduction activation induced by Zn<sup>2+</sup>. To test this hypothesis, we used the Zn<sup>2+</sup> ionophore pyrithione to permeabilize the human bronchial epithelial cell line BEAS and primary human airway epithelial cell cultures (HAEC) to Zn<sup>2+</sup> and measured levels of EGFR phosphorylation. Treatment of BEAS cells with sub- to low-micromolar concentrations of pyrithione in the presence of 250  $\mu$ M extracellular Zn<sup>2+</sup> resulted in dose-dependent increases in Zn<sup>2+</sup>-specific fluorescence as determined by fluorometry using FURA-2. Lysophosphatidylcholine, a physiologically relevant permeabilizing agent, also increased Zn<sup>2+</sup> permeability in BEAS cells. Co-administration of 4  $\mu$ M pyrithione markedly potentiated Zn<sup>2+</sup>-induced EGFR phosphorylation at the auto- and trans-phosphorylation sites Y1068 and Y845, respectively, in HAEC. These findings show that Zn<sup>2+</sup> acts through an intracellular target to effect EGFR phosphorylation. Further, cellular permeability is a critical determinant of cellular responsiveness to signal initiation by exposure to Zn<sup>2+</sup>. Pathophysiological alterations in cellular homeostasis that may result in increased permeability to Zn<sup>2+</sup> influx and sensitize HAEC to the effects of inhaled Zn compounds are presently under investigation. Additionally, the possibility that xenobiotics with pyrithione-like properties exist in PM is also being studied. THIS ABSTRACT OF A PROPOSED PRESENTATION DOES NOT NECESSARILY REFLECT EPA POLICY.

**1451**

ZINC CONTENT IN BALTIMORE PM<sub>2.5</sub> SEAS SAMPLES IS NOT SOLELY RESPONSIBLE FOR CHEMOKINE OR CYTOKINE RESPONSE IN A549 ATII CELLS OR RAW 264.7 MONOCYTES.

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For approximately a decade, metals have been hypothesized to contribute to the relationship between human exposure to fine particulate matter (PM<sub>2.5</sub>) and cardiopulmonary disease. Recently, the metal zinc has been shown to play a pivotal role in inducing cardiac lesions and inflammation *in vivo* (Kodavant *et al.* 2003). In order to test the hypothesis that zinc plays a central role in the release of soluble inflammatory mediators in the lung, we exposed human alveolar type II cells (A549) and monocyte cells (RAW 264.7) to fine particulate matter (PM<sub>2.5</sub>; SEAS samples) collected at the Baltimore Supersite during summer and winter intensive sampling periods in 2002. Median TNF- $\alpha$  levels from RAW 264.7 monocytes were greater following treatment with the November PM<sub>2.5</sub> samples, while MCP-1 release from A549 alveolar epithelial cells was significantly inhibited by the November samples, compared to the July PM<sub>2.5</sub>. A comparison of eleven metal concentrations measured by GFAAS revealed that Al, Fe, and Zn were the most abundant in both the July (Fe>Al>Zn) and November (Fe>Zn>Al) samples. Multiple linear regression revealed that Zn in combination with endotoxin, Cd, Fe, and particle number explained 50% of TNF- $\alpha$  release in November samples, while particle number and Fe explained approximately 44% of the inhibition of MCP-1 release in this same sampling period. Neither zinc, nor any other metal alone, was responsible for the release of the immune mediators. These results challenge prevailing research paradigms that view one or more metals as being solely responsible for the adverse

effects of inhaled PM<sub>2.5</sub> and demonstrate the need for more complex statistical approaches to aid in determining the relative contributions of the various components of PM<sub>2.5</sub>, as well as particle number, to cardiopulmonary inflammation. *Supported by the Baltimore Supersite Program grant R82806301*

**1452**

EFFECT OF PULMONARY EPITHELIAL LINING FLUID ON OXIDATIVE STRESS AND DNA DAMAGE: COMPARISON AMONG ULTRAFINE PARTICLES, FERROUS SULFATE AND DIESEL PARTICLE EXTRACT.

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Previous studies have reported that particulate matter can induce oxidative stress and DNA damage. To protect lung cells against the exogenous oxidative stress, pulmonary epithelial lining fluid (ELF) consists of various antioxidants. However it is not clear which components of PM are affected by ELF. The aim of this study is to determine the effect of ELF on oxidative stress and DNA damage induced by several PM components including ultrafine particles, ferrous sulfate and diesel particle extract. In cell free system, ultrafine carbon black of 15 nm (ufCB; 0, 50, 150 $\mu$ g/ml), FeSO<sub>4</sub> (0, 100, 500 $\mu$ gM), and diesel particle extract (0, 250, 500 $\mu$ g/ml) were suspend in ELF or medium. Subsequently, we used transwell *in vitro* culture system with ELF or medium on the top of A549 cells and medium below. Again, similar concentrations for each component were used. After 4 hr of treatment, ROS was then measured using DCFH assay and DNA single strand breakage was also determined by single-cell gel electrophoreses (Comet assay). The results showed that in cell free system and A549 cells with culture medium, ROS increased with exposure to ufCB and FeSO<sub>4</sub>, but not with diesel particle extract. We also observed DNA SSB increased with FeSO<sub>4</sub> and diesel particle extract exposure in culture medium, but not with ufCB. When ELF was used to replace medium, ROS decreased significantly for both ufCB and FeSO<sub>4</sub> exposure in cell free and A549 cells. There was no increase for DNA SSB for each component in ELF. We conclude that ELF may decrease ROS levels and subsequent DNA SSB formation induced by ufCB and FeSO<sub>4</sub>.

**1453**

EFFECTS OF PM-ASSOCIATED METALS ON MACROPHAGE iNOS AND ERK: A ROLE IN ALTERED IRON HOMEOSTASIS?

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Analyses of fine airborne PM<sub>2.5</sub> samples collected in New York City (NYC), Los Angeles, and Seattle during Fall 2001 indicated that inorganic composition varied from city to city, both in terms of absolute amounts of individual elements and their mass relationships to one another. We hypothesized that relative molar/mass relationships between select PM metals and Fe governs the pulmonary immunotoxic potential of a given day's (or city's) PM by affecting alveolar macrophage (AM) iron status, thereby altering their antibacterial function. To test this, iron response protein (IRP) binding activity in NR8383 rat AM was assessed after exposure to Fe alone or in combination with V, Mn and/or Al. In all studies, relevant molar ratios of the metals, as found in a representative 500  $\mu$ g sample of NYC PM, were used. Results indicated that V and Al, and to a lesser extent Mn, significantly changed IRP activity though the effects were not consistently dose-dependent. This disruption in IRP binding activity was thought to possibly be a result of competition for binding to a transferrin carrier; however, potential effects on IRP activity from the presence of nitric oxide (NO) needed to be ruled out. The results of analyses of concurrent expression of iNOS and phospho-ERK1/2 suggested that while V treatments did not activate NO formation, Al did - most likely via increases in lipid peroxidation that resulted in AP-1 activation. These results confirm that certain metals associated with PM might alter pulmonary immune competence in exposed hosts by impacting upon the Fe status of one major class of deep lung defense cell. This work was supported by EPA/PM Center Grant R82735101.

**1454**

MUTAGENICITY AND CYTOTOXICITY OF MANUAL METAL ARC STAINLESS STEEL WELDING FUMES (MMA-SS) BY ITS PARTICLE SIZE.

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The particle size and metal components of fine particulates have been hypothesized to be important factors in determining in their toxicities and potential adverse health effects. To study cytotoxicity and mutagenicity of MMA-SS by their particle

size fractions, the fume particles were sampled from an exposure chamber and fractioned their size using an 8-stage cascade impactor. The size ranges of fume particles were divided in to 4 groups: group 0, total particles; group 1, 11-2.1  $\mu\text{m}$ ; group 2, 2, 1-0.65  $\mu\text{m}$ ; group 3, below 0.65  $\mu\text{m}$ . The composition of heavy metals in each sample group was analyzed using an atomic absorption spectrophotometry. The bacterial mutation assay using *Salmonella* TA 98 and TA 100 was carried out to compare the mutagenicities of four groups of welding fume particle fraction. The cytotoxicity assay using CHO cells was also performed using a cell counting assay kit. The concentrations of Cr in total suspended particulates of welding fume become higher as the particle sizes become smaller, while those of Mn, Ni and Fe showed similar levels in all size fractions. The mutagenicity in TA 98 without metabolic activation was confirmed in all sample groups but the initial concentration that began to show mutagenicity was lowest in the group 3. The mutagenicity induced by the metabolic activation was only detected in the group 3. TA 98 was shown to be the most sensitive to the finest particles. As for TA100, the mutagenicities were also found in all sample groups with or without metabolic activation. Mutagenicities were clearer in case without metabolic activation in both TA 98 and TA 100. Cytotoxicities also showed increasing patterns as the particle sizes become finer with a statistical significance ( $p<0.05$ ). These results indicated that particle size and Cr components played important roles in inducing mutagenicity and cytotoxicity by MMA-SS fume exposure.

**1455**

DNA DAMAGE IN ALVEOLAR EPITHELIAL INDUCED BY INORGANIC AND ORGANIC SOLUBLE FRACTIONS FROM MEXICO CITY URBAN AIRBORNE PARTICULATE.

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Particulate matter are a complex mixture of inorganic and organic compounds, some with carcinogenic potential (i.e. metals and PAH). We evaluated induced DNA damage and repair using the comet assay in A459 human lung epithelial cells exposed to water and organic soluble fractions from PM10 and PM2.5 of four regions (Xalostoc, Merced, Tlalnepantla and Pedregal) from Mexico City. PM soluble fractions extracted were chemically characterized for metals (Zn, Fe, V, Ni, Cu, Cr, Mn, Pb), ions (sulfate and nitrate), soluble protein and PAH (2 to 6 ring compounds). Results showed low PM levels and high concentration of Zn, Fe, Cu, V and Ni in the water-soluble extracts from Pedregal in comparison with high PM levels and low concentration of water-soluble metals for Xalostoc. Genotoxicity was measured after 48 hr exposures using three non-cytotoxic doses of the inorganic and organic extracts: 0.8, 1.2 and 1.6  $\text{m}^3/\text{ml}$  for PM10 and 0.05, 0.07 and 0.1  $\text{m}^3/\text{ml}$  for PM2.5. Genotoxicity of the two soluble fractions showed a significant increase in DNA migration with respect to non-exposed cells. The water soluble fraction (metals and ions) of PM exhibited higher DNA damage than the organic fraction (PAH) at equivalent doses. Also PM2.5 fractions showed a higher genotoxic damage than PM10, and PM from Pedregal resulted to be the most genotoxic from all regions. The level of induced DNA damage returned to near background levels within 2.5 h in all the conditions previously described. Our data indicates that genotoxicity of PM is determined by the chemical composition and aerodynamic diameter, thus representing an environmental risk factor. (Financed by The Financial Research Training Program from CIIEMAD/COFFA-IPN, CINVESTAV-IPN, SEMARNAT-CONACYT 2002-CO1-1204/A-1 and PAPIIT-UNAM).

**1456**

HEALTH EFFECTS OF ENVIRONMENTAL SHORT-TIME SULFUR DIOXIDE (SO<sub>2</sub>) EXPOSURE EMITTED FROM A VOLCANO IN MIYAKEJIMA ISLAND, SOUTH OF TOKYO.

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Approximately 10% of volcanoes in the world exist in Japan. Mt. Oyama volcano in Miyakejima Island started erupting since June 2000. All the inhabitants evacuated since then. While it calmed down since the last eruption in 2002, the volcanic gas emission (mainly SO<sub>2</sub>) will not end for a while. For several reasons among the inhabitants, the rehabilitation project started in April 2003. About 70% of the former inhabitants expressed their willingness to come back to their home island with some risks of sulfur dioxide exposure. It is very important to monitor the ambient air quality in the island and estimate the future health effects for the inhabitants. We can prepare for measures to minimize the exposure and health effects. We conducted questionnaires among the inhabitants who joined the three-day trial stay project in the island in October, November and December 2003. The question-

naires included symptoms of respiratory tract, nasopharyngeal mucosa, skin and conjunctiva. In addition, we monitored the concentrations of sulfur dioxide in six regions of the island, and divided the inhabitants into two groups by the exposure levels of sulfur dioxide; high exposure group (those exposed to SO<sub>2</sub> higher than 2 ppm in 5-minute period value for more than 1.2 hours per day.) and less exposure group (remaining others). In order to exclude confounding factors sufficiently, such as gender, age and the symptom-related predisposed diseases, we performed analysis evaluating the symptoms as independent variables. These results indicated that all the symptoms were not significantly dependent on concentration of sulfur dioxide levels. In conclusion, the short-term sulfur dioxide exposure to them did not show increase of their symptoms. Finally, we hope that results of this study will be helpful for the risk managers in Japan and other countries that have a volcano emitting sulfur dioxide.

**1457**

MEASUREMENT OF LUNG RESISTANCE AND COMPLIANCE FOR RESPIRATORY SAFETY PHARMACOLOGY STUDIES.

S. Mason, K. Norton and H. Penton. *Safety Pharmacology, CTBR Bio-Research Inc., Senneville, QC, Canada.* Sponsor: C. Banks.

The ICH S7A Guideline for Safety Pharmacology Studies calls for assessment of respiratory parameters to be performed, but only requires that respiratory rate, tidal volume and derived respiratory minute volume (RMV) are measured. While these are sufficient for the majority of new drugs, measurement of ventilatory patterns alone does not assess possible effects on the gas exchange function of the respiratory system, particularly lung mechanics. A method for investigating these effects in conscious animals was therefore developed at this laboratory, based on Murphy (1998). In this, lung compliance is calculated by measuring the pressure required to inflate the lung to maximum volume and lung resistance is calculated as the pressure required for maximum airflow during inspiration or expiration. To demonstrate this technique, three male Sprague-Dawley rats, previously surgically instrumented with DSI TA11PA-C40 telemetry transmitters, were dosed via a tail vein infusion with 30 mg/kg/min methacholine over an approximate 15 minute period. Respiratory rate, tidal volume and intrapleural pressure were continuously monitored using a head-out plethysmograph to record airflow and a DSI OpenArt V2.3 system connected to a Buxco BioSystem XA V2.9 to record pressure. Integration of the pressure and flow signals by the Buxco software provided data on dynamic resistance and compliance. For animals in this study, pulmonary pressure was increased by up to 200% compared to predose and control animal values, this effect being observed within approximately 5 minutes of the start of infusion. Lung compliance also decreased to approximately 70% of predose values and resistance increased by up to 300%. There was no consistent effect on tidal volume, respiratory rate or RMV, indicating that assessment of only ventilatory parameters in this study may not have detected methacholine-related respiratory changes. Measurement of lung mechanics in a conscious animal model such as the one employed here should therefore be considered in testing strategies for new drugs.

**1458**

RELATIVE ACCURACY OF BIDE AND GUYTON FORMULA FOR CALCULATING RESPIRATORY MINUTE VOLUME.

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Theoretical respiratory minute volume (RMV) values have historically been used to calculate achieved dose levels in inhalation toxicology studies. The aim of this investigation was to compare quantitatively measured RMV data to theoretical RMV, within the same population of animals, and determine possible effects on calculated achieved dose levels. RMV was calculated from tidal volume and respiratory frequency data, recorded from animals in a "head-out" plethysmograph using the Buxco Biosystem XA. The test system comprised three groups of Sprague-Dawley rats, Group 1 males aged 6 to 8 weeks weighing ~200-300 g (n=200), Group 2 males aged 11 to 13 weeks weighing ~300 to 450 g, (n=200) and Group 3 females aged 7 to 9 weeks weighing ~150 to 250 g (n=100). The mean 15-minute RMV for each animal was compared to the corresponding theoretical RMV calculated by the Bide or Guyton formula. Bide - Minute Volume (L/min) = 0.499\*[body weight<sup>0.809</sup>] Guyton - Minute Volume (mL/min) = 2.1\*[body weight<sup>0.75</sup>] For Group 1 the mean and standard deviation of the measured RMV was 236  $\pm$  61 mL/min, compared to theoretical measurement of 154  $\pm$  15 or 126  $\pm$  11 mL/min - an underestimation of 35% and 47% for the Bide and Guyton formulae, respectively. Group 2 had a mean and standard deviation measured RMV of 284  $\pm$  70 mL/min compared to a theoretical measurement of 224  $\pm$  12 or 178  $\pm$  9 mL/min - an underestimation of 21% and 39% for the Bide and Guyton formulae, respectively. Group 3 had a mean and standard deviation measured RMV of 214  $\pm$  62 mL/min compared to a theoretical measurement of 132  $\pm$  17 or 109  $\pm$  13 mL/min - an underestimation of 38% and 49% for the Bide and Guyton formulae, respectively. This data shows that although the ranges of Bide or Guyton formula overlaps

with in-life RMV mean values are lower in younger animals. Further investigation of older animals should provide a greater assessment of differences in measured versus calculated RMV and the effects on calculated achieved dose levels.

**1459**

#### COUGHING MODEL BY INHALATION OF CITRIC ACID IN COMMON MAMMOSET (CALLITHRIX JACCHUS).

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Some animal species, such as guinea pigs, rats and cats, have been used as coughing models to evaluate effects of antitussive drugs. However, it is well known that cough reactions to inhaled chemicals are different depending on the species. We investigated the coughing reactions using common marmosets by the inhalation exposure. [Methods] An ultrasonic nebulizer generated a mist from the 2.5% and 7.5% citric acid PBS solution. The mist was exposed to un-anaesthetized common marmosets restrained in a double-chamber for the measurement of respiratory physiology (Buxco Electronics Inc., NC). The exposure duration was set at five minutes for each citric acid concentration. A pressure-transducer and microphone detected the cough reactions. Three female marmosets (67-68 week-old; BW, approx. 350 g) were used in this study. [Results and conclusion] The inhalation exposure to citric acid induced coughing and sneezing among the common marmosets. These reactions increased in proportion to the citric acid concentrations. The marmosets reacted to the citric acid exposure immediately after the commencement of the exposure, and the respiratory reactions continued throughout the exposure duration. The reactions disappeared practically by eight minutes after the termination of the exposure. A previously published paper reported that rats did not reveal coughing reaction by the inhalation challenge. We reported that marmosets have a similar anatomical nasal structure to humans and macaques. It is unquestionable that to use marmosets in the evaluation of antitussive drug by the inhalation route is more advantageous because of its anatomical similarity. Moreover, the metabolism mechanism of antitussive drugs among the marmosets may be similar to the human. Consequently, as the coughing model, the marmosets are considered more advantageous for evaluation of antitussive drugs than the guinea pigs, rats and cats. The results of the present study suggested that the marmosets are useful as a coughing model with the present method.

**1460**

#### APPLICATION OF THE GUINEA PIG COUGH MODEL TO SCREEN FOR TUSSIGENIC AND ANTI-TUSSIVE PHARMACOLOGY.

C. Banks, K. Norton and S. Groom. *Toxicology, CTBR, Senneville, QC, Canada.*

Cough can be an acute or chronic indication. In its acute form, cough is most often caused by upper respiratory tract infection or by irritation. In its chronic form, cough can be useful indicator of diseases such as asthma and COPD. The guinea pig cough model can be used for both of the above indications; screening compounds for either tussigenic or anti-tussive properties. This poster presents data generated for a citric acid induced cough model. Cough was measured using a Buxco plethysmography system in which we defined cough as a sharp increase in the inspiratory flow signal, followed by a rapid expiration at least 1.5 times the height of normal protrusion from the baseline. A representative event trace that captures the cough response is presented. Aerosol exposure to citric acid concentrations of 100, 200 and 300 mg/mL induced a cough response which was not strictly dose-dependent, in part due to the confounding effects of bronchoconstriction at the highest exposure concentration. Pretreatment of the animals with lidocaine hydrochloride at concentrations of 30 and 100 mg/mL reduced citric acid (200 mg/mL)-induced cough by 53 and 42%, respectively. These data are compared and contrasted to data generated for capsaicin-induced cough and are discussed relative to the practical and physiological considerations associated with measuring cough responses in guinea pigs.

**1461**

#### MULTIDRUG RESISTANCE RELATED PROTEINS IN AIR-LIQUID INTERFACE CULTURE OF HUMAN LUNG CELLS.

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Multidrug resistance related proteins (MRP-proteins) are known to mediate drug resistance and xenobiotic tolerance, but localization and regulation of MRP isoforms in the lung are widely unknown. Candidates for regulation of MRP function are glutathione, metals and mediators of inflammation. Some MRPs may be involved in GSH dependent transport processes for metals. Several recent studies have shown that prostaglandins participate in endogenous regulation of the P-glycopro-

tein (mdr1) gene and it is conceivable that this is also true for MRPs. We have used explant cultures from normal human lung tissue from bronchi (NHBEC) and peripheral lung (PLC) in order to prove the cellular distribution of different MRP-isoforms and to prove whether and how prostaglandins modify MRP expression and/or function in normal human lung cells. Cells were maintained either under standard culture condition or in air-liquid interface cultures (dry-wet conditions). At least MRP isoforms 1, 3 and 5 have different distribution in dry-wet cultured cells compared to normal submerge cultures. Normal human lung cells are polarized in dry-wet culture conditions with MRPs expressed at the lateral and basal side of the cells. This was shown in NHBECs and PLCs by indirect immunofluorescence and confocal LASER scanning microscopy. Transport activity of MRP1 was studied using single cell fluorometry. NHBEC and A549 cells, a permanent human lung cell line, showed no alteration in transport activity of MRP1 after PG F2a. On the other hand, an increased transport activity was found after PGE2 (0.01 mM, 1d and 4d treatment). PLCs were much less sensitive towards PGE2 than NHBECs. These effects coincide with the rate of MRP1-mRNA expression obtained by real-time RT-PCR in the same trial. The cyclooxygenase inhibitors Indomethacin and Celecoxib inhibited also the transport activity of MRP1 in NHBEC and A549 cells. These results indicate that a link exists between inflammation reaction and regulation of MRP function in human lung. Supported by Philip Morris External Research Foundation

**1462**

#### AN ASSESSMENT OF THE ESTROUS CYCLES OF SPRAGUE-DAWLEY RATS RESTRAINED IN TUBES FOR NOSE-ONLY INHALATION EXPOSURE.

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For fertility and early embryonic development studies (ICH?1) an assessment of estrous cycles is required. Nose-only inhalation exposure is a possible treatment route for these fertility studies. Therefore, an investigation was undertaken to determine the effects of tube restraint for nose-only inhalation exposure on the estrous cycles of Sprague-Dawley Crl:CD® (SD)IGS BR rats and to examine cyclicity at 7 weeks and 9 weeks of age. Rats were housed individually and were provided with food and water ad libitum. The animal room environment and photoperiod (12 hours light and 12 hours dark) were controlled. Rats were randomly assigned to 3 groups, room control or nose-only exposure restraint tube, at either 7 weeks or 9 weeks of age at the start of cycle assessment. The estrous cycles were assessed for 14 days prior to acclimation to tube restraint to confirm that rats were cycling normally. The estrous cycles were then recorded for an additional 14 days for the room control group. Rats in the tube restraint groups were acclimated to the tubes for 3 days at increasing times, 30, 60 and 130 minutes, respectively. Following the acclimation period, the tube restraint groups were placed in tubes for an additional 11 days for 60 minutes. The estrous cycles were recorded by examination of the vaginal smears. Nose-only tube restraint for 60 minutes per day had no adverse effect on the estrous cycle parameters (cycle length, number of days in estrous and number of cycles) of female CD IGS rats at 9 to 11 and 11 to 13 weeks. Therefore it was concluded that tube restraint for nose-only inhalation exposure does not adversely effect estrous cycles of rats from 9 to 13 weeks of age.

**1463**

#### IMPACT OF LOW FLOW OPERATION IN THREE NOSE-ONLY EXPOSURE SYSTEMS.

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Directed-flow, nose-only (DFNO) exposure systems are designed to deliver a stable concentration of gas or aerosol to the animal with minimum mixing between the inhaled atmosphere and the exhaled breath. Exposure air exits a delivery tube and crosses a cavity before it reaches the animal nose and then reverses direction into the exhaust. Exhaled breath from the animal passes through portions of the same cavity before entering the exhaust. It is expected that the two airstreams mix during each breath. The degree of mixing and thus the reduction in the concentration of test material in the inhaled air is a function of geometry, minute ventilation of the animal and the flow rate of exposure air in the delivery tube. For any DFNO exposure system there is a flow above which dilution of the exposure atmosphere by breath is less than 10%. The impact of low flow was measured in a Cannon (Lab. Products, Seaford, DE), Jaeger Vaccine, and Jaeger 12-Port (CH Technologies Inc., Westwood, NJ) DFNO exposure systems. A Harvard Rodent Ventilator (Harvard Apparatus, S. Natick, MA) was used to simulate a rat minute ventilation of 200 ml/min at 150 breaths per minute. Butadiene (Linde Gas, Maumee, OH) was monitored with a Miran 1A (Foxboro Analytical, S. Norwalk, CT). Exposure air flow rates at each active exposure port were set to 1.5 to 4 times the minute ventilation of the simulated animal. Less than 10% reduction in concentration of test material was achieved when airflows for the Cannon, Jaeger Vaccine, and Jaeger 12-Port DFNO systems were respectively greater than 2.5, 2.5, and 1.7 times the minute ventilation of the animal.

SUPPLEMENTAL DOSING IN PRECLINICAL  
INHALATION STUDIES INCREASES SYSTEMIC  
EXPOSURES.

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Limitations of the exposure generation apparatus and physicochemical properties of the test article can restrict the maximum dose generated by the inhaled route of exposure for preclinical studies. Resultant inhaled doses oftentimes do not achieve adequate systemic exposure to permit the identification of potential extra-pulmonary target organ(s) of toxicity. To optimize systemic exposure and thus help establish target organ toxicity, we have implemented adjunct dosing for preclinical inhalation studies. For two inhaled asthma programs, in which the top inhaled dose was limited due to exposure generation (VLA4 and IL-4 targets), rats and dogs were exposed in 13-week toxicity studies by dry powder inhalation and an adjunctive route (either subcutaneous or oral, based on solubility of test article and oral bioavailability). The studies consisted of an air-sham control and a vehicle control (i.e. received inhaled lactose powder and the adjunct dosing vehicle via subcutaneous or oral route), and a low-, mid- and high-dose inhalation group. An additional high-dose inhalation group received adjunct compound (by the oral route for the IL-4 program and the subcutaneous route for the VLA4 program), at the maximally feasible dose, 1-hour following the daily inhalation session. Results of these studies indicate supplementing compound by the oral or subcutaneous route to the high-dose inhaled group can increase systemic exposures by 2 to 13-fold (Day 90 AUC) over the high-dose dry powder only group. Moreover, dosing dogs and rats to the adjunct vehicle had no deleterious effects on the vehicle control group as compared to the air-sham control group (based on clinical signs, food intake, and body weight). In conclusion, supplemental dosing offers an advantageous method of augmenting systemic exposure when limitations have been imposed by the inhalation exposure system and/or physicochemical properties of the test compound. Achieving higher systemic exposures increases the probability of establishing target organ toxicity.

APPLICATION OF THE AERONEB® PROFESSIONAL  
NEBULISER TO INHALATION DOSING SYSTEMS FOR  
DOGS AND PRIMATES.

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The compact, single-pass nebuliser was mounted close to the breathing zone in face masks and oropharyngeal dosing apparatus for inhalation exposure. No animals were used in this study, which was a system characterisation using aqueous Salbutamol. Delivery to the animal airways via a facemask was modeled by sampling from within a manikin head into a filter with a respiratory pump to simulate the animal breathing. Deposition was also measured in the mask and the exhaust system. Deposition in the dog manikin airway was ca 37%, while into the primate manikin airway this was ca 30%. In a similar experiment modeling delivery to a dog via an oropharyngeal tube, 45% of the generated Salbutamol was drawn into filters by the respiratory pump. The delivered aerosols were highly respirable aerosol, with average Mass Median Aerodynamic Diameter 1.8 micron. Used in conjunction with dog and primate inhalation dosing apparatus, the Aeroneb® Pro delivered respirable aerosols with good efficiency, offering advantages for inhalation toxicology studies.

PERFORMANCE OF FLOW-PAST AND  
CONVENTIONAL NOSE-ONLY INHALATION  
EXPOSURE SYSTEMS WITH POWDER AND DROPLET  
AEROSOLS.

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For snout-only exposure of rodents, two types of inhalation chamber are commonly used: a simple 30-cm cylinder with breathing ports in the side (ADG-type) and a "flow-past" system that delivers fresh aerosol to each animal individually via a manifold. We have evaluated both types of chamber equipped for the exposure of 120 and 128 animals respectively. Nominal aerosol concentrations were between 0.3 and 12 mg/L with 80 L/min air flow rate. Kaolin aerosol from a Rotating Brush Generator was delivered via the flow-past and ADG chambers with ca 25% and 35% efficiency respectively. The delivered Particle Sizes were ca 2.0 and 2.5 $\mu$ m Mass Median Aerodynamic Diameter (MMAD). Consistency of delivered aerosol concentration across all animal ports was e.g. 13% and 7% respectively at the high concentration. Greater variability of Kaolin concentration was observed with the flow-past chamber, possibly due to re-dispersal of impacted powder. An aqueous aerosol of 1% Sunset Yellow in buffer was generated from 1 to 4 Parry LC+ nebu-

lisers. Efficiency of delivery via the flow-past and ADG chambers was ca 60% and 75% respectively at high concentration and a similar 90% efficiency at low concentration. The delivered Particle Size was similar at ca 1.1  $\mu$ m MMAD. Consistency of delivered aerosol concentration was typically ca 10% for both chambers. It was concluded that while either chamber design can give consistent results with liquid aerosols, the advantages of the Flow

ANALYSIS OF UNRESTRAINED WHOLE-BODY  
PLETHYSMOGRAPHY (WBP) DATA BY SIGNAL  
PROCESSING AND MECHANISTIC MODELING.

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Unrestrained WBP provides a measure of respiratory response to irritant gases. We first used WBP and BioSystem XA software (Buxco Electronics, Inc., Wilmington, NC) to collect and analyze respiratory data from rats. The software menu provides the model of "Drorbaugh and Fenn" (Pediatrics, 16:81-87, 1955; D&F) as one option but that model was derived for a completely sealed chamber and the current WBP has continual flow-through of air or exposure atmosphere. Since the software is proprietary, details of the data analysis code are unavailable. Therefore we re-derived the D&F model to include flow and created a program in Matlab (Mathworks, Inc., Natick, MA) to implement the model. Due to high noise in the raw data we also performed a spectrographic analysis to identify the frequency range of primary interest as well as persistent interference from mechanical or electrical noise. Visual investigation of the spectrograms allows for quick identification of periods of high vs. low noise, the general trend of the primary breathing frequency, and constant background noise. A low-pass digital filter was used to remove high-frequency noise from the data before respiration analysis. Criteria for breath length, volume, and volume balance were then applied to identify acceptable breaths, which were analyzed for breathing parameters. BioSystems output and our values for peak inspiratory flow and tidal volume compared well (e.g., 5.7 vs. 5.1 ml/s and 0.90 vs. 0.96 ml, respectively), but our estimated frequencies (e.g., 82 vs. 100 bpm) were substantially lower. Our model derivation for the flow-through WBP uses the same basic assumptions as D&F, is implemented in Matlab, and allows modification of the assumptions and equations as needed for subsequent research.

DESIGN AND CHARACTERIZATION OF A NOVEL  
ROBOTIC WELDING FUME INHALATION AND  
EXPOSURE SYSTEM FOR LABORATORY ANIMALS.

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Respiratory effects observed in welders have included lung function changes, metal fume fever, bronchitis, and a possible increase in the incidence of lung cancer. Many questions remain unanswered regarding the causality and possible underlying mechanisms associated with the potential toxic effects of welding fume inhalation. The objective was to construct a completely automated, computer-controlled welding fume generation and exposure system to simulate real workplace conditions. The system was comprised of a programmable six-axis robotic welding arm, a water-cooled arc welding torch, and a wire feeder that supplied the wire to the torch at a programmed rate up to 300 in/min. For the initial studies, gas metal arc welding was performed using a stainless steel electrode. A flexible trunk was attached to the robotic arm of the welder and was used to collect and transport fume from the vicinity of the arc to the animal exposure chamber. Undiluted fume concentrations consistently ranged from 90-150 mg/m<sup>3</sup> in the animal chamber during welding. Temperature and humidity remained constant in the chamber during the welding operation. The welding particles were comprised of (from highest to lowest concentration) Fe, Cr, Mn, and Ni as measured by inductively coupled plasma atomic emission spectroscopy. Size distribution analysis indicated the mass median aerodynamic diameter of the generated particles to be approximately 0.24  $\mu$ m with a geometric standard deviation of 1.39. As determined by scanning electron microscopy, the generated aerosols were mostly arranged as chain-like agglomerates of primary particles. These characterization studies of the generated welding aerosol have indicated that particle morphology, size, and chemical composition are comparable to stainless steel welding fume generated in the workplace. With the development of this novel system, it will be possible to establish an animal model using controlled welding exposures to investigate how welding fumes affect health.

CONCENTRATION-DEPENDENCE OF STABILITY OF AEROSOLS OF MONOMERIC DIPHENYL-METHANE-4, 4-DIISOCYANATE (MDI).

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Polymeric diphenyl-methane-4, 4-diisocyanate (pMDI) is a reactive chemical intermediate with low vapor pressure. It is a major industrial chemical, mainly used for the production of polyurethanes. pMDI commonly contains 30-50% monomeric MDI (mMDI), the balance are higher oligomers. At room temperature mMDI is a waxy solid, whilst pMDI is a viscous liquid. Its vapor saturation concentration at normal room temperatures is in the range of 0.05-0.1 mg/m<sup>3</sup>, i.e., higher concentrations, as required for inhalation toxicity studies, can only be attained by aerosolization. For MDI, this objective is met by using highly specialized methodologies. The focus of this research is to analyze methodologies for testing of high (approx. 2000 mg/m<sup>3</sup>; the 1-hour LC<sub>50</sub> of mMDI is greater than 2240 mg mMDI/m<sup>3</sup>) and lower concentrations (0.1 mg/m<sup>3</sup>) of mMDI. The results show that mMDI, when evaporated at elevated temperature (90 DegC) has a markedly lower tendency to evaporate than predicted by thermodynamic calculations. In contrast, supersaturated vapor atmospheres could be generated at higher temperatures using bubblers systems and condensation aerosols were formed upon cooling. Aerosols were only detectable at concentrations above the vapor saturation concentration, i.e. 0.1 mg/m<sup>3</sup>. This supports the conclusion that mMDI condensation aerosol is thermodynamically unstable at or, below the vapor saturation concentration. Stable condensation aerosols of mMDI occurred only at supersaturated vapor concentrations in closed systems. Accordingly, for Agencies which mandate a specific physical form for the manifestation of an intrinsic hazard in experimental animals the toxicological significance of findings for humans needs to be challenged. This data suggest, that the intrinsic hazard of mMDI aerosol appears to be dominated by concentration rather than unique features of this chemical. The intrinsic hazards demonstrated in inhalation studies need to be associated with the actual physical form forced by specified testing conditions, which may not reflect the actual physical form present in the workplace.

DEVELOPMENT OF A NOVEL TRIPLE MANIFOLD NOSE-ONLY RODENT INHALATION SYSTEM: ACUTE TOXICITY OF RICIN AEROSOLS IN BALB/C MICE.

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Rodent inhalation nose-only-type exposure systems generally employ a single or double manifold design wherein an airborne substance at a particular concentration is generated and drawn past the snout of the animal before being exhausted from the system. Because of this design limitation, multiple discrete 'runs' of a particular system or concurrent use of many systems using multiple concentration levels are required to accomplish inhalation studies with multiple dosage groups. We have designed a singular tower inhalation system using a triple manifold configuration, electronically actuated directional flow valves, and an automated management platform, allowing simultaneous duration-based exposure of multiple dosage groups from a common aerosol stream. As a demonstration of the new design, groups of BALB/c mice were exposed to ricin toxin aerosols (MMAD=1  $\mu$ m,  $\sigma$ g=1.4) at 5.7  $\pm$  0.3  $\mu$ g/l for either 1, 3, or 15 min for corresponding inhaled doses of 5.4  $\pm$  0.3, 16.2  $\pm$  0.9 or 81.3  $\pm$  4.7  $\mu$ g/kg, respectively. Group survival results indicated consistency with the established ricin inhalation LD<sub>50</sub>, and confirmed the definable dose-response curve. These results suggest that the described system can reduce both inter-experimental aerosol concentration variability and the time required to complete studies involving multiple dosage levels as compared to conventional inhalation systems. These capabilities would allow protocol development for high throughput *in vivo* toxicity testing where inhalation is the desired route of exposure.

ESTABLISHMENT OF A BIOLOGICAL BIOASSAY FOR DETECTION OF LUNG TOXICITY DUE TO FINE PARTICLES.

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In order to establish an appropriate bioassay for detection of lung damage after fine particle inhalation, sequential histopathological changes were examined after intratracheal instillation of quartz, as a typical lung toxic agent, into F344 male rats. Experiment 1: A total of 50, 10-week-old male F344 rats, were separated into two groups. Twenty five were exposed to quartz powder (DQ-12, 4mg/rat) suspended in saline (0.2ml) using an aerolizer and subgroups were killed on Days 1, 3, 7, 14, and 28 thereafter. The remaining 25 rats received the saline vehicle (0.2ml) as a control group and were killed on the same days. Both groups underwent assessment

of lung histopathology and immunohistochemical demonstration of BrdU, iNOS and MMP-3 as end-point markers. Days 1 and 28 after intratracheal instillation of test fine particles were suggested to be most appropriate for detection of acute and subchronic inflammatory changes, respectively. Furthermore, BrdU on Day 1 and iNOS on Day 28 proved to be suitable end-point markers for this purpose. Experiment 2: A total of 108, 10-week-old male F344 rats, were randomly separated into 8 groups. Seventy rats (5 groups) were exposed by intratracheal instillation to quartz, hydrotalcite, potassium octatitanate, palladium oxide or carbon black, 4mg/rat, suspended in 0.2ml control vehicle. Twenty eight (2 groups) animals were exposed by intratracheal instillation to 0.2ml saline and 0.2ml 10% propylene glycol 1% carboxymethylcellulose (PG-CMC) as controls, and the remaining 10 rats were maintained as an untreated group. Subgroups of 7 treated, 7 control and 5 untreated rats, were then killed on Days 1 and 28 and all groups underwent assessment of lung parameters as in Experiment 1. Quartz proved to be the most toxic to lungs and other particles were considered as exerting low toxicity on histopathological and immunohistochemical assessment. This instillation model can be used for detection of acute or subchronic lung toxicity due to inhaled fine particles.

GLASS BEAD INHALATION AND INDUCTION OF SILICOSIS.

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Silicosis is an occupational lung disease caused by the inhalation of silica of respirable size, in crystalline forms. Some have claimed that glass beads, because they too are composed of silica, are associated with silicosis. We reviewed and summarized the primary toxicological literature relating to glass beads, which consisted of 12 *in vitro* studies and 5 *in vivo* studies. The *in vitro* studies used glass beads primarily as negative controls (7/12 studies) in bioassays with alveolar macrophages (7 studies) or respiratory tissue from lungs and/or trachea (6 studies - 1 study used both tissue and macrophages). In the alveolar macrophage studies, glass beads decreased the viability of the macrophages and phagocytosis starting at about 100-1,000  $\mu$ g/mL, probably due to a particle loading effect. Four of the five *in vitro* studies using lung or tracheal tissue, showed few or no differences from untreated controls, and produced no fibrogenic responses and no effects in induction of DNA repair enzymes or cell proliferation. One study showed significant increases in hyperplastic, metaplastic, or dysplastic lesions, but only at the highest dose tested - 10,000  $\mu$ g/mL. In the *in vivo* studies, which were conducted using intratracheal administration of particles in rats, glass beads were used as an inert particle control. These studies showed only minimal (if any) differences in fibrogenicity or lung function parameters. In one study with serial sacrifices, the lungs of rats instilled with glass beads had minimal to moderate inflammation on post-treatment day 3, and in each successive sacrifice the lesions became less severe and affected fewer rats. We also reviewed the relevant occupational guidance numbers (and their basis) set by ACGIH and those promulgated by OSHA. OSHA has classified glass beads as "nuisance dust," since they contain no hazardous materials. Consequently, OSHA and others view glass beads as a safe alternative to the silica used in abrasive blasting. Based on our review of the primary literature, as well as occupational limits and their basis, we conclude that glass beads do not cause silicosis or other respiratory disease.

DEVELOPMENT OF TOLUENE AEGLS USING PBPK MODELING IN RATS AND HUMANS.

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AEGLs have historically been established using empirical approaches for extrapolation across time and applying uncertainty factors (UFs). While PBPK models have been primarily used for cancer and other chronic endpoint risk assessment in the past, we used PBPK models to provide an explicit basis for rat to human extrapolation for the acute endpoint relevant to toluene AEGLs. This context raised a different spectrum of modeling issues, including low- to high-dose extrapolation and the need to consider workload during emergencies. The PBPK model was adapted from existing toluene rat/human PBPK models using previously published physiological and kinetic parameters but adding a second low affinity enzyme. The model was validated with available high dose and workload data and a good fit was obtained to previously published blood and exhaled breath data. As found for some other solvents, blood levels in humans were lower than in rats due to a lower blood:air partition coefficient. AEGL values were calculated by 1) determining the blood level in rats at the point of departure for the AEGL-1, -2 or -3; 2) calculating the equivalent human exposure that resulted in the same blood level during human exposure for 10 and 30 minutes and 1, 4 and 8 hours; 3) applying intraspecies UFs for human variability. Thus, the PBPK approach explicitly dealt with both rat-human and temporal extrapolation. Human equivalent values were generally higher, but assuming a workload of 50W tended to negate the differences. Using

PBPK models provided a basis for setting the toxicokinetic UF in rat-human extrapolation to 1. The PBPK model also showed a significant difference in time-dependant AEGL values from those calculated with the ten Berge equation, which underestimates the equivalent exposure during early time points. For chemicals where PBPK models are available, the approach reduces extrapolation uncertainty and allows workload to be taken into account. [This abstract presents interim AEGL values that are subject to change pending further review.]

#### 1474 INTERIM ACUTE EXPOSURE GUIDELINE LEVELS (AEGLS) FOR XYLEMES.

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AEGLs represent threshold exposure limits for the general public and are applicable to emergency exposure periods ranging from 10 minutes to 8 hours. AEGL-1, AEGL-2 and AEGL-3 are developed for each of five exposure periods (10 and 30 minutes, 1, 4, and 8 hours) and are distinguished by varying degrees of toxicity ranging from mild sensory irritation to irreversible effects to death. AEGLs were developed for mixed xylenes, comprising three isomers: meta-xylene, ortho-xylene, and para-xylene. The AEGL-1 of 130 ppm is based upon the no-effect level for notable discomfort: only slight eye irritation was noted by human volunteers during a 30-minute exposure to 400 ppm mixed xylenes. A total uncertainty factor (UF) of 3 is applied: 1 for interspecies and 3 for intraspecies. Because irritation is considered a threshold effect and should not vary over time, the AEGL-1 threshold value is applied to all times. AEGL-2 values (1100, 590, 400, 400, and 400 ppm, respectively) are based upon the NOEL for the inability to escape: poor coordination was observed in rats 2 hours into a 4-hour exposure to 1300 ppm mixed xylenes. AEGL-3 values (3300, 1700, 1100, 1100, and 1100 ppm, respectively) are based upon a NOEL for death and reversible prostration in rats exposed for 4 hours to 2800 ppm mixed xylenes. A total UF of 3 was applied to both the AEGL-2 and -3 values: 1 for interspecies extrapolation and 3 for intraspecies extrapolation. A validated PBPK model was used to perform the extrapolation of rat to human blood concentrations for setting the AEGL-2 and AEGL-3 values. Using the PBPK model reduced the uncertainty in extrapolation, and allowed direct consideration of the activity level during chemical emergencies. The AEGL-2 and -3 values were set assuming a workload of 50W for 10 minutes, 30 minutes, and 1 hour. The 4- and 8-hour AEGL-2 and -3 values were set equal to the 1-hour AEGL values based on the assumption that this activity level would not be maintained. [This abstract presents interim AEGL values that are subject to change pending further review.]

#### 1475 A REVIEW OF THE BASIS FOR HYDROGEN CHLORIDE OCCUPATIONAL EXPOSURE LIMITS.

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Hydrogen chloride is a corrosive, colorless, water soluble compound that is a widely used commodity chemical in numerous forms and applications. Occupational exposures to hydrogen chloride are predominantly to the gas phase. The main issue related to occupational health is that hydrogen chloride is strong irritant to the eyes, mucous membranes, skin, and respiratory tract. For many decades the acceptable Occupational Exposure Limits (OELs) have ranged from 5-10 ppm as a ceiling or as a time-weighted-average (TWA). The basis of traditional OELs has been the reporting of a 10 ppm lowest observable adverse effect level (LOAEL) in humans for irritation. However, a recent review of the 10 ppm LOAEL showed it to be anecdotal since appearing in the literature about 1931. A more thorough investigation of the LOAEL source report, which dates back to 1889, reveals that the actual LOAEL cited was 100 ppm. It seems that in the 1930s there was a decimal point transposition that propagated the 10 ppm value throughout the literature to present day. Because of the apparent anecdotal nature of the data, limit setting bodies have discounted it in the risk assessment process. This has shifted the basis of hydrogen chloride risk assessment to studies revolving around negative findings in asthmatic subjects, which has lowered one of the OELs. Performing a risk assessment on negative data such as this can result in an unduly low OEL. This report is designed to reintroduce and clarify the reporting of the original data. The use of this original data fills the gap in the concentration-response curve providing a more robust risk assessment for hydrogen chloride. Inclusion of the data suggests that an OEL in the range of 5-10 ppm is acceptable for hydrogen chloride.

#### 1476 DEVELOPMENT OF ACUTE EMERGENCY GUIDELINE LEVELS (AEGLS) USING BENCHMARK DOSE (BMD) METHODOLOGY TO CALCULATE POTENTIALLY LETHAL EXPOSURES.

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The National Advisory Committee for AEGLs for Hazardous Substances (NAC/AEGLs) establishes AEGL-3 values to assist emergency planners in preventing accidental exposures that increase the likelihood of death. Three methods have

been identified to establish a starting point for AEGL-3 values. The preferred method is to use a benchmark dose exposure calculation (BMC) of the 5% and 1% response. We compared the BMC method with the experimentally observed lethality thresholds (AEGL-3 NOAELs), and AEGL-3s estimated by one-third of the LC<sub>50</sub> (LC<sub>50</sub>/3) for 100 data sets using 40 different chemicals. Using U.S.EPA BMD software (BMDS) version 1.3.2, we also compared results of the BMD software to that of the Toxicology-Risk software (TRS) version 3.5. Previously we reported BMC<sub>01</sub>s and BMC<sub>05</sub>s, using TRS, were generally consistent with the NOAELs for lethality. We found a similar conclusion with the BMDS; that is, on average we found NOAELs to be up to 20% greater than the BMC<sub>01</sub> and BMC<sub>05</sub>. While we would have expected the LC<sub>50</sub>/3 method to be conservative, we found that it overestimated the BMDL in 25% of the cases. The occurrence of a shallower slope, i.e., less than 6, appeared to be a major factor in these cases. Finally, we compared the results of the TRS to the BMDS and found a strong correlation between the values. However, in 3% of the comparisons the values were substantially different, probably due to poor determination of the model. In conclusion, the AEGL-3 NOAEL, BMC<sub>05</sub>, BMC<sub>01</sub> and LC<sub>50</sub>/3 generally provided consistent values. However, in cases of slopes less than 6, the default uncertainty factor of 3 is not sufficiently conservative to estimate a non-lethal value.

#### 1477 USING ANIMAL LC50 DATA TO ESTIMATE ACUTE EXPOSURE LETHALITY THRESHOLDS FOR WORKERS.

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Animal toxicology data are used to estimate acute chemical exposures that are safe for humans by applying a widely-used quantitative method to determine concentration-time mortality response relationships as well as uncertainty factors that depend on type and quality of data. We previously identified the quantitative methods for extrapolating from animal LC50s to immediately dangerous to life and health (IDLH) values among key uncertainties in methods NIOSH used for deriving IDLH values. These uncertainties result in part from the paucity of systematic correlations of acute inhalation exposure-response data in animals and humans. Using available data, we studied the following: + Reliability of a calculation method widely used for determining concentration-time mortality response relationships + Quantitative relationships between acute inhalation LC50s and lowest lethal concentrations in animals + Quantitative relationships between acute inhalation lethal exposure data in animals and humans. Our analyses indicate that experimental research to overcome data limitations and inconsistencies between studies is needed. Lethal exposure data in humans always will be limited to what can be deduced for case reports. To eliminate uncontrolled variables in correlations of exposure-response relationships between humans and experimental animals, we encourage future animal studies that attempt to replicate documented exposure conditions that led to human deaths. To improve data for developing acute exposure guidelines, we encourage experimental studies to determine relationships between animal exposures under these conditions and conditions assumed by guideline-setting organizations. The conditions assumed by NIOSH for IDLH values are a single 30-minute inhalation exposure followed by an extended observation period.

#### 1478 ACUTE EXPOSURE GUIDELINE LEVELS (AEGLS) FOR NITROGEN DIOXIDE AND NITRIC OXIDE.

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AEGLs represent threshold exposure limits for the general public and are applicable to emergency exposure periods of 10 min, 30 min, 1 hr, 4 hr, and 8 hr. NO<sub>2</sub> is an irritant to the mucous membranes; death is caused by bronchospasm and pulmonary edema. After the acute phase, apparent recovery may be followed by late-onset injury that manifests as bronchiolitis fibrosa obliterans. Inhaled NO has is used therapeutically but the toxicity is associated with both methemoglobin formation and oxidation to NO<sub>2</sub>. Because NO converts to NO<sub>2</sub> in the atmosphere, and NO<sub>2</sub> is more toxic than NO, the AEGL values for NO<sub>2</sub> are recommended for NO. AEGL-1 (discomfort) values (0.50 ppm for all time-points) were based on exposure of asthmatic volunteers to 0.5 ppm NO<sub>2</sub> for 2 hrs. 7/13 individuals experienced slight burning of the eyes, headache, and chest tightness or labored breathing with exercise but no changes in pulmonary function. No uncertainty factor was applied. AEGL-2 (disability) values (20, 15, 12, 8.2, and 6.7 ppm) were based on exposure of three healthy male volunteers to 30 ppm NO<sub>2</sub> for 2 hrs. The individuals perceived an intense odor upon entering the chamber; after 70 minutes, they experienced a burning sensation and an increasingly severe cough with marked sputum secretion and dyspnea. Towards the end of the exposure, the subjects reported the conditions as bothersome and barely tolerable. AEGL-3 (death) values (34, 25, 20, 14, and 11 ppm, respectively) were based signs of marked irritation and severe lung histopathology in monkeys exposed to 50 ppm NO<sub>2</sub> for 2 hrs. For AEGL-2 and -3 values, extrapolations were made with the equation C<sup>n</sup> × t = k using n = 3.5. The

value of n was calculated using data in five species of laboratory animal. A total uncertainty factor of 3 was applied to the AEGL-2 and -3 values including 3 to account for sensitive individuals and 1 for interspecies extrapolation. [This abstract presents Proposed AEGL values that are subject to change pending further review.] ORNL, managed by UT-Battelle LLC, for the US Department of Energy under contract DEAC0500OR22725.

**1479**

ACUTE EXPOSURE GUIDELINE LEVELS (AEGLS) FOR NITRIC ACID.

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AEGLs represent threshold exposure limits for the general public and are applicable to emergency exposure periods of 10 min, 30 min, 1 hr, 4 hr, and 8 hr. Nitric acid is a highly corrosive, strongly oxidizing acid. The course of toxicity following inhalation exposure to nitric acid is consistent between humans and animals and includes immediate irritation of the respiratory tract, pain, and dyspnea which are followed by a period of recovery that may last several weeks. After this time, a relapse may occur with death caused by bronchopneumonia and/or pulmonary fibrosis. AEGL-1 (discomfort) values (0.53 ppm for all time-points) were based on exposure of five healthy volunteers to 1.6 ppm nitric acid for 10 min. No changes in pulmonary function were observed. An uncertainty factor of 3 was applied to account for sensitive individuals. AEGL-2 (disability) and AEGL-3 values (death) were based on a lethality study in rats. Groups of 5 rats/sex were exposed nose-only for 1 hr to 260-3100 ppm of nitric acid aerosol followed by a 14-day observation period. AEGL-2 values (43, 30, 24, 6.0, and 3.0 ppm, respectively) were based on transient body weight loss following exposure to 460 ppm. AEGL-3 values (170, 120, 92, 23, and 11 ppm) were based on an estimated LC<sub>01</sub> of 919 ppm calculated by a log-probit analysis. For AEGL-2 and -3 values, extrapolations were made with the equation C<sup>n</sup> × t = k using default values of n = 3 for extrapolating to shorter durations and n = 1 for extrapolating to longer durations. A total uncertainty factor of 10 was applied to AEGL-2 and -3 values which includes 3 to account for sensitive individuals and 3 for interspecies extrapolation. [This abstract presents Proposed AEGL values that are subject to change pending further review.] Oak Ridge National Laboratory, managed by UT-Battelle, LLC, for the US Department of Energy under contract DEAC0500OR22725.

**1480**

INTERIM ACUTE EXPOSURE GUIDELINE LEVELS (AEGLS) FOR AMMONIA.

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AEGL values represent threshold exposure limits for the general public applicable to emergency exposure periods of 10, 30 min and 1, 4, and 8 hrs and three severity levels ranging from mild sensory irritation to irreversible/disabling effects to death. Ammonia also has a 5-min value. Ammonia is a corrosive, alkaline gas that has a very strong odor; odor detection ranges from 5-53 ppm. The corrosive and exothermic properties of ammonia cause irritation and burns to eyes, skin, oral cavity, and respiratory tract, the severity depending on the exposure concentration. Low concentrations cause mild upper respiratory tract irritation, high concentrations cause tracheobronchial damage, and extremely high concentrations cause pulmonary edema and death. The AEGL-1 value of 30 ppm for all time points was derived from a human study showing mild respiratory tract irritation after exposure to 30 ppm for 10 min. AEGL-2 values of 380, 170, 160, 110, 110, and 110 ppm, respectively, were derived from a study in human volunteers reporting offensive eyes and respiratory tract irritation after exposure to 110 ppm for 1 hr. The AEGL-2 value for 1 hr was retained for 4 and 8 hrs because of human adaptation to ammonia. AEGL-3 values of 3800, 2700, 1600, 1100, 550, and 390 ppm, respectively, were derived from an estimate of the lethality threshold (LC01 = 3317 and 3374 ppm) calculated from acute lethality data from mice exposed to ammonia for 1 hr. An intraspecies uncertainty factor (UF) of 1 was applied for AEGL-1 and -2 derivations, and intraspecies UF of 1 and an intraspecies UF of 3 were applied for AEGL-3 derivation. The equation, C<sup>n</sup> × t = k, where n = 2, was utilized for time scaling of AEGL-2 and AEGL-3 where applicable. [This abstract presents interim AEGL values that are subject to change pending further review.] Oak Ridge National Laboratory, managed by UT-Battelle, LLC, for the US Department of Energy under contract DEAC0500OR22725.

**1481**

EXTRAPOLATION OF LC50S TO SAFE EXPOSURE LEVELS - INSIGHTS FOR EMERGENCY RESPONDERS.

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Health guidance values provide response managers with useful information for directing evacuations and reentry associated with acute hazardous chemical release. Because health guidance values have not been derived for many hazardous sub-

stances, we investigated the usefulness of extrapolating from published toxicological properties, in order to support response managers. For our dataset, we selected all 74 substances that currently have an inhalation Minimal Risk Level (MRL) from the Agency for Toxic Substances and Disease Registry (ATSDR). LC50 (lethal concentration 50%) values were located for 55 of these; 19 were insufficiently toxic or had no LC50s. A total of 227 LC50s were located for the 55 substances. Concentrations were standardized for LC50s and their respective MRLs. As an initial approach, we plotted LC50s against MRLs. In a log-log plot, a general linear trend was evident, yet the 227 datapoints were widely scattered ( $R^2=0.62$ ). The data were subsetted to control for differences in LC50 species or duration. Substantial variability remained while, at the same time, the number of datapoints was considerably reduced. We also attempted to control for the variability introduced by LC50 exposure time using the ten-Berge et al. (J Hazard Mater 13:301, 1986) modification to Haber's rule, but only a handful of substances had sufficient LC50 data for a reliable concentration exponent. We dropped the log-log plot approach in favor of percentile statistics concerning LC50/MRL ratios. The median LC50/MRL ratio was 14, 000 or, if MRL uncertainty factors are dropped to approximate No Observed Adverse Effect Levels (NOAELs), the median LC50/NOAEL ratio was 800. The 95th percentiles were 880, 000 and 32, 000 for LC50/MRL and LC50/NOAEL ratios, respectively. These results (and additional statistics to be presented) may provide guidance to response managers when handling data-poor substances.

**1482**

ACUTE EXPOSURE GUIDELINE LEVELS (AEGLS) FOR HYDROGEN SULFIDE.

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AEGL values represent one-time exposures for increasingly severe effects with AEGL-1 being least and AEGL-3 being most severe. Hydrogen sulfide is a colorless, flammable gas at ambient temperature and pressure. It has an odor similar to that of rotten eggs and is both an irritant and asphyxiant. It acts similarly to cyanide by interrupting the electron transport chain through inhibition of cytochrome oxidase. AEGL-1 values (0.75 ppm, 0.60 ppm, 0.51 ppm, 0.36 ppm, and 0.33 ppm, for 10-min, 30-min, 1-hr, 4-hr, and 8-hr, respectively) were based on headache in asthmatic humans exposed to 2 ppm H<sub>2</sub>S for 30 minutes. AEGL-1 values were adjusted by a modifying factor of 3. AEGL-2 values (41 ppm, 32 ppm, 27 ppm, 29 ppm, and 17 ppm, for 10-min, 30-min, 1-hr, 4-hr, and 8-hr, respectively) were based on perivascular edema in rats exposed to 200 ppm H<sub>2</sub>S for 4 hours. The AEGL-3 values (76 ppm, 59 ppm, 50 ppm, 37 ppm, and 31 ppm, for 10-min, 30-min, 1-hr, 4-hr, and 8-hr, respectively) were based on a 1-hour lethality threshold in rats of 504 ppm H<sub>2</sub>S. Interspecies and intraspecies uncertainty factors of 3 each were applied to AEGL-2 and AEGL-3 values. AEGL-2 and AEGL-3 values were scaled across time using the exposure-concentration duration relationship, C<sup>4.4</sup> × t = k, shown to be valid for lethality in rats for H<sub>2</sub>S exposures ranging from 10-minutes to 6-hours. Oak Ridge National Laboratory, managed by UT-Battelle, LLC, for the US Department of Energy under contract DEAC0500OR22725.

**1483**

MINIMAL RISK LEVELS FOR HYDROGEN SULFIDE - UPDATE.

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The Agency for Toxic Substances and Disease Registry (ATSDR) derives minimal risk levels (MRLs) for hazardous substances based on available toxicological and epidemiologic information, as detailed in the Agency toxicological profiles. An MRL is an estimate of the daily human exposure to a hazardous substance that is likely to be without appreciable risk of adverse, non-cancer health effects. The toxicological profile for hydrogen sulfide was recently updated; revisions of the acute inhalation MRL and the intermediate inhalation MRL were proposed. The acute inhalation MRL of 0.2 ppm was based on a lowest-observed-adverse-effect level (LOAEL) of 2 ppm for respiratory effects. Two out of 10 persons with asthma exposed to hydrogen sulfide for 30 minutes had changes in excess of 30% in airway resistance or specific airway conductance, suggestive of bronchial constriction. These individuals showed no significant changes in lung function (Jappinen et al. Br J Ind Med. 1990, 47:824-8). To derive the MRL, an uncertainty factor (UF) of 9 was applied, 3 for use of a minimal LOAEL, and 3 for human variability. The intermediate inhalation MRL of 0.02 ppm was based on a no-observed-adverse-effect level (NOAEL) of 10 ppm for respiratory effects in rats exposed to hydrogen sulfide for 6 hours/day, 7 days/week for 10 weeks. Olfactory neuron loss and basal cell hyperplasia in the olfactory epithelium of the nose were seen in rats exposed to 30 ppm and 80 ppm (Brennenman et al. Toxicol Pathol 2000, 28:326-33). To derive the MRL, a human equivalent concentration NOAEL was calculated and an UF of 30 was applied, 3 for animal to human extrapolation, and 10 for human variability. As hydrogen sulfide is a gas, inhalation is the major route of exposure. No federal air

quality standard presently exists for hydrogen sulfide. Health-based guidance values, such as MRLs, are useful for states and local municipalities when addressing community concerns regarding hydrogen sulfide emissions, for example, those from concentrated agricultural feeding operations.

**1484**

ACUTE EXPOSURE GUIDELINE LEVELS (AEGLS) FOR HYDRAZINE (HZ), MONOMETHYLHYDRAZINE (MMH), AND DIMETHYLHYDRAZINE (DMH).

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AEGL values represent one-time exposures for increasingly severe effects with AEGL-1 being least and AEGL-3 being most severe. Hydrazines are highly reactive chemicals used in missile and rocket propellants and in chemical power sources. Their reactivity results in irritation and tissue damage upon exposure. AEGL-1 values for HZ (0.1 ppm) were developed based upon irritation. HZ AEGL-2 values (23 ppm, 16 ppm, 13 ppm, 3.1 ppm, and 1.6 ppm for 10-min, 30-min, 1-hr, 4-hr, and 8-hr, respectively) were based on nasal lesions in rats while AEGL-3 values (64 ppm, 45 ppm, 35 ppm, 8.9 ppm, and 4.4 ppm for 10-min, 30-min, 1-hr, 4-hr, and 8-hr, respectively) were based upon an estimated lethality threshold for rats. Insufficient data precluded the development of AEGL-1 values for MMH or DMH. AEGL-3 values for MMH (16 ppm, 5.5 ppm, 2.7 ppm, 0.68 ppm, and 0.34 ppm) were based upon an estimated lethality threshold for monkeys while AEGL-2 value were derived by a 3-fold reduction of the AEGL-3 values. AEGL-2 values for DMH were 18 ppm, 6 ppm, 3 ppm, 0.75 ppm, and 0.38 ppm using behavioral changes and muscle fasciculations in dogs as the critical effect. DMH AEGL-3 values (65 ppm, 22 ppm, 11 ppm, 2.7 ppm, and 1.4 ppm) were based upon a lethality threshold for dogs. The time-specific AEGLs for MMH and DMH were calculated using the equation,  $c^n \times t = k$ , where  $n$  of approximately 1 was empirically determined for both chemicals. In the absence of an empirically derived chemical-specific scaling exponent, temporal scaling for HZ used default values of  $n=3$  when extrapolating to shorter time points and  $n=1$  when extrapolating to longer time points. All AEGL values were developed using data-justified uncertainty factors. <sup>1</sup>Oak Ridge National Laboratory, managed by UT-Battelle, LLC, for the US Department of Energy under contract DEAC0500OR22725.

**1485**

ACUTE EXPOSURE GUIDELINE LEVELS (AEGLS) FOR 1, 1, 2-TETRAFLUOROETHANE.

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AEGL values represent multiple guidance levels for rare exposures of increasingly severe effects, with AEGL-1 being least and AEGL-3 being most severe. AEGL values were developed for 1, 1, 2-tetrafluoroethane (HFC-134a), a colorless hydrofluorocarbon gas developed as a refrigerant replacement for ozone-depleting chlorofluorocarbons such as CFC-12. Due to its low water solubility and high vapor pressure, uptake is low, but rapid. Elimination is also rapid. Data for development of AEGL values were available from a clinical study and from laboratory studies with multiple species. As a consequence of its proposed use as an aerosol propellant for medicinal inhalers, clinical trials in patients with asthma and chronic obstructive pulmonary disease (COPD) have been performed. The AEGL-1 was based on a 1-hour concentration of 8000 ppm that caused no untoward effects in eight healthy human subjects. An intraspecies uncertainty factor (UF) of 1 was considered sufficient because no adverse effects were reported in therapy tests with sensitive populations (asthmatics and COPD patients) tested with metered dose inhalers. No time scaling was utilized as the clinical study showed that blood levels approached equilibrium after 55 minutes of exposure, and effects are determined by blood concentrations. The AEGL-2 was based on the no-effect concentration of 40,000 ppm for cardiac sensitization in dogs. The dog treated with epinephrine is considered a highly sensitive model for the cardiac effects that could be seen in humans. The levels of administered epinephrine represent an approximate 10-fold excess over the blood concentration that would be achieved endogenously in dogs or humans in highly stressful situations. Therefore, inter- and intraspecies UFs of 1 and 3 were applied. No time scaling was applied: cardiac sensitization is concentration-dependent; duration of exposure does not influence the concentration at which this effect occurs. The AEGL-3 was based on the same study in which 80,000 ppm induced cardiac toxicity in dogs. UFs and time scaling were the same as for the AEGL-2.

**1486**

THE THERAPEUTIC EFFECTS OF LYOPHILIZED BLACK RASPBERRIES ON NMBA-INDUCED CARCINOGENESIS.

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The incidence of squamous cell carcinoma (SCC) of the human esophagus has been linked to diets deficient in fruit and vegetables. Recently, we took a food based approach to prevention of esophageal SCC and found that administration of freeze-

dried strawberries and black raspberries, at concentrations of 5 and 10% in the diet, reduced esophageal tumors induced by the carcinogen, N-nitrosomethylbenzylamine (NMBA), by 40-60% in rats. In the present study, we determined if berries might also exhibit therapeutic effects against esophageal cancer. Six week-old male F344 rats were placed on AIN-76A diet and injected with 0.5 mg/kg NMBA once per week for 15 weeks. Four weeks later, when they had an average of 5 to 6 papillomas per esophagus, the rats were started on diets containing either 0, 5, 10, or 20% freeze-dried black raspberries. For several weeks after initiation of berry treatment, the survival of rats in berry-fed groups was higher than in rats fed control diet. After seven weeks of berry treatment, all surviving rats were sacrificed and esophageal tumor incidence, multiplicity and size determined. In animals fed 10% and 20% black raspberries, there were no significant differences in tumor incidence, multiplicity or size when compared to carcinogen controls. In contrast, rats fed 5% black raspberries had a significant ( $p<0.05$ ) reduction in tumor multiplicity when compared to carcinogen controls. These results suggest that freeze-dried black raspberries may have therapeutic value for the treatment of esophageal cancer. Studies are underway to confirm the therapeutic effect of berries on esophageal tumorigenesis.

**1487**

EFFECTS OF DIETARY SELENIUM ON THE HEPATIC TUMOR PROMOTING ACTIVITIES OF PCBs.

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Polychlorinated biphenyls (PCBs) are persistent organic pollutants that are tumor promoters in the liver. PCBs induce oxidative stress, which may influence carcinogenesis. Due to the antioxidant activities of selenium-dependent glutathione peroxidase and thioredoxin reductase, we hypothesized that selenium could inhibit the promoting activity of PCBs. We examined the effect of dietary selenium on the tumor promoting activities of two PCB congeners, 3, 3', 4, 4'-tetrachlorobiphenyl (PCB 77) and 2, 2', 4, 4', 5, 5'-hexachlorobiphenyl (PCB 153) using a 2-stage carcinogenesis model. An AIN-93 based purified diet containing 0.02 (low), 0.2 (adequate), or 2.0 (high) mg selenium/kg diet was fed to female rats starting ten days after administering a single dose of diethylnitrosamine (150 mg/kg). After being fed the experimental diets for 3 weeks, rats were injected i.p. with either PCB 77 or PCB 153 (300  $\mu$ mol/kg) every other week for 4 injections. Three days before euthanasia, the animals were implanted with osmotic pumps containing bromodeoxyuridine. Our results showed a significant increase in the number of placental glutathione S-transferase-positive foci in liver of animals that received PCB 77. In addition, the focal volume in the PCB 77 treated groups was markedly higher in the adequate selenium group compared with the low selenium group. The number of foci resulting from PCB 153 treatment was neither increased nor decreased; however, the focal volume was significantly increased in rats receiving adequate selenium compared with the low selenium group. Selenium did not affect cell proliferation in hepatocytes of the PCB treated groups. These findings indicate that selenium is not chemopreventive in the promotion of hepatic tumors by PCBs. (Supported by ES07380 and ES07266)

**1488**

EFFECTS OF SELECTED DIETARY ANTIOXIDANT PHYTOCHEMICALS ON THE HEPATIC TUMOR PROMOTING ACTIVITY OF PCB-77.

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Polychlorinated biphenyls (PCBs) have complete carcinogenic and promoting activities in the liver, which may be brought about in part by the induction of oxidative stress. We examined the effects of several antioxidant phytochemicals on the tumor promoting activity of 3, 3', 4, 4'-tetrachlorobiphenyl (PCB-77). Female rats were first injected i.p. with DEN (150 mg/kg) and one week later the rats were fed an AIN-93 based purified diet or the same diet containing ellagic acid (0.4%), beta-carotene (0.5%), curcumin (0.5%), N-acetyl cysteine (NAC) (1.0%), CoQ10 (2.0%), resveratrol (0.005%), lycopene (10% as LycoVit [BASF], which contains 10% lycopene), or epigallocatechin-3-gallate (EGCG; a 1% green tea extract containing 16.5% EGCG and 33.4% total catechins). Rats were fed the diets for the remainder of the study. After 3 weeks, 2/3 of the control and all of the antioxidant diet-fed rats were injected i.p. with PCB-77 (300  $\mu$ mol/kg) every other week for four injections. All rats were euthanized 10 days after the last PCB injection. The rats that received PCB-77 alone showed a large increase in placental glutathione S-transferase (PGST)-positive foci in the liver. Lycopene decreased the number of

foci, while curcumin decreased the size of the foci. Beta-carotene decreased both the number and size of the foci. In contrast Ellagic acid increased both the number and size of the foci. All the other phytochemicals showed only minor effects. Compared with the PCB-77 group, CoQ10 increased cell proliferation in PGST-negative hepatocytes, whereas the other antioxidants had no effect. These findings show that some antioxidant phytochemicals can influence the promoting activity of PCB-77. (Supported by ES07380)

**1489**

CHANGES IN DRUG METABOLISM AS A POTENTIAL MECHANISM FOR THE SYNERGISTIC INDUCTION OF APOPTOSIS IN MDA-MB-231 CELLS BY EGCG AND 4-OH TAMOXIFEN.

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We have previously demonstrated that epigallocatechin gallate (EGCG) and 4-OH tamoxifen (4-OHT) are synergistically cytotoxic toward MDA-MB-231 human breast cancer cells, an ER $\alpha$  negative cell line. The current study was designed to elucidate the mechanisms responsible for this synergistic effect. To determine the role of apoptosis, MDA-MB-231 cells were treated with EGCG (25  $\mu$ M), 4-OH tamoxifen (0.7  $\mu$ M) or a combination of the two for 12, 24, 36 or 48 hours. DMSO (0.001%) served as the vehicle control. Cells were visually analyzed for apoptosis using acridine orange / ethidium bromide staining and this was confirmed by measuring caspase-3 activity via the cleavage of the DEVD moiety from DEVD-pNA. The results demonstrated that at 24 hr the EGCG + 4-OHT group showed condensed chromatin, while this did not occur until 36 hr in the EGCG group and 48 hr in the 4-OHT group. Caspase-3 activity confirmed the early occurrence of apoptosis in the EGCG + 4-OHT group. A potential mechanism for this effect may involve changes in the glucuronidation and/or methylation rate of EGCG. Our evidence that glucuronidation is likely to be of significance is demonstrated by the fact that the cytotoxicity of EGCG toward human breast cancer cell lines (MCF-7, T47D, MDA-MB-231), as determined by the sulforhodamine B assay, is inversely proportional to the glucuronidation capacity of the cell line ( $r^2 = 0.98$ ). We also have evidence that EGCG may form an active methylated metabolite similar to 2-methoxyestradiol. Specifically, the cytotoxicity of EGCG (25  $\mu$ M) toward MDA-MB-231 cells was reduced 14  $\pm$  1% when the cells were also incubated with the catechol O-methyltransferase (COMT) inhibitor pyrogallol (1  $\mu$ M). This indicates that the cytotoxicity of EGCG is increased when COMT is active. In conclusion, our results demonstrate that EGCG and 4-OH tamoxifen synergistically induce apoptosis in MDA-MB-231 cells and the mechanism for this effect is likely to involve tamoxifen-mediated changes in the metabolism of EGCG.

**1490**

PHYTOESTROGENS AND DIETARY FATTY ACIDS INFLUENCE SPONTANEOUS MAMMARY TUMOR DEVELOPMENT IN IN TG.NK (MMTV/C-NEU) MICE.

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Breast cancer is the most frequent tumor type in women worldwide. Epidemiological studies on soy consumption during adulthood and breast cancer risk show either no association or a decreasing risk with increasing soy intake. In addition, high soy consumption during childhood or adolescence has been associated with a reduced breast cancer risk. The timing of phytoestrogen exposure within the life cycle may be critical. Isoflavones have been postulated as breast cancer protective constituents in soy. To investigate this hypothesis, we studied the effect of isoflavones in a high-fat diet on mammary tumor development using female heterozygous MMTV/c-neu mice. On regular chow these mice spontaneously develop mammary tumors at the age of about 6 months. Three different exposure protocols were used, either from conception to weaning, or from weaning onwards, or lifelong. These exposures were conducted on background diets enriched in either n-6 fatty acids or n-3 fatty acids. Mice fed n-3 rich diets appeared to develop mammary tumors 15 weeks later than mice fed the n-6 rich diet. Apart from the fatty acid content of the diets this may be due to the lower food consumption in the former group. In the mice fed the diets enriched in n-3 fatty acids, no significant effect of the isoflavones on mammary tumor development was observed. In the mice fed the diets enriched in n-6 fatty acids, no significant difference in mammary tumor multiplicity or mass at necropsy was found between any of the exposure protocols. However, in this diet group, a significant decrease of tumor incidence and a delay in tumor onset was observed in mice exposed to isoflavones from weaning onwards. Therefore, the effects of phytoestrogen exposure on tumor formation appear to depend on the composition of the background diet.

**1491**

INFLUENCE OF METHOXYCHLOR EXPOSURE ON BREAST CANCER INCIDENCE IN FEMALE SPRAGUE-DAWLEY RATS.

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Endocrine disrupting chemicals may contribute to human breast cancer by altering expression of enzymes involved in estrogen biosynthesis and metabolism. Our objective was to examine the effects of pubertal exposure, or combined *in utero* and neonatal exposure, to an endocrine disrupting chemical, methoxychlor, on spontaneous and 7, 12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumor formation in Sprague-Dawley rats. To achieve *in utero* exposure, methoxychlor was given by gavage, once daily, to pregnant rats at dosages of 5 (n=24) or 50 (n=24) mg/kg, starting at 3 days before mating until 7 days after parturition. The female offspring were monitored for the development of spontaneous mammary tumors and were terminated at 160 days of age. The dams were also monitored and killed at 117 days after parturition. In a second study, methoxychlor (50 mg/kg, n=41) or corn oil (n=41) was given by gavage, once daily, to pubertal rats for 16 consecutive days. At 60 days of age, half of the treated rats received a single dose of 20 mg of DMBA and the rest were treated with vehicle. The corn oil-treated rats also received either DMBA or vehicle. Rats were sacrificed at 160 days of age. No spontaneous mammary tumors were found in rats that received only methoxychlor during puberty or were exposed both *in utero* and neonatally. No tumors were found in control rats. With combined methoxychlor and DMBA treatment, 70% of the rats treated during puberty developed mammary tumors with a mean value of 2.6 tumors/tumor-bearing rat and a mean mass of 3.3 g/tumor. The earliest tumor was palpable on the 5th week after DMBA administration. For rats treated with corn oil and DMBA, the tumor incidence was 68% with a mean value of 1.8 tumors/rat and a mean mass of 1.7 g/tumor. The first tumor was observed on the 7th week after DMBA treatment. Histology analysis indicated that methoxychlor exposure induced mammary gland differentiation and increased the size and number of lobuloalveoli. Studies to measure CYP enzyme expression in mammary tissue are in progress.

**1492**

BREAST CANCER CHEMOPREVENTION WITH THE POLYPHENOL RESVERATROL.

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Even with the advent of new and aggressive therapeutics, breast cancer remains a destructive disease and a leading killer among cancers. In the US alone, there are more than 215, 000 new cases expected this year. There is, and should be, a concerted effort to prevent breast cancer. One polyphenol that has received much attention for health benefits is resveratrol, a polyphenolic phytoalexin found in red grapes (red wine). We have investigated the chemopreventive properties of resveratrol using the dimethylbenz(a)anthracene (DMBA) model for mammary carcinoma in the female Sprague-Dawley rat. Dietary administration of pure resveratrol (1g/kg AIN-76A diet) was provided, starting at parturition. Control animals were fed the phytoestrogen-free diet, AIN-76A. This dose of resveratrol resulted in a serum concentration of 2 +/- 0.64  $\mu$ M. With at least 30 animals per group, tumor multiplicity, and latency were observed. Rats exposed to resveratrol via the diet showed a decrease in tumor multiplicity and had a longer latency as compared to control animals. Resveratrol in the diet resulted in no significant effect on body and uterine weights in 21 and 50 day old rats. In the uteri of 50 day old rats treated with resveratrol, we saw a decrease in protein levels of the steroid receptor coactivators: SRC-1, GRIP-1, and AIB1. While these coactivators are reported to increase the transcriptional activity of steroid receptors including estrogen, progesterone, and androgen receptors, resveratrol may attenuate sex steroid action by down-regulating their concentrations. The elucidation of the mechanisms for the preventive effects that we observe following polyphenol treatment will hopefully pave the way for clinical trials and the prevention of breast cancer. (Supported by NIH NCI P20 CA93753-02 and DOD DAMD17-00-1-0119).

**1493**

RESVERATROL ACTION ON STEROID AND GROWTH FACTOR SIGNALING IN TRAMP MICE.

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Prostate cancer is the most frequently diagnosed cancer in American men and is the second leading cause of death among men in the United States. Resveratrol, a natural polyphenolic phytoalexin, is abundantly present in peanuts, root extracts, grapes, and subsequently red wine. It has previously been shown that resveratrol inhibits the carcinogenic process at the initiation, promotion, and progression stages in cancer cells. We hypothesized that resveratrol will exert a chemopreventive effect via regulation of sex steroid and growth factor pathways. In the current study, five

week old C57BL/6 TRAMP (Transgenic Adenocarcinoma Mouse Prostate) offspring were fed AIN-76A diet supplemented with 625 mg resveratrol/kg diet, starting at 5 weeks of age. Controls received the phytoestrogen free diet, AIN 76A. Resveratrol did not significantly alter body and prostate weights or serum testosterone and estrogen concentrations in 12 and 28 week old mice. Dorsolateral prostates were dissected from 12 week old mice, and Western blot analyses or ELISA were performed to evaluate sex steroid and growth factor signaling. In the dorsolateral prostate, resveratrol significantly decreased insulin-like growth factor-1 (IGF-1) and estrogen receptor beta (ER-beta) and significantly increased androgen receptor (AR) and insulin-like growth factor receptor 1 (IGF-R1). ER-alpha and IGF-binding protein-3 (IGF-BP3) were not altered in resveratrol treated mice. The decrease in the potent growth factor, IGF-1, and the increase in the prostate tumor suppressor, ER-beta, may provide a biochemical basis for resveratrol suppressing prostate cancer without significant toxicity. (Supported by DOD DAMD PC 17-03-1-0153)

**1494** SUPPRESSION OF MMP EXPRESSION AND INVASION OF HUMAN CERVICAL CANCER CELL LINES HEA AND DOTC2 4510 BY NUTRIENTS.

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**Objective:** Cervical cancer is the seventh most common cancer worldwide and the second most common cancer in women. This prompted us to investigate the anti-tumor effect of a unique nutrient formulation (NM) containing lysine, proline, arginine, ascorbic acid, and epigallocatechin gallate on human cervical cancer cells Hela and DoTc2 4510 by measuring: cell proliferation, modulation of MMP-2 and MMP-9 expression, and cancer cell invasive potential. **Materials and Methods:** Human cervical cancer cells Hela and DoTc2 4510 (ATCC) were grown in DME supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml) in 24-well tissue culture plates. At near confluence, the cells were treated with the nutrient mixture (NM) dissolved in media and tested at 0, 10, 100, 500, and 1000 mcg/ml in triplicate at each dose. Cells were also treated with phorbol myristate (PMA) 200 ng/ml. Cell proliferation was evaluated by MTT assay, MMP expression by gelatinase zymography, and invasion through Matrigel. **Results:** NM showed significant antiproliferative effect on both human cervical cell lines. Zymography demonstrated expression of MMP-2 by untreated cervical Hela cells and induced MMP-9 by PMA -treated cells. NM inhibited Hela expression of MMP-2 and MMP-9 in a dose-dependent fashion, with virtual total inhibition of MMP-2 at 1000 mcg/ml and MMP-9 at 500 mcg/ml. Untreated DoTc2 4510 cells demonstrated MMP-9 expression, enhanced with PMA treatment, and inhibited by in a dose-dependent fashion with virtual inhibition at 500 mcg/ml. NM significantly reduced Matrigel invasion of Hela cells in a dose-dependent fashion, with 76% inhibition at 100 mcg/ml and 100% at 500 mcg/ml NM ( $p<0.0001$ ). NM inhibited invasion of DoTc2 4510 cells by 97% at 500 mcg/ml and 100% at 1000 mcg/ml ( $p<0.0001$ ). **Conclusion:** Our results suggest NM has great potential for therapeutic use in the treatment of cervical cancer, by inhibiting critical steps in cancer development and spread, such as cell growth, MMP expression and invasion.

**1495** ALTERED TRANSCRIPTIONAL REGULATION OF GENES INVOLVED IN AFLATOXIN GENOTOXICITY BY SULFORAPHANE (SFN) AND DIINDOLYLMETHANE (DIM).

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Previous work from our laboratory has demonstrated that SFN provides substantial protection against aflatoxin B1 (AFB) genotoxicity, whereas DIM significantly increased AFB-DNA adduct levels in human hepatocytes. We determined the effects of SFN and DIM on mRNA expression of genes involved in biotransformation of AFB in human hepatocyte cultures from nine donors following 48 h incubations with 10 or 50  $\mu$ M SFN or DIM. DIM caused a 625- and 90-fold up-regulation of cytochrome P450 (CYP) 1A1 and CYP1A2 mRNA, respectively, at 50  $\mu$ M DIM, and a significant down-regulation of glutathione S-transferase (GST)M1 mRNA. SFN showed a dramatic and dose-dependent down-regulation of CYP3A4 mRNA (31% and 87% decrease at 10 and 50  $\mu$ M SFN) and, to a lesser extent, of CYP1A2 mRNA. SFN also decreased both GSTT1 and GSTM1 mRNA, but had no effect on hGSTA1. To distinguish between treatment effects on transcription vs. direct effects on enzyme activity, we compared changes in AFB-DNA adduct formation in human hepatocytes under the following conditions: (1) 48h SFN or DIM, then 6h AFB, (2) 48h SFN or DIM, then 6h AFB + SFN or DIM and (3) 6h AFB + SFN or DIM. The results demonstrated that effects on gene expression, but not catalytic activity, are responsible for the observed effects on AFB-DNA adduct formation for

both SFN and DIM. Surprisingly, the data indicated that the transcriptional repression of genes involved in AFB bioactivation but not induction of detoxification pathways contributed to the protective effects of SFN. The increase in AFB-DNA damage following DIM treatment may be explained through its substantial induction of CYP1A2 and/or its down-regulation of GSTM1. The increase in DNA damage by DIM raises potential safety risks for dietary supplements of DIM and its precursor indole-3-carbinol; the apparent down-regulation of CYP3A4 by SFN may have important implications for drug interactions (Supported by NIH grants ES05780 and P30-ES07033).

**1496**

MICROARRAY ANALYSIS OF LPS AND NAC-INDUCED GENE EXPRESSION AND RESULTANT SYNERGISM, POTENTIATION, AND ANTAGONISM IN WEHI-231 B LYMPHOMA CELLS.

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Accumulating evidence suggests that dietary antioxidants can exhibit divergent effects in carcinogenesis possibly through changes in gene expression. In pilot experiments, we selected N-acetyl-L-cysteine (NAC) as a well-recognized antioxidant to test the effect on mitogen-stimulated B lymphoma cells. WEHI-231 murine B cells were cultured with media alone or containing NAC (1 mg/mL), lipopolysaccharide (LPS; 0.1 mg/mL), or both for 72 h to determine independent or combinatorial effects on gene expression using microarray analysis. LPS independently induced the expression of 16 genes including oxyR, Cebpd, Gadd45, Jun, Trafl, etc. NAC inclusion antagonized expression of five genes including Defcr, Krtap6-1, Magea8, Etsrp71, and oxyR. NAC independently induced 8 genes including Hsd3b6, Pit1, Vdr, Tph, etc., and co-incubation with LPS antagonized expression of 4 genes (Entpd5, Akp2, Cbln1, and Sitpec). Twelve genes were not expressed after either LPS or NAC alone but upon co-incubation displayed increased expression of 5 to 20-fold. Conversely, we noted independent LPS and NAC-induced gene expression each of which was antagonized by co-incubation. We observed synergistic interactions of NAC on LPS-stimulated expression of 3 genes (Pkib, Trafl and Jun) and of LPS on NAC-induced expression of two genes (Tph and Vdr). Collectively, we observed independent mitogen and NAC-stimulated gene expression, as well as synergism, potentiation, and antagonism upon co-incubation in a B lymphoma cell line. This supports that a dietary antioxidant can exhibit independent and diverse, interactive effects on gene expression in cancer cells (Supported by an Innovative Biotechnology Research Fund Grant from the Huck Biotechnology Institute, PSU)

**1497**

MODULATION OF ADULT RAT BENZO(A)PYRENE (BAP) METABOLISM AND DNA ADDUCT CONCENTRATIONS BY NEONATAL RESVERATROL (RVT) EXPOSURE.

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This study seeks to elucidate the role of resveratrol (RVT), a phytoestrogen on BaP metabolism in rat. Offspring of F-344 rats were neonatally treated on days 2, 4, and 6 postpartum with subcutaneous injections of RVT (10  $\mu$ mol/kg). Ten weeks after birth, the adult male rats were challenged with <sup>3</sup>H BaP (10  $\mu$ mol/kg) orally, and sacrificed 2 hours after BaP exposure. Prostate, testis, lung, liver, urine and feces samples were collected and extracted using a mixture of water, methanol and chloroform. The extracts were analyzed by reverse phase HPLC. DNA was isolated from the tissues. The DNA samples were digested with spleen phosphodiesterase, micrococcal nuclease and subjected to <sup>32</sup>P-postlabeling. The labeled adducts were separated by TLC and quantified by scintillation counting. The concentrations of BaP organic metabolites in RVT rats were lower compared to controls (vehicle-treated rats). The concentrations of aqueous metabolites were significantly increased in RVT-treated rats. In the organic phase, 3-OH BaP, 9-OH BaP, 4,5-dihydrodiol, 7, 8-dihydrodiol, 9, 10-dihydrodiol and 3, 6-dione were the major metabolites. In the aqueous phase, OH-glucuronides, OH-sulfates, 7, 8-diol GSH, 9, 10-diol GSH, 7, 8-epoxide GSH and 9, 10-epoxide GSH were the major metabolites. The balance between toxication and detoxication processes represented, respectively, by the 7, 8-dihydrodiol 9, 10-epoxide (the reactive metabolite of BaP) and 3(OH) BaP (the detoxication product of BaP) metabolite ratio in organs and body fluids was decreased by 40% in all the samples of RVT-treated rats. The BaP-DNA adducts concentrations showed a trend that mirrored the concentrations of BaP 7, 8-dihydrodiols in the organs studied with low concentrations of DNA adducts in RVT-treated rats compared to controls. Collectively these results suggest that RVT is capable of modulating the metabolic pathway of BaP towards detoxication thereby preventing the manifestation of toxicity (supported by NIGMS-SCORE grant #2S06GM08037-32, NIEHS grant #1R15ES12168-01 and NCRR-RCMI grant #G12RR03032).

EFFECT OF DIETARY FAT ON FLUORANTHENE (FLA) METABOLISM AND DNA ADDUCT FORMATION IN F-344 RATS.

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This study was aimed at measuring the effect of dietary fat on metabolism and DNA adducts formation of fluoranthene (FLA), a polycyclic aromatic hydrocarbon compound, subsequent to acute oral exposure. Ten week-old F-344 male rats were given 50 µg/kg FLA by oral gavage using test meals comprising peanut, corn, and coconut oils as representatives of monounsaturated, polyunsaturated and saturated dietary fats respectively. Control animals were either exposed to vehicle only or unexposed. After exposure for 2 hours, rats were sacrificed. Trunk blood, urine, feces, stomach, intestine, liver, lung, and testis samples were collected. Samples were subjected to liquid-liquid extraction using water, methanol and chloroform. The extracts were analyzed for FLA /metabolites by reverse-phase HPLC with fluorescence detection. DNA was isolated from the tissues of interest. The DNA samples were digested with spleen phosphodiesterase, micrococcal nuclease and subjected to <sup>32</sup>P-postlabeling. The labeled adducts were separated by TLC and quantified by scintillation counting. The FLA metabolite, and DNA adduct concentrations varied with the type of dietary fat intake. For example FLA dosed in test meal containing saturated fat produced the greatest quantity of metabolites and adducts followed by test meals containing polyunsaturated and monounsaturated fat. The FLA 2, 3-diol is the predominant metabolite followed by i) trans-2, 3-dihydroxy-1, 1Ob-epoxy-1, 2, 3, 10b tetrahydro FLA (2, 3 D FLA), ii) FLA-2, 3-dione, iii) 3-hydroxy FLA and iv) 8-hydroxy FLA. Our studies indicate dietary fat type influences the quantitative and qualitative distribution of FLA metabolites in plasma and target tissues. The FLA-DNA adduct concentrations in target tissues were also reflective of the persistence of reactive metabolites of FLA in these tissues. Collectively, our findings suggest that ingested lipid type modulate FLA metabolism and DNA damage in exposed tissues (supported by NIEHS grant #1R15ES12168-01, NIGMS-SCORE grant #2S06GM08037-32, RISE grant #2R25GM 59994, and NCRR-RCMI grant #G12RR03032).

TRANSPLACENTAL GENOTOXICITY OF DIBENZO[A, L]PYRENE (DBP) AND THE EFFECT OF INDOLE -3-CARBINOL (I3C) IN THE MATERNAL DIET.

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The fetus is a sensitive target for chemically induced toxicity including carcinogenesis. The polycyclic aromatic hydrocarbons (PAHs) are known mouse transplacental carcinogens and DBP has been shown to be the most potent of the PAHs in most models, but, as yet, is untested as a transplacental carcinogen. In our study, pregnant mice were administered DBP by gavage (15 mg/Kg) on gestation day 17. DBP-DNA adducts, in putative maternal and fetal target tissues, were analyzed by 33P postlabeling and HPLC from 6 hr to 6 days after DBP dosing. Some pregnant mice were administered 2000 ppm I3C in the diet, beginning at gestation day 9. In a separate study, a single gavage of 14C-I3C (1 mCi/Kg body weight), showed the distribution of I3C in most maternal and fetal tissues peaked at 8 hr. The genotoxicity experiment was designed to examine the effect of the aryl hydrocarbon receptor (AHR) on the maternal and fetal response. Total DNA adducts were highest at day 2 in both maternal and fetal lung. Although similar HPLC adduct profiles were obtained, the fetal AHR genotype affected total binding. I3C in the maternal diet induced the expression, in maternal and fetal liver, of genes (microarray) and proteins (western blot) associated with phase I and phase II metabolism of DBP and this may be a mechanism for the I3C-dependent decrease in DBP-DNA adduction. (Supported by NIH grants CA90890, ES00210 and CA28825)

STUDY OF THE INHIBITING EFFECT OF DIETARY HYDROQUINONE ON ACETYLAMINO-FLUORENE INDUCTION OF INITIATION OF RAT LIVER CARCINOGENESIS.

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Monocyclic phenolics (MPs) have been shown to inhibit the initiation of rat liver carcinogenesis induced by the genotoxic hepatocarcinogen 2-acetylaminofluorene (AAF). Hydroquinone (HQ), the simplest of the MPs, was studied for its ability at low dietary concentrations to inhibit the initiating effects of AAF in the rat liver. Six groups of male F344 rats were used. HQ was given daily ad libitum either at 0.05% (?25 mg/kg bw/d) or 0.2% (? 100 mg/kg bw/d) for 13 weeks, starting one week be-

fore AAF administration, and to 2 groups not receiving AAF. AAF was given intragastrically 3 times a week for 12 weeks at 3 mg/kg bw/time in 0.5% carboxymethyl cellulose and to an untreated group and the two groups receiving HQ. Weekly body weight gain over time, terminal body weights and absolute (mg) and relative liver/body weights were measured. At 13 weeks, DNA adducts (32P-postlabeling), cell proliferation (PCNA immunohistochemistry) and preneoplastic hepatocellular altered foci (HAF) (GST-P immunohistochemistry) were measured. The 2 HQ groups showed body weight gains comparable to controls for the duration of the study, whereas the AAF and HQ/AAF groups had a tendency to reductions in body weight gain. The same pattern was evident in the terminal body weights. These body weight differences were not significant. The absolute and relative liver weight changes showed no significant changes. AAF produced liver DNA adducts, and HQ at both 25 and 100 mg/kg/day significantly reduced adduct levels, by more than 45%. AAF exposure yielded about a 50% increase in hepatocellular proliferation and both HQ exposures significantly reduced the AAF-induced increases in proliferation. Likewise, AAF-induced GST-P+ HAF and these were significantly decreased by both doses of HQ. HQ by itself did not produce adducts and did not affect other parameters. Thus, under the conditions of this experiment, HQ at both 25 and 100 mg/kg/day given in the diet for 13 weeks did not produce any toxicity, but diminished AAF-induced effects in liver.

β-SITOSTEROL IN PSYLLIUM SEED HUSK RESTORES GAP JUNCTIONAL INTERCELLULAR COMMUNICATION IN H4-RAS TRANSFECTED RAT LIVER EPITHELIAL CELLS.

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Psyllium (*Plantago ovata* Forsk; desert Indian wheat) is a common source of soluble fiber derived from the husk of the plant *Plantago ovata*, which is used for colon care with its moderate laxative effect. We previously determined that psyllium seed husk powder restores gap junctional intercellular communication (GJIC) in a tumorigenic WB-F344 rat liver epithelial cell line transfected with the *v-Ha-ras* oncogene (WBras), but not on WB cell lines, transfected with the *src*, *neu* and *myc* oncogenes. We have purified the active compound from the husk with an ethanol extract; HP-20 hydrophobic chromatography; silica gel adsorption column chromatography and TLC. This purified fraction, restores GJIC in WBrs cells, as determined by the scrape loading dye transfer assay. The active compound was identified to be β-sitosterol on the basis of gas chromatographic retention time and EI-MS spectrum of authentic β-sitosterol. β-sitosterol restored GJIC in WBrs cells at the dose of 2.4 µM. In addition, stigmasterol also had some activity to restore GJIC, but the activity shown needed a concentration of 3.6 µM. As determined by Western blot analysis, β-sitosterol and stigmasterol increased the level of connexin43 protein and its active-, phosphorylated, form that correlates with the restoration of GJIC in GJIC-deficient, tumorigenic WBrs cells. The psyllium seed husk has been widely used as a supplement to affect colon care, due to its high fiber content water-insoluble and -soluble dietary fiber. The present study finds another health-promoting ingredient, such as phytosterols (β-sitosterol and stigmasterol), showing an ability to restore GJIC in mutated *Ha-ras* oncogene transformed liver epithelial cells.

THE EFFECTS OF CO-ADMINISTRATION OF ANTIOXIDANTS AND DIMETHYLARSINIC ACID (DMA) ON THE BLADDER EPITHELIUM OF FEMALE F344 RATS.

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Oxidative stress has been increasingly recognized as a possible mechanism in the toxicity and carcinogenicity of various chemicals, including arsenic. Therefore, treatment with antioxidants may afford a protective effect against arsenic-induced cytotoxicity and carcinogenesis. DMA has been shown to be a bladder carcinogen in rats when administered at high doses (100 ppm) in the diet or in the drinking water. The objective of this study was to evaluate the effects of co-administration of antioxidants with DMA on the rat urinary bladder epithelium *in vivo*. In a 10-week study, we co-administered 100 ppm DMA in the diet with 1000 ppm melatonin or 10, 000 ppm sodium ascorbate (NaAsc, the sodium salt of vitamin C) also in the diet or with 0.4 mg/kg N-acetylcysteine (NAC) in the drinking water to female F344 rats. Effects on the bladder epithelium were determined by light microscopy and BrdU labeling index. Mild simple hyperplasia was found in all DMA-treated groups, in 1 of 10 rats treated with melatonin alone, and 3 of 10 rats treated with NAC alone. There was no significant difference in the incidence of simple hyperplasia among the groups. The BrdU labeling index was significantly increased in all DMA-treated groups compared to the control group. Co-administration of NaAsc

significantly decreased the BrdU labeling index compared to the group administered DMA alone. Melatonin and NAC had no effects on the BrdU labeling index when co-administered with DMA. These results suggest that oxidative stress may in part be involved in DMA-induced rat bladder toxicity and proliferation, and therefore, vitamin C may afford inhibitory effects in DMA-induced bladder carcinogenesis in rats.

**1503**

RAPID INDUCTION OF COLORECTAL TUMORS IN RATS INITIATED WITH 1, 2-DIMETHYLHYDRAZINE FOLLOWED BY DEXTRAN SODIUM SULFATE TREATMENT - POSSIBLE APPLICATION FOR A NEW MEDIUM-TERM RAT COLON BIOASSAY.

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To establish a rapid bioassay system with neoplastic end-points for detection of colorectal carcinogenesis modifiers, we evaluated the effects of dextran sodium sulfate (DSS) treatment on the different stages of carcinogenesis in rats initiated with 1, 2-dimethylhydrazine (DMH). F344 male rats were given three subcutaneous injections of DMH (40 mg/kg body weight) in a week, and were administered drinking water containing 1.0% DSS ad libitum either during or after the initiation period for a week, or both during and after initiation periods for 2 weeks. At week 10, the incidences and multiplicities of dysplastic foci/adenomas (Ad)/adenocarcinomas (Ca) were highest in rats treated with DSS after DMH-initiation period. At week 26, the incidences of Ca and their multiplicities were also highest in this group. In the next study, we examined ability of this model to detect modifiers of colon carcinogenesis. Groups of ten or fifteen rats were given the regimen of DMH and DSS treatment followed by free access to basal diet alone, basal diet containing 0.04% nimesulide or 2% lactoferrin as known inhibitors, 0.3% deoxycholic acid (DCA) as a promoter or 1.5% 1-hydroxyanthraquinone (1-HA) as a carcinogen were supplied for 7 or 17 weeks. At week 10, the incidence and multiplicity of combined Ad and Ca were decreased ( $p<0.05$  or 0.01) by nimesulide and lactoferrin, and values for Ad were increased ( $p<0.01$ ) in the 1-HA group. There were no clear changes in these parameters in the DCA group. At week 20, multiplicity and volume of the tumors were decreased ( $p<0.01$  or 0.05) in the nimesulide group, but without effect in lactoferrin group. Multiplicity and volume of tumors were increased ( $p<0.01$ ) in 1-HA group and a similar tendency ( $p=0.08$ ) was apparent with DCA. It is concluded that this medium-term bioassay system possibly offers a useful tool for detection of colorectal carcinogenesis modifiers within 10-20 weeks.

**1504**

LACK OF CARCINOGENIC EFFECTS IN F-344 RATS FED BIS(2-ETHYLHEXYL)-1, 4-BENZENEDICARBOXYLATE (DEHT).

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A carcinogenicity study was conducted in F-344 rats to validate the long-term safety of DEHT, a new plasticizer, with potential for human exposure. Groups of 100 animals (50 males/50 females) were exposed to 0, 1500, 6000, and 12000 ppm DEHT in the diet for 104 weeks. The overall average achieved dosages were 79, 324 and 666 mg/kg/day for males and 102, 418 and 901 mg/kg/day for females. Bodyweight gain was significantly low throughout the treatment period, when compared to the controls, for both sexes receiving 12000 ppm. There were no effects on food consumption. There were no statistically significant differences in percent survival among any of the groups. Hematology evaluations did not show any toxicologically significant differences. A few blood chemistry values were different than controls for high dose animals with no obvious toxicological significance. Relative liver and kidney weights of high-dose females were slightly higher than controls, but males were not affected. A number of other inter-group differences attained statistical significance, when compared to controls, but these were not considered to be toxicologically significant. For animals killed after 104 weeks of treatment, there was a high incidence of opaque eyes for high dose females, but not males. Non-neoplastic findings in high dose females compared with controls included trends such as a decreased incidence of chronic progressive nephropathy, an increased incidence of loss of the outer nuclear layer of the retina in the eyes, and an increase in prominent eosinophilic inclusions in the nasal turbinates; in high dose males a decreased incidence of periportal hepatocyte vacuolation was detected; and a decreased incidence of mineralization of the papillary/pelvic epithelium of the kidney was recorded in both sexes receiving 6000 or 12000 ppm. There was no evidence of liver tumors, pancreatic tumors, or any other tumor type associated with DEHT treatment which is in contrast to results observed in similar studies with some ortho-phthalate plasticizers.

**1505**

DIMETHYLARSINIC ACID (DMA): RESULTS OF CHRONIC TOXICITY/ONCOGENICITY STUDIES IN FISCHER F344 RATS AND B6C3F1 MICE.

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Male and female Fischer F344 rats and B6C3F1 mice were treated with dietary dimethylarsinic acid (DMA) in 2-year feeding studies. Rats were treated with 2, 10, 40, or 100 ppm DMA. Mice were treated with 8, 40, 200, or 500 ppm DMA. Early in the study there was a low incidence of treatment-related mortality in male rats in the two highest dose groups (100 ppm-5/60, 40 ppm-1/60). There was no treatment-related mortality in female rats or in mice. The primary targets for DMA-induced toxicity in the rat and mouse were the urinary bladder and kidney. In the rat, treatment with DMA resulted in a dose responsive increase in urothelial cell hyperplasia at 40 and 100 ppm DMA. Bladder tumors occurred in male and female rats at 100 ppm DMA. The female rat appeared to be more sensitive to the urothelial effects of DMA. In the rat kidneys, there was an increased incidence of medullary nephrocalcinosis, medullary tubular cystic dilation, and hyperplasia of the epithelial lining of the renal papilla in both sexes at doses of 40 and 100 ppm DMA. In mice, treatment with DMA resulted in a dose-dependent increase in vacuolar degeneration of the urinary bladder epithelium without toxicity or proliferation in the 2 highest dose male groups and the 3 highest dose female groups. No treatment-related tumors were found in any mice at any dose of DMA. The NOEL in rats was assessed as 2 ppm in males and 10 ppm in females and in mice as 40 ppm in males and 8 ppm in females. Based on these studies, high doses of DMA are carcinogenic to the rat urinary bladder; DMA is not carcinogenic in the mouse. Subsequent research has demonstrated that the mode of action for the rat bladder carcinogenesis involves cytotoxicity, regenerative proliferation, and ultimately tumors.

**1506**

GINGIVAL CARCINOGENICITY IN FEMALE HARLAN SPRAGUE-DAWLEY RATS AFTER ORAL TREATMENT FOR TWO YEARS WITH 2, 3, 7, 8-TETRACHLORODIBENZO-P-DIOXIN AND DIOXIN-LIKE COMPOUNDS.

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We evaluated gingival toxicities induced by chronic exposure of female Harlan Sprague-Dawley rats to dioxin and dioxin-like compounds (DLCs). This investigation represents part of an ongoing initiative of the National Toxicology Program to determine the relative potency of chronic toxicity and carcinogenicity of polychlorinated dioxins, furans, and biphenyls. For two years, animals were administered by gavage 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD); 3, 3', 4, 4', 5-pentachlorobiphenyl (PCB126); 2, 3, 4, 7, 8-pentachlorodibenzofuran (PeCDF); 2, 2', 4, 4', 5, 5'-hexachloro-biphenyl (PCB153); or a tertiary mixture of TCDD, PCB126, and PeCDF; a binary mixture of PCB126 and 153; or a binary mixture of PCB126 and 2, 3', 4, 4', 5-pentachlorobiphenyl (PCB118); control animals received corn oil-acetone vehicle (99:1) alone. A complete necropsy was performed on all animals and microscopic examination of a full complement of tissues was performed, including upper palates with teeth. In the groups of animals treated with TCDD and the mixtures of TCDD, PCB126, and PeCDF; PCB126 and 153; and PCB126 and 118, the incidences of gingival squamous hyperplasia increased significantly. Moreover, the higher doses of TCDD, PCB126, and the mixture of PCB126 and 153 induced squamous cell carcinoma (SCC) in oral cavities. This investigation constitutes the first report showing that chronic administration of dioxin-like PCBs can induce gingival SCC in rats. These results indicate that dioxin and DLCs target the gingiva of oral cavity, in particular the junctional epithelium of molars.

**1507**

CHRONIC TOXICITY AND ONCOGENICITY STUDY OF OCTAMETHYLCYCLOTETRAZOSILOXANE (D4) IN FISCHER 344 RATS.

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Chronic toxicity and oncogenicity of D4 was evaluated in Fischer 344 rats exposed by vapor inhalation (6 h/d; 5 d/wk) to 10, 30, 150, and 700 ppm D4 for up to 24 months. Effects on survival and body weight were limited to 700 ppm males. Survival was decreased to 38% as compared to 58% for control. Body weight was

reduced ≈6% in the latter months of study. Histomorphologic changes in the nasal cavity at 12 and 24 months were consistent with chronic inhalation of a mild irritant. Lymphocytic leukocytosis was present in 700ppm males and females at 3, 6, and 12 months. Liver weights were increased at 6 (≥30ppm males; 700ppm females), 12 (≥150ppm males and females), and 24 months (700ppm males; ≥150ppm females). Centrilobular hepatocyte hypertrophy was present in 700ppm males at 12 and 24 months. Kidney weight and severity of chronic nephropathy was increased for 700ppm males at 24 months. Kidney weight and incidence/severity of chronic nephropathy was increased for 700ppm (12 and 24 months) and 150 ppm (24 months) females. Uterine weight, incidence of endometrial epithelial hyperplasia, and endometrial adenoma (4 of 35 animals) were increased at 700ppm at 24 months. Endometrial adenomas were not present in unscheduled death animals or animals in the control or intermediate dose groups. Trend analysis identified this incidence profile as statistically significant (Peto, p<0.05). Histomorphological review of endocrine responsive tissues (pituitary, mammary gland, ovary, uterus, and vagina) from treated and control animals indicated that they were cycling normally at 12 months without evidence of abnormal endocrine balance. There were no differences in these endocrine responsive tissues, beyond those already identified for the uterus, to differentiate treated from control at 24 months. The uterine effects associated with chronic D4 exposure have prompted research efforts focused on understanding the mode of action and relevance to humans. (This work was supported in part by the Silicones Environmental, Health and Safety Council of North America)

## 1508

### CHRONIC TOXICITY AND ONCOGENICITY STUDY OF HEXAMETHYLDILOXANE (HMDS) IN FISCHER-344 RATS.

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Chronic toxicity and oncogenicity were evaluated in F344 rats exposed to HMDS by vapor inhalation (6 h/d, 5 d/wk) for 12 or 24 months, or for 12 months with a 12 month recovery period. Males and females were exposed to 100, 400, 1600 or 5000 ppm HMDS. All animals were monitored for mortality, clinical signs, food consumption and body weights. Clinical laboratory studies included hematology, clinical chemistry and urinalysis. There were no test article related mortalities or clinical signs. Slight reductions in female body weights (-2 to -8%) at the mid-high and high dose after 12 and 24 months of exposure were likely associated with reduction in food consumption (-4 to -9%). In female rats, there were no histopathologic findings considered to be test article related. In males, increased liver weights after 12 months at 1600 and 5000 ppm suggested metabolic adaptive changes. Microscopic examination of tissues taken at necropsy identified the kidneys, testes, and nasal cavities as target organs in males. In the kidneys, an increased incidence of intraluminal mineralization was noted in all males exposed to 1600 and 5000 ppm HMDS. Several renal tubular adenomas and carcinomas occurred in males after 24 months of exposure to 1600 (3 of 65) and 5000 ppm (6 of 65). Additional work showed an alpha 2u-globulin mediated mechanism responsible for the observed nephropathy and kidney neoplasia in males. Increased testes weights after 12 and 24 months of exposure correlated with histopathological data. After 12 months, a dose-related increased incidence of Leydig cell tumors was noted in all HMDS-exposed groups (Peto, p<0.05). After 24 months almost all males had this finding since these testicular tumors are spontaneous and common in F344 rats. The data suggest that HMDS exposure accelerated progression of this common tumor. Increased incidence of eosinophilic inclusions in the olfactory epithelium in males at all sacrifice points was consistent with chronic inhalation of a mild irritant and is generally seen in inhalation studies.

## 1509

### CHRONIC TOXICITY AND ONCOGENICITY STUDY OF DECAMETHYLCYCLOPENTASILOXANE (D5) IN FISCHER-344 RATS.

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Chronic toxicity and oncogenicity were evaluated in Fischer-344 rats exposed to D5 by vapor inhalation (6 h/d, 5 d/wk) for up to 24 months. Male and female rats were exposed to 10, 40, and 160ppm D5 for 6, 12 or 24 months, or for 12 months with 12 month recovery period (recovery group). All animals were monitored for mortality, clinical signs, food consumption and body weights. Clinical laboratory investigations included hematology, clinical biochemistry and urinalysis. There were no test article related mortalities, clinical signs or palpable masses. The slight increases in female body weights (0.7 to 9.2%) at 40 and 160ppm after 24 months of exposure and in the recovery group, and in all males exposed to D5 for 24 months (1.4 to 4.3%) lacked a dose relationship. Increased liver weights in males

after 24 months at 160 ppm and females after 6 and 12 months at 10 and 160 ppm showed no clear dose-response and no correlation with any histopathological change. In both sexes, histomorphologic changes in the nasal cavity at high-dose were consistent with chronic inhalation of a mild irritant commonly seen in inhalation studies as non-specific finding. In females, the most significant finding was an increased incidence of endometrial epithelial neoplasia, mainly adenocarcinomas after 24 months of exposure (Peto Mortality Prevalence test, Fisher Exact pair-wise comparison, p<0.05). Endometrial adenocarcinomas were noted in 1 female exposed to 10 ppm (N= 60) and 5 females exposed to 160 ppm D5 (N=60). The uterine effects associated with chronic D5 exposure have prompted research efforts focused on understanding the mode of action and relevance to humans. (This work was supported in part by the Silicones Environmental, Health and Safety Council of North America)

## 1510

### CHRONIC TOXICITY AND ONCOGENICITY STUDY OF POLYDIMETHYLSILOXANE (PDMS) 10 CST FLUID IN FISCHER 344 RATS.

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Chronic toxicity and oncogenicity of PDMS was evaluated in Fischer 344 rats. Polydimethylsiloxane 10 cst fluid was mixed into the feed to provide for dietary exposures of up to 24 months. The diets were prepared to achieve dose levels of 0, 100, 300, and 1000 mg/kg/day. There were no indications of systemic toxicity at any dose level. There were no treatment-related effects on survival, body weight, food consumption, and clinical pathology parameters. Evidence of local irritation was noted in the eye (clinically, macroscopically, and/or microscopically) in males and females from all treatment groups at 24 months and in the nasolacrimal duct (microscopically) of 1000 mg/kg/day oncogenicity group males. These effects were considered to be treatment-related and attributed to deposition/adherence of PDMS-feed on the eye surface. There were no treatment-related neoplastic findings at any dose level. A statistically significant increase in incidence of pancreatic islet cell adenomas was identified in the 1000 mg/kg/day oncogenicity group males. The absence of an increase in relevant preneoplastic changes, the lack of an effect in treated females, the high incidence of islet cell adenomas in the recovery group control animals, and the published incidence range for control animals suggests that the increased incidence of islet cell adenomas in the 1000 mg/kg/day group males was not treatment-related. Based on the results of this study, the no-observed effect level (NOEL) for systemic toxicity of PDMS administered for 12 months was 1000 mg/kg/day for males and females. The NOEL for oncogenicity of PDMS administered for 24 months was 1000 mg/kg/day for males and females. (This work was supported in part by the Silicones Environmental, Health and Safety Council of North America)

## 1511

### HISTOGENESIS OF SPONTANEOUS LESIONS IN THE V-HA-RAS (TG.AC) MOUSE.

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The Tg.AC transgenic mouse carries a v-Ha-ras oncogene linked to a zeta globin promoter. The model has been shown to develop a range of epithelial and mesenchymal tumours, including neoplasms of odontogenic origin and squamous papillomas. Two studies using a total of 120 Tg.AC hemizygous mice were housed for a period of six months as part of a strain assessment. In the first study 30 males and 30 females were housed in groups of 5. There were several incidences of fighting within male groups. In the second study the males were singly housed. Upon histopathological examination, 3 odontogenic tumours and 22 squamous papillomas were observed within the group-housed males with 9 odontogenic tumours and 12 squamous papillomas observed within singly housed males. 15 odontogenic neoplasms and 4 squamous papillomas were observed within both groups of females. In addition there was a 100% incidence of retinal dysplasia. The 27 odontogenic tumours were divided into 3 main types i) Mesenchymal cells in a dense fibrous-like matrix, ii) Loose stroma surrounded by anastomosing cords of epithelial cells that exhibited squamous differentiation, and iii) Odontomas forming mineralised tooth structures surrounded by well-differentiated odontoblasts and ameloblasts. There were similarities between the tumours recorded in this study and human odontogenic tumours. Representative sections of each lesion were selected for various stains, both conventional and immunohistochemical. These included trichrome, reticulin, elastin, cytokeratin, vimentin, desmin, ki 67 and a number of neural markers. The 3 main groups of mouse odontogenic tumour showed variations in their staining characteristics and antigenic expression suggesting progression from type 1 to 3. Evidence of cell proliferation was not apparent. It is suggested that the Tg.AC mouse may prove a suitable model for its human counterpart, in the study of odontogenic tumorigenesis.

**1512****A CARCINOGEN BIOASSAY FOR COMPLEX MIXTURES.**

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Complex mixtures of combustion products contain many carcinogens such as polycyclic aromatic hydrocarbons. Assessing the carcinogenic potency of mixtures by a conventional mouse skin tumor carcinogenesis protocol (CC) is time-consuming and expensive. Initiation protocols are of uncertain accuracy in the prediction of cancer outcome. We evaluated a mouse skin model using a dominant-negative p53 mutant mouse (Vp53) in an initiation-progression protocol (IP), concurrently applying a mixture and a promoter, 12-O-tetra-decanoylphorbol-13-acetate (TPA), for 26 weeks. The mixtures were also tested with Vp53 wild type mice in a CC protocol. Three dose levels of Benzo[a]pyrene (BaP) were first tested to confirm the sensitivity of the models. Four mixtures were then evaluated: a National Institute of Standard and Technology SRM 1597 Coal Tar mix (CT), a 7H-dibenzo[c, g]carbazole (DBC) and BaP mix (M1), two dose levels of coal tar creosote (Cr), and a DBC, BaP and CT mix (M2). No tumors developed in control mice. The percentage of mice with tumors was more than 50% in all mixture treatment groups except M1 and M2 CC. The tumors in CT first appeared at 13 weeks in IP and 15 weeks in CC, with 19 and 23 week latent periods, respectively. The M1 IP tumors first appeared at 10 weeks, with a 17 week latent period; there were no tumors in CC. The first M2 tumors appeared at 15 weeks in IP and 17 weeks in CC, with latent periods of 19 and 17 weeks, respectively. 10mg Cr tumors first appeared at 11 weeks in IP and 17 weeks in CC, with 17 and 21 weeks latent periods, respectively. The 20mg Cr tumors first appeared at 8 weeks in IP and 12 weeks in CC; latent periods were 15 and 16 weeks, respectively. In comparison to conventional protocols the IP protocol results in shorter latent periods and time to first tumor for complex mixtures. [USEPA Star Grant]

**1513****EVALUATION OF K6ODC TRANSGENIC MICE AS A DERMAL CARCINOGENICITY MODEL FOR ONCOGENIC DNA.**

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Transgenic mouse models can serve as sensitive, short-term alternatives to traditional rodent 2-year cancer bioassays. K6ODC transgenic mice develop epidermal tumors when exposed to carcinogens via the promotional stimulus of enhanced expression of ornithine decarboxylase in keratinocytes. K6ODC mice were used in a 36-week study to test their susceptibility to oncogenesis by a plasmid expressing the human T24-H-ras gene, which was developed to evaluate risks posed by residual DNA in vaccines produced in neoplastic cell substrates. Male K6ODC and C57BL/6 mice (15 per group) were treated with H-ras plasmid (2.5, 250, 25000 ng), vector DNA, or saline on scarified skin or by subcutaneous injection. One group of K6ODC mice received a single exposure of 7, 12-dimethylbenz[a]anthracene ([DMBA], 200 nM) dermally. Microscopy of skin, heart, lung, liver, and spleen were performed after unscheduled euthanization or at study completion. DMBA-treated K6ODC mice developed papillomas by 6 weeks that increased in incidence to 25 weeks (9+/0.69 tumors/mouse, < 3mm, 13/15 responded). No tumors were detected in other groups in either mouse strain. Microscopy of skin sections confirmed papillomas in DMBA treated animals with dermal/sebaceous gland hyperplasia and follicular dystrophy in all skin samples, characteristic of this strain. Tissue analysis of K6ODC mice revealed amyloid deposition and neutrophilic infiltration within liver, heart, and spleen, and splenic atrophy regardless of treatment. Pathology was not detected in C57BL/6 mice. After week 15, 94% (126/134) of the K6ODC mice developed unexpected dermal eczema (unrelated to either treatment regimen) requiring unscheduled euthanization. By week 32, six of the eight surviving K6ODC mice showed difficulty in mobility and loss of balance. In this study, the K6ODC failure to develop papillomas to oncogenic DNA, progressive adverse health, and decreased long-term survival suggest that K6ODC mice are an inadequate alternative model for oncogenic DNA and chemical carcinogenicity testing.

**1514****DETERMINATION OF THE SKIN CANCER POTENTIAL OF PETROLEUM-DERIVED MATERIALS USING K6/ODC MICE.**

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ExxonMobil and others in the petroleum industry have historically utilized the mouse dermal carcinogenesis bioassay to assess the carcinogenicity of petroleum-derived materials. Scientists have been searching for a faster, less costly but effective

means to identify carcinogenic compounds. The K6/ODC transgenic mouse shows promise as a replacement for the conventional two-year rodent dermal bioassay. Previously, our laboratory demonstrated that a single low topical dose of benzo(a)pyrene(i.e., 25 µg) could produce skin tumors in 60% of treated mice within 20 weeks. In the current study, ten groups of K6/ODC mice (15/group) were treated once per week as follows: Groups 1, 2 and 3 received 375, 750, or 1875 µg Catalytically Cracked Clarified Oil (CCCO; a high-boiling carcinogenic petroleum material) in acetone and Groups 4-10 received 37.5 microliters (neat) of Blend 1 (refined basestock + 10.5% aromatic extract), Blend 2 (refined basestock + 17.5% aromatic extract), Isodewaxed Hydrocracked Stock, Jet fuel, Deasphalted residual oil, Dewaxed Hydrocracked Lube Basestock and a Lubricant baseoil, respectively. Development of skin tumors was monitored weekly. At study termination (i.e., 6 months post dosing), histological examination revealed that 9/15 animals in Group 1, 12/15 animals in Group 2, 14/15 animals in Group 3, 4/15 animals in Group 4, 8/15 animals in Group 5 and 5/15 animals in Group 6 had developed tumors. Tumors were not observed in any of the animals in Groups 7 through 10. These findings are 100% consistent with the results obtained from 2-year skin painting bioassays with these same test materials. These results, pending QAU verification, provide further evidence of the utility of the K6/ODC mouse as a skin cancer model.

**1515****COMPARATIVE 30-WEEK DERMAL TUMOR PROMOTION STUDY USING SENCAR MICE: EVALUATION OF CIGARETTE SMOKE CONDENSATE FROM A CONTROL CIGARETTE AND TEST CIGARETTES WITH TWO BANDED CIGARETTE PAPER TECHNOLOGIES.**

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A 30-week dermal tumor promotion study was conducted to evaluate the relative tumor-promoting potential of cigarette smoke condensate (CSC) collected from three test cigarettes utilizing banded cigarette paper technologies to that of a control cigarette without banded paper. Mainstream CSC's were collected by cold trap from smoke generators using the Federal Trade Commission puffing regimen. Female SENCAR mice were initiated with a single 75-µg application of 7, 12-dimethylbenz[a]anthracene (DMBA) to the clipped dorsal skin. Beginning one week later, the CSC's were applied to the skin three times per week for 29 weeks. Each CSC was administered at 9, 18, or 36 mg tar/200 µl application to groups of 40 animals. In addition to the appropriate sentinel, vehicle and positive controls, non-initiated mice received high-dose treatments of CSC from the control and test cigarettes. End-points included body weights, clinical observations, organ weights, dermal tumor development data and histopathology. The numbers of dermal tumors and the numbers of tumor-bearing animals for both the control and test CSCs were statistically different from the DMBA-initiated control group and increased with increasing exposure. When corresponding doses of test CSC were compared to the control CSC, no increases were noted. In this assay, the banded cigarette paper technologies associated with the test cigarettes did not adversely affect the dermal tumor-promotion potential of the CSC compared to CSC from a control cigarette that had no banded cigarette paper technology.

**1516****COMPARATIVE 13-WEEK INHALATION STUDY OF MAINSTREAM CIGARETTE SMOKE FROM CIGARETTES CONTAINING CAST SHEET TOBACCO.**

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A subchronic, nose-only inhalation study was conducted to compare the effects of mainstream smoke from a Reference cigarette containing conventional reconstituted tobacco sheet at 30% of the finished blend to mainstream smoke from cigarettes containing 10% or 15% Cast Sheet (a specific type of reconstituted tobacco sheet) substituted for part of the conventional reconstituted tobacco. Male and female Sprague-Dawley rats were exposed for 1 hour a day, 5 days per week, for 13 weeks to mainstream smoke at 0, 0.06, 0.20, or 0.80 mg wet total particulate matter per liter of air. Clinical signs, body and organ weights, clinical chemistry, hematology, carboxyhemoglobin (COHb), serum nicotine, plethysmography, gross pathology, and histopathology were determined. Exposure to cigarette smoke induced a number of changes in respiratory physiology, histopathology, and serum nicotine and COHb levels when compared to sham animals. When corresponding dose groups of Reference and Cast Sheet mainstream smokes were compared, no biological differences were noted. At the end of the exposure period, subsets of rats from each group were maintained without smoke exposures for an additional 13 weeks (recovery period). At the end of the recovery period, there were no statistically significant differences in histopathological findings observed between the

Reference and either Cast Sheet cigarette. Substitution of 10% or 15% Cast Sheet tobacco for conventional reconstituted tobacco sheet in a cigarette does not alter the inhalation toxicology of the mainstream smoke when compared to mainstream smoke from a Reference cigarette containing conventional reconstituted tobacco sheet.

**1517**

DIFFERENTIATING INITIATING FROM PROMOTING EFFECTS OF CIGARETTE MAINSTREAM SMOKE IN THE PRODUCTION OF LUNG TUMORS IN A MOUSE INHALATION BIOASSAY.

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In the present study, we tested a rodent inhalation model to differentiate the effects of mainstream cigarette smoke on tumor initiation and promotion. This study utilized 2R4F cigarette smoke as an initiator or promotor of lung tumors in a rodent model. Balb/c mice were initiated or promoted by 3 h/day, 5 d/wk for eight weeks, to air control or 3 separate exposures (50, 150, and 300 mg of total particulate matter (TPM)/m<sup>3</sup>) of nose-only cigarette smoke inhalation. In group A, 8 weeks of smoke inhalation was followed by 4 weekly injections of BHT (200mg/kg bw), and lungs were harvested 16 weeks after initial exposure. No significant elevation in lung adenomas was observed in group A. In group B, one injection of 3-MC (10mg/kg bw) was followed by 8 weeks of smoke inhalation under exposure conditions noted above, and lungs were harvested 16 weeks after initial injection. Air exposure, 50, 150, and 300 mg TPM/m<sup>3</sup> yielded an average of 0.05, 0.12, 0.20, and 0.22 lung adenomas/animal, respectively. CONCLUSIONS: Eight weeks of cigarette smoke did not display tumor-initiating effects under chemical promotion with BHT. However, cigarette smoke showed a dose-response in lung tumor promotion in mice chemically initiated with 3-MC. These findings suggest that cigarette smoke exposure is associated with low to moderate lung tumor promoting potential but little or no initiating potential in the Balb/c mouse strain under short-term cigarette smoke exposure conditions.

**1518**

CIGARETTE MAINSTREAM SMOKE AND GAS PHASE-DEPLETED PARTICULATE PHASE ENHANCE LUNG TUMORIGENICITY IN A/J MOUSE.

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Despite the established causality between tobacco smoking and lung cancer (IARC 2004), it is difficult to establish reproducible and validated animal inhalation models for this disease. These models are needed for the evaluation of potential risk-reduced products and chemoprevention. In order to further investigate the A/J mouse model for lung tumorigenicity, male A/J mice were whole-body exposed to diluted cigarette mainstream smoke from the standard reference cigarette 2R4F for 6 hours per day, 5 days per week for 5 months followed by 4 months without exposure. The animals were exposed to fresh filtered air, to whole smoke at concentrations of 0.12 or 0.24 mg total particulate matter (TPM)/l, to gas phase depleted particle phase (PP) at 0.24 mg TPM/l, which was generated by an activated charcoal adsorber, or to the gas phase of cigarette mainstream smoke at a concentration equivalent to 0.24 mg TPM/l. Lung tumor incidence and multiplicity were determined at 5 months in the control group and the whole smoke high dose group and at 9 months in all groups. At 5 months, the tumor response was comparable in the two groups. A distinct inflammation was indicated in the whole smoke high dose group by a severe increase in neutrophils and macrophages in bronchoalveolar lavage fluid. At 9 months, both cell types returned nearly to background levels; however, lung tumor incidence and multiplicity in the whole smoke groups were dose dependently higher by a factor of up to 3-fold compared to control. The lung tumor response was similar for PP and the high concentration of whole smoke. GP at the concentration used, failed to enhance lung tumor multiplicity above control level. Step-serial lung section analysis confirmed the macroscopic results. The lung tumor spectrum was the same in all groups with bronchio-alveolar adenoma being the most prominent lung tumor type. Further research is needed to evaluate the relevance of this model with regard to the human disease.

**1519**

INTERSPECIES SITE CONCORDANCE FOR MAMMARY CARCINOGENESIS BY ENVIRONMENTAL TOBACCO SMOKE.

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A problem in extrapolating animal bioassay data to human cancer risk is the variability of site concordance, for a particular carcinogen, between different animal test species and humans. Carcinogens active at the site of first contact often show site concordance, for similar exposure routes, but for systemically distributed car-

cinogens this depends on both the carcinogen and the organ system. This question is important to the study of contributing causes of breast cancer. Various environmental contaminants were tentatively associated with increased breast cancer incidence in ecological studies, but the epidemiological data are often conflicting or equivocal, so correlation with animal carcinogenicity becomes important in their interpretation. An example was identified during our re-evaluation of the health effects of exposure to environmental tobacco smoke (ETS). The summary odds ratio for breast cancer in women exposed to ETS was 1.89 (95% CI 1.52 - 2.36) for six recent case-control studies that enumerated all major sources of lifetime ETS exposure. On the other hand, earlier case-control studies, and a recent prospective cohort study, found lower, often non-significant, risk elevations. This may reflect less complete ETS exposure determination in those studies. Also, earlier studies of active smoking and breast cancer are non-positive, although a recent study reports this association. In this context the correlation with animal carcinogenicity data for identified components of tobacco smoke is useful. Tobacco smoke contains many carcinogens, some of which are up to ten times more abundant in sidestream smoke, the major source of ETS. We found 20 compounds in tobacco smoke that are identified (IARC 1, 2A or 2B) mammary gland carcinogens, including several polycyclic aromatic hydrocarbons and nitrosamines. These data suggest site concordance for mammary carcinogenesis in man and rodents, and provide substantial support for the conclusion that ETS exposure may be associated with increased incidence of breast cancer.

**1520**

DEVELOPMENT OF QUANTITATIVE QSAR MODELS TO PREDICT CANCER POTENCY OF CHEMICALS.

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Two important disadvantages of long-term animal bioassays are that testing involves substantial amounts of time and money, and that high doses are usually used in the testing process. These disadvantages can be circumvented using Quantitative Structure-Activity Relationships (QSARs). There are a limited number of QSAR models to predict the carcinogenicity of various chemicals, a majority of which relate the carcinogenic potency to measures of carcinogenicity such as mutagenicity, lethal dose (LD50) or the maximum tolerated dose (MTD). In one such QSAR, Krewski et al. demonstrated a good correlation ( $r = 0.952$ ,  $N = 191$ ) between tumor dose that causes cancer in 50% of an exposed population (TD50) and the MTD. The objective of this study was to predict the carcinogenic potency of a wide variety of chemicals. Initially, this study aimed to duplicate the results of Krewski et al. using 467 rat TD50s of either sex from the carcinogenic potency and genotoxicity databases maintained by Gold et al. The MTDs for the 467 chemicals were calculated using the rat Maximum Tolerated Dose model in TOPKAT, a commercial QSAR software for toxicity prediction. Since the results from the previous analysis indicated that MTD was a poor predictor for TD50, this study aimed to develop a pilot QSAR model to predict the cancer potencies for 70 chemicals from the Integrated Risk Information System (IRIS) database using their physicochemical properties as predictor variables or descriptors. Initially, the molecular structures of the 70 IRIS chemicals were optimized to their lowest energy geometries using the CONFLEX module in CAChe (Computer Aided Chemistry) software. Three descriptor generating programs are being used to generate the predictor variables for the QSAR model: Molconn-Z, CAChe and AMPAC/CODESSA. Results from the final QSAR for the pilot study involving 70 chemicals indicate that electrostatic variables such as nucleophilicity and electrophilicity from the three programs mentioned above are better descriptors of cancer potency than MTD.

**1521**

CANCER RISK ESTIMATION FOR EXPOSURE TO NAPHTHALENE.

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Naphthalene is a common air and soil pollutant. It is an industrial intermediate, a component of some fuels and a combustion byproduct. Naphthalene is identified as a California Toxic Air Contaminant (TAC) and a US Hazardous Air Pollutant. Naphthalene is listed as a chemical known to the State of California to cause cancer, and was recently classified as Group 2B (possibly carcinogenic to humans) by the International Agency for Research on Cancer. Recent National Toxicology Program (NTP) studies in rats showed clear evidence of a carcinogenic effect and provided data that allow satisfactory cancer risk assessment. Accordingly, and in view of the importance of naphthalene as an air pollutant, we developed a cancer risk assessment for the Hot Spots, TAC and Proposition 65 programs. Human cancer potencies and unit risk estimates were derived based on data from the NTP (2000) rat studies and the earlier NTP mouse studies, using both the linearized multistage model and the benchmark dose procedure, which avoids reliance on a specific tumor causation model. Available data on metabolism and genotoxicity supported the use of either the linearized multistage model or linear extrapolation from a benchmark in order to predict risk at low doses. Pharmacokinetic effects were examined but found not to have a substantial effect on the risk estimate. Since naphthalene induced significant increases in two different tumor types in the NTP male

rat study, a Monte Carlo procedure was used to provide a combined potency estimate. The recommended unit risk value for naphthalene was  $3.4 \times 10^{-5}$  ( $\mu\text{g}/\text{m}^3$ )<sup>-1</sup> and slope factor was  $1.2 \times 10^{-1}$  ( $\text{mg}/(\text{kg}\cdot\text{day})$ )<sup>-1</sup>. These values are based on the incidences of nasal respiratory epithelial adenoma and nasal olfactory epithelial neoplasia in male rats.

**1522**

PROTECTIVE EFFECT OF NRF2 ON THE MUSCLE OF MICE WITH AMYOTROPHIC LATERAL SCLEROSIS.

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Muscle atrophy is a primary characteristic of end-stage amyotrophic lateral sclerosis (ALS). This wasting effect stems from unknown mechanisms of motor neuron death in the spinal cord. A majority of ALS research has been performed in mice containing G93A mutant superoxide dismutase (SODG93A), which serve as excellent models for demonstrating consistent pathology compared to human patients. The mouse pathology displays increased oxidative stress similar in timing to our previous findings of the activation of the antioxidant response element (ARE) in ARE-hPAP mice crossed with SODG93A mice. The ARE is a DNA sequence present in the promoter of many detoxification enzymes and is activated by the transcription factor nuclear factor E2-related factor (Nrf2). To investigate the role of Nrf2 in the prevention of ALS, ARE-hPAP mice were injected with adenovirus containing Nrf2 (Ad-Nrf2) in the muscle at 45 days of age. They displayed sustained hPAP activity at 52 and 120 days of age that was strongest in the muscle followed by the spinal cord and brainstem, respectively. Further, retrograde transport of the hPAP activation demonstrates a possible protective effect on the motor neurons by Ad-Nrf2. By injecting Ad-Nrf2 in SODG93A mice at 45 days of age (a presymptomatic stage), the addition of Nrf2 was able to significantly delay downstream paralysis. The molecular basis for these effects was further explored using microarray technology of the infected muscle and suggests that novel secretory proteins and transport pathways are augmented to confer motor neuron protection. While the motor neurons in ALS are primarily targeted for death, we show that the muscle offers a unique supporting environment for protecting motor neurons and delaying paralysis. Support contributed by NIEHS: ES08089 and ES10042, ALSA, and the Robert Packard Center for ALS Research at Johns Hopkins. JCK is supported by NIEHS: ES013691 and ES0715-25

**1523**

SENSITIVITY TO MITOCHONDRIAL COMPLEX II INHIBITORS, MALONATE AND 3-NITROPROPIONIC ACID IN NRF2 KNOCKOUT MICE.

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Huntington's disease (HD) is caused by a CAG repeat expansion in the first exon of the huntingtin gene resulting in massive striatal degeneration. Although the mechanism of cell death is unclear, there is evidence that this cell loss is in part due to mitochondrial dysfunction. Complex II inhibitors 3-nitropropionic acid (3NP) and malonate cause striatal damage reminiscent of HD and have been used extensively as Huntington's models. Previous research has shown that complex II inhibition involves oxidative stress and secondary excitotoxicity. Because Nrf2-dependant transcriptional activation via the antioxidant response element (ARE) is known to coordinate the upregulation of genes involved in combating oxidative insults and cytotoxicity, we investigated the significance of Nrf2 in 3NP- and malonate-induced toxicity. We found that Nrf2-deficient cells and knockout mice are significantly more vulnerable to malonate and 3NP. Additionally, complex II inhibition resulted in increased ARE-regulated transcription mediated by astrocytes. Taken together, these observations implicate Nrf2 as an essential inducible factor in the protection against complex II inhibitor-mediated neurotoxicity.

**1524**

NEUROPROTECTIVE EFFECTS OF METALLOTHIONEIN-III IN MUTANT EXPANDED POLYGLUTAMINE EXPRESSED NEUROBLASTOMA CELLS.

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Neuronal loss and intraneuronal protein aggregates are characteristics of Huntington's disease (HD), which is one of neurodegenerative disorders caused by an expanded polyglutamine [poly(Q)] tract in the disease protein. N-terminal fragments of mutant huntingtin produce intracellular aggregates and cause toxicity. Metallothioneins (MT) is a low molecular weight proteins characterized by its abundant content of cystein. MT-III, a number of MT family, is expressed in brain in contrast to MT-I and MT-II, those are found in most tissue. In the present study,

we identified MT-III inhibits aggregation of poly(Q) and suppress the poly(Q)-mediated cell death, using a cellular model of HD. We tested the hypotheses that MT-III may reduce poly(Q)-mediated cell death by inhibiting the mitochondrial death pathway. In neuroblastoma cells expressing mutants poly(Q), MT-III treatments resulted in increase of cell viability. Moreover, we identified that MT-III decreased release of cytochrome c and p53 expression. We propose that a poly(Q) can induce mitochondrial death pathway that directly contribute to cell death and that MT-III is an antagonist of this process.

**1525**

METALLOTHIONEIN III ATTENUATES 6-HYDROXYDOPAMINE-INDUCED APOPTOTIC CELL DEATH IN SH-SY5Y CELLS.

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Metallothioneins (MTs) are low molecular weight, metal-binding proteins with established antioxidant capabilities. MT-III, a number of MT family, is expressed in brain in contrast to MT-I and MT-II, those are found in most tissue. In the brain, MT-III exhibits a free radical scavenging activity. Enhanced oxidative stress is implicated in the pathogenesis of neurodegenerative diseases such as Parkinson's disease. The catecholaminergic neurotoxin 6-Hydroxydopamine (6-OHDA) induces the production of reactive oxygen species (ROS), leading to neuronal cell death. In the present study, we investigated the effects of MT-III on 6-OHDA-induced apoptotic cell death using the human dopaminergic neuroblastoma cell line, SH-SY5Y. The co-treatments of cells with MT-III significantly attenuated 6-OHDA-induced ROS generation and subsequent apoptotic cell death. We also demonstrated that pretreatment alone with MT-III for 24 h prior to the exposure confers resistance against 6-OHDA-induced cell toxicity. These finding suggested that the MT-III acts principally as a radical scavenger to suppress the level of ROS, whereas the other mechanisms might be involved in the protective effects.

**1526**

MITOCHONDRIAL-DEPENDENT INITIATION OF APOPTOSIS AND FEEDBACK REGULATION BY PKC $\delta$  IN PROTEOSOME INHIBITOR MG-132-INDUCED DOPAMINERGIC DEGENERATION.

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Ubiquitin-proteosome dysfunction is implicated in the development of Parkinson disease (PD) due to the accumulation of ubiquitin and  $\alpha$ -synuclein in Lewy bodies, the defining pathological change of PD patients. To understand the underlying mechanisms by which dysfunction of this system contributes to the dopaminergic degeneration, we investigated the effects of the proteosome inhibitor MG-132 on apoptotic cell death in rat mesencephalic dopaminergic neuronal (N27) cells. Treatment with 2.5, 5.0, and 10  $\mu\text{M}$  MG-132 resulted in dose- and time-dependent cytotoxicity. Exposure to 5.0  $\mu\text{M}$  MG-132 rapidly and progressively inhibited the proteosomal activity, with almost 50% proteosomal inhibition within 5 min. This potent proteosomal inhibition by MG-132 was accompanied by rapid releases of the proapoptotic molecules cytochrome c and Smac from the mitochondria to the cytosol. Caspase-9 and -3 were activated dramatically in a time-dependent manner, whereas caspase-8 was increased moderately. The caspase-9 inhibitor LEHD-fmk completely inhibited MG-132-induced caspase-3 activity, indicating that caspase-9 is a major upstream caspase responsible for caspase-3 activation. MG-132-induced caspase-3 proteolytically activated a key proapoptotic kinase, protein kinase C delta (PKC $\delta$ ), in a time-dependent manner. Suppression of PKC $\delta$  by the caspase-3 cleavage-resistant mutant (PKC $\delta$ -CRM) or the kinase inhibitor rottlerin significantly attenuated MG-132-induced DNA fragmentation, suggesting that PKC $\delta$  is a key downstream mediator of proteosome inhibitor-induced apoptotic cell death. Furthermore, PKC $\delta$ -CRM and rottlerin significantly attenuated caspase-9 and -3, suggesting the existence of a possible feedback amplification loop. Collectively, these results demonstrate that dysfunction of the ubiquitin-proteosome system initiates apoptosis through an intrinsic apoptotic pathway involving PKC $\delta$ , which has a significant regulatory role in the dopaminergic cell death process [supported: ES10586 & NS45133].

**1527**

PROTEOSOME INHIBITOR ACLARUBICIN INDUCES DOPAMINERGIC CELL DEATH AND PARKINSON'S-LIKE SYMPTOMS IN MICE: IMPLICATIONS FOR UBIQUITIN-PROTEOSOME DYSFUNCTION IN PARKINSON'S DISEASE.

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The ubiquitin-proteosome pathway has recently been suggested to be critical in the pathogenesis of Parkinson's disease (PD). Impairment of proteosome function promotes aggregation of key proteins, including  $\alpha$ -synuclein and ubiquitin, which

subsequently leads to development of Lewy bodies, a pathological hallmark of PD. In order to understand the cellular and molecular mechanisms of proteosomal dysfunction in PD, we tested aclarubicin, an anthracycline anticancer drug obtained from *Streptomyces galilaeus* known to inhibit proteosome activity, in both cell culture and animal models. Aclarubicin (6 mg/kg i.p.) was administered to C57 black mice for 6 consecutive days. Neurobehavioral analysis revealed that both the horizontal and vertical motor activity of aclarubicin-treated animals started to decrease 3 days post-treatment and progressed over time. Aclarubicin treatment did not alter the body weight of the animals. Neurotransmitter analysis revealed a significant depletion of dopamine and DOPAC in the striatum of aclarubicin-treated animals. Proteosome activity was increased in the substantia nigra, suggesting that proteosome levels may be upregulated during the early phase of aclarubicin treatment. To better understand the mechanism of aclarubicin-induced dopaminergic neurotoxicity, we treated mesencephalic dopaminergic neuronal (N27) cells, *in vitro* model of PD, with aclarubicin. Treatment of N27 cells with aclarubicin (0.1, 1, and 3  $\mu$ M) for 1-12 hr induced dose- and time-dependent cytotoxicity. The cytotoxicity was accompanied by a time-dependent decrease in proteosome activity. The known proteosome inhibitor MG 132 (positive control) dramatically reduced the proteosome activity. Together, these results indicate that aclarubicin can produce PD-like motor deficits and neurochemical changes in the C57 black mouse model, which may be a useful animal model for investigation of ubiquitin-proteosome impairment associated with PD. [supported:ES10586 & NS45133].

**1528**

THROMBIN PRECONDITIONING PREVENTS STRIATAL ATROPHY AND DOPAMINERGIC TERMINAL LOSS, BUT NOT DOPAMINE DEPLETION IN A 6-HYDROXYDOPAMINE PARKINSON'S DISEASE MODEL.

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Administration of low dose thrombin several days before lesioning is neuroprotective in stroke models, an effect termed thrombin preconditioning (TPC). Recently, we found that TPC reduces behavioral deficits in the 6-hydroxydopamine (6-OHDA) Parkinson's disease (PD) model. Here, the ability of TPC to prevent striatal atrophy, dopaminergic terminal loss, and catecholamine depletion in the 6-OHDA model was evaluated. All animals received 10  $\mu$ g of 6-OHDA onto the right medial forebrain bundle. Three days prior to lesioning, animals received either 1 U thrombin (n=17) or saline (n=14) 1 mm dorsal to lesion. All animals were sacrificed 21 days after 6-OHDA for either histology or striatal catecholamine determination, using HPLC with electrochemical detection. Coronal (20  $\mu$ m) sections at 1.7, 1.2, 0.7, 0.2, and -0.3 to bregma underwent standard H & E staining, and using NIH Image, lateral ventricular volume was determined for both the lesioned and nonlesioned sides. Additional striatal sections were stained for tyrosine hydroxylase, here a marker for dopaminergic terminals. Optical density in the striatum was used to quantify terminals. TPC animals exhibited significantly smaller ventricular volumes on the lesioned side than those pretreated with saline (1.45 $\pm$ 0.22 vs. 0.94 $\pm$ 0.08 mm<sup>3</sup> $\pm$ SE; SPC vs. TPC; Student's *t*-test, p<0.05), indicating decreased striatal atrophy in TPC animals. Additionally, TPC prevented dopaminergic terminal loss as evidenced by optical density ratios (lesioned/nonlesioned; 0.80 $\pm$ 0.09 vs. 0.51 $\pm$ 0.06; TPC vs. SPC; p<0.05). However, neither TPC nor saline prevented massive striatal dopamine depletion (>95%), a striking finding, considering morphological and behavioral protection. The results indicate that TPC is able to prevent striatal atrophy and dopaminergic terminal loss in a 6-OHDA PD model and may explain the observed behavioral protection. Mechanistic studies may reveal novel therapeutic pathways.

**1529**

ROLE FOR PROTEASE-ACTIVATED RECEPTOR 1 (PAR1) IN MPTP-INDUCED DOPAMINERGIC NEUROTOXICITY.

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Protease-activated receptor 1 (PAR1) is a G-protein coupled receptor best-known for its role in blood coagulation. PAR1 is expressed in the CNS, particularly in the Substantia Nigra pars Compacta (SNc) and in astrocytes throughout the brain (J Neurosci 1995 Apr;15(4): 2906-19. and Exp Neurol. 2004 May; in press). Activation of this receptor has been implicated in neuronal death during transient focal ischemia and hypoxia, as well as in microglial activation, and in astrocyte proliferation. Due to its expression in the SNc and its putative role in neurodegeneration, we hypothesized that PAR1 activation might contribute to dopaminergic cell death in response to neurotoxins. To test our hypothesis, we used PAR1-/- and wild-type animals injected with the dopaminergic neurotoxin, 1-methyl-4-phenyl-

1, 2, 3, 6-tetrahydropyridine (MPTP). Wild-type and PAR1-/- animals received two subcutaneous injections of either vehicle or 10mg/kg of MPTP separated by 12 hours. After 48 hours, animals were sacrificed and the striata were removed and prepared for Western blot and HPLC. We observed that PAR1-/- mice appeared to be partially protected from dopaminergic cell loss following MPTP administration, as wild-type mice showed significantly greater loss of tyrosine hydroxylase (TH) protein. MPTP treatment reduced TH protein levels by 42.1 $\pm$ 4.1% in wild-type mice, but by only 8.1 $\pm$ 6.7% in PAR1-/- mice (ANOVA p<0.01 n=7-8). Similar results were seen for dopamine transporter (DAT) protein levels. Dopamine levels were reduced by 54.0 $\pm$ 5.4% in wild-type animals while there was only a 35.0 $\pm$ 4.0% decrease in PAR1-/- animals (ANOVA p<0.05 n=7-8 mice), further suggesting a protective phenotype in PAR1-/- animals. These results implicate the PAR1 receptor in the degeneration of the nigrostriatal dopamine system following neurotoxin exposure and indicate that there may be a link between blood-derived serine proteases and dopaminergic degeneration.

**1530**

NICOTINE STIMULATION INDUCES TH MESSENGER RNA EXPRESSION AND TRANSCRIPTION RATE IN MN9D CELLS.

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Although the transcriptional regulation of tyrosine hydroxylase (TH) gene expression has been widely studied in adrenal medulla and locus coeruleus (LC) models that express epinephrine or norepinephrine, very little is known about how the gene is regulated in dopaminergic neurons. Alterations in the normal function of mid-brain dopaminergic neurons occur in drug addiction, as well as movement disorders such as Parkinson disease and Tourette syndrome. Since dopamine biosynthesis is largely controlled by the activity of TH, information about the regulation of this enzyme may potentially be important for understanding the etiology of these pathologies. The goal of this study is to determine whether TH is transcriptionally regulated in dopamine neurons of the midbrain. Using the MN9D cell line, which is derived from midbrain dopaminergic neurons, we demonstrate that treatment of these cells with 100 micromolar nicotine for 15 minutes results in a 2.5-fold increase in TH gene transcription rate, as measured by an increase in the levels of TH gene primary transcripts. TH mRNA levels also increase in response to nicotine at later time points. These data suggest that acute exposure of midbrain dopaminergic neurons to nicotine induces TH mRNA via transcriptional mechanisms.

**1531**

NEUROPROTECTION AND NEURORESCUE AGAINST BETA-AMYLOID TOXICITY BY METALLOTHIONEIN III.

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Alzheimer's disease (AD) is characterized by senior plaques, containing aggregated beta-amyloid peptide (A-beta), and neurofibrillary tangles in the AD brains. Metallothioneins (MTs) are low molecular weight, metal-binding proteins with established antioxidant capabilities. MT-III, a number of MT family, is expressed in brain in contrast to MT-I and MT-II, those are found in most tissue. The central hypothesis guiding this study is that MT-III may play an important role in amyloid precursor protein (APP) secretion and protection against toxicity induced by A-beta. The present study shows that MT-III enhances the release of the non-amyloidogenic soluble form of the alpha-amyloid precursor protein (sAPPalpha) into the cultured media of mouse neuro 2A neuroblastoma cells. MT-III is not only able to protect, but it can rescue neuro 2A cells against the A-beta toxicity. These results suggested that MT-III has protective effects against A-beta-induced neurotoxicity and regulates secretory processing of non-amyloidogenic sAPPalpha.

**1532**

CHARACTERIZATION OF CEREBRAL SP1, A $\beta$  AND APP IN RATS: AN IMMUNOHISTOCHEMICAL STUDY OF AGING AND NEURODEGENERATION.

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A hallmark of Alzheimer's disease (AD) is the presence of plaques containing aggregated beta-amyloid peptides (A $\beta$ ), derived from the amyloid precursor protein (APP). APP over-expression has been associated with premature accumulation of A and plaque formation and has been linked to learning deficits in transgenic mice. We have found that APP and A $\beta$  over-expression as well as elevated Sp1 DNA-binding activity occur in rats which have been developmentally exposed to lead

(Pb). Recently *in vitro* studies in transfected cells demonstrated that over-expression of Sp1 results in an elevation of APP and overproduction of A $\beta$  (Christenson et al., 2004), while findings from our lab showed that the depletion of Sp1 greatly diminished the responsiveness of the APP promoter. These molecular studies implicate Sp1 as a primary regulator of the APP gene and a mediator of Pb-induced changes in APP expression. To determine whether the relationship between Sp1, APP, and A $\beta$  in the brain is consistent with these findings, we analyzed brain sections of 20 month old rats for Sp1, APP and A $\beta$  immunoreactivity. We found that the overall distribution of Sp1 and APP in various brain regions is similar and appears to be neuronal in origin. In some regions of the brain such as the cerebellum we found differential expression of Sp1, APP, and A $\beta$  which was restricted to the Purkinje cell layer. Consistently, Purkinje cells which over-expressed Sp1 also contained an over-abundance of APP and A $\beta$ . These *in vivo* immunohistochemical findings confirm the existence of a relationship between APP expression and Sp1, and suggest that overproduction of A $\beta$  occurs in cells where Sp1 is strongly present. Therefore, environmental models of AD should consider Sp1 as a potential target that could influence the process of amyloidogenesis.

### 1533

#### THE CHOROID PLEXUS EXPRESSES THE ENZYMES NECESSARY FOR BETA-AMYLOID PRODUCTION.

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Aggregation of beta-amyloid peptides are central in the formation of insoluble plaques seen in brains of Alzheimer's disease patients. Amyloid precursor protein (APP) cleavage by alpha-secretase leads to the release of a soluble peptide fragment, whereas cleavage by beta-secretase promotes the formation of insoluble beta-amyloid peptides. Gamma-secretase cleaves the carboxyl-terminus resulting in variable lengths of the peptides. While a role for the choroid plexus, which separates the blood from cerebrospinal fluid (CSF), in transporting amyloid peptides has been suggested, the question as to whether the tissue itself produces amyloid proteins was unanswered. The objective of this study was to test the hypothesis that the choroid plexus possesses the capacity to produce beta-amyloid. Proteins and total RNAs were extracted from rat choroid plexus tissue and Z310 cells, an established rat choroid epithelium cell line. Quantitative real-time RT-PCR revealed the presence of APP mRNA as well as those of two processing enzymes, alpha- and beta-secretase in both choroid plexus tissue and Z310 cells. We also compared the plexus expression of APP and secretases with brain capillaries isolated from various brain regions. All values were normalized to the mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Results showed that the choroid plexus expressed the same levels of mRNAs encoding APP, alpha-secretase, and beta-secretase as capillaries of hippocampus, frontal cortex, and cerebellum. Capillaries from the striatum had significantly higher APP and beta-secretase mRNAs than did the choroid plexus. Western blot analysis to confirm the presence of alpha- and beta- secretase, as well as APP proteins is currently in progress. In conclusion, our data indicate that APP and enzymes involving in its metabolism, i.e., alpha- and beta-secretase, appear to be present in the choroid plexus and brain capillaries, suggesting that the blood-CSF barrier and blood-brain barrier may participate in the production and secretion of beta-amyloid in the central nervous system.

### 1534

#### THE CHOROID PLEXUS ACCUMULATES BETA-AMYLOID FROM BRAIN CSF: IMPLICATIONS FOR ALZHEIMER'S DISEASE.

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Beta-amyloid (A $\beta$ ) peptide accumulates and precipitates as insoluble plaques in the brains of Alzheimer's disease (AD) patients. A $\beta$  circulates throughout and between the blood and cerebrospinal fluid (CSF) by crossing the choroid plexus (CP), which comprises the blood-CSF barrier. This study examined the role of the CP in A $\beta$  movement in normal brain. CP tissue isolated from the brains of male Sprague-Dawley rats was weighed and incubated in an artificial CSF (aCSF) containing radiolabeled A $\beta_{1-40}$  ( $^{125}\text{I}$ A $\beta$ ) and a marker for extracellular space and diffusion,  $^{14}\text{C}$ sucrose. Uptake was determined as the ratio of radioactivity in tissue to aCSF.  $^{125}\text{I}$ A $\beta$  uptake into isolated rat CP was linear to 5 minutes, after which uptake leveled. Uptake of total  $^{125}\text{I}$  labeled species was not saturable, while the uptake of the unchanged  $^{125}\text{I}$ A $\beta$  was saturated only at the highest A $\beta$  concentration tested (1000 ng/mL). Low temperature inhibited  $^{125}\text{I}$ A $\beta$  uptake, while A $\beta$  binding partners transthyretin and RAGE (receptor for advanced glycation endproducts) had no significant effect. Apolipoprotein E3 inhibited uptake of total  $^{125}\text{I}$  label, but not that of  $^{125}\text{I}$ A $\beta$ . A primary culture of rat CP epithelial cells seeded onto Transwell chamber devices and grown to confluence was used to study the transepithelial transport and accumulation of A $\beta$ .  $^{125}\text{I}$ A $\beta$  and  $^{14}\text{C}$ sucrose were added to the donor chamber, and aliquots of receiver chamber aCSF were collected for 48 hours.  $^{125}\text{I}$ A $\beta$  permeability across the CP cell monolayers, determined from the slope of receiver chamber concentration over time, appears to be higher in efflux than influx studies. A $\beta$  accumulation into CP cell cultures was higher from the CSF-facing side

than the blood-facing side, suggesting that the CP is more effective in exporting A $\beta$  from the brain. These data suggest that the CP sequesters A $\beta$  from CSF and may be a site of normal brain A $\beta$  clearance. Whether a dysfunction of CP may affect A $\beta$  homeostasis in the brains of AD patients deserves further investigation.

### 1535

#### DIFFERENTIAL EXPRESSION OF GRP78 AND HSP70 IN PRIMARY BRAINSTEM AND CORTICAL ASTROCYTES EXPOSED TO 1, 3-DINTROBENZENE.

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Exposure to 1, 3-dinitrobenzene (1, 3-DNB) results in a gliovascular lesion within specific brainstem nuclei. Although these regions have high energy requirements, this alone does not describe the observed pathology due to the absence of lesion in areas of similar energy demand. We investigated the possibility that acute *in vitro* neurotoxicity of 1, 3-DNB may be mediated through a disruptive effect by the compound on two key molecular chaperones, glucose regulated protein 78 (GRP78) and heat shock protein 70 (HSP70). Real time RT-PCR analysis of primary brainstem and cortical astrocyte cultures revealed GRP78 mRNA expression increased approximately two-fold in the brainstem, 2 hours post 1mM 1, 3-DNB exposure. Cortical GRP78 mRNA expression increased approximately two-fold, 4 hours after exposure. Regional differences in sensitivity were also observed with the positive control. Thapsigargin (1 $\mu$ M) induced a five-fold increase in brainstem GRP78 expression, in contrast, cortical expression levels were not significantly affected. Immunoblot analysis of brainstem astrocytes showed down-regulation of GRP78 protein expression at 0.5 and 2 hours and up-regulation to basal levels at 4 hours. No significant changes in GRP78 protein expression were observed in the cortical astrocytes after 6 hours of exposure. Immunofluorescence was used to monitor changes in HSP70 expression after 1, 3-DNB exposure in primary brainstem and cortical astrocytes. HSP70 was up-regulated at 2 hours in the cortical astrocytes and at 4 hours in the brainstem astrocytes. This data indicates that primary brainstem and cortical astrocytes respond differentially to oxidative cues after 1, 3-DNB exposure: brainstem astrocytes are significantly more sensitive. Temporal regulation of regional molecular responses to oxidative stress may underlie the differential susceptibility of brainstem astrocytes to 1, 3-DNB. This work was supported by NIH T32 ES07062 (SAR) and RO1 ES08846 (SRS & MAP).

### 1536

#### MIXED INHIBITION OF THE PYRUVATE DEHYDROGENASE COMPLEX BY M-DINITROBENZENE.

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Pyruvate dehydrogenase (PDH), an essential multi-component enzyme complex responsible for the production of acetyl-CoA from glycolytically derived pyruvate, links glycolysis to the TCA cycle. The normal brain relies almost exclusively on the complete oxidation of glucose as its sole fuel source making PDH function critical in maintaining energy metabolism. Disturbances in the function of this enzyme complex frequently result in ensuing pathological states. m-Dinitrobenzene (DNB) has been shown to selectively affect glia-vascular components in distinct brainstem regions resembling the early effects of other acute energy deprivation syndromes. Previously we have shown the rapid and dose dependent inhibition of bovine heart PDH by DNB in purified enzyme reactions. To determine the nature of the inhibition we employed a standard spectrophotometric assay in which the production of the reduced form of nicotinamide-adenine dinucleotide (NADH) by bovine heart PDH was monitored at different substrate concentrations. Through examination of the double reciprocal plots of control versus enzyme incubated with DNB concentrations that have been shown to inhibit in the range of 20 to 60%, it was determined that DNB does not compete for either pyruvate or CoA but results in a mixed type inhibition mechanism. Lipoamide dehydrogenase, the E3 component of the enzyme complex, has been shown to possess nitro-reductase activity. To determine whether or not DNB acts as a specific E3 component inhibitor, oxidation of NADH was measured in the presence of lipoamide. No effect of varying the concentration of DNB was observed on the activity of the E3 component of the complex. In conclusion, DNB exerts its inhibitory effects on PDH via a mixed mechanism. One potential mechanism is through active site modification of either the E1 or E2 components of the complex but not through E3 effects. Further investigation into the mechanism of this inhibition is required. This work was supported by the NIH grant R01-ES08846.

### 1537

#### NITRIC OXIDE MEDIATES MITOCHONDRIAL DYSFUNCTION IN NEUROENDOCRINE TOXICITY.

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Nitric oxide (NO) produced by neuronal nitric oxide synthase, is critical for activity of the neuroendocrine axis, mediating the functioning of the hypothalamic-pituitary-gonadal axis and the hypothalamic-pituitary-adrenal axis. However, NO released from neuroendocrine cells has also been implicated in disorders of these

conduits. In addition, reactive oxygen species (ROS) generated by dysregulation of mitochondrial oxygen sensing may also play a role in pituitary injury. To evaluate the role of NO in the pituitary, we first determined the levels of nitric oxide, and its effective radius, following release from GH3 adenocarcinoma pituitary cells. In these cells nitric oxide release is mediated by activation of L-channel agonists, therefore nitric oxide release was stimulated by membrane depolarizing concentrations of K<sup>+</sup> (25 mM) and by the L-channel calcium agonist Bay kappa 8644 (Bay K, 3  $\mu$ M) and measured using a NO self-referencing sensor. NO was released from GH3 cells immediately following treatment with K<sup>+</sup> and Bay K and could be detected at distances of up to 1 mm from the cells. Mitochondrial function, as measured by cellular oxygen uptake, was not altered by nitric oxide release. Potentially, nitrosation of critical mitochondrial proteins mediated by peroxynitrite, an intermediate formed by interaction of nitric oxide with ROS, is critical to tissue injury. In this regard, we found that O<sub>2</sub> uptake was diminished by the presence of peroxynitrite (10-100  $\mu$ M). In addition, dysregulation of mitochondrial O<sub>2</sub> sensing using nitroarginine methyl ester (1 mM) enhanced oxygen uptake and ROS generation. Taken together these data demonstrate that peroxynitrite, but not agonist-stimulated NO release, alters mitochondrial function in GH3 cells. Our findings also indicate that inhibition of O<sub>2</sub> sensing, through limiting mitochondrial NO production, results in ROS formation. We speculate that peroxynitrite formed in the degenerating pituitary mediates, in part, neuroendocrine toxicity. Supported by NIH grants ES05022, ES007148 and CA093798.

**1538** NITRIC OXIDE ACTIVATES P53 AND INDUCES APOPTOSIS IN NEURONAL PC12 CELLS.

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These experiments tested the hypothesis that the p53 tumor suppressor protein and its regulatory elements are involved in nitric oxide (NO)-mediated toxicity in PC12 cells. In order to closely model physiological dopaminergic neurons, PC12 cells were differentiated with nerve growth factor (NGF) and exposed to the nitric oxide donor sodium nitroprusside (SNP). It was shown that differentiated PC12 neurons (dPC12) undergo marked decreases in cell viability following SNP treatment as determined by the MTS dye reduction assay. Pretreatment with the selective p38/MAPK inhibitor, SB202190, partially protected cells from SNP-induced death, implicating p38/MAPK in NO-mediated neuronal toxicity. Despite p53 accumulation and phosphorylation resulting from NGF-induced cell cycle arrest and senescence, additional modest p53 protein accumulation, acetylation and phosphorylation occurs following SNP treatment. Demonstrated NO-induced cytosolic cytochrome c release and caspase activation in dPC12 cells confirms the loss of dPC12 viability occurs through an apoptotic mechanism. Cell death following SNP treatment can be partially attenuated by transcriptional and translational inhibition with actinomycin D and cyclohexamide, respectively. Caspase activity profiling shows marked increases in caspase-1 activity, while several other caspases are significantly inactivated. Apoptosis following SNP treatment does not involve caspase 3/7 activation in differentiated PC12 cells. While p53 is transcriptionally active during NGF-mediated senescence, further study is underway to describe the p53-mediated events leading from senescence to apoptosis. We conclude that differentiated PC12 cells undergo apoptosis in response to SNP treatment, and that p53 is involved in this NO-induced neuronal toxicity. Further study of transcription complexes by p53-specific chromatin immunoprecipitation screening will define its role in neuronal cell death.

**1539** EVALUATION OF THE INTERACTION BETWEEN CAVEOLIN AND GLIAL FIBRILLARY ACIDIC PROTEIN IN C6 GLIA.

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Plasma membrane organelles known as caveolae (CAV) are involved in signal transduction events and subcellular transport. These roles of caveolae make it relevant in pathogen, drug entrance to the cell, and as targets for toxicity. The principal components of caveolae are the caveolins (cav), which exist in three different isoforms: cav-1, cav-2, and cav-3. These have been identified in glial cells, particularly the C6 astroglial cell line. In the evaluation of the role of Cav/caveolins in glial cells, here we seek to study their putative interactions with intermediate filaments (IF) proteins. This evaluation is being done in differentiated C6 astrocytes where glial fibrillary acidic protein (GFAP) is the major IF protein. The interaction of caveolin-1 and GFAP was evaluated via laser scanning confocal microscopy, which revealed discrete patches of cav-1/GFAP co-localization in plasma membrane CAV. Subcellular fractionation of CAV-rich microdomains using sodium bicarbonate su-

crose gradients shows co-fractionation of caveolin-1 and GFAP. Preliminary immunoprecipitation results using anti-cav-1 antibodies coupled to agarose beads also support a potential interaction between cav-1 and GFAP. GFAP and IF sequence analysis lead us to propose a novel caveolin-binding motif in the rod 1A region of IF proteins. Studies to confirm these preliminary finding are being undertaken. IF proteins may interact directly or indirectly with glial cell CAV, playing a potential role in their physiological functions, such as signal transduction, transcytosis, potocytosis, and endocytosis. In addition, the interaction between CAV and GFAP can be related with pathological disorders, such as Alexander's disease.

**1540** REACTIVE GLIOSIS IN NEUROTOXIC AND MECHANICAL INJURY MODELS.

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Reactive gliosis is a hallmark of disease-, trauma-, and chemical-induced damage to the CNS. This response is characterized by activation of microglia and astrocytes at sites of damage. Signaling pathways associated with the induction of gliosis remain elusive, but recent evidence implicates the Janus kinase (JAK)-signal transducer and activator of transcription-3 (STAT3) pathway. This pathway is a down-stream effector of cytokines linked to injury-induced astrogliol activation and subsequent expression of GFAP, a cellular response that appears to require STAT3 phosphorylation. Here, we seek to further characterize gliosis by elucidating ligands involved in STAT3 phosphorylation and the induction of GFAP. Two injury models were employed. First we administered, *in vivo*, saline or the dopaminergic neurotoxin, 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP, 12.5 mg/kg, sc). 12 hours post-treatment, coronal slices of striatum were examined for STAT3 phosphorylation and cytokine production. pSTAT3 was present in MPTP treated animals while absent in controls. Messenger RNA for gp130 cytokines [leukemia inhibitory factor (LIF) and oncostatin M (OSM)] as well as a glial-derived chemokine (CCL-2/MCP-1) was induced in MPTP treated animals. Striatal slices were then incubated in oxygenated buffer. Within 45 minutes, control striata also expressed p-STAT3 indicating that STAT3 activation can result from slice injury. To confirm the induction of pSTAT3 *in vivo*, stab wounds were made in striata of mice. GFAP was induced 48 hours after stab and this effect was preceded by pSTAT3 expression. In addition, mRNA for LIF, OSM, and MCP-1 was induced by 24 hours. Since these three ligands were upregulated in both damage models, they were incubated 45 minutes with untreated slices and slices were then assayed for pSTAT3. LIF and OSM promoted glial activation as evidenced by increased pSTAT3 and GFAP protein whereas CCL-2 inhibited gliosis. Together, these data suggest that glial chemokines and gp130 cytokines mediate/modulate reactive gliosis and that pSTAT3 is an early event in glial activation in both neurotoxic and mechanical injury.

**1541** ERK1/2-RSK2 STIMULATION OF MEF2C TRANSCRIPTION PROMOTES CORTICAL NEURON SURVIVAL.

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Extracellular signal-regulated protein kinase (ERK) 1/2 pathway has been implicated in neuronal protection against several forms of neuronal apoptosis. However, the downstream mechanisms are not completely understood. Here we report that brain-derived neurotrophic factor (BDNF) protects embryonic cortical neurons from apoptosis after serum withdrawal. Furthermore, BDNF neuroprotection was mediated in part by activation of ERK1/2 signaling pathway. Importantly, we discovered that ERK1/2 activates ribosomal S6 kinase 2 (RSK2), which phosphorylates and activates myocyte enhancer factor 2C (MEF2C). We also provide evidence that RSK2/MEF2C are responsible for the neuroprotection afforded by ERK1/2. Together, our data identify ERK1/2-RSK2-MEF2C as a novel mechanism of neuroprotection.

**1542** DEVELOPMENTAL LEAD EXPOSURE AFFECTS THE SURVIVAL OF GRANULE CELLS IN THE RAT HIPPOCAMPUS.

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Environmental factors can modify cognitive function and synaptic plasticity by altering neurogenesis in the dentate gyrus (DG) of the hippocampus. We have previously shown that developmental lead (Pb<sup>2+</sup>) exposure impairs neurogenesis in the DG at postnatal day (PN) 50 (Verina, et al., *Toxicol. Sciences.*, 78(Suppl.):109, 2004), a time point in which Pb<sup>2+</sup>-exposed rats exhibit deficits in spatial learning

and hippocampal LTP. In the present study, we examined whether developmental Pb<sup>2+</sup> exposure influences the survival of newborn cells in the DG. Newborn cells were detected using the proliferation marker bromodeoxyuridine (BrdU). Daily injections of BrdU (100mg/kg) were administered to each rat from PN45 to 49. Animals were sacrificed 4 weeks after the last BrdU injection. BrdU-labeled cells were detected by immunohistochemistry and survival of newborn cells was measured after four weeks in the granule cell layer of DG (GCL) in controls and Pb<sup>2+</sup>-exposed rats. BrdU-positive cells were counted bilaterally at the level of the dorsal hippocampus. Cell counts were obtained individually from suprapyramidal (SP) and infrapyramidal (IF) blades of GCL and analyzed using a Poisson regression model with count as the response variable, area as an offset, and treatment group as a factor. There was a highly significant 27.5% decrease of BrdU-positive cells in GCL of Pb<sup>2+</sup>-exposed rats, which was more pronounced in the IF blade (34.2%). To identify potential mediators of the Pb<sup>2+</sup> effect on granule cell survival we assess levels of neurotrophic factors. Preliminary results indicate that the marked decrease in the survival of granule cells in the DG of Pb<sup>2+</sup>-exposed rats might be attributable to altered levels of neurotrophic factors. In summary, our present and previous study indicates that developmental Pb<sup>2+</sup> exposure reduces granule cell proliferation and survival and that this effect may be mediated by altered levels of neurotrophic factors. [Supported by grant number ES06189]

#### 1543

#### PPAR ALPHA ACTIVATION ENHANCES CYANIDE-INDUCED NEUROTOXICITY BY UP-REGULATION OF UCP-2.

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The activation of peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ) enhances cerebellar granule cell death, but the mechanism underlying PPAR $\alpha$  involvement in neuronal cell death is not known. Previous studies have shown that the PPAR $\alpha$  activator WY14, 643 induces up-regulation of Uncoupling Protein-2 (UCP-2) in rodent hepatocytes. To further explore the role of PPAR $\alpha$  in neuronal cell death and its potential function in the nervous system, we examined the effects of WY14, 643 on potassium cyanide (KCN)-induced cell death in rat dopaminergic N27 cells and expression levels of UCP-2. When cells were treated with either WY14, 643 (100  $\mu$ M) or KCN (300  $\mu$ M) alone, no significant cell death was observed. However, when WY14, 643 and KCN treatments were combined, the cells exhibited a significant level of necrotic cell death as compared to controls. Cell death was preceded by a marked decrease of cellular ATP levels and mitochondrial membrane potential ( $\Delta\psi$  m). The results also showed that cell death was blocked by pretreatment with MK-886, a non-competitive inhibitor of PPAR $\alpha$ . To determine the involvement of UCP-2 in the response, western blot analysis showed that KCN had no influence on expression of UCP-2, whereas pretreatment with WY14, 643 significantly increased UCP-2 expression. Inhibition of UCP-2 translation by transfection with UCP-2 siRNA (RNA-interference) protected cells from death. These results suggest a possible role of PPAR $\alpha$  in enhancement of toxicant-induced neuronal cell death in which PPAR $\alpha$  activation leads to up-regulation of UCP-2 and thus sensitizing the cells to mitochondrial dysfunction. (This study was supported by NIH grant ES04140)

#### 1544

#### NEUROPROTECTION AGAINST METHAMPHETAMINE (METH) AFFORDED BY PRETREATMENT WITH INCREASING DOSES OF THE DRUG IS DEPENDENT ON THE DOSING SCHEDULE OF METH USED DURING THE DRUG CHALLENGE.

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Because repeated intake of methamphetamine (METH) leads to the development of tolerance to its behavioral effects, human addicts usually take higher doses of the drug to achieve consistent euphoric effects. Although evidence exists that METH taken in this manner can cause degeneration of monoaminergic terminals in humans, it is still not clear if this is the case in animal models because the most common ways of assessing METH toxicity involve a METH challenge in animals that have not been exposed previously to the drug. Thus, the purpose of the present study was to evaluate the effects of two types of METH challenges on METH-induced monoamine depletion in the rat brain after pre-treatment of the animals with increasing doses of the drug. Adult male Sprague-Dawley rats were injected with METH or saline according to an escalating dose (ED) schedule for two weeks, followed by a challenge with either saline or one of two METH doses (10 mg/kg every 2 hrs X 3 injections or 5 mg/kg every hr X 6 injections). Brain regions were extracted at 2 or 24 hrs following the final injection. HPLC analyses revealed that

METH causes significant depletion of dopamine (DA) and serotonin (5-HT) in the striatum of saline-treated rats using either of the two challenge approaches. In addition, while there was significant protection from the 3 x 10 mg/kg METH challenge, there was no such protection in the animals treated with the 6 X 5 mg/kg challenge schedule after the animals were pre-treated with escalating doses of the drug. These results are compatible with the documentation of monoamine depletion in the brains of METH abusers.

#### 1545

#### SUBCHRONIC EXPOSURE TO LOW LEVELS OF POLYCHLORINATED BIPHENYLS REDUCES DOPAMINE TRANSPORTER AND VESICULAR MONOAMINE TRANSPORTER 2 LEVELS.

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Polychlorinated biphenyls (PCBs) are ubiquitous environmental contaminants which have been shown to target the dopaminergic system, causing reductions of dopamine levels both in cell culture and in animal models. However, the mechanisms behind the actions of PCBs on the dopaminergic system are unclear. Previously, we reported that high-level acute exposure to Aroclor 1016 or 1260 reduces striatal dopamine and levels of the dopamine transporter (DAT) and vesicular monoamine transporter 2 (VMAT2) (Richardson and Miller, 2004). In this study, we examined the effects of daily exposure of mice to a 1:1 mixture of Aroclor 1254 and 1260, which is similar in composition to PCBs found to contaminate fish in the Great Lakes. Oral exposure of mice to 7.5 or 15 mg/kg/day for 14 days resulted in a dose-related decrease in the levels of DAT as determine by immunoblotting (15 and 60%), [<sup>3</sup>H]DA uptake (15 and 50%) and [<sup>3</sup>H]WIN 35, 428 binding (15 and 40%). Exposure of mice for 30 days resulted in a dose related decrease in levels and function of DAT as determined by immunoblotting (18 and 40%), [<sup>3</sup>H]DA uptake (40 and 60%) and [<sup>3</sup>H]WIN 35, 428 binding (25 and 50%). Reduction of VMAT2 (15 and 20%) was also observed. No changes were observed in tyrosine hydroxylase (TH) levels, suggesting that the decreases in DAT and VMAT2 were not the result of loss of dopamine neurons. These data suggest that low-level exposure to PCBs alters DAT and VMAT2 levels. Since DAT and VMAT2 play significant roles in the regulation of dopamine levels, alterations of these transporters may contribute to the reduction of dopamine levels observed in animals chronically exposed to PCBs. Funding: ES012068 (GWM) and F32ES013457 (JRR)

#### 1546

#### ACRYLAMIDE DISRUPTS UPTAKE OF DOPAMINE INTO RAT STRIATAL SYNAPTIC VESICLES.

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Acrylamide (ACR) exposure impairs calcium-dependent neurotransmitter release from rat brain synaptosomes (Neurotoxicology 25: 349-363, 2004). Although the corresponding site and mechanism of action are unknown, it is possible that ACR decreases the presynaptic pool of releasable neurotransmitter by inhibiting synaptic vesicle (SV) transport and storage. To investigate this possibility, SVs were prepared from rat striatum and were exposed to graded concentrations of ACR (0.4-2.0M). Results show that ACR caused concentration-dependent decreases in vesicular <sup>3</sup>H-dopamine (DA) uptake (IC<sub>50</sub> = 243 mM). *In vitro* exposure of SVs to the sulphydryl reagent, N-ethylmaleimide (NEM), produced similar inhibitory effects (IC<sub>50</sub> = 0.006 mM). Kinetic analysis revealed that *in vitro* exposure to either ACR or NEM increased Km and decreased V<sub>max</sub>. Analysis of SVs from ACR-intoxicated rats (50 mg/kg x 8d or 21 mg/kg x 21 d) demonstrated similar alterations in kinetic parameters. These *in vitro* and *in vivo* changes are consistent with ACR addition of SV proteins resulting in mixed noncompetitive inhibition. Additional *in vivo* studies showed that ACR did not affect protein content or synaptosomal GSH levels, whereas *in vitro* ACR exposure caused no changes in vesicular DA retention. These results suggest that ACR causes direct inhibition of SV neurotransmitter storage and thereby decreases the releasable presynaptic pool. We hypothesize that ACR forms cysteine adducts, which inhibit the function of SV proteins that mediate DA uptake (VMAT2, v-ATPase). Sponsored by ESO3830-18.

#### 1547

#### INHIBITION OF HUMAN $\alpha$ 7 NEURONAL NICOTINIC ACETYLCHOLINE RECEPTORS BY THE VOLATILE ORGANIC SOLVENT TRICHLOROETHYLENE.

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Volatile organic solvents (VOCs) such as toluene and perchloroethylene block currents mediated by neuronal nicotinic acetylcholine receptors (nAChR), an effect hypothesized to be a mode of action for these compounds. Trichloroethylene

(TCE) is a neurotoxic VOC that has behavioral effects similar to toluene and perchloroethylene. The present study examined the hypothesis that TCE will inhibit nAChR function in a manner similar to other VOCs. TCE effects on acetylcholine-induced currents through human  $\alpha 7$  receptors expressed in Xenopus oocytes were measured using two-electrode voltage clamp techniques. Acetylcholine-induced currents had an average amplitude of  $-257 \pm 121$  (n=7) nA. TCE caused a concentration-dependent inhibition of nAChR current amplitude. At a nominal TCE concentration of 250  $\mu$ M, TCE inhibited currents through  $\alpha 7$  receptors by 88% (n=4), while at a nominal concentration of 2500  $\mu$ M, inhibition was 93% (n=4). Effects of TCE on  $\alpha 7$  nAChR were rapid and completely reversible. Actual TCE concentration in the test medium was determined by gas chromatography of samples (nominally 50 - 5000  $\mu$ M) taken directly from the recording chamber. Linear regression indicated that nominal TCE concentrations of 250 and 2500  $\mu$ M corresponded to actual TCE concentrations of 50 and 500  $\mu$ M, respectively. These results indicate that TCE inhibits nAChR receptor function at low concentrations and suggests that disruption of nAChR function may be a common mode of action for neurotoxic VOCs. (This abstract does not reflect EPA policy).

**1548 XYLENE BLOCKS VOLTAGE-GATED CALCIUM CURRENTS IN PHEOCHROMOCYTOMA CELLS.**

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Volatile organic compounds such as toluene, trichloroethylene and perchloroethylene are potent and reversible blockers of voltage-gated calcium current ( $I_{Ca}$ ) in nerve growth factor (NGF)-differentiated pheochromocytoma (PC12) cells. It is hypothesized that effects of VOCs on  $I_{Ca}$  contribute to the acute neurotoxicity of VOCs. The present experiments examined the ability of xylene, a VOC that is structurally similar to toluene, to block  $I_{Ca}$  in PC12 cells. Whole-cell patch clamp techniques were utilized to examine xylene effects on currents elicited by a 200 ms voltage-step from a holding potential of -70 to +10 mV. Following exposure to 3000  $\mu$ M xylene (as mixed xylene isomers), peak  $I_{Ca}$  was reduced to  $21 \pm 9$  % of control amplitude while current amplitude immediately prior to the end of the voltage step was reduced to  $12 \pm 6$  % of control (n = 3). Xylene, at nominal concentrations between 150 and 3000  $\mu$ M, reduced  $I_{Ca}$  in a concentration-dependent manner. In addition to decreasing amplitude, xylene also altered the inactivation kinetics of  $I_{Ca}$ . In the absence of xylene, currents decayed with a single exponential and had a tau value of  $133 \pm 39$  msec (n = 3). In the presence of 1500  $\mu$ M xylene, inactivation kinetics were best fit by two exponential components with tau values of  $17 \pm 9$  and  $57 \pm 10$  msec (n=3). These data indicate that voltage-gated calcium channels are sensitive to the effects of xylene, and that xylene produces the same characteristic alterations in calcium channel function that are observed following exposure to toluene, perchloroethylene and trichloroethylene. Thus, VOCs have a common ability to disrupt calcium channel function, and this action may contribute to the neurotoxic effects of VOCs. (This abstract does not reflect EPA policy).

**1549 NEUROTOXIC (1, 2, 4-) AND NON-NEUROTOXIC (1, 3, 5-) TRIETHYLBENZENES.**

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We have reported the protein-reactive, chromogenic and neurotoxic properties of the aromatic and aliphatic gamma-diketone solvent metabolites 1, 2-diacetylbenzene (1, 2-DAB) and 2, 5-hexanedione (2, 5-HD), respectively; by contrast their corresponding non-chromogenic isomers, 1, 3-DAB & 2, 4-HD, respectively, fail to induce murine neuropathy, the hallmark of which is proximal (1, 2-DAB) or distal (2, 5-HD) axonal swellings (with distal atrophy) filled with maloriented neurofilaments (NF). We asked whether 1, 2, 4-triethylbenzene (1, 2, 4-TEB), the probable precursor of 1, 2, 4-triacylbenzene (1, 2, 4-TAB) shows comparable structure-dependent chromogenic and neurotoxic properties. Twelve week-old male C57BL/6 mice were dosed orally with 300 mg/kg (n=3), 600 mg/kg (n=3) or 900 mg/kg (n=3) body weight of 1, 2, 4-TEB, equivalent doses of 1, 3, 5-TEB (which lacks the 1, 2-diacetyl moiety), or an equal volume of the TEB Vehicle (olive oil), for 3 evenly spaced days/week for up to 12 weeks. Mice treated with 1, 2, 4-TEB excreted greenish urine after 5 (900 mg/kg), 14 (600 mg/kg) and 49 (300 mg/kg) days and, thereafter, developed muscle spasm and hind-limb weakness. Blue-greenish tissue from the central nervous system (frontal cortex, hippocampus, basal ganglia, cerebellum, medulla oblongata, lumbar and cervical spinal cord) and the peripheral nervous system (lumbar spinal ventral roots, dorsal spinal roots and ganglia, sciatic nerves) were sampled for light and electron microscopy. Mice treated with 1, 2, 4-TEB showed a dose-dependent 1, 2-DAB-like pattern of intramedullary, intraspinal and spinal root NF-filled axonal swellings presumably arising from proximal blockade of NF anterograde transport. Mice treated with 1, 3, 5-TEB or Vehicle lacked urine and tissue discoloration, hindlimb weakness, and

neuropathology. These results show that 1, 2-spaced ethyl moieties on a benzene ring are required to induce a 1, 2-DAB-like pattern of murine CNS-PNS proximal axonopathy. [Supported by NIEHS grants ES10338 and ES 11384 and Oregon Workers' Benefit Fund]

**1550 RAPID-ONSET GAMMA-DIKETONE AXONOPATHY IN HIPPOCAMPAL NEURONS IN CULTURE.**

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Aromatic and aliphatic hydrocarbons with a 1, 2-diketo side chain react with neuronal proteins and cause proximal axon swellings filled with neurofilaments and distal retrograde degeneration, respectively, in the peripheral and central nervous system (CNS) of rodents. Critical protein targets of these gamma-diketones have yet to be identified. We used low-density primary cultures of rat hippocampal neurons to examine responses to neurotoxic 1, 2-diacetylbenzene (1, 2-DAB, gamma-diketone) vs. non-neurotoxic 1, 3-diacetylbenzene (1, 3-DAB, delta-diketone). These largely (85-90%) pyramidal nerve cells develop single elongate axons and dendritic arrays in which protein transport, delivery and fate can be characterized. Hippocampal neurons were incubated at 37 degrees C for up to 90 min. with concentrations of 1, 2-DAB (10, 250, 500, 1000, 2000 or 4000 micromolar), 1, 3-DAB (10, 250 micromolar) or DAB Vehicle (-1 micromolar acetone) in serum-free media. Cells were fixed at 30, 60 or 90 min. with 4% paraformaldehyde/4% sucrose in phosphate-buffered saline (pH 7.4) and examined by phase-contrast and/or Nomarski microscopy. 1, 2-DAB induced concentration- and time-dependent axonal beading reminiscent of wallerian-like degeneration. Distal, retrograde axonal beading was seen with 250 micromolar 1, 2-DAB but not with an equivalent concentration of 1, 3-DAB. High concentrations of 1, 2-DAB were acutely neurotoxic. No axonal beading was seen with 10 micromolar DAB or with Vehicle. These findings demonstrate a rapid alternative approach to study molecular mechanisms of gamma-diketone axonal degeneration. [Supported by NIEHS grants ES10338 and ES 11384 and Oregon Workers' Benefit Fund]

**1551 GAMMA-DIKETONE TOXICITY: A ROLE FOR STATHMIN IN NERVE AND TESTES DAMAGE?**

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Stathmin is a ubiquitous, brain-enriched phosphoprotein that binds to tubulin and inhibits microtubule (MT) assembly. Preliminary studies show stathmin modulation in brains of mice treated with single doses of neurotoxic 1, 2-DAB (gamma-diketone) relative to non-neurotoxic 1, 3-DAB (delta-diketone). Stathmin is of special interest in relation to 1, 2-DAB-induced axonopathy because aged stathmin(-/-) mice also develop axonal degeneration. Another neurotoxic gamma-diketone, 2, 5-hexanedione, promotes MT assembly and acts as a Sertoli cell toxicant. To examine the effect of DAB isomers on stathmin, slices of rat testes were incubated with 1-10 mM 1, 2-DAB, 1, 3-DAB, or Vehicle, for 30 min at 37 degrees C. Tissues were homogenized in sample buffer and 6-12 microgram protein aliquots subjected to SDS-PAGE. Electrophoresed proteins were transferred to PVDF membranes and probed with anti-stathmin antibody. Stathmin was visualized (a) colorimetrically by applying secondary antibody conjugated with horse radish peroxidase (HRP) and streptavidin-HRP amplifying reagent or (b) fluorometrically by secondary antibody tagged with IR Dye-800. 1, 2-DAB reduced the intensity of the native stathmin band in a concentration-dependent manner: ~15%, ~20%, ~35% and ~50% at 1, 2, 5, and 10 mM respectively, with corresponding increases in high-molecular-weight protein adducts. By contrast, 1-10 mM 1, 3-DAB under similar conditions had no effect on stathmin, and adducts were not observed. Rat brain slices incubated with 1-10 mM 1, 2-DAB, but not equivalent concentrations of 1, 3-DAB, showed a similar pattern of stathmin loss. Stathmin, a MT-destabilizing cytosolic protein, is of potential mechanistic relevance to the tissue damage elicited by gamma-diketones. [Supported by NIEHS grants ES10338 and ES 11384 and Oregon Workers' Benefit Fund]

**1552 EFFECTS OF SUBACUTE AND CHRONIC ACRYLAMIDE DOSING ON FAST-TRANSPORTED PROTEINS INTO RAT NEUROMUSCULAR JUNCTIONS.**

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Reductions in fast axonal transport and inhibition of the motor protein kinesin have been identified as molecular sites of action of the neurotoxicant acrylamide (Sickles, Neurotoxicology 23:223, 2002). We are determining whether these effects

reduce vital macromolecules in the distal axons sufficient to produce ataxia and paralysis. Previous work has identified specific reductions in ion channels to CNS and PNS nodes of Ranvier. Male, Sprague-Dawley rats (n=5/group;225g) were either given subacute exposure of acrylamide (50mg/kg/d ip for 10d) or were chronically exposed (2.8mM in drinking water; effective daily dose of 20-21mg/kg/d for 49d); saline-injected or regular drinking water served as controls. The extensor digitorum longus (EDL) and extensor carpi radialis (ECR), representing distal and more proximal NMJs, homogenates were used for semiquantitative dot blot analysis for synaptophysin, synaptotagmin and syntaxin content. Total muscle protein content was reduced 36 (EDL) - 39% (ECR) in chronic animals and 21 (EDL)-23% (ECR) by subacute treatment. These reductions did not parallel changes in NMJ proteins. Chronic treatment produced significant reductions in EDL synaptotagmin (-54%; relative units = 11.5±1.3 vs 5.25±2.4), and syntaxin (-30%; 23.3±4.2 vs. 16.3±2.6 but no significant change in synaptophysin. EDL NMJ proteins were always reduced more than ECR, the only significant change was a 32% reduction in synaptotagmin. Subacute ACR produced significant reductions in EDL synaptophysin (-62%), synaptotagmin (-57%) and syntaxin (-27%) while no significant changes were observed in the ECR muscle. These results clearly identify reductions in vital NMJ proteins following both subacute and chronic ACR exposure that are most pronounced in distal, in contrast to more proximal, NMJs. The spatial pattern is consistent with reduced fast transport as the cause of deficiencies. Reductions in these proteins are anticipated to compromise neurotransmission and produce symptoms, despite the absence of neurodegeneration. Supported by NIH # ES011223.

### 1553

#### REDUCTIONS IN NEUROMUSCULAR JUNCTION CHOLINESTERASE ACTIVITY BY SUBACUTE AND CHRONIC ACRYLAMIDE EXPOSURE.

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Acrylamide has been demonstrated to inhibit fast anterograde motor protein kinesin and produce reductions in fast transport (Sickles, *Neurotoxicology* 23:223, 2002). To determine whether the reductions are sufficient to produce deficiencies in vital nerve terminal proteins, we have used semiquantitative analysis of a silver cholinesterase stain in distal neuromuscular junctions of the extensor digitorum longus (EDL) and the more proximal forearm extensor carpi radialis (ECR). Male, Sprague-Dawley rats (225g; n=5/group) were exposed to either: subacute ACR@ 50mg/kg/d for 12 days, chronic ACR @ 2.8mM in drinking water for average of 20-21mg/kg/d for 49d, saline ip control or regular drinking water control. Fifty NMJ per animal were photographed and analyzed using Zeiss Image manual outline by an investigator blind to experimental group. Chronic ACR treatment produced a significant 33% reduction in EDL NMJ cholinesterase (54.6±6.6 vs 36.5±2.9 relative units) with a non-significant 20% reduction in the ECR (41.2±5.6 vs 33.0±2.6). Acute treatment to ACR for 12 days was sufficient to initiate forelimb splay with severe splay in the hindlimb. Following this prolonged treatment, both the EDL and ECR NMJ cholinesterase were reduced by 49% and 38%, respectively. In all cases, EDL was reduced more than ECR. We conclude that ACR, at two different dosing rates that produce neurological symptoms, also produces reductions in NMJ cholinesterase. Significant reductions in cholinesterase were not observed in symptom-free muscles. The absence of neurodegeneration in acute animals indicate that protein deficiencies such as cholinesterase or other vital fast transported proteins may be sufficient to produce symptoms. The greater effect on EDL cholinesterase in comparison to ECR indicates that direct actions are not the cause of the reductions in cholinesterase. This spatial pattern is consistent with ACR-induced reductions in axonal transport. The reductions in cholinesterase are less than observed with other NMJ proteins (see accompanying abstract). Supported by NIH ES012233

### 1554

#### HUMAN FLAVIN-CONTAINING MONOOXYGENASE: QUANTIFICATION IN HUMAN TISSUES.

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The flavin-containing monooxygenases (FMOs) comprise a family of FAD-, NADPH- and oxygen-dependent microsomal enzymes. The FMO constitute the second most important human monooxygenase system behind cytochrome P450 (CYP), attaining expression levels approaching 60% of CYP 3A in adult human liver. FMO catalyzes the oxygenation of many nitrogen-, sulfur-, phosphorous-, selenium-, and other nucleophilic heteroatom-containing chemicals and drugs. Five functional forms of FMO (i.e., FMOs 1-5) are present in humans, but it is FMO3 in adult human liver that has previously been identified as the major enzyme responsible for hepatic drug metabolism. Complete characterization of the FMO genes is needed to place this monooxygenase in context with other enzyme systems. The scope of this study was to quantify all the major FMO genes in the following

tissue: adult liver, kidney, lung, small intestine, and brain as well as fetal liver and fetal brain. The experimental procedure involved QPCR. The data gave a complete quantitative description of the copy number of each FMO gene in the tissues examined. The principal conclusions were surprising: FMO5 was prominent in adult and fetal liver and small intestine, FMO1 was prominent in adult kidney and FMO2 was prominent in adult lung. Evidence obtained showed that FMO1 was prominent in fetal brain while FMO2 was prominent in adult brain but the copy numbers were quite low and the result may be due to inadvertent sampling error. The data suggest that revision of our current ideas about the type and amount of FMO in the above-studied tissues is in order. For example, it is apparent that FMO5 is a major form of FMO present in important human tissue that does drug metabolism. However, our lack of understanding of FMO5 in so far as enzyme characterization and substrate specificity considerably confounds our understanding about the way FMO5 participates in human chemical or drug metabolism. Supported by NIH Grant DK59618.

### 1555

#### OXIDATION OF SELENOMETHIONINE BY FLAVIN-CONTAINING MONOOXYGENASES (FMOS).

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Selenium biotransformation into organoselenium forms by primary producers and subsequent accumulation by higher food chain organisms such as fish are critical for mechanistically understanding the adverse effects of selenium in aquatic organisms. Although previous laboratory and field studies have both demonstrated a good correlation between adverse effects and total selenium in fish tissue, direct confirmation of the most toxicologically important forms of organoselenium is lacking, with nearly all measurements being total selenium. To illustrate this, we hypothesize that following accumulation by fish organoselenides are oxidized to selenoxides by flavin-containing monooxygenase (FMO) upon the consumption of NADPH and oxygen. The generated selenoxide can be reduced to organoselenide by consuming the antioxidant glutathione. With the continuous redox cycling between organoselenide and selenoxide we hypothesize, glutathione will be depleted, eventually resulting in cellular toxicity. Therefore, in this study, an *in vitro* substrate dependent NADPH oxidation assay was performed to test the first step of the hypothesis, the oxidation of organoselenium by FMO. A series concentration of the organoselenium selenomethionine (0.05 — 2.0 mM) was incubated with 0.2 mM NADPH and 100 µg human microsomal FMO1 or FMO3 proteins in 50 mM KPO<sub>4</sub> buffer (pH 8.4) at 37 °C. The absorbance of NADPH (340 nm) of the reaction mixture was measured over 30 min at 5 min intervals, and a NADPH extinction coefficient of 6220 M<sup>-1</sup> cm<sup>-1</sup> was used in calculating catalytic constants. The results show that both FMO1 and FMO3 demonstrated catalytic activity for selenomethionine, with FMO3 having a lower Km than FMO1 (i.e., 172 µM and 617 µM, respectively), indicating that FMO3 is more reactive in catalyzing the oxygenation of selenomethionine to the oxide. Future study is required to directly detect the generation of selenomethionine oxide from selenomethionine oxidation by FMO.

### 1556

#### COMPARISON OF MAJOR PHASE I AND PHASE II METABOLISM REACTIONS IN CRYOPRESERVED CYNO MONKEY AND HUMAN HEPATOCYTES.

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Cryopreserved hepatocytes are a powerful tool for *in vitro* metabolic profiling and toxicity assessment of New Chemical Entities (NCEs). *In vitro* studies of NCEs' metabolism and toxicity in hepatocytes are predictive of *in vivo* reactions and are recommended precursors to *in vivo* studies. Cryopreserved cynomolgus monkey and cryopreserved human hepatocytes were thawed and assessed for Phase I and Phase II metabolic activity, viability over a four-hour incubation period, and lactate dehydrogenase (LDH) leakage when exposed to known toxins. Phase I reactions measured included 6b-hydroxylation of testosterone, O-deethylation of phenacetin, 1'-hydroxylation of bufuralol, and 2-hydroxylation of p-nitrophenol. Glucuronidation and sulfation of 7-hydroxycoumarin were assessed as representative Phase II reactions. Thawed cryopreserved hepatocytes from both species maintained all Phase I and Phase II metabolic activity tested over a four-hour incubation period and demonstrated only a small decline in viability. Both species were comparable in 6b-hydroxylation of testosterone and glucuronidation of 7-hydroxycoumarin, while on average cynomolgus monkey hepatocytes were more active than human in O-deethylation of phenacetin (5-fold) and 1'-hydroxylation of bufuralol (9-fold). Both species hepatocytes demonstrated comparable LDH leakage when exposed to increasing Tamoxifen concentrations. These data demonstrate conservation of Phase I and Phase II metabolic activity in cryopreserved cyno monkey and human hepatocytes and that both species' cryopreserved hepatocytes are useful for *in vitro* metabolism and toxicity studies of NCEs.

## COMPARISON OF CELLULAR MODELS FOR ASSESSING LIVER TOXICITY.

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Prediction of toxicity through *in vitro* modeling can reduce cost of drug development. *In vitro* models while used in exploratory toxicology, vary considerably in terms of their lineage, properties, and applicability. For example, most hepatocyte cell lines have limited expression of P450 enzymes, and as such, have limited capability to transform xenobiotics. By contrast, freshly isolated primary hepatocytes have abundant expression of most P450 enzymes but rapidly lose this function during culture. However, primary cells are diverse in their genetics and their metabolism is affected by underlying physiological conditions of donors. We investigated several hepatic *in vitro* models - human HepG2 and THLE-3 cells, rat H4IIE-C3 cells, as well as, freshly isolated primary rat (PRH) and human (PHH) hepatocytes for their comparative sensitivity to the model hepatotoxicant, acetaminophen (APAP). APAP remains the main cause of acute liver failure in the US due to over-dosage. Cellular toxicity, as determined by suppression of relative intracellular ATP level, was recognized in every model, implying specific toxicity to APAP. Resistance to APAP correlated negatively with reported expression of CYP2E1, the purported major form of P450 involved in APAP-metabolism with HepG2 cells demonstrating maximum resistance to the test agent. By contrast rat H4IIE-C3, PHH and PRH; cells expressing CYP2E1 were sensitive to toxicity with lower IC50 values. Interestingly in PHH, APAP toxicity positively correlated with degree of liver steatosis in donors. Our results indicate that cells can be used for predictive toxicology but are subject to the same sources of variability, such as genetics and underlying disease conditions, as intact subjects. These limitations should be taken into consideration when choosing an appropriate model system for predictive toxicology.

## PANCREATIC AND HEPATIC ETHANOL TOXICITY IN HEPATIC ALCOHOL DEHYDROGENASE DEFICIENT DEER MICE: MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION.

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Chronic alcohol abuse is one of the major causes of pancreatic and hepatic diseases and many chronic alcoholics die before reaching the clinical stage of the diseases. The mechanism and etiologic agent(s) involved in alcoholic pancreatitis and hepatitis are not known. Oxidative metabolism of ethanol catalyzed by alcohol dehydrogenase (ADH) is generally impaired during chronic alcohol abuse and inhibition of hepatic ADH is shown to facilitate nonoxidative metabolism of ethanol to fatty acid ethyl esters (FAEEs). FAEEs are known to cause pancreatic and hepatic toxicity *in vitro* and pancreatitis-like injury in rat model. In order to understand the mechanism and etiology of alcoholic pancreatitis and hepatitis, hepatic ADH deficient (ADH-) deer mice and ADH normal (ADH+) deer mice were fed ethanol (4%, wt/v) via Lieber-DeCarli liquid diet. Control animals were pair-fed with isocaloric liquid diet. Following 60 days of ethanol treatment, 11 out of 18 ADH-deer mice died as compared to 1 out of 16 ADH+ deer mice. All surviving animals were sacrificed, whole blood was collected to obtain plasma and organs were excised for histopathology. Pancreatic damage was evident by vacuolization, edema, necrosis and loss of acinar cells in some areas. Extensive vacuolization, displacement or absence of nucleus was seen in some hepatocytes in ethanol-fed ADH- deer mice. No significant histopathological changes were found in other organs of both ethanol-fed deer mice strains. There was no death in control groups of both strains, and minimal change in histology. Correlation of the histopathological changes seen in the pancreas and liver will be established with biochemical changes in the plasma with an ultimate aim to develop biomarkers of alcoholic pancreatitis and hepatitis.

## VARIABILITY IN METABOLISM OF CHLORAL HYDRATE IN HUMAN HEPATOCYTES.

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Chloral hydrate is a sedative drug that is also a CYP 450-derived metabolite of the suspect human carcinogen, trichloroethylene (TCE). Chloral hydrate is metabolized in the liver to the rodent hepatocarcinogen, trichloroacetate (TCA), by aldehyde dehydrogenase (ALDH), and to the non-carcinogenic metabolite, trichloroethanol (TCE-OH), by alcohol dehydrogenase (ADH). Both ALDH and

ADH are polymorphic in humans, which predicts that human disposition of chloral hydrate into the carcinogenic vs. non-carcinogenic pathways will show inter-individual variation. This raises the possibility that sub-populations of humans will produce greater amounts of TCA relative to TCE-OH and hence have a greater risk of developing liver tumors after exposure to TCE. To assess the variability of chloral hydrate metabolism in humans, chloral hydrate (0.5-2.0 mM) was added to human hepatocyte suspensions obtained from commercial sources. Incubations were carried out for 10 min at 37°C, and the reactions were stopped by the addition of an esterizer solution. The formation of TCA and TCE-OH was measured using head-space gas chromatography with electron-capture detection. Initial velocities of chloral hydrate metabolism (pmol metabolite/10<sup>6</sup> cells/min) were used to calculate the Km and Vmax values for each metabolite. We report significant variability in chloral hydrate metabolism in 10 human hepatocyte samples. TCE-OH was the major metabolite (Km of 7.64 mM ± 2.97 SEM) among donors; variability in TCA formation (Km of 7.72 mM ± 4.99) was also observed. These results indicate that humans may not be uniform in their capacity to metabolize chloral hydrate to TCA and TCE-OH. Supported by DOE cooperative agreement DE-FC09-02CH11109.

## TOXICOKINETIC ASPECTS OF HEPATOTOXIC INTERACTIONS BETWEEN BENZENE AND TCE.

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In an ongoing 3-year project, mechanistic models are being established for both, toxicokinetic and toxicodynamic interactions within mixtures. One of the focus areas is the interaction of benzene and trichloroethylene (TCE) in the liver of rats treated repeatedly with both compounds. *In vivo* microarray data of mixture experiments have shown, that peroxisome proliferation caused by TCE alone was increased by concomitant benzene dosing. To establish the nature of the interaction, *in vitro* experiments were performed in rat hepatocyte sandwich cultures to study the impact of each of the two compounds on liver enzyme activities of CYP2E1 and GST, both of which are known to be relevant for the metabolism of benzene and trichloroethylene. A computational PBTK (physiologically based toxicokinetic) model was used to connect *in-vitro* data with *in vivo* experiments. It was found that repeated benzene dosing increased the activity of CYP2E1 in the hepatocyte cultures. Using a PBPK model for TCE, it could be shown that at higher oral doses of TCE such an increase of activity leads to marked increases of trichloroacetic acid levels. Using this combined approach, the nature of the experimentally observed interaction between TCE and benzene could be explained as a toxicokinetic interaction.

## IN VITRO AND IN VIVO KINETICS OF MIDAZOLAM IN COMMERCIALLY RAISED GAMEBIRDS.

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Species differences in drug metabolism can affect therapeutic efficacy and residues in food animals. To examine species differences with regard to cytochrome P450 3A capabilities, *in vitro* and *in vivo* studies were conducted with a marker substrate (midazolam) in commercially raised pheasant, turkey, chicken and bobwhite quail. Microsomal incubations with midazolam (1.25-20 uM) were analyzed using HPLC with UV detection. *In vitro* kinetic parameters for oxidation of midazolam to both the 1-hydroxymidazolam (1-OH) and 4-hydroxymidazolam (4-OH) metabolites were estimated. The apparent Vmax for the major metabolite, 1-OH, in pheasant, turkey, chicken and bobwhite quail was 419.8+21.7, 209.6+11.6, 167.2+11.3 and 120.4+7.0 pmoles product/mg x min, respectively. The apparent Michaelis constant, Km, was 10.5+1.5, 3.0+0.7, 1.8+0.6 and 1.8+0.6 uM in pheasant, turkey, chicken and bobwhite quail, respectively. The apparent Vmax values calculated for the 4-OH metabolite showed a similar pattern of activity between the species examined, but with more variability and much less affinity. Values were 187+63.4, 84.6+17.4, 26.1+7.0 and 26.2+0.7 pmole product/mg x minute, for pheasant, turkey, chicken and bobwhite quail, respectively. The calculated Km values were 75.2+37.9, 84.6+17.4, 17.8+11.4, and 26.6+1.6 uM. *In vitro* inhibition studies were also conducted with a prototypical inhibitor of CYP3A, ketoconazole, at 3.125-100 uM and at a substrate concentration of 25 uM. Pheasant were most sensitive to inhibition, quail were least sensitive and chicken and turkey were intermediate. *In vivo* pharmacokinetic studies were conducted in all species at a dose of 5 mg/kg. Results indicate that pheasant and turkey are similar in their kinetic profiles of midazolam even though turkeys appear to achieve much deeper levels of sedation. These studies suggest that extrapolating dose regimens from one species to another, even within the same family (ie. Galliformes), should be done conservatively at best.

## HEPATIC MICROSOMAL METABOLISM OF LITHOCHOLIC ACID.

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The purpose of the present study was to investigate the *in vitro* metabolism of lithocholic acid (3 $\alpha$ -hydroxy-5 $\beta$ -cholanoic acid, LCA). The metabolites formed were detected using liquid chromatography-electrospray mass spectrometry (LC-MS). LCA was incubated with a reaction mixture containing rat hepatic microsomes. A liquid-liquid double extraction method with d4-cholic acid as an internal standard was utilized. Metabolites were separated using a C18 (octadecyl) reversed-phase column and detected in single ion recording (SIR) mode. Assay conditions were validated and optimized for effective analysis. The limit of detection was 50 ng/ml. The recovery of the authentic metabolite standards averaged 80-100%. The identities of five metabolites of LCA were confirmed using authentic standards. The major metabolites were found to be murocholic acid (MUCA) and isolithocholic acid (ILCA). Minor metabolites were identified as  $\beta$ -muricholic acid ( $\beta$ -MCA), 6-keto lithocholic acid (6KLCA) and ursodeoxycholic acid (UDCA). In addition, six other unidentified metabolites were also detected. Metabolite (MUCA, ILCA, UDCA and 6KLCA) formation followed typical Michaelis-Menten kinetics and kinetic constants ( $K_m$  and  $V_{max}$ ) for these metabolites were calculated from triplicate measurements. The possibility that  $\beta$ -MCA could be metabolized to secondary metabolites is under investigation. The identities of specific CYP enzymes involved in the formation of LCA metabolites and the effect of treatment of cytochrome P450 enzyme (CYP) inducers such as dexamethasone, 3-methylcholanthrene, and phenobarbital on the metabolism of LCA in rats are currently underway.

## IN VITRO METABOLISM OF 4-VINYLPHENOL AND STYRENE IN MOUSE, RAT AND HUMAN MICROSOMES.

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4-Vinylphenol (4VP) has been identified as a minor urinary metabolite of styrene in rats and human volunteers. This compound has been shown to be both a hepatotoxin and pneumotoxin in mice. 4VP is thought to be a transient metabolite of styrene, since it is not seen in *in vitro* incubations of styrene with microsomes from lung or liver of the rat and mouse. This lack of measurable 4VP is attributed to the rapid metabolism of this compound to other, unidentified products. The current study was designed to characterize reactive metabolites of styrene, arising via the 4VP pathway, in the lung tissue of the mouse, rat and human donors. Incubations of radiolabeled 4VP with mouse lung tissue microsomes afforded two major metabolites. The mass spectrum for both products was consistent with incorporation of one oxygen atom. Coincubation with glutathione (GSH) was performed to trap reactive metabolites of 4VP. LC/MS/MS analysis afforded identification of these metabolites as GSH conjugates of ring-hydroxylated- and side-chain epoxide products of 4VP. MRM-LC/MS/MS analysis of incubates of styrene, GSH and lung tissue microsomes afforded identification of these two GSH-containing metabolites in tissue from mouse, rat or human. Relative formation rates for both 4VP-GSH conjugates were mouse > rat > human. These results should provide useful data in characterizing the relative toxicity of styrene in these three species. (This work was funded by the Styrenics Steering Committee of the European Chemical Industry Council- CEFIC).

## COVALENT MODIFICATION OF AMINO ACID NUCLEOPHILES BY STYRENE OXIDE.

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Styrene is widely used as one of the most important industrial materials for production of synthetic rubbers, plastic, insulation, fiberglass, automobile parts. Its major metabolic route in rats, mice and human involved conversion of styrene to styrene oxide by cytochrome P450. This fairly reactive metabolite has been reported to form covalent bonding to cellular macromolecules, such as proteins. Among 21 amino acids, Cys, Lys, Arg, His are the amino acids possibly responsible for nucleophilic protein modification. The purpose of this study is to compare the susceptibility of these nucleophilic amino acid residues to electrophilic styrene oxide. Model peptides, including PFECG, PFFHCG, PFVCG, PFKCG, PFRCG, were incubated with styrene oxide and analyzed by liquid chromatography/electrospray tandem mass spectrometry. All Cys residues of the peptides were found to be modified by styrene oxide. Little modification was observed at Lys, Arg, His and Glu of the corresponding peptides. Kinetic studies showed that the neighboring amino acid residues have little effect on the reactivity of cysteine. Only Lys slightly increased the reactivity of cysteine towards styrene oxide. In conclusion, Cys is the

most reactive amino acid residue responsible for peptide modification by electrophilic agents. Neighboring amino acid residues have little influence on the reactivity of cysteine.

## NITROREDUCTION OF TATTOO PIGMENT YELLOW 74 BY XANTHINE OXIDASE: FORMATION OF A DNA ADDUCT AND MUTAGENICITY OF THE NITROSO REDUCTION PRODUCT.

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Pigment Yellow 74 (PY74), a monoazo pigment used in paints, dyes and printing inks, is also found in some tattoo inks. We have previously shown that PY74 photodecomposes in simulated sunlight to various products. We have also shown that PY74 is metabolized primarily by cytochromes P450 (CYP) 1A1 and 1A2 to a 4'-phenol metabolite, which is further metabolized by CYP 1A1 or 1A2 through *O*-demethylation to a bis-phenol. PY74 is synthesized through reaction of the diazonium salt of 2-amino-5-nitroanisole with *o*-acetoacetaniside, and as such, has a nitro group that should be available for nitroreductive metabolism. The incubation of PY74 with a nitroreductase, xanthine oxidase, under hypoxic conditions leads to the reduction of the nitro group to an amine (NH<sub>2</sub>-PY74). Since the formation of NH<sub>2</sub>-PY74 would proceed through initial reduction of the nitro to a nitroso group, we synthesized the nitroso derivative (NO-PY74) to investigate its reactivity. The reduction of NO-PY74 with ascorbate in the presence of DNA resulted in the formation of a DNA adduct that was detected using <sup>32</sup>P-postlabeling. NO-PY74 mutagenicity was determined using the L5178Y *Tk*<sup>+/+</sup> mouse lymphoma assay; a clear dose response in cytotoxicity and mutagenicity was detected with up to 14  $\mu$ M NO-PY74. <sup>32</sup>P-Postlabeling analysis of the DNA isolated from L5178Y *Tk*<sup>+/+</sup> cells treated with 14  $\mu$ M NO-PY74 confirmed the presence of the same DNA adduct. These results indicate that nitroreduction of the tattoo pigment PY74 may be a bioactivation pathway. Further studies are focused on identification of the DNA adduct and determining if nitroreduction occurs *in vivo*.

## REACTIONS OF NITRIC OXIDE-DERIVED OXIDANTS WITH 4-ACETAMIDOPHENOL: FORMATION OF ELECTROPHILIC INTERMEDIATES.

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Nitric oxide ( $\cdot$ NO) is an important bio-molecule eliciting hormonal and toxic properties. The toxic effects of  $\cdot$ NO manifest as a result of the formation of peroxynitrite (PN; ONOOH  $\leftrightarrow$  ONOO $^-$  + H $^+$ ; pKa $\approx$ 6.2) and its free radical products  $\cdot$ NO $_2$ , CO $_3$  $^{2-}$  and  $\cdot$ OH, the latter being formed in yields up to 40 mol% via H $^+$ -assisted or CO $_2$ -catalyzed isomerization to NO $_3$  $^-$ . The information on the involvement of  $\cdot$ NO-derived oxidants (NOx) in xenobiotic metabolism resulting in electrophilic intermediates is limiting. It was reasoned that NOx can cause metabolic activation, especially in xenobiotics that have nitrogen or sulfur in a low oxidation state. In an effort to establish this concept, we conducted studies of oxidative transformation of 4-acetamidophenol (4-AP) by PN and PN/CO $_2$  adducts at pH 7.2. The 4-AP, a popular over-the-counter drug (which causes hepatic failure in accidental overdosing) was chosen for its toxicity. Based on the initial concentration of PN in the reaction, the yields of oxidation of 4-AP were 34 $\pm$ 1.0 mol%. One of the major products of 4-AP oxidation was 3-nitro-4-acetamidophenol, identified by co-chromatography with standard and by fragmentation pattern in GC/MS and FAB/MS analysis. It accounted for 30 $\pm$ 1.8 mol% of the oxidation of 4-AP. Another product identified was N-acetyl-1, 4-benzoquinoneimine (NBQI). This decays in phosphate buffer at pH 7.2, with a half-life of 2 min, and can be trapped as stable adducts of N-acetyl-L-cysteine and glutathione. In these reactions, the NBQI formed can arylate the thiol/thiolate functionality and inhibit glyceraldehyde-3-phosphate dehydrogenase, a key enzyme of glycolysis. These findings suggest that xenobiotic activation by nonenzymatic, putative oxidants from  $\cdot$ NO metabolism may contribute to long-term toxicity due to alkylation of proteins and, possibly, nucleic acids. (Funding from NIEHS ES10018 and Louisiana Biomedical Research Network is acknowledged).

## THE ROLES OF LIVER AND TARGET-TISSUE P450-MEDIATED DMBA METABOLISM IN DMBA-INDUCED DNA ADDUCT FORMATION IN THE MAMMARY GLAND.

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 7, 12-Dimethylbenz[a]anthracene (DMBA) is a potent mammary gland carcinogen in rodents. The aim of this study was to determine the roles of liver and target-tissue cytochrome P450-mediated DMBA metabolism in DMBA-induced DNA

adduct formation in the mammary gland. The role of microsomal P450 in DMBA-induced DNA adduct formation was confirmed using a mouse model (designated the Cpr-low mouse) that has a globally suppressed expression of the NADPH-cytochrome P450 reductase (Cpr) gene (Wu et al., *J. Pharmacology Expt. Ther.* In press, 2005). The role of hepatic P450-mediated metabolism in mammary gland DMBA-induced DNA adduct formation was determined using a mouse model (designated the liver-Cpr-null mouse) that has liver-specific deletion of the Cpr gene (Gu et al., *J. Biol. Chem.*, 278, 25895-25901, 2003). Mice were treated orally with DMBA (50 mg/kg/day for 3 days). The level of DNA adducts in the mammary gland, determined by  $^{32}\text{P}$ -postlabeling, and DMBA blood concentrations following DMBA treatment were monitored. We found that the level of total DNA adducts in the mammary gland was about 72% and 80% lower in the liver-Cpr-null mice and the Cpr-low mice, respectively, than in wild-type mice. In the liver-Cpr-null mice, DMBA was eliminated more slowly than in the wild-type mice, with a three-fold difference in area under the curve (AUC) between the two strains. Thus, in spite of a greater bioavailability of DMBA, the level of DNA adducts was significantly reduced in the mammary gland of the mutant mice than in the wild-type mice. These results suggest that, following systemic exposure to DMBA, hepatic P450-mediated generation of either intermediate metabolites or the ultimate carcinogenic metabolite plays a critical role in the formation of DNA adducts in the breast tissue. Additional studies designed to directly examine the role of local metabolic activation in the formation of DNA adducts in the mammary gland are underway. (Supported in part by grant DAMD17-02-1-0404 from USAMRAA)

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### VINCLOZOLIN *IN VITRO* METABOLISM BY RAT LIVER MICROSONES.

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Vinclozolin (V) is a fungicide used in agricultural settings. V administered to rats is hydrolyzed to 2-[(3, 5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenoic acid (M1) and 3', 5'-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2). V, M1 and M2 have antiandrogenic properties by interacting with the androgen receptor. Data on V, M1 and M2 biotransformation is limited. Our objective was to study the *in vitro* metabolism of V by rat liver microsomes. V (12.5-200  $\mu\text{M}$ ) was incubated in 0.1 M phosphate buffer pH 7.4, 5 mM MgCl<sub>2</sub>, non-treated adult male Long-Evans rat liver microsomes (1-2 mg) and 1 mM NADPH for 30 min at 37°C. V metabolites from incubation media were extracted in acetonitrile and analyzed by HPLC/DAD/MS. Three metabolites were detected from rat liver metabolism. On the basis of their  $t_{\text{ret}}$ , UV/Vis spectrum and negative ESI mass spectra, two metabolites were identified as 3', 5'-dichloro-2, 3, 4-trihydroxy-2-methylbutylanilide (M4) ( $[\text{M}-\text{H}]^-$  at  $m/z$  293) and *N*-(2, 3, 4-trihydroxy-2-methyl-1-oxo)-3, 5-dichlorophenyl-1-carbamic acid (M5) ( $[\text{M}-\text{R}]^-$  at  $m/z$  246). Based on UV/Vis spectrum alone, a third unidentified metabolite (M6) co-eluted with M4. The  $K_m$  for co-eluted M4/M6 was 53.6  $\mu\text{M}$  and the  $V_{\text{max}}$  was 0.810 nmoles/min/mg protein. The  $K_m$  for M5 was 142.3  $\mu\text{M}$  and the  $V_{\text{max}}$  was 0.700 nmoles/min/mg protein. Extracting at a lower pH altered the  $K_m$  and  $V_{\text{max}}$  values for the 3 metabolites indicating the recovery of V metabolites is pH dependent. These results indicate that V is efficiently metabolized by rats liver microsomes. Determination of the metabolites of V will provide further insight into the relationship between toxicity and tissue dose of V and its metabolites. (Funded in part by NRC CR 828790. This abstract does not represent USEPA policy).

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### HYDROLYtic METABOLISM OF PYRETHROIDS BY HUMAN CARBOXYLESTERASES AND RODENT AND HUMAN LIVER MICROSONES.

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The mammalian carboxylesterases (CESs) are a multigene family of enzymes that have an important role in detoxifying pesticides, such as pyrethroids. The identities of the CES isozymes responsible for detoxification are poorly characterized. The objective of this study was two-fold: first, to determine the substrate specificity and kinetic properties of the major human liver CES enzymes (hCES1 and hCES2) using *cis*- and *trans*-permethrin, and second, to compare rat, mouse, and human liver microsomes for their ability to cleave several pyrethroid compounds, including permethrin. Recombinant CES enzymes and rodent and human liver microsomes were utilized so that rates of pyrethroid hydrolysis could be compared, as determined by HPLC analysis of metabolites. The results demonstrated that *trans*-permethrin was hydrolyzed markedly faster by hCES1 than hCES2 ( $k_{\text{cat}} = 13.9 \text{ min}^{-1}$  and  $1.2 \text{ min}^{-1}$ , respectively). However, the  $K_m$  for hCES2 was 56-fold lower than

for hCES1 ( $K_m = 103.5 \mu\text{M}$  and  $1.8 \mu\text{M}$  for hCES1 and hCES2, respectively), thus the apparently high affinity of *trans*-permethrin for hCES2 suggests that this isozyme may contribute to the detoxification of permethrin at low substrate concentrations ( $<10 \mu\text{M}$ ). In contrast, *cis*-permethrin was hydrolyzed much slower than *trans*, both by hCES1 and hCES2. The hydrolytic activity of liver microsomes toward *trans*-permethrin followed the species order mouse>rat>human. The  $V_{\text{max}}$  values of six individual human liver microsomes ranged from 0.36-1.50 nmol/min/mg protein, indicating interindividual differences in *trans*-permethrin hydrolysis proficiency. There were no marked differences in  $K_m$  values (14.7-21.0  $\mu\text{M}$ ) between rodent and human liver microsomes. Taken together, the kinetic parameters for the human CESs are consistent with *trans*-permethrin being more efficiently hydrolyzed than *cis*-permethrin, and both liver enzymes likely have a role during *in vivo* permethrin metabolism. (Supported by NIH P20RR017661).

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### METABOLISM OF ARSENICALS MAY INFLUENCE THEIR PROLIFERATIVE EFFECTS ON HUMAN CACO-2 CELLS.

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Roxarsone (4-hydroxy-3-nitrobenzenearsonate) is used as a poultry feed additive for its antimicrobial and growth promoting effects. More than 90% of the additive is excreted unchanged. Recently, both USGS and USEPA have expressed concern due to the more than 2 million pounds/year, excreted into waterways. The reduction product, 3-amino-4-hydroxy-benzenearsonate (AHBA) has been identified as a metabolite from animal, microbial, and geologic reactions found in groundwater and sediments and its proposed oxidation product, an azobenzene has been described. The potential toxic effects of these organoarsenicals as water contaminants should be evaluated for impact on humans. The human intestinal line, Caco-2, was exposed to Roxarsone, AHBA, and its acetylated product 3-N-acetylamo-4-hydroxybenzenearsonate (Acetarsone). At micromolar levels, proliferation, determined by [<sup>3</sup>H]- thymidine incorporation into DNA, was increased significantly with AHBA and Acetarsone, as compared to controls. Arsenate (iAs V) and arsenite (iAs III) were tested, but gave variable results. These observations on the organoarsenicals are supported by flow cytometric data, whereby cell cycle studies revealed an accumulation of cells in the S phase from both AHBA and Acetarsone. *In vitro* studies show that the N-acetylation of AHBA to Acetarsone is catalyzed by human N-Acetyltransferase (NAT) and that human CYP3A4 catalyzes an apparent oxidation of AHBA. The mRNAs for these key enzymes, NAT and CYP3A4, are expressed in Caco-2 cells, as shown by real-time PCR analysis. The aromatic amine AHBA then, has the potential both for acetylation and oxidation. The dynamics of the acetylation vs P450 pathways are established as important in considering potential toxicity of such compounds. The variable Caco-2 cell proliferation in response to different metabolites therefore may be dependent upon metabolic activation by competing acetylation and oxidation reactions. (Support:RIMI MD00215-01;RCMI RR03032;MBRS/GM-028248;MIE/NASA NCC8-227).

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### A NOVEL HALOENOL LACTONE DERIVATIVE POTENTIATES CYTOTOXICITY INDUCED BY CISPLATIN RETARDING THE GLUTATHIONE-MEDIATED DETOXIFICATION OF CISPLATIN IN HUMAN RENAL TUMOR CELL LINES.

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Drug resistance remains one of the primary causes of suboptimal outcomes in cancer chemotherapy. Over-expression of glutathione S-transferases (especially GST-Pi) has been found to play a significant role in multiple drug resistance in cancer chemotherapy. Our laboratory has designed and synthesized a haloenol lactone (HEL) derivative as a mechanism-based inactivator of GST-Pi. We found that pretreatment of 10 uM HEL potentiate the cytotoxicity induced by cisplatin in both human renal tumor cell line (UOK-130) and acquired cisplatin-resistant UOK130 cell line. The mechanism studies of HEL demonstrate that cytotoxic potentiation by HEL results from retarding the GSH-mediated detoxification of cisplatin. This compound depletes intracellular GSH as well as inactivates cytosolic GST thus reduces GSH-cisplatin conjugation. This compound is also shown to be an inhibitor of multidrug resistance-associated protein 1 (MRP-1) mediated drug resistance which is observed increased in acquired cisplatin-resistant UOK130 cell line. As a result, the metabolic inactivation of cisplatin was reduced and more DNA-DNA crosslinking was found in nucleus of UOK130 cells. In summary, a novel haloenol lactone was identified as a lead compound, capable of potentiating cytotoxicity by cisplatin at low concentration, through a mechanism retarding the total GSH-mediated detoxification of cisplatin.

BIOACTIVATION OF 1-CHLORO-2-HYDROXY-3-BUTENE TO 1-CHLORO-3-BUTEN-2-ONE, A NOVEL CROSS-LINKING AGENT.

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1, 3-Butadiene (BD) has been classified as "known to be a human carcinogen" primarily because epidemiological studies showed increased incidences of lymphohematopoietic cancers among workers occupationally exposed to BD. Previously we identified 1-chloro-2-hydroxy-3-butene (CHB) as a primary metabolite of BD by human myeloperoxidase, an enzyme located mostly in the bone marrow. In this study, we examined the *in vitro* metabolism of CHB to 1-chloro-3-buten-2-one (CBO) by purified liver alcohol dehydrogenase (ADH) and by rat bone marrow post-mitochondrial fractions (S9) and liver cytosol. Because CBO is expected to be highly reactive, we included glutathione (GSH) in the incubations as a trapping agent. In incubations with ADH and NAD, we detected three product peaks by HPLC that were both time- and NAD-dependent. These peaks co-eluted with peaks that were formed in the chemical reaction of CBO and GSH. The major peak was identified by mass spectrometry (MS) as the di-GSH conjugate of CBO. These results and the instability of the other two peaks suggest the minor peaks were the mono-GSH conjugates. In incubations with rat bone marrow S9 fractions or liver cytosol, the di-GSH conjugate peak was detected and its formation was substrate- and protein-dependent. Since CBO readily formed the di-GSH conjugate, we examined its ability to form hemoglobin crosslinks in freshly isolated mouse erythrocytes. Red blood cells were incubated with 5 or 10 mM CBO for 1 h, the globin isolated, and run on SDS-PAGE. Globin dimers and trimers were detected using silver staining with both CBO concentrations. The presence of crosslinks was also confirmed by MS. These results show that CHB is a substrate for ADH. CBO formed in bone marrow or liver could readily react with GSH or form crosslinks with macromolecules. The high reactivity of CBO suggests a role for this compound in the toxicity and carcinogenicity of BD. (Supported by ES06841)

4'-FLUORO SUBSTITUTION OF DESMETHYLATED ARZOXIFENE COULD DECREASE TOXICITY WHILE MAINTAINING ANTIESTROGENIC ACTIVITY.

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The SERM, aroxifene is currently in clinical trials for the treatment of breast cancer. The active form of aroxifene *in vivo*, desmethylated aroxifene (DMA), can be metabolized to electrophilic/redox-active quinoids by rat and human liver microsomes which might contribute to the cytotoxicity and/or genotoxicity of DMA through depletion of GSH, alkylation of proteins, and induction of DNA single strand breaks. In the current study, a new fluorinated derivative unable to form a di-quinone methide, 4'-F-DMA, was synthesized. 4'-F-DMA showed similar ER binding affinity as compared to DMA. The antiestrogenic activity as measured by inhibition of estradiol-mediated induction of alkaline phosphatase activity in Ishikawa cells showed 10-fold lower activity for 4'-F-DMA compared to DMA; however, the antiestrogenic activity was comparable to raloxifene. Using MCF-7 cells transfected with the ERE-luciferase reporter system, antiestrogenic activity for 4'-F-DMA, DMA and raloxifene parallels that observed with alkaline phosphatase assay in Ishikawa cells. In microsomal incubations of 4'-F-DMA in the presence of GSH, no GSH adducts were detected. 4'-F-DMA and DMA were incubated with cryopreserved rat hepatocytes, only one glucuronide adduct was detected using LC-MS-MS for 4'-FDMA. In contrast, two glucuronide adducts and two GSH adducts were detected using LC-MS-MS for DMA. 4'-F-DMA showed less cytotoxicity compared with DMA, and induced less DNA single strand breaks than DMA in cryopreserved rat hepatocytes. Finally, the effect of 4'-F-DMA and DMA on GSH content in rat hepatocytes was examined. DMA significantly decreased the GSH levels within half an hour. In contrast, 4'-F-DMA did not cause any change in GSH levels in rat hepatocytes. These data suggest that 4'-F-DMA might be a promising SERM with similar activity to DMA and raloxifene and less toxicity. Supported by NIH grant # CA 798700

REDUCTION OF 1-FURAN-2-YL-3-PYRIDIN-2-YL PROPENONE, AN ANTI-INFLAMMATORY AGENT, BY CARBONYL REDUCTASE IN RAT LIVER MICROSONES.

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Although oxidative biotransformation of xenobiotics by cytochrome P450s is the primary metabolic process for a number of xenobiotics, the reduction is also a significant step in the phase 1 biotransformation for a variety of aromatic, alicyclic and

aliphatic compounds bearing a carbonyl group. 1-Furan-2-yl-3-pyridin-2-ylpropenone (FPP-3) has recently been synthesized by our group. From serial studies, FPP-3 has been characterized to have an anti-inflammatory activity through the inhibition of the production of nitric oxidation and tumor necrosis factor- $\alpha$ . In the present studies, the metabolic fate and a possible involvement of carbonyl reductase in the metabolism of FPP-3 were partially investigated in rat liver microsomes. When FPP-3 was incubated with rat liver microsomes in the presence of NADPH, 2 major peaks were detected on an LC/MS/MS. Two peaks (i.e., M1 and M2) were believed to be the reduction on the propenone: M1 (1-furan-2-yl-3-pyridin-2-ylpropen-1-one) was a initial metabolite and M2 (1-furan-2-yl-3-pyridin-2-ylpropan-1-ol) was a secondary alcohol believed to be formed from M1 by carbonyl reductase. This biotransformation was a NADPH-dependent but a cytochrome P450-independent reaction, not only because the production of metabolites was not decreased by SKF-525A, a well-known cytochrome P450 inhibitor, but also because this reaction was inhibited by the *D, L*-glyceraldehyde, a substrate of carbonyl reductase. (Supported by a grant for the Institute for Drug Research, Yeungnam University, from the Korea Research Foundation).

PHASE 1 AND PHASE 2 METABOLISMS OF RUTAECARPINE IN MICE AND RATS.

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Recently, we have reported the *in vitro* metabolism of rutaecarpine, a new cytochrome P450-2 inhibitor, in rat liver microsomes. In the present studies, phase 1 and phase 2 metabolisms of rutaecarpine were compared in mice and rats using liquid chromatography-electrospray ionization tandem mass spectrometry. When urine, feces, sera and bile were analyzed following rutaecarpine administration to rats, 4 glucuronide and 4 sulfate conjugates were identified. In mice, the same 8 conjugates of rutaecarpine were detected. Sulfate conjugated 4 metabolites were either on the aromatic ring of the indole moiety (S1, S2 and S3) or on the aromatic ring of the quinazolinone moiety (S4). Other three metabolites (U1, U2 and U3) were believed to be glucuronidated on the aromatic ring of the indole moiety and last one (U4) was on the phenyl group of the quinazolinone moiety. The *in vitro* incubation of rutaecarpine in murine hepatocytes showed similar results. Our present results indicated that the metabolism of rutaecarpine in mice and rats is very similar. (Supported by a grant from Korea Research Foundation for the Institute for Drug Research, Yeungnam University)

METABOLIC SHIFT FOLLOWING MULTIPLE DOSES OF [14C]-1, 3-DINITROBENZENE TO RATS.

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While ample data is available regarding the CNS toxicity of 1, 3-dinitrobenzene (1, 3-DNB) following multiple-dosing regimens, studies regarding the time-course of brain uptake into, as well as metabolism and disposition within the CNS have not been forthcoming. We previously proposed that metabolism is an essential determinant of the CNS toxicity observed following 1, 3-DNB administration and that neuronal nitric oxide synthase produces peroxynitrite when exposed to DNB isomers. In the present study, rats received either a single dose (10 mg/kg; ip in corn oil containing 20 microCi radiolabel) or were dosed dose q 12h X 3 doses. Forebrain, brainstem and cerebellum were collected at various times post-dose and analyzed for metabolites by HPLC. 1, 3-DNB reached peak brain levels one hour after ip injection and elimination data revealed apparent half-lives in brain of 3h in pons/medulla and cerebellum and 4h in brainstem. Brainstem and cerebellum contained double radiolabel of forebrain (2 vs 0.5 pmol/mg). 1, 3-DNB and 3-NA were the major compounds present in forebrain, cerebellum and brainstem following either single or multiple dosing regimens. Compounds present in brainstem 90 min after dosing were (expressed as % of the radiolabel present in the brain region): 1-nitroso-3-nitrobenzene-NNB (10%), 3-nitroacetanilide (13%), 3-nitroaniline-3-NA (21%) and 1, 3-DNB (58%). Following 3 doses, NNB (4%), 3-NA (58%), 1, 3-DNB (35%) and an unidentified fourth peak were detected in brainstem, however by 90 min 3-NA was no longer detectable. Cerebellum contained 2 major and 2 minor peaks at the 45 min time-point while only 3-NA (31%) and 1, 3-DNB (69%) were detected at 90 min. These data confirm that 1, 3-DNB is reductively metabolized *in vivo* and enters the CNS. Following either 1 or 3 doses the major metabolites were 1, 3-DNB and 3-NA. Additionally, brainstem and cerebellum are exposed to twice the 1, 3-DNB levels compared to forebrain. Furthermore, NNB was present cerebellum and brainstem in both single and multiple dose groups. Furthermore, an apparent shift in metabolism occurs following multiple doses of 1, 3-DNB.

METABOLISM OF METHACRYLONITRILE TO CYANIDE: INHIBITION BY CAFFEINE AND POTENTIATION BY ALCOHOL.

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We have reported earlier that caffeine inhibited and alcohol enhanced the toxicity of methacrylonitrile (MeAN) in male Sprague-Dawley rats. We have now studied the relationship between the toxicity of MeAN and its metabolism to cyanide using normal rats and those pre-treated with caffeine or alcohol or both. One group of experimental rats received an oral dose of 6 % MeAN solution in corn oil (equivalent to 0.5 LD50). Other three groups of rats were pre-treated with alcohol (2 ml of 50 % solution in water), caffeine (1 ml of 2 % solution in water) or both alcohol and caffeine twelve hours before receiving MeAN dose. The rats were closely observed for known signs of toxicity of nitriles. Three, six and twenty four hours after the treatments the rats were sacrificed under ether anesthesia for collection of blood, liver, kidney and brain. The concentration of cyanide was determined in blood, liver, kidney and brain. In the rats pre-treated with alcohol and alcohol + caffeine the cyanide concentration increased in the blood and other organs two to three fold whereas in rats pre-treated with caffeine alone the concentration of cyanide was not significantly different from controls. These results suggest that caffeine inhibited and alcohol enhanced metabolism of MeAN resulting in a significant increase in cyanide concentration in blood and organs. When rats were pre-treated with both caffeine and alcohol, caffeine could not provide this protection. The opposite effects of caffeine and alcohol may be through opposite modulation of microsomal activity. (Supported by NIH South Texas Doctoral Bridge Program Grant # 2 R25GM50080).

METABOLISM OF GERANYL NITRILE AND CITRONELLYL NITRILE BY PRIMARY HEPATOCYTES FROM MICE, RATS AND HUMANS.

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Geranyl nitrile (GN) and citronellyl nitrile (CN) are fragrance components used in consumer and personal care products. The metabolic clearance and biotransformation of GN and CN were compared in primary hepatocytes from mice, rats and humans. For determination of intrinsic clearance, GN and CN (25  $\mu$ M) were incubated with hepatocytes in sealed vials, and the headspace above the reaction was sampled periodically by solid phase microextraction and analyzed by GC/MS. For metabolite identification, GN and CN (250  $\mu$ M) were incubated with hepatocytes from each species for 60 min. Reaction mixtures were extracted and analyzed by GC/MS and LC/MS. Both GN and CN were rapidly metabolized in hepatocytes from all species. Within a species, intrinsic clearance was similar for both compounds, and increased in the order human < rat << mouse. For all species, estimated *in vivo* intrinsic clearance is well in excess of hepatic blood flow, so clearance in whole animals is expected to be flow-limited. Major common pathways for biotransformation of GN and CN involved 1) epoxidation of the 6-alkenyl moiety followed by conjugation with GSH, 2) hydroxylation of the terminal methyl group followed by direct conjugation with glucuronic acid in rodents or by further oxidation to the corresponding acid and formation of an acid glucuronide in humans. It is unclear if epoxidation of the 2-alkenyl group of GN occurs. The data from these experiments demonstrate significant species differences in hepatocyte clearance rates and suggest that metabolism of GN and CN is qualitatively similar across species. These data will be valuable in planning and interpretation of whole animal ADME studies and extrapolation of the results of rodent ADME studies to humans. This work was supported by RIFM.

KINETICS AND SCALING OF METABOLIC BIOTRANSFORMATION IN FISH: A COMPILATION OF RATE AND AFFINITY VALUES MEASURED UNDER PHYSIOLOGICAL CONDITIONS.

P. N. Fitzsimmons and J. W. Nichols. *USEPA/ORD/NHEERL/MED, Duluth, MN.*

Literature from the past twenty-five years was searched to obtain *in vitro* and *in vivo* metabolism information for fish collected under physiological conditions. Scaling factors were then used in conjunction with a venous equilibrium liver model and log Kow-dependent fat:blood partitioning relationship to convert *in vitro* hepatic metabolism data into whole-body clearance rates. In general, whole-body clearance values predicted by *in vitro* data under-predict reported levels of *in vivo* activity. This discrepancy is particularly apparent for high log Kow compounds due to the predicted effects of chemical hydrophobicity on plasma binding and steady-state volume of distribution. The data compiled in this study provide a basis for comparing metabolic activity within and among fish species, between fish and mammals, and between *in vitro* and *in vivo* test systems. This information can be used to im-

prove ecological risk assessments for metabolized compounds, parameterize chemical kinetic models, and optimize the design of field sampling efforts. (This abstract does not necessarily reflect EPA policy)

KINETICS AND SCALING OF METABOLIC BIOTRANSFORMATION IN FISH: EFFECTS ON BIOACCUMULATION PREDICTED BY INCORPORATING *IN VITRO* DATA INTO TWO KINETIC MODELS.

J. W. Nichols and P. N. Fitzsimmons. *USEPA/ORD/NHEERL/MED, Duluth, MN.*

Whole-body clearance rates based on *in vitro* hepatic metabolism data for fish were incorporated into a one-compartment bioaccumulation model. Using this model, metabolism was predicted to have little effect on chemical bioaccumulation due to: 1) high rates of branchial uptake for low log Kow compounds; and 2) low rates of metabolic clearance for high log Kow compounds. In a second model-based effort, *in vitro* metabolism data were used as inputs to a physiologically based toxicokinetic (PBTK) model for fish. This model provides for first-pass hepatic clearance of dietary contaminants and, at a fixed rate of metabolic activity, results in lower steady-state bioaccumulation of high log Kow compounds than levels predicted by a one-compartment model. In either case, however, model-predicted effects of metabolism on chemical bioaccumulation in fish underestimate impacts suggested by measured concentrations of well-metabolized compounds in field-collected animals. This discrepancy may be due to several factors including: 1) underestimation of true *in vivo* hepatic metabolism by *in vitro* assays; 2) failure of the scale-up procedure to account for non-hepatic metabolism; and 3) overestimation of chemical binding in blood. *In vivo* metabolism rates obtained by modeling to field data may be overestimated if models do not account for presystemic clearance (branchial or following dietary exposure) and metabolism at all relevant trophic levels. (This abstract does not necessarily reflect EPA policy)

MECHANISMS OF INTERACTIVE DEVELOPMENTAL TOXICITY OF POLYCYCLIC AROMATIC HYDROCARBONS IN ZEBRAFISH.

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Polyyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants and their prevalence in aquatic ecosystems warrants effective methods for assessing their risk. Some PAHs can mimic the embryotoxic effects of planar halogenated hydrocarbons (pHAHs) that are agonists for the aryl-hydrocarbon receptor (AHR), notably cranial deformities and cardiovascular defects. Studies have shown that pHAH toxicity is AHR-mediated and evidence suggests that some of this toxicity may be due to cytochrome P4501A (CYP1A) activity. Studies in our laboratory with killifish embryos have shown that co-exposure to the PAH-type AHR agonist  $\beta$ -naphthoflavone (BNF), and the partial AHR agonist and CYP1A inhibitor  $\alpha$ -naphthoflavone (ANF), significantly increased toxicity greater than that predicted from an additive PAH toxicity model. This enhanced toxicity contrasts to protective effects reported for CYP1A inhibition on the developmental toxicity of pHAH. Thus, the role of the AHR pathway and CYP1A activity in PAH toxicity is less clear. We repeated these experiments in zebrafish embryos to determine if PAH interactions observed in killifish were species-specific and, more importantly, to explore the mechanism by which inhibition of CYP1A activity leads to increased embryotoxicity in light of the molecular and genetic tools available for this model species. Zebrafish embryos were exposed to BNF alone and in combination with ANF at doses that by themselves were non-embryotoxic. As observed in killifish co-exposures, ANF decreased BNF-induced CYP1A activity and also caused greater pericardial edema and cranial deformities over those observed in BNF-alone, ANF-alone, or control groups. These data clearly show that enhanced toxicity observed between PAH-type AHR agonists and CYP1A inhibitors is not species-specific. However, the mechanism by which these interactions are greater-than-additive with respect to embryotoxicity remains unknown. We are currently using an antisense morpholino approach in zebrafish to elucidate the role of the AHR and CYP1A activity in the developmental toxicity of PAHs.

ALTERED GENE EXPRESSION AND DEVELOPMENT OF POTENTIAL BIOMARKERS IN MEDAKA (*ORYZIAS LATIPES*) BRAIN, LIVER, AND TESTIS FOLLOWING EXPOSURE TO FIBRATE PHARMACEUTICALS.

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To help address the consequences of increasing levels of environmental contaminants and to identify potentially novel markers of toxicity, we examined gene expression profiles from medaka (*Oryzias latipes*) exposed to a prototypical fibrate

pharmaceutical. Changes in gene expression in male medaka brain, liver and testis following 48 h exposure to 15  $\mu$ g (injected) of the antihyperlipidemic agent ciprofibrate (CF) were analyzed using suppressive subtractive hybridization. From a total of 1152 clones, 288 were sequenced and 100 were identified as differentially expressed. Furthermore, only 1 gene was shared among all three organs and less than 5% between any two organs, suggesting organ specific responses at the level of gene expression. The results of this CF and other xenobiotic induced gene expression studies were used to develop macroarrays specific for brain, liver, or testis containing 200-300 non-redundant cDNAs. Following 7 day water exposures to 10, 100, and 1000  $\mu$ g/l of CF or clofibrate, compound, dose, and organ specific altered gene expression was determined. Highly responsive genes were further validated using real-time QPCR. In order to associate changes in gene expression with altered phenotype, compound- and dose-dependent histopathology samples were also examined. A combination of macroarray, real-time QPCR, and histopathological techniques will aid in linking molecular events and biochemical alterations following exposure and provide a mechanism to evaluate exposure and effects at the individual and population levels. Research supported in part by grants from Duke University Integrated Toxicology Program (T32 ES07031), NCBC (ARG0030), and WERF (01HHE21T). Although this work was reviewed by the USEPA and approved for publication, it may not necessarily reflect official Agency policy.

### 1583 A CROSS-SPECIES APPROACH TO USING GENOMICS TOOLS IN AQUATIC TOXICOLOGY.

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Microarray technology has proven to be a useful tool for analyzing the transcriptome of various organisms representing conditions such as disease states, developmental stages, and responses to chemical exposure. Most commercially available arrays are limited to organisms that have complete, or near complete, genome information; few of which are relevant for non-mammalian toxicology. However, with the imminent completion of the zebrafish genome project, and the availability of high density microarrays, the zebrafish (*Danio rerio*) represents a useful species for focused toxicological research. An approach involving the transcriptional mining of the zebrafish for potential biomarkers to be validated for use in another cyprinid, the fathead minnow (*Pimephales promelas*), will be described as a useful cross-species comparison for using genomics in an organism without a defined genome. The high degree of evolutionary conservation in vertebrates of many biochemical and developmental pathways strongly supports the extrapolation of molecular data from one fish species to another. Linkage of other molecular measures and phenotypic effects are also required to interpret transcriptional comparisons between the species. Although this work was reviewed by EPA and approved for publication, it may not necessarily reflect official Agency policy

### 1584 MICROARRAY ANALYSIS OF HEPATIC GENE EXPRESSION IN COHO SALMON (*ONCORHYNCHUS KISUTCH*).

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Pacific salmon populations have declined markedly in the western United States, and exposure to agricultural and urban chemicals has been identified as a key determinant of salmon injury. Salmon undergo complex physiological transformations during the transition from juveniles to migratory adult stages that may affect susceptibility to chemical injury. In the current study, transcriptome profiling using a 16K salmonid microarray was conducted on liver mRNA samples from smolt-stage juveniles, adult males, and adult female Coho salmon (*Oncorhynchus kisutch*). The microarrays included ESTs and related sequences of genes pertaining to toxicological pathways of interest including oxidative stress, immune system regulation, cellular structure and function, and biotransformation. These genes also included several glutathione S-transferases (GST) with some structural similarity to -omega, -zeta, -pi, and -theta class GST. However, although over 90% of the cDNAs on the chip were homologous to known *Oncorhynchus* genes, less than 1% of the genes originated from *O. kisutch*. Accordingly, a subset of transcript expressions was validated using real-time quantitative PCR (QPCR). This subset included a GST pi homologue, which was cloned and sequenced from coho liver. Coho liver GST pi expression did not differ among the life stages and was well correlated with the gene array information. Preliminary screening of the baseline life-stage transcriptomes revealed several potential genes with ontology related to chemical injury and immune system regulation that were differentially expressed among the life stages. Following further validation of key genes by QPCR, the utility of this array platform will be evaluated in support of studies addressing mechanism-based markers of exposure, susceptibility, and biological effects in Coho salmon. Supported by P42-ES07375, P30ES07033, and Genome Canada.

### 1585

### THE USE OF RNA MICROARRAY TECHNOLOGY AS A BIOMARKER FOR EXPOSURE OF FATHEAD MINNOW (*PIMEPHALES PROMELAS*) TO 2, 4-DINITROTOLUENE.

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Production and use of 2, 4-dinitrotoluene (2, 4-DNT) and 2, 6-dinitrotoluene (2, 6-DNT) for military and civilian purposes has resulted in their release to the aquatic environment through various surface water pathways. The aquatic toxicology of the DNT homologues is poorly understood and this lack of knowledge contributes to uncertainty when assessing potential risks involved in exposure of aquatic species to these compounds. The purpose of this study is to characterize the effects of 2, 4-DNT and the associated mechanisms of toxicity in the fathead minnow (*Pimephales promelas*), through the use of RNA microarray technology. Larval ( $2.8 \pm 1.4$  mg) and adult ( $2.9 \pm 0.9$  g) minnows were exposed to 2, 4-DNT and the herbicide diquat dibromide for 10 days in independent static renewal exposures. Diquat dibromide exposures were conducted as a positive control for oxidative stress, the hypothesized mechanism of action of DNT. At the completion of the experiments survival and growth were assessed and tissues were collected for gene expression and chemical analyses. Significant mortality resulted in the  $51 \pm 7$  and  $80 \pm 4$   $\mu$ mole/L 2, 4-DNT treatments for the larval and adult experiments, respectively, with the larval life stage demonstrating the most sensitivity to 2, 4-DNT. No-observed-effect-concentrations (NOEC) were determined to be  $23 \pm 2$  and  $37 \pm 3$   $\mu$  mole/L 2, 4-DNT in the larval and adult experiments, respectively. The larval experiment produced a median lethal toxicant concentration (LC50) of  $32$  (28-37 95% Confidence Interval (CI))  $\mu$  mole/L 2, 4-DNT and a median lethal tissue residue (MLR) of  $155$  (142-169 95% CI)  $\mu$  mole/kg. Tissue residues increased in a concentration dependent manner in both the larval and adult experiments. Assessment of exposure and associated mechanisms of toxic action will be further investigated using microarray technology to explore gene expression profiles in collected tissues of exposed fish.

### 1586

### GENE EXPRESSION PROFILING IN RAINBOW TROUT (*ONORHYNCUS MYKISS*), EXPOSED TO A VARIETY OF MODEL TOXICANTS.

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The increased availability and use of DNA microarrays has allowed the characterization of gene expression patterns associated with different toxicants. An important question is whether toxicant induced changes in gene expression in fish are sufficiently diverse to allow for identification of specific modes of action or specific contaminants. In theory, each class of toxicant may generate a gene expression profile unique to its mode of toxic action. The latter will be influenced by dose, route of administration and developmental state among other potential modifying factors. We exposed isogenic (cloned) rainbow trout *Onorhyncus mykiss*, to sublethal levels of a series of model toxicants with varying modes of action, including ethynodiol (xeno-estrogen), trenbolone (anabolic steroid; model androgen), tetrabromodiphenyl ether (BDE-47, thyroid active), diquat (oxidant stressor), chromium VI, and benzo-a-pyrene (BaP) for a period of 1-3 weeks. Following exposure, fish were euthanized, livers harvested and RNA extracted. Fluorescently labeled cDNA were generated and hybridized against a commercially available Atlantic Salmon / Trout array (GRASP project, University of Victoria) spotted with 16, 000 cDNAs. The slides were scanned to measure abundance of a given transcript in each sample relative to controls. Data were analyzed via Genespring (Silicon Genetics) to identify a list of up and down regulated genes, as well as to determine gene clustering patterns that can be used as expression signatures. Initial analysis indicates each toxicant generated specific gene expression profiles. Most genes exhibiting altered expression responded to only one of the toxicants. Relatively few genes are co-expressed in multiple treatments. For example, BaP and Diquat, both of which exert toxicity via oxidative stress, up-regulated 28 of the same genes. Other genes associated with steroidogenesis, p450 and estrogen responsive genes appear to be useful for selectively identifying toxicant mode of in fish (Supported by the U. S. Department of Energy under contract DE-AC06-76RLO 1830 with Battelle PNNL).

### 1587

### NONYLPHENOL POLYETHOXYLATE INHIBITS TRANSPORT OF ESTROGENS INTO BILE IN THE CHANNEL CATFISH, *ICTALARUS PUNCTATUS*.

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P-glycoprotein (Pgp) has been implicated in multixenobiotic resistance in aquatic species, including the channel catfish *Ictalurus punctatus*. This ABC transporter effluxes amphiphatic compounds, including environmental pollutants such as

nonylphenol polyethoxylate (NPE), from cells in tissues such as the liver. Recent studies suggest that Pgp also may play a role in regulating levels of steroid hormones. The interactions of NPE, the Pgp substrate rhodamine 123 (Rh123) and estradiol (E2) were assessed as they relate to Pgp transport, using an *in situ* -prepared isolated perfused catfish liver model. Our results demonstrate that catfish livers perfused with 1  $\mu$ M of Rh123 and 20  $\mu$ M NPE transport less Rh123 to bile than do untreated controls (con=11.43+3.44 nmol/kg body weight [bw]; NPE=3.43+1.66 nmol/kg bw,  $p<0.007$ ). Livers perfused with increasing doses of E2 (0.5-20  $\mu$ M) and either 1 or 5  $\mu$ M Rh123 showed a similar dose-dependent reduction in Rh123 transport. Dixon plot analysis (Ki=6.48) suggests a complex E2/Rh123 interaction that includes both competitive and uncompetitive components. Biliary transport of [<sup>3</sup>H]-E2 (1 nM; specific activity=41.3Ci/mmol) was examined alone, or with NPE (20  $\mu$ M), the Pgp-inhibitor verapamil (VRP; 20  $\mu$ M), lucifer yellow (LY; 5  $\mu$ M; a MRP substrate in fish), or VRP/LY (20  $\mu$ M/5  $\mu$ M). NPE, VRP, and VRP/LY reduced transport of [<sup>3</sup>H]-E2 molar equivalents (ME) by 51.7%, 45.7%, and 64.7%, respectively. LY alone did not reduce E2 ME movement. HPLC analysis of biliary [<sup>3</sup>H]-E2 ME indicates that E2-glucuronides were the major species transported. Treatment groups exhibiting decreased E2 ME transport (NPE, VRP, VRP/LY) showed an altered biliary metabolite profile compared to the control and LY groups. VRP inhibition of E2 ME transported into bile, E2/Rh123 interactions, and a lack of a LY effect suggest that Pgp is involved in E2 disposition. The changing character of E2 ME entering the bile and dixon analysis results may be indicative of additional mechanisms involved in transport. (NIEHS ES-07375)

**1588** ESTROGEN EQUIVALENTS AND CORRESPONDING CHEMICAL ANALYSES OF MARINE WATER SAMPLES COLLECTED ALONG A CORAL REEF.

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The goal of this research is to assess the potential toxicological impacts of degraded water quality due to human derived activities in marine coastal areas along the Florida Keys. Sensitive marine species such as coral, sea grasses, and fish and invertebrates in their early stages of life may be at risk due to the introduction of pollutants, in particular those compounds with potential to disrupt the endocrine system. In this study, forty water samples were collected at Looe Key before, during and after the annual Underwater Music Festival. Samples were chemically analyzed for 8 pharmaceuticals and 14 steroids and screened for estrogen equivalents using the yeast estrogen screen. Estrogen equivalents ranged from nondetectable (nd) to 1300 ng/L. Even though recreational activities peaked during the day of the festival, higher average estrogen equivalents ( $730 \pm 125$  ng/L) were found in samples collected the day prior to the festival. This trend was supported by the analytical data where the sum of contaminant concentrations of these samples was statistically higher compared to data from the other collection times. Estrone (0.74 – 0.82 ng/L), equilin (nd – 0.88 ng/L), cholesterol (180 – 1250 ng/L), caffeine (nd – 31 ng/L) and DEET (nd – 17 ng/L) were detected in the majority of samples. However, no nonylphenol, bisphenol A, or ethynodiol was detected. Although the results do not indicate high potential risk to the ecosystem from acute recreational exposure, the presence of human-derived contaminants should not be downplayed. The estrogen equivalents detected at Looe Key were significantly lower than what we have found at waste water impacted marine sites (up to 6.6  $\mu$ g/L). (Supported by NOAA/NIUST).

**1589** MERCURY DISTRIBUTION IN SEDIMENTS AND UPTAKE BY PLANTS ALONG SNOW AND CHOCOLLOCCO CREEKS IN NORTHEAST ALABAMA.

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The lower part of the Snow Creek Watershed drains an urbanized area within the city of Oxford, AL. Approximately one mile before Snow Creek empties into Choccolocco Creek, it flows through a city park and a nearby shopping mall parking lot. The city park is in the floodplain of Snow Creek and contains baseball fields, playground areas, and tennis courts. Fill deposits used as landscape material in the construction of both the park and the shopping mall may have been contaminated with mercury (Hg) from industrial sources. This land now serves as a non-point source of Hg contaminated soil. Gradual erosion has redistributed contaminated sediment from this site downstream into Choccolocco Creek and its associated floodplain. Sediment samples were collected downstream from the confluence of Choccolocco and Snow Creeks for a distance of 25 miles. Mercury

contamination was detected a distance of fourteen miles downstream from Snow Creek. Sediments taken from Snow Creek upstream of the park and shopping mall had Hg levels of 0.06 ug/g, which appear to be the background level. Channel deposits from Snow Creek and Choccolocco Creek below the contaminated site had Hg levels that ranged from 0.08 to 2.46 ug/g. Soil samples taken from the city park had values as high as 6.58 ug/g. Three different plant species were collected from the contaminated area of Snow Creek and analyzed for total Hg. The metal was not detected in either Indian mock strawberry (*Duchesnea indica*) or Johnson grass (*Sorghum halepense*). However, Hg was detected in wild hops (*Humulus lupulus*), which grows abundantly in sun exposed areas along the creek. Hop samples had average Hg concentrations of 0.09 ug/g in leaves and 0.21 ug/g in stems. Soil concentration at the collection site was 0.55 ug/g. The periodic harvesting of this fast growing plant could provide a relatively inexpensive means of removing Hg from the contaminated soil.

**1590** IDENTIFICATION OF SELENOPROTEINS IN SELENIUM-SENSITIVE TISSUES IN JAPANESE MEDAKA (*ORYZIAS LATIPES*) USING TANDEMLY COUPLED HPLC-ICPMS.

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Selenium (Se) is an essential micronutrient for many organisms, but for oviparous vertebrates, Se at slightly elevated levels becomes highly toxic. Sulfur (S), a fundamental constituent of many proteins, is found in the disulfide bond linkages (S-S linkages) between amino acid strands. The similarities in chemical and physical properties of Se and S has been used to support the hypothesis that Se exerts its toxicity by interfering with disulfide bonds. This can result in distorted, dysfunctional proteins and enzymes, which ultimately could impair normal cellular biochemistry. Studies are currently underway to determine proteins in *O. latipes* that may be affected by Se toxicity after dietary exposure to the metal. Brine shrimp (*Artemia*) used for the dietary exposures to *O. latipes* were exposed to 100 ppm selenomethionine (SeMet) for 1 hour. Se levels in exposed *Artemia* averaged 5.82 nmol Se/mg dry weight. Assimilation of Se into proteins from *O. latipes* liver, gonad, and egg samples following dietary exposure were evaluated by tandemly coupled size-exclusion HPLC (SE-HPLC) ICPMS (Perkin Elmer 6100 DRC). Chemical resolution of Se from the polyatomic Ar<sub>2</sub><sup>+</sup> dimer at m/z 80 was accomplished using methane as a reactant gas. The identification of these Se-affected proteins could aid in better understanding of Se toxicity within oviparous organisms.

**1591** ACUTE TOXICITY AND BIOACCUMULATION OF TRIBUTYLTIN IN THE REEF BUILDING CORAL SPECIES ACROPORA SP. (STAGHORN CORAL).

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Tributyltin oxide (TBTO) is used as a biocide by manufacturers of antifouling marine paint. Previous studies of marine species including gastropods, fish, algae and marine mammals, have shown the detrimental effects of TBTO exposure to non target organisms in the environment. Ship groundings result in the deposition of TBTO in the form of paint chips and contaminated debris. Two such groundings have occurred on the Great Barrier Reef, Australia. To better understand the impacts of acute TBTO exposure to reef building corals, coral fragments of *Acropora* sp. (staghorn coral) were housed in glass aquaria static tank systems with appropriate aeration. Metal halide/power compaq lighting systems provided dawn/dusk and daytime lighting during a 12 hour photo period. Individual 15 cm fragments were exposed to 100ng/l, 500ng/l or 1000ng/l of TBTO via water column. Non treated fragments were housed as controls. After 12hr exposure, fragments were fixed in 3% gluteraldehyde, and processed for scanning electron microscopy. Samples for light and transmission electron microscopy were decalcified after gluteraldehyde fixation and appropriately processed for sectioning. Graphite furnace atomic absorption spectrophotometry (GFAAS) was used to determine tin concentration. Chlorophyll extraction/colorimetric analysis were used to quantify loss of zooxanthellae (bleaching). Electron microscopy results demonstrate a dose dependant swelling and exfoliation of epithelium. GFAAS results show significant increase in tin concentrations of treated coral compared to controls. Colorimetric analysis of chlorophyll concentration exhibits a dose dependant release of zooxanthellae among treatment groups.

HYPERSensitivity OF THE ELASMOBRANCH  
UROLOPHUS JAMAICENSIS (YELLOW STINGRAY) TO  
TRIBUTYLTIN EXPOSURE.

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Tributyltin oxide (TBTO) is the main constituent of tin based antifouling marine paint. It is used on the hulls of ships to prevent the growth of fouling organisms. Upon leaching from a variety of sources, TBTO binds strongly to suspended material. Elasmobranchs play an important role in the health and stability of the marine ecosystem. *Urolophus jamaicensis* represents the ideal study organism for the toxic effects of TBTO to these marine vertebrates. Previous work has shown that the elasmobranch gill is more sensitive to TBTO exposure than many species of teleost fish. This work is a continuation of a study to demonstrate the toxicity and accumulation of tin in gill tissue of the stingray *U. jamaicensis* after a single exposure of TBTO. This work demonstrates the alterations in the morphological architecture of the gill using LM, TEM, and SEM, the induction of stress proteins, and peroxidative damage in response to TBT exposure in doses much lower than previously reported. Animals were exposed to one of five experimental doses of TBTO (4ug/L, 2ug/L, 1ug/L, .5ug/L or .05ug/L). A sixth group served as a control population. Three hours following treatment animals were sacrificed, gill tissue removed, processed and stored for analysis. Results indicate that *U. jamaicensis* is hypersensitive to TBT exposure. The elasmobranch gill showed a distorted, swollen epithelium with exfoliation following acute exposure to as little as .05ug/l TBTO for 3 hours. TEM revealed swollen mitochondria with membrane disruption. Differential staining confirms no mucous production, and no inflammatory response. GFAAS results indicate that tissues of treated animals contained a significantly increased tin concentration as compared to controls. Western Blot analysis demonstrates the induction of the stress proteins Hsp 70 and HO1. 4HNE adduct formation determined by Western blot analysis provides further evidence that observed membrane degradation is a result of lipid peroxidation.

IN SITU BIOMONITORING TO ASSESS THE  
GENOTOXICITY OF PAH-CONTAMINATED  
SEDIMENTS TO JUVENILE COHO SALMON  
(ONCORHYNCHUS KISUTCH).

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Polycyclic aromatic hydrocarbons (PAH) are ubiquitous marine and freshwater sediment contaminants. However, limited information is available regarding the bioavailability and genotoxicity of sediment PAHs to aquatic organisms. We investigated an integrated biomonitoring approach using chemical analyses and biomarkers to characterize the bioavailability and genotoxicity of a complex PAH mixture in freshwater lake sediments associated with a former manufactured gas plant (MGP). Sediment PAH genotoxicity was assessed by flow cytometry, DNA adduct 32P-postlabeling, and erythrocyte micronuclei in juvenile coho salmon (*Oncorhynchus kisutch*) caged in the water column. Significant PAH-induced genotoxicity was observed with flow cytometry and DNA adduct 32P-postlabeling, but not with erythrocyte micronuclei. Chromosome damage measured by flow cytometry and hepatic DNA adducts correlated with sediment PAH concentrations. Total hepatic DNA adducts in salmon caged nearest the former MGP facility was 28.1 (SD = 4.7) RALx10E9, while salmon caged in a reference lake had 21.2 (SD = 2.4) RALx10E9 DNA adducts. These data indicate aged sediment PAHs can migrate into the water column and be genotoxic to caged salmon. The data are insufficient to determine if such genotoxicity will reduce salmon viability or reproductive capability, but show *in situ* biomonitoring using biomarkers and caged fish can be effective in sediment ecotoxicology studies.

EXPOSURE OF FATHEAD MINNOW TO FULLERENE  
AND SINGLE-WALLED CARBON NANOTUBES.

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A water-soluble fullerene derivative (nC60) has been shown to induce lipid peroxidation in the brains of juvenile largemouth bass (*Micropterus salmoides*). In addition, proteins related to an inflammatory response were up-regulated by nC60, as

were enzymes related to metabolism of organic molecules, most notably CYP2K1. This current study was done as a follow-up, and to expand the exposure regime to polypeptide-coated and uncoated single-walled carbon nanotubes (SWNTs). For this study, the nC60 was dissolved by stirring for two weeks in milliQ water. SWNT were water-solubilized using the synthetic helical peptide Nano-1. Adult male fathead minnows (*Pimephales promelas*) were dosed individually via the water column with either a) 0.5 ppm nC60 (positive control); b) 0.2 ppm Nano-1 wrapped SWNT; or c) 0.2 ppm SWNT (added to water, but did not disperse) in Reconstituted Hard Water (RHW) in 1L aquaria for 48 hours, with a 50% water volume change at 24 hours. Control fish were exposed to only RHW, and n=10 for each group except nC60, where n=5. At 48 hours, there was no mortality. Fish were euthanized using MS-222, rinsed and dried, weighed, and ten tissues were harvested from each fish. For both the Nano-1 SWNT and un-coated SWNT exposed fish, SWNTs were detected in the fecal material collected from the digestive tract, as was verified by Raman spectroscopy. For the Nano-1 SWNT exposures, clumps of SWNTs were visible on the gill, but similar clumps were not visible for the un-coated SWNTs. Tissues will be further analyzed for the presence of either SWNTs or the fullerene, and for possible oxidative damage. Livers are being processed by RT/RT-PCR for the induction of CYP2K1 mRNA to identify whether nano-1 wrapped SWNTs, unwrapped SWNTs, or nC60 can induce this enzyme in fathead minnows. This is only the first trial with this exposure regime, and will be repeated.

TEMPORAL PATTERNS OF MICROCYSTIN-LR  
CONTAMINATION IN LAKES OF WEST TEXAS.

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Microcystin-LR (MCLR) is a predominant cyanotoxin which contaminates fresh water sources all over the world. MCLR is a hepatotoxic and a potent tumor promoter in animal models. In addition to linking to elevated incidence of gastrointestinal cancers in humans, ecotoxic effects of MCLR contamination are predominant in recent years, as demonstrated by growing reports on bloom outbreaks and large scale fish killed in many water bodies of USA, including lakes from west Texas. In this study, temporal patterns of MCLR contamination in Buffalo Springs Lake and Lake Ransom Canyon of west Texas were monitored over a year. Relationship between production of MCLR and various physicochemical parameters of water was investigated. MCLR in water samples were determined by ELISA and protein phosphatase inhibition assay and confirmed by HPLC. Results showed that in Buffalo Springs Lake, seasonal averaged concentration of MCLR was found to be  $1.78 \pm 1.43$  ppb ranged at 0.18 - 4.91 ppb in spring samples,  $0.41 \pm 0.09$  ppb ranged at 0.19 - 0.5 ppb in summer samples,  $0.46 \pm 0.41$  ppb ranged at 0.21 - 1.6 ppb in fall samples, and  $1.04 \pm 0.71$  ppb ranged at 0.09 - 2.43 ppb in winter samples, respectively. In Lake Ransom Canyon, the averaged concentration of MCLR was found to be  $1.08 \pm 1.29$  ppb ranged at 0.44 - 3.7 ppb in spring samples,  $0.83 \pm 0.46$  ppb ranged at 0.34 - 1.45 ppb in summer samples,  $0.44 \pm 0.03$  ppb ranged at 0.41 - 0.48 ppb in fall samples, and  $0.78 \pm 0.52$  ppb ranged at 0.30 - 1.76 ppb in winter samples, respectively. Seasonal fluctuation of MCLR concentrations were found to be correlated with values of water pH, temperature and dissolved oxygen. (Supported by the NIH grant CA94683)

GLUCURONIDATION OF DESMETHYL-  
METHOXYCHLOR IN THE CHANNEL CATFISH  
INTESTINE AND LIVER.

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The chlorinated pesticide methoxychlor (MXC) is a Superfund toxic chemical whose mono- and bis-desmethylated metabolites (OH-MXC and HPTE respectively) interact with estrogen receptors and may be endocrine disruptors. Slow phase II metabolism of OH-MXC or HPTE may delay their excretion and contribute to MXC toxicity. The glucuronidation of OH-MXC was examined in detergent-treated microsomes from liver and intestine of control catfish, fish exposed to MXC, 2 mg/kg i.p. for 6 days before sacrifice and fish exposed to 3-methylcholanthrene (3-MC), 10 mg/kg i.p. 4 days before sacrifice. The assay used 14C-UDPGA and ion-pair extraction of product, and was optimized for concentration of detergent, microsomal protein and incubation time. Varying concentrations of OH-MXC or UDPGA were used to examine reaction kinetics. Hepatic microsomes from control and 3-MC-treated catfish had similar Km values of 250  $\mu$ M, however Vmax was significantly ( $p < 0.05$ ) higher in 3-MC-treated fish than controls (426 vs 277 pmol/min/mg protein). Microsomes from MXC-treated fish had a lower apparent Km value of 143  $\mu$ M but similar Vmax to controls, 291 pmol/min/mg protein. Product was formed in microsomes from MXC-treated fish without added substrate, suggesting the lower apparent Km was an artifact due to

residues of OH-MXC or HPTE. Analysis of these microsomes revealed residues of OH-MXC and HPTE, but not parent MXC. Intestinal microsomes from control and 3-MC-treated fish glucuronidated OH-MXC more rapidly ( $p<0.05$ ) than liver. Intestinal Vmax values were 536 and 1026 pmol/min/mg protein in control and 3-MC-induced fish respectively. Our results show that although OH-MXC was readily glucuronidated in catfish liver and intestine, the Km values for glucuronidation were 50-fold higher than for CYP-dependent formation of the OH-MXC and HPTE. This suggests that glucuronidation may be inefficient at environmental exposure concentrations. Supported in part by ES-07375.

**1597**

DECABROMODIPHENYL OXIDE/ETHER (BDE-209): INVESTIGATION OF PHOTOLYTIC DEGRADATION IN A CONSUMER PRODUCT.

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Decabromodiphenyl oxide/ether (DBDPO) is a highly effective flame retardant used typically in styrenic resins requiring significant ignition resistance, e.g. US V-0 rating. Consumer products that may be flame retarded with DBDPO include electrical and electronic equipment such as television cabinet backs and wire and cable insulation, and upholstery textiles used in commercial applications. DBDPO represents over 80% of the global production of the flame retardant class known as polybrominated diphenyl ethers (PBDEs). PBDEs have received worldwide attention due to their detection in the environment. Those isomers most frequently detected contain four (mainly BDE-47), five (mainly BDE-99, 100) and six (mainly BDE 153, 154) bromine atoms, and appear to originate from the use of the pentabromodiphenyl ether (Penta) product. DBDPO's reductive debromination *in situ* within consumer products, with subsequent migration of breakdown product(s) out of the appliance and into the environment, has been suggested as a possible source of these environmental PBDEs. Using an accelerated aging process, we investigated DBDPO's potential to photodegrade in its most common US application, e.g. a TV cabinet back. The aging process exposed a sample cut from the back of retail TV to a Xenon Arc lamp for up to 900 hours, and was approximately equivalent to 16 years of indoor exposure to sunlight. Our results showed no generation of BDE-47 within the plastic, and potential generation of a maximum of  $8.6 \times 10^{-5}$  % of the BDE-99 contained in one year's global production of the Penta product. We also demonstrated photolytic degradation of BDE-47 and BDE-153, dissolved in organic solvent, via reductive debromination as well as by a competing process; that is, solvent trapping of the BDE radical. Based on these results, degradation of DBDPO in consumer products is an insignificant source of environmental PBDEs: 47, 99, 100, 153, and 154.

**1598**

HIGH-THROUGHPUT CHEMICAL SCREENING USING PROTEIN PROFILING OF FISH PLASMA.

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Compounds that affect the hormone system, referred to as "endocrine-disrupting chemicals" (EDCs) cause human and animal health problems. It is necessary to test new chemicals for EDC effects, though current testing is time/animal intensive and costly. To screen the EPA inventory of ~ 87,000 chemicals, other methods must be developed. Protein profiling can be used for high-throughput ID of protein patterns indicative of a change in physiological status. This "fingerprint" can be used diagnostically to screen for chemical exposure. Using Surface Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF MS), we profiled plasma obtained from Sheepshead minnows (*Cyprinodon variegatus*) for endocrine-related changes in protein patterns. The fish were placed into flow-through aquaria consisting of duplicate tanks for treatment with each of the following: vehicle control (triethylene glycol, TEG), estrogen (17  $\mu$ -estradiol), EDC positive controls (methoxychlor and bisphenol) and EDC negative controls (endosulfan and chlorpyrophos). Test concentrations were maintained by intermittent seawater flow and by injection of the chemical and control treatments. After treatment, fish were sampled for their plasma. The plasma was applied to ProteinChip® arrays and analyzed. No significant difference was found between protein profiles of seawater control and TEG-treated fish. Proteins in the <20 kDa range had altered expression in response to estrogen exposure, with respect to controls. An estrogen-specific spectral fingerprint was found using the pattern recognition software, Biomarker Patterns. Using this pattern diagnostically to determine estrogenicity, the two EDC positive controls were classified by the software as estrogenic. The two EDC negative controls were classified as non-estrogenic. SELDI represents a viable and rapid means for assaying chemicals for estrogenic effects. Also, this protocol should be readily transferable to other species and other mechanisms of interest.

**1599**

LOSS OF GLUTAMATE-CYSTEINE LIGASE MODIFIER SUBUNIT SENSITIZES CELLS TO CADMIUM TOXICITY.

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Glutamate-cysteine ligase modifier subunit (GCLM) has the overall effect of increasing the efficiency of glutathione (GSH) synthesis, an important antioxidant. *Gclm*(-/-) knockout cells have an approximately 80% lower level of GSH, and thus a compromised oxidant stress response. Numerous studies have demonstrated cadmium (Cd) as being a toxic metal which causes oxidative stress. To examine the role of *Gclm* in Cd toxicity, we exposed mouse fetal fibroblasts (MFFs) derived from wild-type [*Gclm*(+/+)] or *Gclm* null [*Gclm*(-/-)] mice to ranging doses (0-30  $\mu$ M) of cadmium chloride for 24 hours. *Gclm*(-/-) MFFs were nearly 15-fold more sensitive to Cd, EC50 of 1.2  $\mu$ M, compared with 17.5  $\mu$ M for *Gclm*(+/+) MFFs. Differential toxicity did not reflect a difference in  $^{109}$ Cd uptake between *Gclm*(+/+) and *Gclm*(-/-) cells. GSH binds Cd with high affinity ( $K_d = 10^{-10}$  M) and protection likely reflects Cd sequestration from sensitive cellular targets which may be decreased with GSH loss. Such cellular targets may include those that control transcriptional response of the Cd-binding and protective *Mt* gene products. Interestingly, accumulation of MT mRNA by Cd in *Gclm*(-/-) cells closely paralleled that of *Gclm*(+/+) cells. Thus, although *Gclm*(-/-) cells are sensitized to Cd toxicity, their protective response is similar to wild-type. In fact, at Cd concentrations in which *Gclm*(-/-) cells experience dramatic toxicity, induction of the *Mt* protective response is marginal. These data suggest that *Gclm*, and by extension GSH, is essential for protection against Cd toxicity and that "buffering" of Cd by GSH may be necessary for cells to mount a protective response. Supported in part by NIH grants R01 ES012463 and R01 ES010416-05.

**1600**

CADMUM AND TERT-BUTYL HYDROPEROXIDE-INDUCED DIFFERENT PATTERNS OF CHANGES IN ENERGY AND REDOX STATES IN HEPG2 CELLS - THEIR CORRELATION WITH THE MODE OF CELL DEATH.

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Cadmium (Cd) and tert-butyl hydroperoxide (t-BHP) are both toxic chemicals. These toxicants were used as models for studying cellular response to chemical stresses. The parameters examined included lipid peroxidation, energy state, redox state, mitochondrial membrane potential change and morphological appearance of apoptotic bodies in the cell nucleus. t-BHP caused lipid peroxidation in HepG2 cells in a dose-dependent manner. Compared to t-BHP, Cd caused a much lower dose-dependent increase in lipid peroxidation. At a higher concentration, Cd caused a dose-dependent decrease in GSH/GSSG while t-BHP caused only a small change in redox state. The two toxicants exerted different patterns of change in energy state. While both Cd and t-BHP caused a gradual decrease in the levels of total adenosine nucleotides (TAN=ATP+ADP+AMP), the relative amount of ATP/TAN increased and ADP/TAN and AMP/TAN decreased. The change resulted in a continuous increase in energy charge potential (ECP=[ATP+0.5ADP]/TAN). On the other hand, increasing concentrations of t-BHP initially caused a significant increase in ECP. As the dose increased, there was a decrease in ATP/TAN and increase in ADP/TAN and AMP/TAN. These changes resulted in a decrease in ECP. Under the specified culture conditions, increasing concentrations of t-BHP but not Cd caused a change in mitochondrial membrane potential, but apoptotic bodies appeared only in cells treated with Cd and not with t-BHP. The present study demonstrated that toxicants of different classes could cause different patterns of metabolic changes. These changes may reflect the modes of cell death.

**1601**

IDENTIFICATION OF DOSE- AND TIME-DEPENDENT CHANGES IN CELLULAR METABOLIC EVENTS IN HEPG2 CELLS UPON EXPOSURE TO CADMIUM.

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Cadmium (Cd) is a toxic metal. The mechanisms of its action have been attributed to its ability to alter cellular energy metabolism, damage DNA, generate free radicals and oxidize proteins. Along the process leading to cell death, a number of events occur, including protective gene induction and attempt to repair. Failure of these processes ultimately resulted in cell death via apoptosis. Cd induces cellular apoptosis through either the caspase-dependent or -independent pathway. Apoptosis could also be a result of free radical generation or changes in energy metabolism. The present study aims to investigate the action of Cd on cellular metabolism of the HepG2 hepatoma cell line by examining the time-and dose-dependent changes in cellular energy state and redox state. The results demonstrated the 3-hr

LC50 for Cd was approximately 0.7 mM. There was a dose-dependent decrease in total adenosine nucleotides, which was correlated to a dose-dependent increase in percentage of cells with apoptotic nucleus. Upon analyzing the different adenosine nucleotides content, relative amount of ATP increased as the concentrations of Cd increased. On the other hand, there was a dose-dependent decrease in GSH/GSSG suggesting the occurrence of oxidative stress. Over a 3 hr exposure to 0.7 mM Cd, there was an initial fluctuation in GSH/GSSG ratio at 0.5 to 1.5 hr, which corresponded to a time-dependent change in intracellular free radical generation as detected by fluorescent staining with 2', 7'-DCF. Cellular energy state varied at different times. In addition to a gradual increase in relative ATP and energy charge potential over the 3 hr study period, there was a significant increase in relative ADP level after 1.5 hr, which was reverted to normal at 4 hr. It was suggested that this change may be related to cellular repair. The present study demonstrated the use of appropriate markers to identify the sequence of events occurred in cells following Cd exposure.

## 1602

### CADMIUM DISRUPTS N-CADHERIN-DEPENDENT CELL-CELL JUNCTIONS AND ACTIVATES $\beta$ -CATENIN MEDIATED NUCLEAR SIGNALING IN ROS 17/2.8 CELLS.

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The N-cadherin/ $\beta$ -catenin complex serves as an important structural component of adherens junctions in various types of epithelial cells. In addition,  $\beta$ -catenin can function as a nuclear signaling molecule and regulate the expression of genes that are involved in cell-cycle control and apoptosis. The recent finding that the environment pollutant Cd can disrupt cadherin-dependent junctions in various types of epithelial cells has led us to examine the effects of Cd on N-cadherin-dependent cell-cell junctions and  $\beta$ -catenin regulated gene expression in ROS 17/2.8 cells. Results of immunofluorescent labeling studies showed that exposure to 5-10  $\mu$ M Cd for 0.5-3 hours caused the cells to separate from each other without detaching from the growing surface. This effect coincided with the loss of N-cadherin and  $\beta$ -catenin from the cell-cell contacts and the translocation of  $\beta$ -catenin to the cell nucleus. Additional studies utilizing the TOPFLASH  $\beta$ -catenin reporter gene construct showed that Cd caused a 2-3 fold increase in the expression of the  $\beta$ -catenin regulated luciferase reporter gene. These results provide additional evidence that the N-cadherin/ $\beta$ -catenin complex may be an important early target of Cd toxicity and they suggest that Cd can activate endogenous  $\beta$ -catenin nuclear signaling via TCF/LEF1 transcription factors. Supported by NIH Grant # R01 ES006478.

## 1603

### UPREGULATION OF $\gamma$ -GLUTAMYL CYSTEINE SYNTHETASE EXPRESSION IN CADMIUM-TREATED RAT FETAL LUNG FIBROBLASTS.

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Cadmium (Cd), a toxic metal of environmental and occupational health concerns, damages the microtubule cytoskeleton initiated by binding to critical tubulin sulphydryls and causes a biphasic response in total cellular glutathione (GSH). Since GSH is critical in protecting protein sulphydryls and providing cellular defense against Cd insult, we have explored mechanisms of Cd-mediated GSH elevation in rat fetal lung fibroblasts (RFL6) focusing on  $\gamma$ -glutamylcysteine synthetase ( $\gamma$ -GCS), the rate limiting enzyme in GSH biosynthesis. As measured by the Tietze assay, 4  $\mu$ M CdCl<sub>2</sub> treatment for 16 h increased total cellular GSH to 241% of the control (100% = 611 $\pm$ 73 ng GSH/mg total cell protein). These cells also showed a typical biphasic GSH response as evidenced by a decrease to 57% at 2 h followed by a steady recovery and overshooting reaching 134% and 249% (100% = 484 $\pm$ 56 ng GSH/mg total cell protein) at 4 h and 16 h, respectively, post incubation with Cd. Western blots showed enhanced  $\gamma$ -GCS protein expression (156% of control) in RFL6 cells treated with 4  $\mu$ M CdCl<sub>2</sub> for 16 h. Furthermore, as measured by the reverse transcription-polymerase chain reaction (RT-PCR) assays, the steady-state  $\gamma$ -GCS mRNA level was elevated to 280% of the control level under the same conditions. Notably, previous work has suggested that the Cd-elevated cellular GSH probably resulted from an increased synthesis based on studies using buthionine sulfoximine (BSO), which inhibits GSH biosynthesis. Since the rate-limiting  $\gamma$ -GCS is under feedback inhibition by GSH itself, the initial GSH reduction due to Cd binding would lessen its inhibition on  $\gamma$ -GCS thus enabling an upregulation of  $\gamma$ -GCS mRNA and protein expression resulting in enhancement of new GSH biosynthesis to replenish the initially reduced cellular GSH pools. These studies provide direct evidence for Cd stimulation of new GSH biosynthesis in RFL6 cells. (Supported in part by NIH grants R01 ES 11164 and ES 11340 and Philip Morris External Research Program.)

## 1604

### MECHANISMS OF CADMIUM TRANSPORT IN MDCK CELLS.

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In humans exposed to cadmium (Cd) via oral and/or pulmonary routes, the kidney is by far the primary organ affected adversely by Cd. Although there are data indicating that proximal and distal portions of the nephron handle Cd, the mechanisms involved in this transport are poorly understood. Thus, the principal aim of the present study was to investigate mechanisms of Cd transport in cultured epithelial cells derived from proximal (NRK-52E cells) and distal (Madin-Darby Canine Kidney; MDCK) portions of the nephron. The rates of luminal uptake of Cd in NRK-52E cells (28.4 $\pm$ 4.2 pmol x mg protein<sup>-1</sup> x min<sup>-1</sup>) were significantly greater (2.7 fold) than those the MDCK cells (10.4 $\pm$ 0.12 pmol x mg protein<sup>-1</sup> x min<sup>-1</sup>). The calcium (Ca<sup>2+</sup>) channel blockers, verapamil or diltiazem, significantly inhibited the uptake of Cd only in the MDCK cells, but not in NRK-52E cells. This indicates that Cd transport in the distal nephron is mediated in part through the actions of Ca<sup>2+</sup> channels. Another important difference noted between these two cell lines is that under normal extracellular Ca<sup>2+</sup> concentrations, in the presence of ferrous iron (Fe<sup>2+</sup>), the uptake of Cd was significantly inhibited only in MDCK cells. These findings indicate that the Fe<sup>2+</sup> transporter, Divalent Metal Transporter 1 (DMT1), also transports Cd. Moreover, additive effects were documented in the MDCK cells when both Ca<sup>2+</sup> channel antagonists and Fe<sup>2+</sup> were present in the extracellular compartment. Overall our results indicate that at least two different transporters (Ca<sup>2+</sup> channels and DMT1) participate in the luminal uptake of Cd in distal portions of the nephron.

## 1605

### CADMIUM NEPHROTOXICITY IS ASSOCIATED WITH A LOSS OF N-CADHERIN MEDIATED ADHESION AND ALTERATIONS IN EPITHELIAL POLARITY IN THE PROXIMAL TUBULE.

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Recent studies from our laboratory have shown that the nephrotoxic metal Cd can selectively disrupt N-cadherin dependent cell-cell junctions in the proximal tubule of rat kidney. The objective of the present study was to determine whether or not the Cd-induced loss of N-cadherin-mediated adhesion resulted in any changes in the polarity of the epithelial cells of the proximal tubule. Male Sprague-Dawley rats received subcutaneous injections of Cd (0.6 mg/kg in isotonic saline, 5 days per week for up to 6 weeks). One day each week, 24-h urine samples were collected and analyzed for protein and creatinine. After 5-6 weeks, the Cd-treated animals developed significant proteinuria, with no change in creatinine excretion. Epithelial polarity was evaluated by examining the histochemical localization of the apical, brush border marker enzyme, alkaline phosphatase, and the basolateral marker protein, Na<sup>+</sup>, K<sup>+</sup>-ATPase. Results of immunofluorescent labeling studies revealed that the kidneys from the Cd treated animals exhibited a marked reduction in N-cadherin labeling in proximal tubule, along with pronounced changes in the localization of alkaline phosphatase and Na<sup>+</sup>, K<sup>+</sup>-ATPase. In the samples from control animals, the alkaline phosphatase labeling was localized at the apical cell surface, and the Na<sup>+</sup>, K<sup>+</sup>-ATPase labeling was concentrated at the basolateral cell surface. In the samples from the Cd<sup>2+</sup> treated animals, both molecules appeared to be diffusely distributed over the entire surface of the proximal tubule epithelial cells. These findings indicate that the Cd-induced loss of N-cadherin mediated adhesion results in alterations in renal epithelial polarity and the redistribution of cell surface proteins in the proximal tubule. Supported by NIH Grant R01 ES006478.

## 1606

### NECROSIS AND NOT APOPTOSIS IS THE PREDOMINANT MODE OF CELL DEATH IN MORTAL HUMAN PROXIMAL TUBULE CELLS EXPOSED TO CADMIUM CHLORIDE.

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The third isoform of metallothionein, MT-3 was discovered recently and was thought to be expressed only in the brain. However, we have previously shown that MT-3 is expressed in the human kidney in situ, including the cells of the proximal tubule. A subsequent analysis of MT-3 expression demonstrated that cell cultures of mortal human proximal tubule cells also expressed MT-3. However, an immortalized human proximal tubule cell line, HK-2 did not express the MT-3 gene. Transfection of the MT-3 gene into the HK-2 cells resulted in an increase sensitivity of these cells to the cytotoxic effects of cadmium and this was correlated to an alteration in the mechanism of cell death, being changed from apoptosis in the HK-2 cells to necrosis in the MT-3 transfected cells. The goal of this study was to determine if the human proximal tubule cell (HPT) cultures that normally express the MT-3 gene had a mode of cell death similar to the HK-2 MT-3 transfected cells in response to cadmium. For this purpose, the HPT cells, HK-2 cell line and the

MT-3 transfected HK-2 cells were exposed to lethal and sub-lethal concentrations of cadmium chloride for various time periods. Apoptosis was measured by the release of cytochrome c from the mitochondria to the cytosol, activation of caspase 3 and 9, and DNA laddering. The release of lactate dehydrogenase from the treated cells was also measured. There was no activation of caspase 3 and 9 in the HPT and HK-2 MT-3 transfected cells in response to cadmium unlike the HK-2 cells which showed activation of caspases and DNA laddering in response to cadmium treatment. Furthermore, the release of LDH was highly elevated for the HPT and MT-3 transfected cells compared to the HK-2 cells. These results indicate that human proximal tubule cells do not undergo apoptosis in response to cadmium, rather necrosis seems to be the predominant mode of cell death.

**1607 CADMIUM-INDUCED CELL CYCLE ARREST IN RAT KIDNEY EPITHELIAL CELLS IS MEDIATED THROUGH P53 ACTIVATION.**

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Cadmium (Cd) induces apoptosis in different cell types, including the kidney cells. This study was designed to investigate the Cd-induced p53-mediated cell cycle arrest, which may lead to apoptotic cell death. Subconfluent cultures of a normal rat kidney proximal tubular epithelial cell line, NRK-52E, were treated for up to 24 h with 5-20  $\mu$ M CdCl<sub>2</sub> in DMEM containing 5% calf serum. By electrophoresis mobility shift assay, wild-type p53 was found to be up-regulated upon Cd exposure. Flowcytometric analysis of the cells revealed a significant time- and concentration-dependent increase in cells in G2/M phase of the cell cycle. Exposure to 20  $\mu$ M Cd for 24 h doubled the number of cells in the G2/M phase. The cell cycle arrest was closely related to the repression of cyclins A and B that control the progression of cells in different phases of the cell cycle. Additionally, Cd treatment led to down-modulation of cyclin-dependent kinases, cdk2 and Cdc2. Cells treated with Cd also showed elevated expression of cyclin-specific inhibitor p21 (WAF1/Cip1), a p53-controlled downstream gene product. These observations suggest that Cd-mediated cell cycle dysregulation is mediated via modulation of cyclin inhibitor, cyclin and cdk machinery, and that this process is p53-dependent. (Supported in part by NIH grant P20 RR016457)

**1608 CADMIUM-INDUCED CARCINOGENIC TRANSFORMATION OF HUMAN PANCREATIC DUCTAL CELLS.**

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Human pancreatic cancer is a deadly disease with a poorly defined etiology. Cadmium is thought to be a plausible human pancreatic carcinogen. Thus, this study was designed to determine whether a nontumorigenic human pancreatic ductal epithelial cell line (H6c7) could be transformed *in vitro* by chronic low-level cadmium exposure. H6c7 cells were cultured in 0.5 or 1.0  $\mu$ M cadmium (as CdCl<sub>2</sub>) and compared to passage-matched control cells. At 11 weeks of continuous cadmium exposure the cells underwent a distinct morphological change from a round, epithelial-like form with consistent size to a fibroblastoid shape with heterogeneous sizes. After 21 week, these chronic cadmium exposed human pancreatic ductal epithelial (CCE-HPDE) cells showed atypical foci of cell mounding, indicative of a loss of contact inhibition, and had a marked increase in matrix metalloproteinase-9 secretion, which is common in cancer cells. CCE-HPDE cells were hyperproliferative with a growth rate about 2.5-fold higher than passage-matched control cells. Moreover, CCE-HPDE cells were able to grow much better in media containing suboptimal growth supplement (pituitary extract plus EGF) than control cells, indicating less dependence on exogenous growth factors for continued proliferation, a characteristic common in tumors. Marked over-expression of the oncogenes c-myc and c-jun and the positive cell-cycle regulatory gene cyclin D1 occurred in CCE-HPDE cells, as evidenced by up to 5-fold increases in both transcript and protein levels compared to control. Genomic DNA methylation, determined by methyl acceptance assay, was significantly reduced in CCE-HPDE cells. Taken together, these results indicate cadmium may well be able to induce carcinogenic transformation in human pancreatic ductal epithelial cells. CCE-HPDE cells are now being tested for tumor-forming ability after inoculation into nude mice.

**1609 CADMIUM MAY AFFECT THE LIGAND INDUCED MIGRATORY ABILITY OF IMMORTALIZED EXTRAVILLOUS TROPHOBLAST CELLS.**

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A group of proliferative and migratory/invasive cytotrophoblast cells known as "extravillous trophoblast" (EVT) is the most important cell type in human placenta for maintaining attachment with the uterus and establishing easy uteroplacental blood

flow. Exposure of these cells to exogenous, toxic elements may disturb the function of the placenta, resulting in deleterious effects to the fetus. One element filling this description is cadmium (Cd<sup>2+</sup>), the concentration of which can be high in placenta of mothers who are exposed to tobacco smoke and those who work in welding and battery manufacturing. A large proportion of absorbed Cd<sup>2+</sup> cannot cross the placental barrier and as a result accumulates in the placenta. This metal may cause toxic effects to the cells or alter their normal function, possibly altering the migratory/invasive capabilities of these cells. Calcium (Ca<sup>2+</sup>) is an important element in cell motility. Impaired mobility/invasion may be the result of interference in Ca<sup>2+</sup> kinetics in these cells. As a first step in identifying the actions of Cd<sup>2+</sup> on EVT cell function we used immortalized, pure, first-trimester human EVT cells to study the effects of Cd<sup>2+</sup> on migration induced by insulin-like growth factor II (IGF-II), endothelin-1 (ET-1), prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), urokinase plasminogen activator amino terminal fragment (uPA ATF), and epidermal growth factor (EGF). Using transwell migration assays it was found that 0.5 to 1.0  $\mu$ mol/L of CdCl<sub>2</sub> abrogates EVT cell migration induced by IGF-II, PGE<sub>2</sub> and uPA ATF. Fluorometric studies show that intracellular calcium induction by different ligands may be impaired by small amounts of Cd<sup>2+</sup>.

**1610 CHRONIC EXPOSURE TO CADMIUM INDUCES ANEMIA WITH HIGH IRON STORAGE THROUGH DEFECTIVE IRON UTILIZATION AND ENHANCED IRON ABSORPTION IN RATS.**

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Chronic exposure to cadmium (Cd), one of the toxic heavy metals, can induce anemia, which has been considered to be derived from the setting of iron deficiency, hemolysis, and insufficient production of erythropoietin (EPO) from the kidney. In order to know detailed mechanism of the Cd-induced anemia, we investigated the relationship between anemia and iron metabolism in the rats exposed to Cd with or without iron deficiency. We injected rats with saline or Cd at 2.0 mg/kg s.c. twice a week for 3 months, with normal diet or low-iron diet (the iron contents are 60 ppm and 10-20 ppm, respectively), resulting in four groups of rats: saline with normal diet, saline with low-iron diet, Cd with normal diet, and Cd with low-iron diet. The group of saline with low-iron diet developed typical iron deficiency anemia, evidenced by the increased TIBC and EPO levels as well as the low levels of plasma iron and ferritin. On the other hand, the group of Cd with normal diet showed a different kind of anemia: a decreased plasma iron level along with highly elevated TIBC and ferritin (3 and 6 times higher than the group saline with normal diet, respectively) and a suppressed level in EPO. This result indicates that Cd would induce anemia by interfering with iron utilization for erythropoiesis while it enhances iron absorption, leading to a large amount of iron accumulation in a body. Furthermore, the group of Cd with low-iron diet showed the same degrees of anemia and EPO as those of the group of Cd with normal diet, although the magnitudes of elevation in TIBC and ferritin were lower than the group of Cd with normal diet. This result indicates that defective iron utilization, not iron deficiency, would contribute to the development of Cd-induced anemia significantly. In conclusion, chronic exposure to Cd induces anemia with high iron storage, which would be derived from defective iron utilization as well as enhanced iron absorption, not from iron deficiency.

**1611 BEHAVIORAL ALTERATIONS OF C57BL AND METALLOTHIONEIN KNOCK-OUT MICE PERINATALLY EXPOSED TO LOW LEVEL OF CADMIUM.**

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While the renal toxicity is the cardinal feature of chronic cadmium (Cd) toxicity, little information is available regarding the developmental toxicity of Cd, especially at low exposure level. Since prenatal exposure to low level of Cd has been shown to accelerate sexual maturation presumably through endocrine mechanism, such exposure to Cd might result in developmental abnormality. To test this possibility, we have exposed wild-type C57BL and its metallothionein-I, II-null (MT-null) mice to 10 microg/mL Cd through drinking water from the beginning of gestation to 10 days after parturition. Behaviors of the offspring born to these mice were examined at the age of 8 to 9 weeks. Regardless the mice strain, the Cd-exposed females, compared to the non-exposed females, (i) were significantly less active in the open-field test [to examine the emotionality/activity] and (ii) exhibited shorter step-through latency (time to enter the dark compartment) in the 'pre-learning' session of the passive avoidance test [to assess the learning ability]. Neither of these effects was observed in males. In a separate experiment, Cd-exposed MT-null females showed

poorer performance than the non-exposed counterpart in Morris water maze, a test for spatial learning capacity, under the circumstance in which the difficulty of the task was increased. At postnatal day 10, right after the end of exposure, although the brain levels of Cd was as low as 0.1 ng/g, plasma thyroxine level was significantly decreased in Cd-exposed offspring, an effect being more distinct in the MT-null groups. These results suggested that perinatal, low-level exposure to Cd could lead to subtle alterations in the behaviors of matured mice. The mechanism for these effects, including the reason for the susceptibility of females and the possible contribution of the altered thyroid hormone level is warranted to be examined.

## 1612

### MODULATION OF ACUTE HEPATOTOXICITY OF CADMIUM BY SIMULTANEOUS ADMINISTRATION OF COBALT IN MICE.

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Acute toxicity of cadmium is characterized by hepatic lesion and testicular hemorrhage. Although the effects of metallothionein on cadmium toxicity have been well investigated, other mechanisms modifying cadmium toxicity have not been fully elucidated. To explore metallothionein-irrelevant modification of cadmium toxicity, we examined the effects of simultaneous administration of cobalt, which can hardly induce metallothionein, on acute toxicity of cadmium. Male ICR mice were injected with cadmium chloride and cobalt chloride simultaneously, and blood and liver samples were collected at 0, 0.5, 1, 3, 6, 12, 18, 24, 36 and 48 hrs after the treatment. The increase in GPT activity 24 hr after cadmium treatment was lowered by cobalt treatment dose-dependently. At the same time, cadmium-induced increase in concentrations of serum amyloid A (SAA), an acute phase protein, was decreased by cobalt dose-dependently. Cobalt did not induce metallothionein nor reduced tissue cadmium concentrations. Since SAA is regulated by interleukin-6 (IL-6), plasma concentrations of inflammatory cytokines were examined by ELISA. Cadmium treatment caused increases in plasma IL-6, the peak being both at 3 and 24 hr, accompanying a gradual increase in SAA. Although simultaneous cobalt treatment almost completely blocked the cadmium-induced increase in SAA, administration of cobalt alone or in combination with cadmium increased plasma IL-6 concentrations at 3 hr. Quantitative RT-PCR showed increases in mRNA levels of IL-6 in the liver of mice treated with cadmium and/or cobalt. Thus, cobalt is an inducer of IL-6, but reducer of cadmium-induced SAA. These results suggest that cytokines and acute phase proteins may be involved in the modulation of cadmium toxicity by cobalt.

## 1613

### LIVER INJURY IN METALLOTHIONEIN-NULL MICE AFTER TREATMENT WITH THIOACETAMIDE.

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This study was undertaken to understand the role of metallothionein (MT) on liver injury after chemical injury. Thioacetamide (TAA) which causes liver damage by oxidative stress was used in Wild-type (WTM) and MT-null (MT-N) mice. Both types of mice were injected with i.p. with 3 doses (150, 200 & 300 mg/kg) of TAA, and the serum enzymes alkaline phosphatase (ALP) and glutamate/pyruvate transaminase (GPT) were measured. The enzyme levels were highest at 24 hrs in MT-N, and were similar in both types of mice at 48 hrs. Histopathology examination showed necrotic hepatocytes around the central vein with infiltration of macrophages at 150 mg/kg of TAA in MT-N mice, and 200 mg/kg in WTM. Alaysis of glutathione (GSH) levels showed decrease in hepatic levels for both groups of mice 12 to 24 hrs after injection of 150 mg/kg. The hepatic MT levels were increased in WTM after injection of TAA and was highest at 12 hrs. The highest number of apoptotic bodies (TUNEL) was found in MT-null mice at 24 hrs after TAA (150 mg/kg) injection. The apoptosis was much less in wild type mice as compared to MT-null mice. These results confirm that MT-null mice with no MT was more susceptible to hepatotoxic effects of TAA than wild type mice. (supported by grants from CIHR).

## 1614

### THE NORMAL HUMAN PROSTATE EPITHELIAL CELL LINE, RWPE-1, AS AN *IN VITRO* MODEL SYSTEM OF METALLOTHIONEIN REGULATION IN THE PROSTATE.

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The prostate gland contains the highest content of zinc of any tissue in the body and this organ is sensitive to the development of tumors by the heavy metal cadmium. The intracellular handling of both these metals is controlled mainly by the

metal sequestering protein, metallothionein. In the human, this protein is produced by at least 10 closely related genes. The normal human prostate epithelial cell line, RWPE-1, has recently been utilized as an excellent model to study cadmium carcinogenesis *in vitro*. The purpose of this study was to characterize the expression of metallothionein in response to zinc and cadmium exposure. RWPE-1 cells were exposed to cadmium (3, 6 and 12  $\mu$ M) and zinc (75 and 100  $\mu$ M) for periods from 1 to 13 days. Cells were harvested for RNA and metallothionein mRNA levels were assessed with real time PCR. Cultured cells were also harvested for protein and metallothionein-1 and -2 protein levels assessed by immunodot blot with the E9 antibody. Levels of metallothionein protein attained tremendously high levels, up to 10 to 30 percent of the total soluble protein by day 4 in cadmium exposed cells, and up to 10 to 30 percent by day 1 in zinc exposed cells. Metallothionein protein expression was supported by the induction of MT-2A, -1E-, 1X, and -1A mRNA. The extreme levels of metallothionein attained during these metal exposures may relate to the zinc accumulating ability of the prostate gland.

## 1615

### METALLOTHIONEIN SUSTAINS METALLOPROTEINASE2 GENE EXPRESSION IN IMMORTALIZED FIBROBLAST CELLS.

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The metallothionein (MT), a cysteine rich and metal binding protein, is induced by various stress conditions. Recent study shows that MT, a cytosolic protein in resting cells, can be translocated transiently to the cell nucleus during cell proliferation and differentiation. These results indicate that MT may be associated with nuclear functions. However little is known the role of MT in cell nucleus. In the present study, we examined the gene expression profiles in two embryonic fibroblast cells isolated from wild-type and MT null mice using oligonucleotide micro-arrays (Intelligene mouse CHIP Set I Version 1.0, TAKARA Co.), containing 564 functionally characterized mouse genes and 301 Expressed Sequence Tags (EST). Both of cells were immortalized by SV40 large T-antigen. Three genes including matrix metalloproteinase2 (MMP2) gene were lower expressed, but other 3 genes including cryac crystalline  $\alpha$ C gene were rather higher expressed in MT null cells than wild-type cells. Lower expression of MMP2 gene in MT null cells was further confirmed by RT-PCR (Reverse Transcription- Polynucleotide Chain Reaction). The wild-type cells secreted more a 68 kDa type IV collagenase encoded by MMP2 gene than the MT null cells. These results represent that a stimulation of MMP2 gene expression by MT results in a significant secretion of a 68 kDa type IV collagenase in the wild-type cells, namely MT might regulate MMP2 gene expression. (Supported by a Grant-in-aid for General Research from the Ministry of Education, Sciences, Sports and Culture of Japan.)

## 1616

### POST-TRANSLATIONAL STABILIZATION OF METALLOTHIONEIN ISOFORM 3 IN THE NORMAL BLADDER CELL LINE, UROTS.

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Metallothionein isoform 3 (MT-3) is overexpressed in most human bladder cancers but is not expressed in normal bladder epithelium. Previous work has shown that most tumor cell lines can support the expression of MT-3, unlike nontumorigenic lines which cannot support its expression. The normal bladder cell line, UROtsa, stably transfected with MT-3 under control of the cytomegalovirus promoter makes ample mRNA for MT-3, but very little MT-3 protein. To test the hypothesis that the lack of MT-3 protein expression is due to MT-3 mediated cell toxicity during the antibiotic selection of transfected clones, the MT-3 gene was expressed under the regulatable vector system, LacSwitch. Inducing expression of the cloned MT-3 gene with isopropyl- $\beta$ -D-thiogalactoside (IPTG) gave only a modest induction of MT-3 protein and clones did not exhibit slower growth rates in the presence of this inducer; however, a basal expression of MT-3 mRNA was detected without the inducer indicating that the inhibition of clone formation by MT-3 early in the transfection can not be ruled out. To test the hypothesis that inhibitors of protein degradation could stabilize the MT-3 protein leading to accumulation similar to that found in cancer cells, MT-3 stably transfected UROtsa cells were exposed to inhibitors of the proteosome (MG-132 and lactacystin), lysosomal acidification (chloroquine), calpain family of proteases (PD150606, MDL28170, and calpeptin), cysteine proteases (leupeptin) and serine proteases [phenylmethylsulfonyl fluoride (PMSF)]. These agents were added to cultures for 8 and 24 hr, and the cells were harvested for protein. MT-3 protein levels were measured using immunodot blot utilizing an MT-3 specific antibody. MG-132 increased MT-3 at 24

hr and lactacystin did so only at 8hr. PD150606 and MDL28170 were able to induce only at very high concentrations. These data suggest that control of MT-3 levels either directly or indirectly involve the proteosome and possibly calpain proteases

**1617**

ACTIVITY OF METAL-RESPONSIVE TRANSCRIPTION FACTOR 1 IS MODULATED BY METALLOTHIONEIN.

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Zinc is an essential micronutrient involved in structural and regulatory cellular functions. Cytosolic zinc-binding protein, metallothionein (MT) is normally saturated with zinc. Zn-MT has the potential to act as an intracellular zinc pool. Destruction of Zn-MT might have implications for the bioavailability of zinc and signal transduction. Metal-responsive transcription factor 1 (MTF-1) plays an important role in the zinc-mediated transcriptional induction. In this study, we examined whether destruction of MT activates MTF-1. Activity of MTF-1 was determined by electrophoretic mobility shift assay. It is known that inhibition of protein synthesis by cycloheximide induces activation of MTF-1 and the inhibition increases degradation of protein. Using interleukin-6-treated cells and MT-overexpressing cells, we found that MTF-1 activation by cycloheximide depended on MT protein level. Also, oxidative stresses such as H<sub>2</sub>O<sub>2</sub> and *tert*-butyl hydroperoxide were used for degradation of Zn-MT. MTF-1, synthesized *in vitro* using reticulocyte lysate system, was not activated in the presence of H<sub>2</sub>O<sub>2</sub> alone. Activation of MTF-1 by H<sub>2</sub>O<sub>2</sub> required the presence of Zn-MT. In primary hepatocytes, oxidative stress activated MTF-1-DNA binding activity. This activation was not observed in MT-null hepatocytes. These findings suggest that destruction of Zn-MT activates MTF-1, and that MT plays an important role in zinc-mediated signal transduction.

**1618**

THE EFFECT OF METALLOTHIONEIN ISOFORM-3 MUTANTS ON THE DIFFERENTIATION OF BREAST CANCER MCF-7 CELLS.

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The third isoform of metallothionein, MT-3, was isolated as a down-regulated metal binding protein in Alzheimer's disease brain. The MT-3 protein was shown to possess a neuronal growth inhibitory activity that was not shared by any other member of the MT family. Studies from this laboratory have expanded the role of MT-3 as a growth regulating protein to cells outside the neural system. This was accomplished by showing that stable transfection of MT-3 into the PC-3 prostate cancer cell line or the MCF-7 and Hs578T breast cancer cell lines resulted in a marked inhibition of cell growth. Furthermore, studies from this laboratory also suggested that MT-3 might have a role in cell differentiation. This was suggested by studies which demonstrated that the stable expression of MT-3 restored active vectorial ion transport to a proximal tubule cell line that had lost this differentiated function. Active vectorial ion transport was monitored by the formation of out-of-focus areas of the cell monolayer known as domes, a hallmark of cultured renal epithelial cells that retain the *in situ* property of vectorial active transport. In the present study, site directed mutants of MT-3 were generated in an attempt to determine which epitope of the MT-3 protein was responsible for the growth inhibition noted when MCF-7 cells were stably transfected with the MT-3 gene. The first set of MT-3 mutants assessed possessed alteration in the  $\beta$ -domain of the protein, specifically, proline 7 and 9 were mutated to threonines and a mutant with the  $\beta$ -domain totally deleted from the protein. However, instead of these mutants having an effect on MCF-7 cell growth, transfection of either  $\beta$ -domain mutation into MCF-7 cell resulted in dome formation by the cells, indicating a gain-of-function for the differentiated property of vectorial active ion transport. These results suggest that the  $\beta$ -domain of MT-3 might suppress the ability of the  $\alpha$ - domain to generate a signal for cellular differentiation

**1619**

PRODUCTION OF METALLOTHIONEIN POLYCLONAL ANTIBODIES USING CHICKENS AS MODEL.

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Frequently the production of polyclonal antibodies (pAbs) against metallothioneins (MT) has been done in mouse, rabbits and lambs. In this work we describe the production of liver rat MT-pAbs in chickens. Liver MT-1 and 2 isoforms isolated from

Wistar rats (200g) treated by 3 days with cadmium chloride were used as immunogens. MT-1 and 2 were purified by exclusion (Sephadex G-75) and ionic exchange (DEAE Sephadex A-25) chromatography. Leghorn White chickens (28 weeks) were immunized with 3 doses of each isoform (100  $\mu$ g) emulsified with complete Freund adjuvant by two weeks. MT-pAbs were isolated from yolk eggs and purified by ammonium sulphate and IgY affinity chromatography. MT-pAbs were characterized by PAGE-SDS electrophoresis, western-blot and ELISA assays. MT-Abs were detected 20 days after the first immunization. Western-blot analysis showed a cross-reaction between MT-1 and 2 isoforms, however the Abs obtained did not react with other non-MT proteins in hepatic homogenates. Sensitivity assays showed that MT-pAbs detected MT-1 and 2 at nanogram levels. Data obtained suggest that chickens can be a useful model to raise MT-pAbs and is a good alternative to obtain Abs against mammal high homology proteins such as MT.

**1620**

INTERACTION OF PB AND DEET, ALONE AND IN COMBINATION WITH P-GLYCOPROTEIN EXPRESSED IN E. COLI LEAKY MUTANT.

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P-glycoprotein (P-gp), the most extensively studied ATP-binding cassette transporter, functions as a biological barrier by extruding toxic substances and xenobiotics out of cells. This study was carried out to determine the effect of DEET and PB alone and in combination on P-gp expression using *E. coli* leaky mutant transfected with *Mdr1* gene (*pT5-7/mdr1*), which codes for P-gp or lactose permease (*pT5-7/lacY*) as negative control. An *in vitro* cell resistant assay was used as an initial method for monitoring the potential of test drugs to interact P-gp. Cells were exposed to DEET, PB, daunomycin (a known P-gp substrate; was used as positive control) and reserpine (a known P-gp inhibitor; was used as negative control), it is readily apparent that P-gp confers significant resistance against PB and daunomycin while, reserpine and DEET significantly inhibited P-gp. Cells were grown in the presence of non-cytotoxic concentrations of daunomycin, PB, reserpine or DEET and membrane fractions were examined by Western immunoblotting for expression of P-gp. Daunomycin induced expression of P-gp more than PB, while reserpine and DEET significantly inhibited P-gp expression in cells harboring *mdr1*. Competitive photoaffinity labeling experiment performed with P-gp ligand [<sup>125</sup>I]-iodoarylazidoprazosin demonstrated that drugs that induced or inhibited P-gp transport activity actually bound P-gp. DEET was also found to be a potent inhibitor of P-gp-mediated ATPase activity, whereas, pyridostigmine bromide slightly increased P-gp ATPase activity. Cells expressing P-gp or *lac* permease as negative control were exposed to PB and DEET, alone and in combination. It is interesting that non-cytotoxic concentration of DEET significantly inhibited P-gp-mediated resistance against PB. This resulted in a reduction of the number of effective drug interactions with biological targets.

**1621**

HORMESIS EFFECT OF TRACE METALS ON CULTURED NORMAL AND IMMORTAL HUMAN MAMMARY CELLS.

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An *in vitro* study was conducted to determine the effects of variable concentrations of trace metals on human cultured mammary cells. Monolayers of human mortal (MCF-12A) and immortal (MDA-MB231) mammary epithelial cells were incubated in the absence or presence of increasing concentrations of arsenic (As), mercury (Hg) and copper (Cu) for 24-h, 72-h, 4-d, and 7-d. The MTT assay was used to assess viability for all time periods and cell proliferation was monitored for 4-d and 7-d studies. Monolayers were also labeled with 3 fluorescent dyes, in 24-h studies, as indicators for intracellular esterase activity, nucleic acid staining, and metabolic reduction. In addition, test medium was removed from select groups of cultures on day 4, replaced with fresh medium in the absence of chemical, and assays were performed on day 7 as above (recovery studies). The data suggest that there is a consistent protective and/or stimulating effect of metals at the lowest concentrations in MCF-12A cells that is not observed in immortal MDA-MB231 cells. In fact, cell viability of MCF-12A cells is stimulated by otherwise equivalent inhibitory concentrations of As, Cu, and Hg on MDA-MB231 cells at 24-h. Whereas As and Hg suppress proliferation and viability in both cell lines after 4-d and 7-d of exposure, Cu enhances these indicators in MCF-12A cells. MDA-MB231, however, recover better after 4-days of toxic insult. In addition, switching of media between the cell lines, or pretreatment with penicillamine, did not alter the hormesis effect displayed by MCF-12A, although proliferation of these cells was not maintained in the alternative medium. The study demonstrates that a hormesis effect from trace metals is detectable in cultured mammary cells using cell proliferation or MTT; fluorescent indicators, however, were not as sensitive. In addition, sensitivity

of mammary cells to lower concentrations of Cu, a biologically important trace metal, may play an important role in controlling cellular processes and proliferation. (Supported by NIH/NIEHS/AREA R15 ES012170-01).

1622

#### INTERACTIVE TOXICITY OF MERCURY AND POLYCHLORINATED BIPHENYL IN THE NEUROELECTROPHYSIOLOGY OF RAT EMBRYO.

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Interactive neurotoxic effects of methylmercury (MeHg) and polychlorinated biphenyls (PCB) have been difficult to monitor due to multifarious neurobiochemical responses and mechanistic differences in mode of action. This study monitored neuroexcitotoxicity of MeHg and PCB 128 during ontogenesis in rat embryo. Non-specific neuroexcitotoxicity as electrochemical (EC) potential was monitored in cultured whole rat embryo exposed to MeHg and PCB. EC potentials were generated using the micro-needle platinum electrode inserted in the diencephalon region of embryonic brain at gestation day (GD) of 19.5. The shift in amplitude of the EC potential due to exposure to MeHg and PCB at different binary combinations in saline base was observed. Total voltages developed during the binary mixture exposure concentrations were (1) MeHg (10nM) + PCB (90nM) = 60.5 (SD 6.2), (2) MeHg (50nM) + PCB (50nM) = 108.7 (SD 8.7), and (3) MeHg (90nM) + PCB (10nM) = 111.5 (SD 12.4) on GD 19.5 day. These mixtures generated higher voltage potentials than the corresponding concentrations exposed to individual chemicals at similar exposure conditions. The increase in the EC potential due to binary combinations of PCB and MeHg are predominant synergistic responses of neuronal network stress and also attributed to specific uptake and ionization of these compounds. This methodology can be used as a quantitative risk assessment tool for studies on neurotoxic chemical mixtures and the quantification of the ion regulation expressed in mV also provides an insight for the mechanisms of neuro-response to the mixture of PCB and MeHg.

1623

#### COMPARATIVE EVALUATION OF SURFACTANTS ON IN VITRO RABBIT AND HUMAN CORNEAS.

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**Purpose:** To develop an *in vitro* technique for comparison of corneal toxicity of surfactants in isolated rabbit and human corneas. **Methods:** Rabbit eyes were obtained from Pel-Freeze and human corneas, stored in Optisol GST<sup>TM</sup>, were obtained from the Georgia Eye Bank. Corneas were isolated and mounted for endothelial perfusion in an *in vitro* specular microscope. Surfactants (cetyl alcohol, CA; sodium lauryl sulfate, SLS; benzalkonium chloride, BAC; sodium sulfolaurate mixture, SSL) were applied to corneas with and without an intact epithelium. These 4 surfactants represent a range of Draize ocular irritation. The corneal endothelium was continuously perfused with a balanced salt solution. The effects of 3% SLS, 39% SSL and 1% BAC on the rabbit corneal tissue were evaluated after 3-, 9- and 20-min exposures and on the human corneal tissue after 3-min. At the end of the exposures, SLS, SSL and BAC were removed and the corneas perfused for 3 hr before being fixed for histological evaluation by light and electron microscopy. The effect of CA was similarly evaluated immediately after a 4-hr exposure in both species. **Results:** In the rabbit cornea, SSL, SLS and BAC showed time dependent corneal swelling and structural changes with associated damage to the endothelial layer. With both SSL and SLS, the 3-min exposure to the intact cornea caused epithelial damage and minimal stromal and endothelial changes. By comparison, when the epithelium was removed, SSL and SLS caused marked stromal and endothelial changes. After 9- and 20-min exposures (with and without epithelium), SSL and SLS caused marked changes in all layers of the cornea. At all time points, BAC caused extensive epithelial, stromal and endothelial damage. In both species, CA showed minimal corneal swelling and marked epithelial changes with no change to the endothelium. Human corneas exposed to the other 3 surfactants for 3-min showed the same corneal alterations as observed in the rabbit corneas. **Conclusion:** In both species, the rank order of corneal damage paralleled the Draize ranking: CA(SLS=SSL)(BAC).

1624

#### ESTABLISHMENT OF AN IN VITRO BRAIN BARRIER EPITHELIAL TRANSPORT SYSTEM FOR TOXICOLOGICAL STUDIES.

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The blood-cerebrospinal fluid barrier (BCB), which possesses tight junctions (TJ) between adjacent epithelia, is located in the choroid plexus in brain ventricles. An immortalized Z310 murine choroidal epithelial cell line was created in this labora-

tory (Zheng & Zhao, *Brain Res.*, 2002). The purposes of this study were (1) to identify representative TJ proteins in Z310 cells and (2) by using this cell line to develop an *in vitro* BCB transport model. Both RT-PCR and Western blot confirmed the presence of occludin, claudin-1 and ZO1, but not claudin-2 in Z310 cells. When Z310 cells were cultured on a two-chamber Transwell device, the permeability of [<sup>14</sup>C]sucrose across the cell monolayer, which allows for quantification of paracellular diffusion, was  $(5.98 \pm 0.27) \times 10^{-4}$  cm/min and TEER (transepithelial electrical resistance), which is an instantaneous measurement of ionic conductivity, was  $61.3 \pm 4.5$  ohm-cm<sup>2</sup>. Four approaches were used to improve the tightness of Z310 barrier: (1) Culturing the cells in astrocyte-conditioned medium increased TEER by  $33.0 \pm 2.7\%$  of controls (Z310 cells without treatment); (2) introduction of dexamethasone (1  $\mu$ M), which increases recruitment of TJ proteins, to the culture medium increased TEER by  $50.4 \pm 7.6\%$ ; (3) the presence of eicosapentaenoic acids (EPA) (10  $\mu$ M), an inducer of TJ proteins, increased TEER by  $38.4 \pm 2.3\%$ ; and (4) addition of EGF (100 ng/ml) to improve the cell differentiation increased TEER by  $40.3 \pm 8.9\%$ . Among tested chemicals, only dexamethasone significantly reduced [<sup>14</sup>C]sucrose paracellular permeability about 200-300% of controls. These data suggest that culture of Z310 cells on Transwell device in the presence of dexamethasone significantly increases tightness of Z310 cell monolayer (TEER and space marker permeability), which is highly comparable to primary culture of choroid plexus epithelial cells. This *in vitro* model appears to be suitable for transepithelial transport study of drugs and toxicants. Validation by using model drugs is in progress. (Support by Johnson & Johnson Focused Giving Program)

1625

#### A GERM-LINE STEM CELL LINE AS A MODEL FOR EVALUATING THE CYTOTOXICITY OF NANOPARTICLES *IN VITRO*.

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The purpose of this study was to assess the suitability of a spermatogonial stem cell line as a sensitive model for toxicity studies in the male germ line, in particular for the cytotoxicity of nanoparticles. Gametogenesis is a complex biological process that is particularly sensitive to environmental and exogenous chemical insults. Because germ line stem cells self-renew and require specific conditions for their growth, many chemicals have a negative impact on their normal function. Ultimately, these effects can inhibit fertility, may have negative consequences on the development of the offspring, or can generate carcinoma. Recently, new nanomaterials such as diverse nanoparticles, fabric with nanotubes, nanowire for efficient energy transfer, lubricants with fullerene derivatives, and targeted quantum dots have received enormous attention to create new types of engineering tools including those for direct use in life systems. Nanomaterials, which are by definition of 1-100 nanometer in length, are being actively pursued to create materials with even more novel physical/chemical properties. However, there is a serious lack of information concerning their impact on human health and the environment. In the present study, the effects of different types (CdO, Ag, Mb and Al) and different concentrations of nanoparticles on spermatogonial stem cells were evaluated using light microscopy, cell proliferation and standard cytotoxicity assays. Our results demonstrate a concentration-dependent toxicity for all types of particles tested. As expected, CdO nanoparticles were highly toxic (EC50=0.5  $\mu$ g/ml), and were used as a positive control for system validation. Of the novel nanoparticles studied, Ag particles were the most toxic (EC50=7.5  $\mu$ g/ml) while Mb nanoparticles were the least toxic (EC50=75  $\mu$ g/ml). Further, our data suggests that spermatogonial stem cells provide a much more sensitive model than somatic cells to assess the cytotoxicity of nanoparticles *in vitro*.

1626

#### VALIDATION OF AN IN VITRO CELL LINE FOR SCREENING MYELOTOXICITY.

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Screening for potential bone marrow toxicants early in drug discovery may be useful in reducing compound attrition. Currently, *in vitro* colony-forming unit (CFU) assays, which are low throughput, are used to evaluate hematotoxicity prior to *in vivo* toxicology studies. The purpose of the present work was to develop a 96-well assay using murine M1 and FDCP1 myeloid cell lines as potential surrogates for the mouse CFU-GM assay; such an assay would significantly improve throughput. Relative cell number was determined by a luminescent-based assay that measures intracellular ATP. The assay was linear at seeding densities of 400 to 40,000 cells/well with a density of 4,000 cells/well chosen to ensure linearity of the signal after 4 population doublings or 96h. To validate the cell line assay, known myelotoxicants were used as positive controls and hepatotoxicants were used as negative

controls. Cells were treated with compound (0.3 $\mu$ M to 300 $\mu$ M) for 72h and ATP measured. Some of the compounds were also analyzed for hematotoxicity with the CFU-GM assay and comparisons were made with data reported in the literature where possible. The myelotoxicant carboplatin was potent in CFU-GM (IC50; 5.54 $\mu$ M), M1 (IC50; 4.5 $\mu$ M), and FDCP-1 (IC50; 30 $\mu$ M) assays, but less potent in erythroid cells (IC50; 113 $\mu$ M). M1 cells were generally found to be more sensitive than FDCP-1 cells and were used for subsequent experiments. Chloramphenicol, an erythrotoxicant, was not toxic in the M1 assay (IC50; >300 $\mu$ M), but was toxic to erythroid cells (IC50; 8 $\mu$ M), further indicating selectivity of the M1 assay. The hepatotoxins were not toxic to M1 cells. There was a good correlation ( $r^2=0.91$ ) between M1 and CFU-GM assays for 6 tested compounds. 13 other compounds screened through the M1 assay were compared with literature mouse CFU-GM data and there was a similar rank order of toxicity for the 2 assays, again showing a good correlation. In conclusion, the M1 cell line assay is a potential surrogate for the CFU-GM assay in predicting myelotoxicity.

## 1627 CORRELATION OF *IN VITRO* CYTOTOXICITY WITH PARACELLULAR PERMEABILITY IN CACO-2 CELLS.

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This *in vitro* study aims to develop a cell culture model that compares paracellular permeability (PP) with acute cytotoxicity (AT). Caco-2 cells were seeded in 96-well plates and on polycarbonate filter inserts. Confluent monolayers were exposed to increasing concentrations of 20 reference chemicals for 24-h and 72-h. Cytotoxicity was determined using MTT and NRU cell viability assays in 96-well plates. Transepithelial electrical resistance (TEER) for PP was measured in culture inserts. Inhibitory concentrations 50% (IC50s) suggest that there were good correlations between 24-h and 72-h exposures. NRU IC50 values correlated better with TEER, which is consistent with the Registry of Cytotoxicity (RC; ICCVAM) database report. Both cell viability assays were more sensitive than TEER measurements. In fact, 24-h and 72-h NRU assays, and 72-h TEER measurements displayed the highest correlations with established rodent LD50s. In addition, [<sup>3</sup>H]- and [<sup>14</sup>C]-mannitol, lucifer yellow (low mw indicators), and FITC-dextran (higher mw indicator) were used to fully understand the influence of chemical agents on PP. In our experiments, passive paracellular transport of the tight junction markers parallels the IC50s determined with the viability assays and TEER measurements. Our AT/PP model thus allows for the differentiation between the concentrations necessary for AT and those needed to interfere with PP. Development of an *in vitro* test system for PP, in combination with acute toxicity, strengthens the reliability of the intestinal cell assay. Thus, we propose that the AT/PP model be used to compute a formula that can "normalize" and improve the predictive ability of *in vitro* acute cytotoxicity assays for *in vivo* lethality. (Supported by NIH/NIEHS/AREA R15 ES012170-01).

## 1628 PROTOCOL OPTIMIZATION FOR THE EVALUATION OF *IN VITRO* CYTOTOXICITY ASSAYS FOR ESTIMATING RODENT AND HUMAN ACUTE SYSTEMIC TOXICITY.

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NICEATM and ECVAM initiated a three-phase multi-laboratory validation study to evaluate the usefulness of two *in vitro* basal cytotoxicity assays for estimating human acute toxicity and starting doses for acute rodent lethality testing. Seventy-two coded chemicals exhibiting a wide range of toxicity were tested in mouse 3T3 fibroblasts and normal human keratinocytes (NHK) using neutral red uptake (NRU) to assess cytotoxicity. A phased approach optimized the protocols between phases to enhance intra- and inter-laboratory reproducibility. Phase Ia established historical databases for the positive control for each of the 3 labs. Protocols were modified to prevent NR crystal formation and improve cell growth. Following Phase Ib testing of 3 coded chemicals, NR concentration was further reduced in the 3T3 assay to prevent crystals. Phase II testing of 9 coded chemicals prompted adoption of plate sealers to improve testing of volatile chemicals and more rigorous solubility procedures to dissolve less soluble chemicals. For Phase III, a procedure to prequalify NHK medium to assure it promoted adequate cell growth was added. As the phased data were evaluated, test acceptance criteria were also revised. This study highlights the value of a phased approach that allows data evaluation and protocol optimization prior to each subsequent phase. The authors recommend more and

smaller phases at the beginning of such studies to quickly and efficiently optimize a standard test method protocol for use in the main study. Supported by: NIEHS contracts N01-ES-85424 and N01-ES-75408; EPA IAG DW-75-93893601-0; European Commission contract No. 19416-2002-04 F2ED ISP GB

## 1629 ETHANOL-INDUCED TOXICITY AND APOPTOSIS IN HEPG2 CELLS AND VA-13 CELLS: ROLE OF FATTY ACID ETHYL ESTERS.

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Oxidative metabolism of ethanol is primarily catalyzed by hepatic alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (cyp 2E1). Earlier, we reported significantly higher synthesis of fatty acid ethyl esters (FAEEs, nonoxidative metabolites of ethanol) in hepatocellular carcinoma (HepG2) cells (ADH deficient) as well as HepG2 cells transfected with human cyp 2E1 (E47 cells) than that in HepG2 cells transfected with murine ADH I (VA-13 cells). Therefore, we determined apoptosis in HepG2 and VA-13 cells by ELISA and DNA ladder formation after ethanol exposure (100-800 mg %). Individual FAEEs were analyzed at 6 hr (optimal time for FAEE formation) and toxicity was evaluated by MTT assay and lactate dehydrogenase (LDH) leakage. Although no significant change in viability of the cells was observed at all the concentrations of ethanol, apoptosis was significantly higher in HepG2 cells than VA-13 cells exposed to 800 mg % ethanol. Surprisingly, LDH release was diminished with increasing concentration of ethanol in HepG2 cells as compared to none in VA-13 cells. Formation of FAEEs was found to be dose-dependent, and 16:0, 18:0 and 18:1 FAEEs were major FAEEs synthesized in HepG2 cells (5.3, 8.2 and 34.1 nmol/25 million cells, respectively) as compared to only trace amounts in VA-13 cells. The levels of FAEEs in HepG2 and VA-13 cells and extent of apoptosis and diminished LDH release indicate role of FAEEs in ethanol-induced toxicity and apoptosis. Therefore, lack of ADH in HepG2 cells favors nonoxidative metabolism of ethanol to FAEEs and be responsible for the ethanol-induced toxicity and apoptosis in HepG2 cells.

## 1630 OPTIMIZATION OF AN *IN VITRO* LONG TERM CORNEAL CULTURE ASSAY.

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The long-term culture of corneas has been proposed as an *in vitro* model to evaluate potential eye irritation and post-treatment recovery following chemical exposures. Techniques used were modifications of those of Foreman, DM (1996), Xu, KP (2004) and Boulton, M (2003). Porcine eyes were obtained from Sioux-Preme within 24 hours of sacrifice. The eyes were disinfected by immersion in 1% povidone iodine followed by 0.1% Gentamicin in PBS. Excised corneas were filled with an agar/gelatin gel in M199 medium to support the corneas, and were cultured at 37°C, 5% CO<sub>2</sub>, 90% RH in M199 medium to the limbus, leaving the epithelium exposed to air. The corneas were moistened by brief immersion in medium every 2.7 h using a modified plate rocker. To induce damage, corneas were treated with either 1 or 3% SLS, or H<sub>2</sub>O (controls) for exposures of 2, 5 or 10 min. The corneas were rinsed with PBS, cultured for 6, 24 or 48 h, and then fixed in buffered formalin to determine their responses to damage and potential recovery. H&E-stained preparations of control corneas showed normal morphology throughout the 3-day assay, and were comparable to excised/immediately fixed corneas. Controls were characterized by an intact epithelium with viable squamous, wing, and basal cells. The stroma showed frequent viable keratocytes, and minimal swelling indicative of a functional endothelium. The endothelium was typically intact. Corneas treated with 1% SLS for 2 min showed no pathological changes, while exposure to 3% SLS for 2 min induced just slight hyper-eosinophilia in the squamous epithelium. However, corneas treated with 3% SLS for 5 or 10 min showed complete epithelial cell damage or loss 24 h after treatment, as well as loss of viable keratocytes in the upper stroma. By 48 h after treatment, evidence of epithelial cell sheet migration along the basal membrane into the damaged zone was observed. These results confirm the ability to culture porcine corneas for at least 72 hours, and demonstrate the potential for further optimization for the evaluation of recovery after induction of chemical damage.

## 1631 AN EXPANDED REFERENCE DATA-BASE OF *IN VITRO* OCULAR IRRITATION SCORES FOR MARKETED COSMETIC AND PERSONAL CARE PRODUCTS USING A TISSUE EQUIVALENT MODEL.

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A significant advantage of the MatTek EpiOcular OCL-200 tissue model is that it can be used to discriminate between the eye irritation potential of extremely mild products (e.g., most cosmetic and personal care products). We have previously re-

ported how we have used the MatTek model (comprised of human epidermal keratinocytes which form a stratified squamous epithelium similar to corneal tissue) to establish ranges of *in vitro* ocular irritation scores for several categories of cosmetic and personal care products (McCain et al., 2002). These data provided us with a reference database of *in vitro* ocular irritation scores for a cross-section of their currently marketed cosmetic and personal care products. Comparison of the ET50 for prototype formulations with the appropriate range of ET50 scores for marketed products of similar type has proved a useful benchmark of the anticipated consumer acceptability of new products in development. We now report data resulting from our efforts to expand and refine our database as a result of testing over 80 additional marketed products. As reported previously, materials were tested using either the standard (4 hours) or extended (>20 hours) exposure protocol based on their expected irritation potential (i.e., consideration of formula composition, product type, etc.). Cellular viability was used as a marker for irritation potential and measured at various time points by a MTT metabolism colorimetric assay. MTT was quantitated spectrophotometrically at 570nm and an ET50 (time to 50% loss of viability) calculated for each product and a range of *in vitro* ocular ET50 scores was then determined for each product category. Our results provide additional confidence in the data previously determined and establish ranges of *in vitro* ocular irritation scores for several new categories (e.g., eye makeup removers) and sub-categories of products (e.g., adult shampoos, baby shampoos, dandruff shampoos) which can be used as benchmarks for the evaluation of similar, prototype formulations.

### 1632

#### EFFECT OF THE PROTEASOME INHIBITOR PS-341 ON NEURONAL CELLS *IN VITRO*.

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Peripheral neuropathy is a common adverse effect of many chemotherapeutic drugs. The proteasome inhibitor PS-341 for treatment of multiple myeloma, has induced peripheral neuropathy in a subset of patients. The mechanism of PS-341-induced peripheral neuropathy is unknown. The objective of this study was to investigate the effect of PS-341 on neuronal cells *in vitro*. Unlike myeloma cells, GT-1 murine neuronal cells that were exposed to high concentrations of PS-341 for 16 hours showed increased cell death via a non-apoptotic mechanism as assessed by TUNEL and DNA fragmentation assays. Immunofluorescence, Western, and Immunostaining analysis showed non-ubiquitinated actin microfilament and microtubule organization and relocation to the perinuclear region in PS-341 treated cells. At the same time, other proteins became ubiquitinated or upregulated including ubiquitin and other stress proteins. In the PS-341 treated cells there was also perinuclear-centrosomal accumulation of  $\gamma$ -tubulin, c-myc, and vimentin, which are known to be regulated by proteasomal degradation. There was also an accumulation of RelA, GRP78,  $\gamma$ 1-adaptin, and LAMP proteins in the perinuclear region. Biochemical analysis indicates that PS-341 induced accumulation of protein aggregates in GT-1 cells are detergent - and mechanical - disruption resistant. Similar results were observed in GT-1 cells treated with Lactacystin. In conclusion, PS-341 causes cytoskeletal reorganization and intracytosolic accumulation of diverse ubiquitinated and non-ubiquitinated proteins in the perinuclear region and potentially overloads the endoplasmic reticulum-dependent quality control mechanism. These processes acting alone or in combination are hypothesized to affect axonal transport or other aspects of cellular homeostasis and thus result in neuropathy.

### 1633

#### DEVELOPMENT OF *IN VITRO* CARDIOTOXICITY ASSAY USING RAT CARDIOMYOCYTES CULTURED ON COLLAGEN GEL.

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In early stages of drug discovery, highthroughput *in vitro* culture systems are very important for the evaluation of toxicity. However, the functions of the cells rapidly diminish in conventional monolayer cultures. To establish an *in vitro* cardiotoxicity evaluation system that maintains cardiomyocyte (CMC) functions, collagen gel cultures of rat CMCs were investigated. SD rats (0-day- to 5-week-old) were anesthetized; the ventricle was isolated, minced, and digested by collagenase and trypsin. The prepared CMCs were suspended in culture medium and cultured on non-treated plates (monolayer) or plates with type I collagen gel (CG) or CG + Matrigel (MG). The cultured cells were morphologically observed and immunohistochemically stained with anti-connexin 43 (specific to gap junctions in the heart) to characterize each culture condition. Doxorubicin was added to the cultures and the lactate dehydrogenase in the cells and the supernatant were measured to compare the susceptibility to known cardiotoxic compounds. In the monolayer, fibroblast-like cells gradually proliferated and, within 1 week, became the major population in the culture. In the culture on CG, the proliferation of fibroblasts was less

severe and higher numbers of the cells were beating than in the monolayer. By the addition of MG in the CG, cell-cell contacts were augmented and the number of beating cells increased. The staining patterns of connexin 43 in the cultured CMCs on CG+MG (1-week culture) were similar to the intact heart, but there was very little positive staining in the monolayer. This suggests that the monolayer is not suitable for long term culture of CMCs. The cells were more susceptible to doxorubicin in CG and CG+MG than in monolayer. These results show that rat CMC cultures on the CG (+MG) would be effective to maintain cellular functions of CMCs. This culture condition would be suitable for the evaluation of cardiotoxicity as it more resembles *in vivo* conditions than the monolayer.

### 1634

#### EVALUATION OF CIGARETTE SMOKE CYTOTOXICITY UNDER *IN VITRO* CONDITIONS.

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*In vitro* assays could be used to examine toxicity of cigarette ingredients, design, and prototypes. Several accepted smoking regimens (i.e., ISO and Canadian Intense (CINT) are used to prepare extracts that could be used to determine their biological effects. The first goal of this study was to compare the cytotoxicity of frozen cigarette smoke condensate (CSC) as determined by the neutral red uptake (NRU) assay in 3 different types of mammalian cell lines (HepG2 and BALB/c 3T3 and CHO) under *in vitro* conditions. The second goal was to compare the cytotoxicity of fresh CSC (also referred to as particulate phase or PP), gas vapor phase (GVP), and whole smoke (WS) fractions, prepared under ISO and CINT smoking regimens, in HepG2 cells. Samples were generated on a smoking machine. The PP was collected on a filter pad and extracted in DMSO. GVP was smoke fraction that was trapped in phosphate buffered saline after passing through a filter pad. WS was collected by trapping cigarette smoke in PBS and DMSO. Smoke samples were used to expose cells 1h following extraction. NRU assay was performed after 24 and 48 h incubation for HepG2 and 3T3 cells, respectively. NIH has established a standard operating protocol for the NRU using BALB/c 3T3 cells, however, it did not exclude the use of other cell lines. CSC was more cytotoxic in HepG2 cells than in 3T3 and CHO cells. Comparing cigarette smoke fractions obtained under ISO and CINT conditions showed that smoking under the later conditions has resulted in a more cytotoxic extract than ISO. PP was more toxic than GVP or WS under both regimens. No significant differences between the GVP and WS under the ISO regimen were seen. Under CINT regimen WS was only moderately more cytotoxic than the GVP. Much of this may be attributable to the collection method. Chemical analysis of the gas vapor phase indicated that the majority of the volatile compounds were not captured in the PBS solution. New trapping solutions will be developed in order to enhance capture of volatile and semi-volatile compounds without adversely affecting cell growth.

### 1635

#### *IN VITRO* MODEL FOR VASCULAR DEVELOPMENT IN MOUSE EMBRYONIC STEM CELLS.

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Embryonic stem (ES) cells serve as a most popular model system to study early mammalian development. The objective of this study is to drive more simple and efficient vascular development without genetically modified ES cells and to examine the specific gene expression pattern during the differentiation, finally to finding biomarker can be applied for toxicological evaluation. To induce vascular development, embryoid body was formed for 3 days by hanging drop culture and replated on gelatin-coated plate in EGM-2 medium containing VEGF, ascorbic acid, bFGF, IGF, and EGF. When placed in matrigel, these mouse ES cell-derived endothelial cells formed networks similar to vascular structures and observed the expression of Flk1, PECAM1 and VE-cadherin by immunocytochemical analysis within 4-11 days. The gene expression of Flk1, PECAM1 and VE-cadherin were confirmed by RT-PCR analysis. At different time points during the differentiation process, cell surface differentiation antigen such as Flk1 and PECAM1 expression were analyzed by flow cytometric analysis. When sorted Flk1+ cells were replated in 10 % FBS medium containing VEGF(50ng/ml), Flk1+ cells formed vessel-like structure in three-dimensional culture. These preliminary data suggest that Flk+ cells are suitable candidate for studying specific marker genes during vascular development.

### 1636

#### HISTOPATHOLOGICAL BACKGROUND FINDINGS IN THE COMMON MARMOSET (CALLITHRIX JACCHUS).

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The marmoset model is being used increasingly in toxicological studies with biotech compounds as a second non-rodent species. Compared to other non-rodent species the benefits of this primate model are its small size, and easy and less

hazardous handling. Whereas much information is available for cynomolgus monkey and rhesus monkey, little is known about the incidence and occurrence of spontaneous histopathological lesions in marmosets used in toxicological research. In total, 110 marmosets from 10 different toxicological studies performed during 1995 - 2000, were examined. All animals were control animals. A complete necropsy was performed on all animals and organs were examined histopathologically. Spontaneous histopathological lesions in marmosets that were not seen in cynomolgus monkeys or rhesus monkeys included: Extramedullary haematopoiesis in brain, lung, adrenals, liver, spleen, kidney; C-cell hyperplasia in thyroid; ectopic parathyroid gland in thymus; myocardial fibrosis in heart; osseous metaplasia in lung; microgranuloma in liver; Ito-cell vacuolation in the liver; cholecystitis in the gallbladder; Brunner's gland dilatation in duodenum; chronic enteritis in large intestine and luteal hyperplasia in ovary. The interpretation of the histopathological results of toxicological studies requires a precise knowledge of the background pathology of the laboratory animal species used. It is important for the study pathologist working with marmosets to know the background lesions described, to avoid misinterpretation as treatment-related changes. In conclusion, marmosets show a number of spontaneous histopathological background findings that appear restricted to this laboratory primate species.

**1637 HISTOPATHOLOGY DATABASE FOR CARCINOGENICITY STUDIES WITH SPRAGUE-DAWLEY RATS.**

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From recent carcinogenicity studies in Sprague-Dawley (SD) rats, Charles River Laboratories DDS - Arkansas Division has compiled the incidence of neoplasms in routinely examined tissues. The SD is an outbred strain that is meant to change over time. Therefore, more recent studies with adequate statistical power were selected to reveal such trends. Neoplastic data were exported from a validated pathology software (LABCAT 4.33) as alpha-numeric fields, imported into linked-query tables (Microsoft ACCESS 2003) then sorted using organ name as the primary key. The results were reported as tissue/organ, histopathologic classification, number of rats, total number of neoplasms and incidence. The tissue/organ incidence was calculated for each sex. In males (n = 330) the highest incidences of background histopathologic neoplasms were pituitary gland adenoma (61.8%), adrenal gland benign pheochromocytoma (13.0%), and pancreas islet cell adenoma (7.2%). In females (n = 260) the highest incidence of background histopathologic neoplasms were pituitary gland adenoma (90.4%), mammary gland fibroadenoma (40.4%), and mammary gland adenocarcinoma (29.2%). Laboratory-specific incidences of neoplasms from recent studies are important for distinguishing background lesions from test article-related findings in toxicology studies with outbred animals.

**1638 A COMPARISON OF PATHOLOGICAL CHANGE AND SURVIVAL IN DIFFERENT RODENT STRAINS OVER A TWO-YEAR STUDY DURATION.**

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We have undertaken a comparison of a number of rat and mouse strains commonly used in the assessment of oncogenicity and chronic toxicity. Specifically, two mouse strains (CD-1 and B6C3F1 from Charles River UK) and two rat strains (HsdBrHan<sup>TM</sup>:WIST and Hsd:Sprague-Dawley<sup>®</sup> SD<sup>®</sup> from Harlan UK) have been evaluated along side the most frequently used strains at these laboratories (C57BL10JAlpk mouse and the Alpk:APrSD Wistar Rat). The purpose of this work was to describe and understand the spontaneous biology of a variety of rodent strains in order to be able to use them in toxicology studies in our laboratory. The strains were assessed over a two-year study duration using routine toxicological parameters that included clinical observation, bodyweight, food consumption and ophthalmoscopy. For rats functional observation battery assessments and motor activity were also assessed. At a number of intervals, corresponding to routine regulatory study durations (28 and 91 days, 6 months, 80 and 104 weeks for mice, and 28 and 91 days, 52 and 104 weeks for rats), animals were killed and blood samples collected for clinical pathology evaluation. A full list of organs was weighed and a detailed examination post mortem was completed. Tissues were also examined by light microscopy. Overall there were no significant differences in clinical condition and husbandry requirements from those of the most frequently used strains at our laboratory. Although there were differences in the absolute bodyweights of each strain, overall the strains had good bodyweight development. The survival of both rat strains was as good or better than that of the Alpk:APrSD Wistar. The survival of the B6C3F1 mouse was better than that of the C57BL10JAlpk mouse, and although the survival of the CD-1 mouse was generally poorer, it was generally within the range of survival reported by Charles River. This data provides a unique description of the survival characteristics and comparison of pathological change seen over time in these rat and mouse strains.

**1639**

**IN-LIFE PARAMETERS AND TUMOUR DATA FROM CHARLES RIVER CRL:CD-1 ® (ICR) BR MOUSE DIETARY AND ORAL GAVAGE TUMORIGENICITY STUDIES TERMINATED AFTER TWO YEARS.**

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The in-life and tumour data have been analysed from the control groups of up to 25 tumorigenicity studies to confirm the profile of the Charles River Crl:CD-1 ® (ICR) BR mouse and to compare any possible differences between dietary and oral gavage routes of administration. The mice were housed one or two per cage and the studies were completed over 1995 to 2001. Previous analyses showed mortality values at 2 years of  $52 \pm 10.4\%$  for males and  $56 \pm 8.1\%$  for females, with the values being similar for dietary and oral gavage studies (1). Analysis of bodyweight gain over the first year revealed lower values for oral gavage studies (both sexes) when compared with dietary studies. There was no similar difference in food consumption. Assessment of the tumour profile, from studies terminated at 2 years, has shown that the most prevalent tumours were hepatocellular adenoma/adenocarcinoma (higher in males, with a notably higher incidence of adenoma in dietary studies), bronchioalveolar adenoma/adenocarcinoma and Harderian gland adenoma (males and females), haemopoietic tumours (higher in females), and uterine polyps (females). With the exception of the latter, these tumours were also the major factors contributory to death (FCTD). Other major FCTD included urinary tract lesions (males) and ovarian haemorrhagic cysts (females), with a slightly higher incidence of these findings in oral gavage studies. Additionally, when dietary studies terminated at 18 or 24 months were compared, the longer duration studies showed a higher incidence of the major tumour types and in the total number of tumour types identified. In conclusion, although there were some differences in the in-life parameters, tumour profile and FCTD between the routes of administration, the profiles were largely similar and that continuing the studies to 2 years results in an acceptable, and perhaps a more developed, tumour profile. (1) Hooks, W.N. *The Toxicologist*, 72: 1863 (2003).

**1640**

**SPONTANEOUS LESIONS IN CRJ:CD-1(ICR)-NU/NU MICE BY 106 WEEKS OF AGE DURING THE OBSERVATION PERIOD.**

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[Backgrounds] Spontaneous lesions of nude mice, especially those in the life-span period, have not been fully reported. We examined the incidences and types of spontaneously developed lesions of Crj:CD-1(ICR)-nu/nu nude mice in the lifetime periods. One hundred and sixteen male and 117 female nude mice of five weeks of age were purchased from Charles River Japan, Inc. and were kept in the air-conditioned barrier system room. All the animals were allowed free access for the food (radiation sterilized CRF-1 LID6, Oriental Yeast Co.) and sterilized water. Mice were monitored for body weight, survival period, clinical signs, and post-mortem pathology. [Results] Body weights increased for 31 weeks, and attained at plateau thereafter. Mean survival time was  $65.3 \pm 1.8$  weeks in the males and  $61.3 \pm 8.8$  weeks in the females and 94% of males and 99% of females died in 106 weeks of age. Moribund animals showed emaciation, abdominal distension and subcutaneous mass or nodule. Swelling of spleen, lymph node and liver and discoloration of kidneys were observed in the autopsy. In the histopathological observation, these lesions were diagnosed as malignant lymphoma or glomerular lesions. In the course of the observation period, incidences of malignant lymphoma attained to 71% in males and 80% in females. Moreover, hepatocellular adenoma and carcinoma in the liver, glandular dilatation in the seminal vesicle, glandular hyperplasia and polyp in the uterus, atrophy and follicular cyst in the ovaries also increased in number with age. [Conclusion] In the present study, predominant neoplastic lesion was found as malignant lymphoma in the higher frequency from Crj:CD-1 (ICR) mice (8% in the males and 22% in the females, in the literature), which strongly suggests the close relevance with T-cell deficiency in the nude mice.

**1641**

**THROMBOCYTOPENIC PURPURA IN GOTTINGEN MINIPIGS.**

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Thrombocytopenic purpura syndrome has been reported to occur spontaneously in sexually mature Gottingen minipigs at a low incidence (0.1%, 11/11, 637) in a closed breeding colony. [Carasco et al, 2002]. Thrombocytopenic purpura, also referred to as hemorrhagic syndrome, is of unknown etiology and is also seen in the normal pig. The corresponding syndrome in humans is von Willebrands disease. In

our laboratory, this syndrome was noted in two Gottingen minipigs of 306 (0.65%) tested between 2000 and 2002, a high incidence relative to that reported in the literature. The minipig is a non-rodent laboratory animal used extensively in safety evaluation of dermally-administered materials and is increasingly being used as an alternative to dogs and non-human primates for evaluation of drugs administered by a variety of routes. The Gottingen minipig is popular for safety evaluation because of its small size, slow growth rate and non-pigmented skin. Therefore, as use of this animal increases, it is likely that an increasing number of cases of thrombocytopenic purpura will be observed. This poster describes observations for the two cases seen in Gottingen minipigs in our laboratory. The disease had a rapid onset and was characterized by disseminated hemorrhage associated with marked reductions in numbers of circulating thrombocytes (platelets). Both animals were 5 month-old females. Clinical signs included pallor, lethargy, coalescing petechiae on the abdomen, face, neck and limbs, palpable swelling under the jaw and blood beneath the cage. Both animals showed rapid deterioration and were euthanized shortly after the first appearance of signs. Clinical pathology evaluations revealed marked thrombocytopenia, anemia, hematuria, proteinuria and glycosuria. Microscopically, interstitial hemorrhages were present in most organs and most lymph nodes contained large amounts of free erythrocytes in the subcapsular and medullary sinuses. The bone marrow was hypercellular and had numerous degenerating megakaryocytes with granulocytic hypereosinophilic cytoplasm and pyknotic nuclei. Kidneys had minimal focal membranous glomerulonephritis.

## 1642

### CLINICAL PATHOLOGY DATABASE FOR NON-CLINICAL GLP STUDIES WITH LANDRACE -CROSS SWINE.

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The objective of this study was determine the clinical pathology reference ranges for control male and female Landrace-Cross swine used on non-clinical GLP studies conducted at Charles River Laboratories Discovery and Development Services - Arkansas Division. The Landrace-cross pig has been used as a biomedical research model for urolithiasis, myocardial disease and transplantation, bone and cartilage repair, phototoxicity, and many other conditions. The pig has been used on a limited basis as a non-rodent regulatory model in non-clinical drug toxicity studies. Clinical pathology data were exported from a validated pathology software (LAB-CAT 4.43) as alpha-numeric fields, imported into query tables then sorted by parameter name and individual reference ranges were calculated. Six studies were selected with animals of approximately 2 – 6 months of age (males 8 to 60 kg, females 9 to 55 kg). The hematology (n = 85), coagulation (n = 40) and clinical chemistry (n = 93) parameters were reported for each sex as a range of two standard deviations from the mean along with total number of pigs sampled. Laboratory specific background of clinical pathology reference values for the Landrace-cross pig, provides guidance for interpreting test article-related findings in toxicology studies and to determine the appropriateness of this species for regulatory risk.



## 1643 DEVELOPMENTAL TOXICOLOGY OF THE LUNG.

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Respiratory problems account for a large proportion of hospital admissions to pediatric wards, and the incidence of childhood asthma is increasing for unknown reasons. A number of toxicants have been demonstrated to adversely affect lung development in humans and/or laboratory animals. Lung development begins early in gestation and continues well after birth. The molecular control of normal lung development has been intensely studied and is becoming well elucidated, involving multiple complex signaling networks. In contrast, mechanisms of toxicity to the developing lung are poorly understood. This symposium will present an overview of normal lung development and examples in humans and laboratory animal species of chemicals and conditions that adversely affect lung development. Effects of tobacco smoke and constituents have been well-studied and will be highlighted. Possible modes of action for developmental toxicity to the lung will be presented and research needs will be discussed.



## 1644 GENETIC PATHWAYS CONTROLLING LUNG MORPHOGENESIS.

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Sponsor: J. Rogers.

The respiratory tract begins as an outpouching of foregut endoderm on day 9-10 of mouse development. Respiratory tubules undergo stereotypic branching to form the bronchi, bronchioles, and acinar regions of the lung. Sacculations, septation, and vascularization ultimately produce the alveolar gas exchange region typical of

the mammalian lung. Transgenic mice were developed in which lung epithelial cell specific gene addition or deletion is accomplished under conditional control of doxycycline, using the reverse tetracycline transactivator protein expressed by SP-C or CCSP promoters. Conditional expression of cre-recombinase was accomplished in transgenic mice using alkaline phosphatase or green fluorescent protein (GFP) as reporters. Highly efficient recombination of lung progenitor cells was observed prior to lung bud formation on PC6.5-8.5, resulting in the lung-specific gene targeting of virtually all peripheral lung cells. Distinct subsets of proximal and distal lung progenitor cells were identified by the Cre-dependent labeling experiments, demonstrating that the establishment of proximal-distal subsets of cells had occurred remarkably early in foregut endoderm development, commitment being established even before lung bud formation. Gene addition and deletion experiments were then utilized to clarify the role of epithelial mesenchymal interactions involved in lung morphogenesis. FGF signaling was required at precise times during development for lung morphogenesis, supporting the concept that FGF signaling is required for commitment of subsets of stem cells but is not required for ongoing proliferation of epithelial cells in the postnatal lung. The roles of signaling molecules and transcription factors involved in lung morphogenesis, including beta-catenin, Shh, TTF-1, Stat-3 and Foxa2 were studied after Cre-dependent gene targeting. We hope that the ability to precisely control gene expression in the lung will provide insights into the pathogenesis of acute and chronic lung diseases in neonates and adults.



## 1645 EFFECTS OF ENVIRONMENTAL TOBACCO SMOKE (ETS) ON THE DEVELOPING LUNG.

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Infants raised in homes of smokers have more cough and wheeze. These symptoms may arise as a result of abnormal neural and epithelial development of the airways. One study examined whether perinatal exposure to ETS alters the innervation and epithelial composition of the trachea. Rhesus monkeys were exposed to filtered air (FA) or ETS (1 mg/m<sup>3</sup> total suspended particulates, 6 hr/day, 5 days/week) from day 50 of gestation to 75 days postnatal age (n=4 each group). All mothers gave birth to infants within the normal gestational period (165+/-10 days). The trachea of each infant was examined for epithelial composition and mucin content. Innervation of the trachea was determined using protein gene product 9.5 (PGP9.5), a general nerve marker, and substance P (SP), a marker for a subset of nerves including C-fibers. Four areas of the trachea immediately above the carina were imaged and morphometric analysis performed. Epithelial volume/basal lamina surface area (um<sup>3</sup>/um<sup>2</sup>) was 31.6+/-3.4 for infants exposed to FA and 22.6+/-1.3 for infants exposed to ETS (p<0.05). The percentage of epithelial mucin was significantly increased following exposure to ETS (41.6+/-3.3%) compared to FA (23.0+/-3.4%). The volume percent of PGP9.5-immunoreactive (IR) nerve fibers within the tracheal epithelium was unchanged following ETS exposure. In contrast, ETS exposure significantly increased the volume percent of SP-IR fibers in the epithelium (4.0+/-0.1x 10<sup>-3</sup>) compared to FA (1.0+/-0.4x10<sup>-3</sup>). This study demonstrates that exposure of rhesus monkeys to ETS during the perinatal period significantly alters the epithelial composition, mucin content, and presence of SP-IR nerves in the neonatal trachea.



## 1646 EFFECT OF MATERNAL NICOTINE EXPOSURE DURING DIFFERENT PHASES OF LUNG DEVELOPMENT ON LUNG GROWTH IN THE OFFSPRING: PROTECTIVE EFFECT OF COPPER.

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Development of the lung is "programmed" to go through distinct phases of growth. Maternal smoking increases the incidence of respiratory disease in the offspring and some of the adverse effects are associated with nicotine, probably due to interference with "programming" of lung growth. A study was conducted to determine the effect of maternal nicotine exposure during different phases of lung development on the integrity of the lungs of the offspring, and to develop strategies to prevent the adverse effects of nicotine. Pregnant Wistar rats were given nicotine subcutaneously (1mg/kg/day) during (a) all the phases of lung development, that is from day 4 of gestation and throughout lactation ( gestation was 22 days and weaning 21 days), and, (b) from postnatal day 3, one day before the onset of the alveolar phase of lung development. This means that the latter group received nicotine only via the mother's milk. After weaning on postnatal day 21 the offspring received no nicotine. Lungs from 14, 21, and 42 day old neonates were evaluated from at least 9 rats from at least 3 litters of each age group. Maternal nicotine exposure during all the phases of lung development, as well as from the onset of alveolarisation had no effect on lung parenchyma up to postnatal day 21. On postnatal day 42, that is 3

weeks after weaning, the lungs of all the nicotine exposed animals showed signs of premature aging. Changes included a decrease in alveolar number and internal surface area, and an increase in alveolar volume. Microscopic emphysema also occurred. On postnatal day 42 no significant differences occurred between the groups exposed to nicotine during all the phases of lung development and those exposed from one day before the onset of the alveolar phase of development. Maternal copper supplementation (1mg/kg/day) during all the phases of lung development prevented all the adverse effects of maternal nicotine exposure on lung development. Copper supplementation from one before the onset of alveolarisation did not prevent the development of microscopic emphysema. Lung volume and body weights were not affected by maternal nicotine exposure. Therefore, it appears that maternal nicotine exposure changes the "programming" of the lungs of the offspring and thereby induce premature aging which renders the lungs more susceptible to damage. It appears that copper supplementation may prevent the premature aging phenomenon by maintaining the connective tissue framework of the lungs.

 **1647** PRENATAL PERFLUOROOCTANE SULFONATE EXPOSURE AFFECTS PERINATAL LUNG DEVELOPMENT AND CAUSES RESPIRATORY DISTRESS IN RATS.

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Perfluorooctane sulfonate (PFOS) is an environmentally stable industrial and household compound used as a protectant and surfactant. Prenatal exposure to PFOS at cumulative dosages of 50-100 mg/kg in rats leads to offspring mortality within hours to days after birth; the underlying pathophysiology remains unknown. Studies have shown that a critical period occurs late in gestation, with treatment on gestation days 19-20 (25 or 50 mg/kg/d) sufficient to induce neonatal death. Rat pups exposed to PFOS prenatally presented with labored breathing that worsened until death. The lungs of PFOS-exposed pups were pale and unable to expand fully upon perfusion. Histology and morphometry suggested that the lungs of the treated pups were immature. Therefore, it was hypothesized that PFOS lead to respiratory distress in the rat neonates. Attempts were made to rescue with therapeutic agents (dexamethasone and retinoic acid) to accelerate lung maturation and to combat the PFOS effects. These agents lengthened the time to death, but all exposed animals died, indicating that lung immaturity was not the major defect. Transmission electron microscopy of lungs of PFOS-treated pups revealed that surfactant was present in the alveolar type II cells, with an increase in lamellar bodies present in the 50 mg/kg/d group. Additionally, the alveolar cell membranes in the treated animals were irregular and lacked cell-cell contact. In addition, pronounced filamentous structures were located within the type II cells of treated animals. Further studies will identify the components of these structures and determine whether these structures block surfactant secretion. Microarray studies indicate changes in fatty acid synthesis. Investigations are ongoing to assess the integrity of surfactant in PFOS-exposed rat pups by measuring the ratios of various phospholipid species. The developing lung appears to be a primary target of PFOS, and further studies contribute to elucidating the mechanisms underlying this toxicity.

 **1648** EFFECTS OF ENVIRONMENTAL AIR POLLUTANTS ON POSTNATAL LUNG DEVELOPMENT.

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The respiratory system is a target for a wide range of toxic environmental contaminants. While the acute and chronic effects of a large number of these substances have been well characterized for the respiratory system of adult mammals, there is significantly less known about the impact of these lung-targeted compounds on the developing respiratory system. The pattern of lung development itself may play a significant role in modulating toxic responses, since significant portions of lung morphogenesis and cytodifferentiation occur postnatally. The risk of injury from an environmental contaminant which is known to target the lung in adults must be evaluated with two considerations: (1) the toxicant may have its impact by altering the processes of morphogenesis and cytodifferentiation resulting in differential expression or organization of the lung in the adult and (2) the stage of morphogenesis and differentiation of various lung subcompartments at the time of exposure may significantly modulate the severity of the toxic response. While there is extensive literature on the toxic potential of a wide range of environmental contaminants when the exposure is directed towards adults, it is obvious that there is a dearth of information regarding the toxic response of the respiratory system during development. The majority of studies suggest that: 1) the respiratory system in pre- and postnatal animals is more susceptible to injury from lung-directed toxicants than it is in adults of the same species; 2) the differences in toxic response to respiratory-targeted compounds among species are amplified when responses are evaluated during lung development.

 **1649** ROLE OF CELL-CELL AND CELL-MATRIX INTERACTIONS IN REGULATION OF TOXICANT-MEDIATED CELL DEATH.

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Cell-cell interactions are key for the regulation of cell survival, cell death and cell proliferation. Thus, an understanding of the interdependency between different tissue cellular compartments is key to elucidating mechanisms of cell death in response to toxicants. This symposium will provide a timely update on the molecular mechanisms of cell death and its regulation, including the role played by cell-cell and cell-matrix interactions. This will be illustrated by three examples of the role these pathways play in toxicant response in diverse tissues. The first speaker will consider how hepatic Kupffer cells are implicated in normal homeostasis and in hepatocarcinogenesis and can mediate both hepatocyte survival or cell death, depending on the toxic insult. The second speaker will address how gap junctional intercellular communication and cell adhesion are interrelated processes using the example of hexachlorobenzene which induces a down-regulation of connexins and E-cadherin in the liver of female but not male rats. The third speaker brings an external perspective and will discuss how the cell detects and signals damage leading to survival or cell death. The fourth speaker will address how neuronal trophic and communication functions are maintained by intricate coupling with associated astroglial cells with emphasis on neurotoxicants that selectively target astroglial cells, rendering neurons vulnerable to both physiologic and pathophysiologic stresses. The forth speaker will also summarise the symposium by uncovering similarities and common themes in the role of the cellular environment in determining cell fate after toxicant insult. This symposium will be of interest both to non-experts looking to understand this field and those with a specific interest in cell biology, carcinogenesis, neurotoxicology, apoptosis or cell-cell communication.

 **1650** CELL-CELL INTERACTIONS AND THEIR ROLE IN TOXICANT-INDUCED HEPATIC CELL DEATH.

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Diverse hepatotoxins induce differing responses; peroxisome proliferators (PPs) induce hepatocyte proliferation, suppression of apoptosis and ultimately liver tumors. In contrast, acetaminophen causes oxidative stress, cell death and irreversible necrotic liver damage. How are these different responses orchestrated and controlled? Increasing evidence suggests that hepatic nonparenchymal cells (NPCs), particularly Kupffer cells, play a major role in directing the response of the hepatocyte via synthesis, storage and release of cytokines such as tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), Interleukin 1L1 $\alpha$  (IL1 $\alpha$ ) and interleukin 6 (IL6). In the case of TNF $\alpha$ , binding to a specific receptor can activate death-signalling pathways leading to apoptosis but also others that lead to survival via p38 MAP kinase and NF- $\kappa$ B. The choice of outcome depends on cellular context; for example, inhibition of translation switches the TNF $\alpha$  signal from survival to death. Additionally, there may be a role for an excessive release from Kupffer cells and adjacent endothelial cells of reactive oxygen species and/or nitric oxide that cause oxidative damage in neighbouring hepatocytes and other liver cells. Although the responses of the rodent liver to PPs are entirely dependent on the PP activated receptor, (PPAR $\alpha$ ), the removal of NPCs abrogates the response to PPs both *in vivo* and *in vitro*, demonstrating that cell-cell interactions and cytokine release plays a key role. Interestingly, NPCs isolated from PPAR $\alpha$  null mice, like those isolated from the wild type NPCs, can restore the hepatocyte response to PPs. However, as expected, PPAR $\alpha$  null hepatocytes remained non-responsive to PPs, irrespective of the genotype of the added NPCs. These data support a role for NPCs in facilitating a response of hepatocytes to PPs that is ultimately dependent on the presence of PPAR $\alpha$  in the hepatocyte. Overall, hepatic Kupffer cells are implicated in normal homeostasis, in acute response to toxicants and in hepatocarcinogenesis and can mediate both hepatocyte survival or cell death, depending on the toxic insult and the cellular context.

 **1651** MODULATION OF CELL-CELL INTERACTIONS BY EPIGENETIC HEPATOCARCINOGENS.

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Mechanisms of epigenetic carcinogenesis involve disruption of cell or tissue homeostasis, such as cell-cell interactions mediated by various types of junctions. Gap junctions are formed by channels that allow direct communication between cells (GJIC), while adhering junctions maintain tissue integrity by linking cells together. Gap and adhering junctions are physically and functionally interrelated membrane structures. Connexins and cadherins are key proteins in gap and adhering junctions, respectively. While the effects of hepatocarcinogens on these proteins in ro-

dent livers are well documented and will be discussed in this presentation, the pathways involved in their regulation are less understood. Consequently, a particular emphasis will be made on hexachlorobenzene (HCB), an important environmental contaminant, that modulates intracellular pathway involved in cell junctions. HCB causes liver tumor formation and down-regulation of connexins and E-cadherin in the liver of female but not male rats. A sexual dimorphism in the activation of the ILK pathway is believed to be responsible for these down-regulations; inactivation of GSK3- $\beta$  and nuclear translocation of both Akt and  $\beta$ -catenin are important events. *In vitro* studies, using specific inhibitors and overexpression systems confirmed not only the role of ILK pathway in cell-cell interactions, but also the functional interrelationships between connexins and cadherins. Down-regulation of proteins forming gap and adhering junctions allow initiated cells to escape control from neighboring cells, thus promoting their clonal expansion. The importance of such mechanisms in carcinogenesis is illustrated by the fact that human tumors of various origins show low levels of immunoreactive connexins and cadherins. Moreover, studies suggest that loss of cell-cell interaction proteins is an early event in the carcinogenesis process. Overall, data support the contention that gap and adhering junctions are at the basis of important cell-cell interactions disrupted in epigenetic hepatocarcinogenesis.

duces progressive dyskinesia, emotional lability, and certain neurological deficits resembling Parkinsons disease. It is postulated that astroglial-derived NO mediates neuronal injury induced by manganese exposure and that manganese potentiates the effects of pro-inflammatory cytokines on induction of nitric oxide synthase in astroglial cells. This hypothesis is tested utilizing: 1) subchronic *in vivo* exposure to manganese in mice; 2) an astroglial-neuronal co-culture system; and 3) primary astrocyte cultures to examine molecular signaling events relevant to inflammatory gene expression. Manganese potentiates cytokine-induced expression of nitric oxide synthase in astrocytes that increases apoptosis in co-cultured neurons in an NF- $\kappa$ B-dependent fashion. Dysregulation of intracellular calcium and mitochondrial dysfunction in astroglia mitochondria appear to be pivotal to induction of nitric oxide production. Thus, therapeutic strategies that target the molecular signaling pathways regulating expression of nitric oxide synthase in astroglia may be effective in mitigating neuronal injury in degenerative conditions of the basal ganglia.

## 1652 THE REGULATION OF APOPTOSIS: SURVIVAL SIGNALLING AND THE IMPORTANCE OF CELL TO CELL COMMUNICATION.

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Apoptosis is a physiological death process that is conserved from worms and flies to man. All cells will undergo apoptosis by default if not supplied with appropriate survival signals, ensuring that the correct numbers of healthy cells are found in the right place at the right time. Two general pathways to apoptosis have been characterised. The extrinsic pathway, activated by ligation of plasma membrane death receptors (e.g. Fas), drives the formation of a Death Initiating Signaling Complex (DISC) that in turn activates a proteolytic caspase cascade. The intrinsic pathway is activated by drug-induced damage and via loss of cell-cell or cell-matrix contacts. These apoptotic signals are relayed to mitochondria where interactions between pro- and anti-apoptotic proteins of the Bcl-2 family determine the release of apoptotic factors including cytochrome c and Smac. Cytosolic cytochrome c activates the apoptosome complex and a caspase cascade and Smac binds to inhibitor of apoptosis proteins (IAPS) to remove their restraint on caspase activity. The cellular microenvironment plays a major role in regulating the induction of apoptosis. For example, the hypoxia experienced by tumour cells within a solid mass has an impact on the Bcl-2 family of proteins with consequences for tumour cell survival and resistance to therapy. Specifically, the role of hypoxia inducible factor  $\alpha$  in promoting cell survival will be examined *in vitro* and *in vivo*. A second example is provided by studies that ask how the Schwann cell population is lost in patients with diabetic neuropathy. Given the intimate interplay between Schwann cells and the axon they surround, we ask how this Schwann cell loss influences the observed axonopathy associated with this disease. Specifically, the impact of chronic hypoxia combined with hyperglycaemia on Schwann cell Bcl-2 family proteins and apoptosis will be examined.

## 1653 NEURO-GLIAL INTERACTIONS IN BASAL GANGLIA DYSFUNCTION: INSIGHTS FROM MANGANESE NEUROTOXICITY.

R. Tjalkens. *Toxicology Section, Department of Environmental and Radiological Health Sciences, Colorado State University, Fort Collins, CO*.

During periods of stress or injury, astroglia can undergo phenotypic transformation into an activated state whereupon expression of inflammatory mediators is dramatically increased, to the detriment of associated neurons. Neuronal injury in several disorders of the basal ganglia, including manganism and Parkinsons disease, is associated with regional increases in expression of inflammatory mediators and activation of astroglia, with subsequent overproduction of nitric oxide (NO). The present studies explore the role of astroglial activation in basal ganglia dysfunction by examining a prototypic neurotoxicant of the basal ganglia, manganese, and its capacity to elicit expression of inflammatory genes in astroglia. Although playing various essential physiological roles in the central nervous system, manganese in excess is the cause of an extrapyramidal neurodegenerative disorder in humans that pro-

## 1654 THE UBIQUITIN-PROTEASOME SYSTEM AS A BIOLOGICAL TARGET IN TOXIC RESPONSES AND DISEASE.

R. S. Pollenz. *Biology, University of South Florida, Tampa, FL*.

The ubiquitin-proteasome system is an essential pathway involved in covalently modifying proteins to influence their function and turnover. The cascade is initiated by an activating enzyme (E1), that binds to the 76 amino acid ubiquitin protein (UB). The E1 then transfers the UB to an E2 carrier protein. Following this transfer, a UB ligase enzyme (E3) covalently links the UB to a target protein. It is the E3, or E3/E2 complex that supplies the target specificity of the ubiquitination event. Genome mining has identified 530 possible genes encoding E1, E2, E3, UB and deubiquitinating enzymes (DUB). Ubiquinated substrates are usually recognized by the 20S or 26S proteasome complexes and destroyed, although recent studies suggest that UB or UB-like modification does not always result in destruction. The proteasomes are huge multiprotein complexes that can be found in both the cytoplasm and nucleus. Overall, the proteasome accounts for 1% of a cells protein. Due to the number of gene products required for the ubiquitin-proteasome system, it is a prime target in various human diseases states and cancer. Defects in the system have been implicated in neurodegenerative diseases such as Parkinsons and Alzheimers. In addition, studies continue to suggest that that the various enzymes of the pathway may be targets for toxicologically relevant compounds typified by arsenic, cadmium, TCDD and ethanol. In addition, the ubiquitin-proteasome system is implicated in the ligand mediated degradation of important transcription factors such as p53, steroid hormone receptors and the aryl hydrocarbon receptor (AHR). Due to these findings, the enzymes of the ubiquitin-proteasome pathway have become important targets in toxicology screens and in drug discovery paradigms.

## 1655 TOXICANT AFFECTS ON UBIQUITIN-PROTEOSOME SYSTEMS: LESSONS FROM CROSS-COMPOUND AND CROSS-SYSTEM ASSESSMENTS.

E. Faustman, X. Yu, J. Sidhu and J. Robinson. *Department of Environmental and Occupational Health Sciences, University of Washington, Seattle, WA*.

This talk will highlight mechanistic research from our laboratory which examines the similarities and dissimilarities of metal effects on the Ubiquitin-proteasome (UB) pathway. The effects of three metals were evaluated in mouse embryonic fibroblast cells (MEF) using environmentally relevant concentrations of MeHg (2.4  $\mu$ M), Cd (5.0  $\mu$ M) and As (5.0  $\mu$ M) for 24 hours. MG132 (0.5  $\mu$ M), a classical proteasome inhibitor, was also included to compare the effects of these metals with a well-characterized inhibitor of the UB pathway. These concentrations were chosen as they were consistent with our previous studies where minimal impacts were observed on cytotoxicity, stress signaling and apoptotic pathways under these treatment conditions. Gene expression array analysis was done using CodeLink Mouse Uniset 10K oligonucleotide based platforms. To determine the role that specific cell signaling pathways might play in mediating these metal effects, we evaluated these responses in both wild-type as well as P53 knockout cells. We developed pathway specific analysis approaches using GenMAPP (Gene MicroArray Pathway Profiler) on normalized data. Our results have allowed us to compare both molecular and cellular level responses across these four agents and have facilitated our identification of common and unique targets within the UB pathway for these agents. The dependence of these targets upon the P53 cell cycle control checkpoint pathway was determined. These studies were supported by USEPA and NIEHS ES08601, ES07033, ES 11387, ES 10613 and R 826882.



## 1656 ENVIRONMENTAL FACTORS, UBIQUITIN-PROTEOSOME DYSFUNCTION AND PARKINSON'S DISEASE.

A. G. Kanthasamy, *Biomedical Sciences, Iowa State University, Ames, IA.*

Exposure to pesticides and metals has been linked to the etiopathogenesis of Parkinson's disease. Recently, we showed that the organochlorine pesticide dieldrin induces apoptosis in dopaminergic cells via caspase-3-dependent proteolytic activation of the proapoptotic kinase PKC $\delta$ . Abnormal accumulation and aggregation of  $\alpha$ -synuclein and impairment of the ubiquitin-proteosome system have been suggested to contribute to the dysfunction, degeneration and ultimate death of dopaminergic neurons in PD. To further understand the cellular mechanisms underlying dieldrin-induced dopaminergic neurodegeneration, we examined the effects of dieldrin on the ubiquitin-proteosomal function in rat mesencephalic dopaminergic cells overexpressing human  $\alpha$ -synuclein ( $\alpha$ -SYN cells). Low dose (30 microM) dieldrin induced time-dependent cytotoxicity over a 24 hr period in  $\alpha$ -SYN cells compared to vector expressing cells.  $\alpha$ -SYN cells were slightly resistant to dieldrin toxicity up to 12 hr post exposure but were more sensitive at 24 hr. Dieldrin treatment for 24 hr also resulted in a significant increase in caspase-3 enzyme activity in  $\alpha$ -SYN cells. Dieldrin treatment significantly increased the proteosomal activity at 3 hr in vector cells but not in  $\alpha$ -SYN cells. At a later time point (24 hr), a significant reduction in proteosomal activity was observed in both vector and  $\alpha$ -SYN cells, however, the impairment of proteosomal function was more pronounced in  $\alpha$ -SYN cells than in vector cells. Dieldrin also increased the accumulation of ubiquitin. Immunocytochemical analysis of dieldrin-treated  $\alpha$ -SYN cells showed aggregation of  $\alpha$ -synuclein in a time-dependent manner, with small aggregates appearing as early as 6 hr and progressively increasing over a 24 hr. The proteosomal inhibitor lactacystin also induced a profound aggregation in  $\alpha$ -SYN cells. Overall, these results suggest that impairment of the ubiquitin-proteosome function following environmental neurotoxin exposure may play a role in  $\alpha$ -synuclein aggregation in Parkinson's Disease [NIH grants NS 045133 and ES 10586].



## 1657 EFFECT OF ETHANOL ADMINISTRATION ON PROTEASOME ACTIVITY IN LIVER AND IN CULTURED HEPATOMA CELLS.

T. M. Donohue, N. A. Osna and D. L. Clemens. *Research, VA Medical Center, Omaha, NE.* Sponsor: R. Pollenz.

Chronic ethanol consumption by heavy drinkers and alcoholics or exposure of liver cells to ethanol *in vitro* can cause liver cell death and liver injury, leading to the complications of hepatic fibrosis and cirrhosis in humans and laboratory animals. The loss of cell viability has been associated with a decline in proteasome function in cells, because of the pivotal role of this proteolytic enzyme in cell regulation and function, including cell proliferation, enzyme regulation and antigen presentation. Ethanol is a hepatotoxin that, when administered to animals to produce blood ethanol levels of 40 mM (i.e 184 mg/dL) or more results in a 36-40% decline in liver proteasome activity. Our aim is to determine the mechanism(s) responsible for the alcohol-elicited decline in proteasome activity and its association with liver injury. Our studies to date have revealed that ethanol-induced oxidative stress contributes significantly to this decline in enzyme activity and that the level of oxidative stress correlates with the severity of liver pathology. Oxidative stress is regulated not only by the levels of ethanol and its metabolism but also by the type of dietary fat that is included in the diet of ethanol-fed experimental animals. At this time, we know that the ethanol-elicited decline in proteasome activity is correlated with liver damage, but we do not know whether such a decline is causally related to liver injury. However, because the proteasome degrades the majority of oxidized proteins and proteins modified by ethanol-derived aldehydes and reactive oxygen and nitrogen species, we hypothesize that the partial loss of proteasome activity would augment the accumulation of potentially cytotoxic proteins to enhance the onset of cell death. In this presentation, I will provide evidence from both *in vivo* and *in vitro* studies to suggest that proteasome activity and proteolytic systems in general, influence cell viability in response to ethanol consumption *in vivo* or ethanol exposure *in vitro*.



## 1658 THE UBIQUITIN-PROTEASOME SYSTEM IN REGULATION OF NUCLEAR TRANSCRIPTION FACTORS AND SIGNAL TRANSDUCTION PATHWAYS.

R. S. Pollenz, J. Popat, M. de la Pena and J. McQuown. *Biology, University of South Florida, Tampa, FL.*

The concentration or subcellular location of the key proteins involved in signal transduction pathways have been shown to impact gene regulation. The aryl hydrocarbon receptor (AHR) is one protein that appears to be controlled by the ubiquitin-proteasome system. It is clear that binding of ligands typified by 2, 3, 7, 8 tetra-

chlorodibenzo-p-dioxin (TCDD) result in nuclear accumulation and degradation of the AHR. However, the sequence of events following ligand binding that specify degradation are poorly understood. Studies indicate that there is a 60-minute lag between ligand exposure and the detection of AHR degradation. This implies a regulatory event. Analyses of receptors that are defective in nuclear import reveal that the lag time is not reduced and the kinetics of degradation are not changed when compared to wild type AHR proteins. AHRs that are nuclear prior to ligand exposure also are degraded along the same time frame as wild type receptors. During the first 60 minutes of exposure, the AHR becomes localized within the nucleus and appears to exist as either a dimer with ARNT or in a complex with hsp90 and XAP2. Since the AHR is a phosphoprotein, studies were initiated to determine whether changes in the phosphorylation state were important in the degradation events. The analysis of endogenous AHR turnover in the presence of phosphatase inhibitors did not reveal any reductions in AHR turnover. Interestingly, stimulation of various cell lines with TCDD, results in a transient reduction in estrogen receptor protein levels and gene-chip studies suggest that several genes of the ubiquitin-proteasome system are upregulated by TCDD. Thus, while the degradation switch of the AHR remains elusive, it is intriguing to consider that enzymes of the ubiquitin-proteasome system may be impacted by ligands typified by TCDD to affect other pathways. Supported by NIEHS 10991.



## 1659 WHY METALS BECOME NEUROTOXIC.

W. Zheng. *School of Health Sciences, Purdue University, West Lafayette, IN.*

Neurodegenerative diseases are characterized by progressive atrophy and dysfunction of anatomically or physiologically related neurological systems. Cumulative evidence suggests a role of metals in the etiology of numerous such diseases. For example, excess manganese (Mn) and iron (Fe) in particular brain regions have been associated with Parkinsonism; copper (Cu) and zinc (Zn) have been implicated in extracellular deposits of amyloid plaques in Alzheimer's brains; overload of aluminum (Al) in the brain has also been controversially related to Alzheimer's disease. Moreover, exposure to organic metals such as methylmercury (MeHg) has been linked to persistent psychomotor disturbances. However, the imminent question remains as to what factors may render metals, either essential or xenobiotic, more prone to being harmful in sporadic or hereditary neurodegenerative diseases. Understandably, interactions of these metals with genetic components, proteins, metal transport machineries, and cellular redox mechanisms, may signify some of the key factors in metal-induced neurotoxicities. This symposium will address the current understanding of biochemical characteristics of metals that are implicated in neurodegenerative disorders, including (1) metal-protein interaction such as Cu and Zn in beta-amyloid aggregation, (2) metal-metal interaction such as Mn in alteration of brain Fe functions, (3) metal-subcellular structure interaction such as MeHg and microtubules, (4) metal-transporter interaction such as Al speciation in brain Al metabolomics, and (5) metal-redox pathway interaction implicated in metal-induced oxidative stress. The symposium will be of interest to those who are engaged in metal toxicology, neuroscience, neurotoxicology, risk assessment, regulatory management, occupational health, and toxicology education.



## 1660 SELECTIVE BLOOD-BRAIN BARRIER TRANSPORT OF ALUMINUM, MANGANESE, AND OTHER METALS IN METAL-INDUCED NEURODEGENERATION.

R. A. Yokel. *College of Pharmacy, University of Kentucky Medical Center, Lexington, KY.*

Excessive concentrations of aluminum (Al), copper (Cu), iron (Fe), mercury (Hg), manganese (Mn), tin (Sn) and zinc (Zn) have been shown or hypothesized to contribute to one or more neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), Wilson's disease (WD) and Friedreich's ataxia. The uptake of metals into the brain is often mediated by selective carriers or transporters localized at the blood-brain barrier (BBB). Thus, the regulation of metal transport by the BBB is fundamental to subsequent metal-induced neurotoxicities. This presentation will first review the evidence supporting and refuting roles of Mn in PD and Al in AD, including disparities between Mn-induced Parkinsonism and idiopathic PD, epidemiological evidence of Al neurotoxicity, clinical studies of brain Al in AD patients, and biochemical effects produced by Al that mimic AD. It will then focus on the selective metal transporters at the BBB as a key factor in the potential for metals to produce neurotoxicity. The specific interaction of a unique chemical species (form) of a neurotoxic metal with a particular membrane transporter at the BBB serves as a good example of selective transport of metals by the BBB. The ability of the methyl-Hg-cysteine complex to mimic methionine and to serve as a substrate for an amino acid transporter illustrates this mechanism of brain metal entry. Another mechanism that may mediate brain entry of several metals is transferrin-receptor mediated endocytosis. This presentation will further discuss the role of similar mechanisms in Al and Mn brain influx and efflux. The evidence for carrier-

mediated influx and efflux of Al across the BBB will be presented, as will the evidence for carrier-mediated influx, but only diffusion-mediated efflux, of Mn. Our incomplete knowledge of the mechanisms mediating Al and Mn flux across the BBB will be contrasted to the understanding of the Cu transporter that plays a central role in WD and the paucity of data on BBB flux of Sn and Zn.

#### 1661 INTERACTION OF COPPER AND ZINC WITH $\beta$ -AMYLOID IN PATHOGENESIS OF ALZHEIMER'S DISEASE.

A. I. Bush. *Genetics and Aging Research Unit, Harvard Medical School and Massachusetts General Hospital, Charlestown, MA.* Sponsor: W. Zheng.

$\beta$ -Amyloid ( $\text{A}\beta$ ) is a ubiquitous metalloprotein with selective high-affinity binding sites for zinc (Zn) and copper (Cu). Studies have shown an elevation of Zn and Cu in the plaques in AD. Furthermore, genetic ablation of the ZnT3 transporter abolishes amyloid deposition in the Tg2576 mouse model for AD.  $\text{A}\beta$  binds Cu and Zn through a site that involves oligomeric peptide assembly, and resembles the structure of SOD1. When synthetic  $\text{A}\beta$  binds Cu in the absence of Zn, it is highly redox active. Biological reducing agents such as dopamine, cholesterol and vitamin C can act as a reservoir of electron donation, so that the  $\text{A}\beta/\text{Cu}$  complex acts as a catalyst generating  $\text{H}_2\text{O}_2$  ( $\text{Km}=5 \mu\text{M}$ ,  $\text{Vmax}=30 \text{nM}/\text{min}$ ). The toxicity of  $\text{A}\beta$  species is proportional to the peptide's ability to reduce Cu or iron (Fe) and generate  $\text{H}_2\text{O}_2$  ( $\text{A}\beta42 > \text{A}\beta40 > \text{rat A}\beta$ ).  $\text{H}_2\text{O}_2$  is a freely permeable pro-oxidant and is the chemical source of much of the oxidation damage that is abundantly evident in AD brain. Where cholesterol is the substrate for  $\text{H}_2\text{O}_2$  production, it is specifically oxidized by  $\text{A}\beta/\text{Cu}$  complexes into forms that are markedly increased in AD post-mortem brain tissue compared to age-matched normal and neurological disease control tissue. Chelators of Cu and Zn both disaggregate  $\text{A}\beta$  deposits from post-mortem human brain and also inhibit redox activity and  $\text{H}_2\text{O}_2$  production. We have found that one such orally bioavailable chelator, clioquinol, markedly inhibits brain amyloid pathology in Tg2576 mice. This compound has recently completed a successful pilot Phase 2 clinical trial in AD, where plasma  $\text{A}\beta$  levels were lowered and cognitive decline significantly arrested. Cu and Fe levels rise in several tissues with aging for reasons that are still unclear. We hypothesize that some metalloproteins are liable to becoming abnormally decorated with extra Cu and Fe ions leading to a gain of redox-activity. The interactions described for  $\text{A}\beta$  may be applicable to other protein targets implicated in other age-related degenerative diseases such as cataracts, Parkinson's disease, and amyotrophic lateral sclerosis.

#### 1662 BINDING TO SUBCELLULAR STRUCTURES IN METHYLMERCURY-INDUCED NEURODEGENERATIVE DAMAGE.

K. R. Reuhl. *Pharmacology & Toxicology, Rutgers University, Piscataway, NJ.*

Binding of metals to subcellular structures represents a crucial factor in the molecular and cellular events that prompt metals to generate neurotoxicities, such as in the case of methylmercury (MeHg). MeHg is a potent neurotoxicant. During the last three decades, studies have attempted to identify organelar targets within neurons to account for the compound's regional selectivity. Two organelles - the mitochondrion and the microtubule (MT) - have been shown to be highly vulnerable to MeHg. Recent studies indicate that these structures have a complex pattern of structural and functional co-dependency. Exposure of mature neurons to MeHg results in a rapid disassembly of perikaryal microtubules and almost coincident loss of mitochondrial membrane potential and increases in cytosolic markers of oxidative stress. A subpopulation of tyrosinated MTs immediately adjacent to the mitochondria is first to disassemble, followed by other perikaryal MT and later, axonal MTs. The slow degeneration of axonal MTs likely contributes to the delay in the clinical expression of neuronal dysfunction in adults. Pretreatment with taxol, a microtubule stabilizing agent, protects against MeHg damage and inhibits loss of mitochondrial membrane potential. Inhibition of mitochondrial activity slows, but does not prevent, MT disassembly. In contrast, MeHg treatment of undifferentiated neurons causes a prompt and extensive loss of MT in neurons, but only modest increases in mitochondrial permeability and oxidative stress. However, as the neuron develops neurites, it rapidly assumes an adult pattern of response, including increasing reliance on mitochondrial respiration. Taken together, these data suggest that MT represent an early target of MeHg in both immature and mature neurons, while mitochondria are most vulnerable once they assume mature respiratory function. (Supported by NIH ES-05022, ES11256 and USEPA R829391).

#### 1663 METAL-METAL INTERACTIONS IN MANGANESE-INDUCED PARKINSONISM.

W. Zheng. *School of Health Sciences, Purdue University, West Lafayette, IN.*

Metals, by their chemical nature, share many similarities, such as the valence charge, ionic radius, and electronic polarity. These characteristics comprise the molecular basis on which one metal can interact with another in biological reactions.

For example, lead toxicity has been linked to its interaction with calcium; arsenate, by mimicking phosphate, causes a disruption in energy production. This presentation will discuss how the chemical similarity between manganese (Mn) and iron (Fe) leads to a distorted Fe homeostasis in the CNS following Mn exposure, which may contribute to the etiology of Mn-induced Parkinsonism. Systemic and cellular Fe metabolism is regulated by the iron regulatory protein-1 (IRP1), which binds to the iron responsive element (IRE) in mRNAs of transferrin receptor (TfR) and ferritin via the [4Fe-4S] structure of IRP1's active center. Mn competes with Fe for the binding of IRP1, resulting in an altered binding affinity of IRP1 to IRE-containing mRNAs. A gel shift assay demonstrated that Mn exposure increased the binding of IRP1 to the stem loop-containing mRNAs encoding the TfR and the divalent metal transporter (DMT1) in cultured choroidal epithelial cells, which constitute the blood-cerebrospinal (CSF) barrier. Consequently, a real-time PCR analysis revealed an increased density of TfR and DMT1 mRNAs after Mn treatment, with the subsequent elevated cellular protein concentrations of TfR and DMT1 in cultured choroid plexus cells. Studies utilizing RT-PCR and nuclear run off techniques further showed that Mn treatment did not affect the level of heterogeneous nuclear RNA (hnRNA) encoding TfR and DMT1, nor did it affect the level of nascent TfR mRNA. Taken together, these results suggest that Mn-Fe interactions at the blood-CSF barrier prompt Fe transport at the blood-CSF barrier; the effect is likely due to Mn action on translational events relevant to the production of TfR and DMT1, but not due to its action on transcriptional, gene expression of TfR. The disrupted Fe homeostasis in the brain upon Mn exposure may explain Mn-induced neurodegenerative toxicity. (Supported by ES08146)

#### 1664 ADVANCES IN MATERIAL SAFETY DATA SHEET COMMUNICATION.

M. McDiarmid<sup>1</sup> and L. Frazier<sup>2</sup>. <sup>1</sup>*Occupational Health Project, University of Maryland, Baltimore, MD* and <sup>2</sup>*Department of Preventive Medicine, University of Kansas School of Medicine, Wichita, KS.*

OSHA is currently reviewing its Material Safety Data Sheets (MSDS) requirements, as is the American National Standards Institute (ANSI). Employers using hazardous chemicals are required by OSHA's Hazard Communications Standard to have MSDSs available to workers. Developing and providing effective communication is a major challenge within the public health and occupational health communities. Some MSDSs are difficult for workers to read; some MSDSs are outdated; and some MSDSs omit needed health information. On the other hand, some manufacturers produce what might be considered examples of "best practice" MSDS writing: excellent, readable, and timely information. Another source of information, the New Jersey Hazardous Substances Fact Sheets, covers only about 1, 600 workplace chemicals. However, the program receives 80, 000 hits per month on its website for its fact sheets, which are considered by many environmental and labor advocates to be good sources of essential health and safety information. The message that reproductive health includes both men and women, and that it can be affected by their workplace exposures, needs to reach the workers and their employers through MSDS communication. Paul and Kurtz surveyed Massachusetts MSDSs in 1994 for products containing two known reproductive toxicants, lead and glycol ethers, and found that over 60% did not mention possible reproductive health effects. They also found that where reproductive hazards were mentioned in MSDSs, they were 18 times more likely to address developmental effects than male reproductive risks. How can MSDS writing be improved to a best practice standard while avoiding national or international standards conflicts? The NIOSH Reproductive Health Research Team is interested in finding ways to improve the quality of MSDSs in general, and particularly the quality of reproductive health information. Representatives from NIOSH, industry, academia, and the New Jersey Right to Know Program will present and discuss multiple facets of this issue.

#### 1665 ISSUES WITH MSDS COMMUNICATION OF REPRODUCTIVE HAZARDS.

L. Frazier<sup>1,2</sup>, M. J. Rall<sup>2</sup> and D. B. Fromer<sup>1</sup>. <sup>1</sup>*Preventive Medicine and Public Health, University of Kansas School of Medicine-Wichita, Wichita, KS* and <sup>2</sup>*Obstetrics and Gynecology, University of Kansas School of Medicine-Wichita, Wichita, KS.* Sponsor: M. McDiarmid.

MSDS have multiple audiences, ranging from professionals with technical expertise to workers. To help improve the readability of MSDS, the American National Standards Institute emphasizes communication principles in its newly revised standard (ANSI Z400.1). OSHA plans to provide guidance on preparing MSDS, and has partnered with the Society for Chemical Hazard Communication. Implementation of the Globally Harmonized System (GHS) of Classification and Labeling of Chemicals is being considered. The GHS International Chemical Safety (ICS) Cards contain standardized risk phrases for 1600 chemicals. These phrases may need further development to ensure that male reproductive hazards are stated. For dibromochloropropane (DBCP), the reproductive risk phrases on the

ICS card are "Avoid exposure to (pregnant) women" and "Causes toxicity to human reproduction or development." The reading grade levels of these phrases are 9.9 and 14.04 (college level), respectively. Reading grade levels can be calculated using software like MS Word or calculated manually. The following sentence has a reading grade level of 18.7, PhD level: "DBCP is a specific male gonadotoxicant in humans and laboratory animals, leading at high doses to azoospermia which may not be reversible." The following are examples of simpler language: "At high levels, this chemical may cause damage to the male reproductive system" (reading grade level 8.5; source: ToxFAQ from the Agency for Toxic Substances and Disease Registry). "Reproductive Hazard. DBCP may damage the testes (male reproductive glands). DBCP may decrease fertility in males and females. Infertility and lack of sex drive have been noted in exposed males" (reading grade level 7.3, source: New Jersey Right to Know Hazardous Substances Fact Sheets). In conclusion, chemical toxicity phrases on MSDS need to be both accurate and comprehensible. Efforts to develop simpler language about health hazards for MSDS are similar to efforts in the medical field to improve patient education.

## 1666 DEVELOPING GOOD MSDS COMMUNICATIONS IN INDUSTRY.

J. S. Bus. *The Dow Chemical Company, Midland, MI.*

Material Safety Data Sheets (MSDS) are an important mechanism by which the potential developmental and reproductive health hazards of chemicals are communicated to workers and other health professionals. Generation and maintenance of consistently and clearly worded MSDS documents by industry is supported by internal processes that encompass several key functions. These functions include: 1) generation, archiving and retrieval of relevant toxicology and human health information from standardized, globally accessible database(s). The data may be from both company- and literature generated studies; 2) designated Subject Matter Experts (SMEs) use professional judgment to interpret toxicological data gathered from a broad spectrum of sources, tapping additional specific expertise from with the laboratory research functions as necessary; 3) SMEs from disciplines of toxicology, industrial hygiene and occupational medicine develop standardized policies, practices and descriptive phrases that promote globally consistency language describing effects within and between documents. The OSHA Hazard Communication Standard and ANSI Z400.1-2004 provide valuable guidance in preparation of such standardized descriptors. Consistency in application of appropriate standardized MSDS descriptor phrases to specific developmental or reproductive toxicity responses is facilitated by SME cross-reference to rationale guidance statements for selection of specific phrases. For example, selection of the standardized phrase *Has been toxic to the fetus in laboratory animal tests* is guided by the rationale statement *Fetotoxic; no information as to maternal toxicity available. Does not address birth defects (teratogenicity). May be used with standardized statement XXX.* Additional guidance is available to SMEs on standardized definitions such as *fetotoxicity, birth defects, maternal toxicity*, etc. Thus, effective and consistent communication of MSDS health information is facilitated by SME access to well-maintained databases whose basic toxicology findings are accurately linked to standardized communication phrases.

## 1667 MSDS IN CANADA AND THE COMMUNICATION OF REPRODUCTIVE TOXICITY.

P. Demers. *School of Occ/Env Hygiene, University of British Columbia, Vancouver, BC, Canada.* Sponsor: M. McDiarmid.

In Canada the content and availability of Material Safety Data Sheets (MSDSs) is regulated under Hazardous Products Act (HPA) and the associated Controlled Products Regulations (CPR). The program is administered by the Workplace Hazardous Materials Information System (WHMIS), a federal program housed within Health Canada. However, actual enforcement and responsibility of workplace inspection is delegated to the 13 provinces and territories, which are also responsible for other health and safety regulation in Canada for the great majority of workplaces. Disclosure is required for toxicological information regarding both reproductive and developmental toxicity. For the purpose of MSDS disclosure, any indication of an adverse effect on fetal development or reproductive parameters must be disclosed on the MSDS irrespective of whether or not there is an adverse effect on the pregnant female. However, the guidelines state "it is important to focus on any effects that were found at low doses or in the absence of maternal toxicity." In general, the Hazardous Products Act requires chemical ingredients to be specifically listed if they are present at 1.0% or more in a product. However, that threshold is reduced to 0.1% or more for teratogens, embryotoxins, and reproductive toxins (as well as carcinogens, respiratory tract sensitizers and mutagens). While regulations list the areas that must be covered on MSDSs, there is no approval mechanism or systematic review of the content or completeness of the information on the sheets. Enforcement focuses on the availability of MSDSs at the workplace and meeting of training requirements. While enforcement of violations in omitting

information from the sheets is in theory possible, there is no well-established process or program for doing so. Because of this the content is highly variable. For example, in a recent review of Canadian MSDSs for products containing toluene diisocyanate it was discovered that 25% of sheets failed to indicate the asthma was a potential effect of exposure.

## 1668 STRENGTHS OF THE NJ HAZARDOUS SUBSTANCE FACT SHEET PROGRAM: HOW TO WRITE A BETTER MSDS.

R. Willinger, JD MPH and A. Sobieszczyk, MD PhD. *Right to Know Program, New Jersey Department of Health and Senior Services, Trenton, NJ.* Sponsor: B. Grajewski.

The New Jersey Department of Health and Senior Services, Right to Know Program, pursuant to the Worker and Community Right to Know Act, prepares Hazardous Substance Fact Sheets on individual chemicals. They are similar to Material Safety Data Sheets (MSDS). This effort is unique among the States. The Fact Sheet Group includes a toxicologist, physician, industrial hygienist, word processor and editor. The Fact Sheets are prepared in a standardized format and use standardized phrases that are written in a language understandable to non-health professionals. Under the section entitled Health Hazard Information, the toxicologist prepares the Chronic Health Effects subsection that includes Reproductive Hazards. Staff conducts a comprehensive search for information about reproductive hazards using a variety of authoritative textbooks and databases. Hazards searched for include teratogenicity, fetotoxicity, embryotoxicity, and damage to the male and female germ cells and reproductive systems in general. Teratogenicity is defined by regulation and requires "epidemiological evidence of teratogenicity in humans or positive teratogenic evidence in at least two different animal species." A Decision Logic determines the use of eleven standard reproductive hazard phrases. If no standardized phrase is applicable, text specific to the hazard is written. When reproductive hazard or teratogenicity is present, standardized questions and answers about reproductive hazard and teratogenicity also appear in the Question and Answer section of the Fact Sheet. Since the inception of this effort in 1984, over 1,700 Fact Sheets have been prepared (and revised) and included on the Department's website. The Fact Sheets are used by public and private employers, employees, emergency responders, health care providers and agencies, and members of the public for hazard communication.

## 1669 THE SAFETY ASSESSMENT OF NUTRITIONALLY IMPROVED FOOD AND FEED CROPS.

B. G. Hammond. *Product Safety Center, Monsanto Company, St. Louis, MO.*

The first generation of food and feed crops developed through modern agricultural biotechnology were designed to improve agronomic characteristics such as control of noxious weeds (herbicide tolerance) or protection against insect pests. The next generation of products under development include crops that have improved nutritional characteristics. Speakers for this workshop will discuss (1) various biotechnology approaches that are being used to improve nutritional characteristics of feed/food crops, (2) a case study for the safety and nutritional assessment of a nutritionally improved food/feed crop (3) recommendations of the EU ENTRANSFOOD working group on safety assessment of foods derived from genetically modified crops (4) conclusions from a recent ILSI workshop entitled Nutritional and Safety Assessments of Foods and Feeds Nutritionally Improved through Biotechnology. At the end of the presentations, a panel discussion will compare and contrast recommendations from the SOT, ENTRANSFOOD and ILSI work-groups regarding the safety assessment of nutritionally improved crops.

## 1670 IMPROVED NUTRITION THROUGH MODERN BIOTECHNOLOGY.

M. Newell - McGloughlin. *University of California Systemwide Biotechnology Research and Education Program, U.C. Davis, Davis, CA.* Sponsor: B. Hammond.

Innovation is essential for sustaining and enhancing agricultural productivity. Biotechnology has introduced a new dimension to such innovation, offering efficient and cost-effective means to produce a diverse array of novel, value-added products and tools. It has the potential to improve qualitative and quantitative aspects of food, feed and fiber production, reduce dependency on chemicals, and lower the cost of raw materials, in a sustainable manner. Commercialization of the products of recombinant DNA technology is just another facet in a long history of human intervention in nature and as such the same parameters of risk-based assessment should apply. It must be undertaken within a regulatory framework that ensures adequate protection of the consumer and the environment while not

stymieing innovation. The first generation of such crops focused largely on input agronomic traits, the next generation will focus more on value-added output traits such as improved nutrition and food functionality. Functional foods are defined as any modified food or food ingredient that may provide a health benefit beyond basic nutrition. From a health perspective, plant components of dietary interest can broadly be divided into four main categories, macronutrients (proteins, carbohydrates, lipids [oils], and fiber), micronutrients (vitamins, minerals, phytochemicals), anti-nutrients (substances such as phytate that limit bioavailability) and allergens & toxins. Developing plants with improved quality traits involves overcoming a variety of technical challenges inherent to metabolic engineering. Both traditional and biotechnology techniques are needed to produce plants carrying the desired quality traits. Continuing improvements in molecular and genomic technologies are contributing to the acceleration of product development. These new products and new approaches on the horizon require a reassessment of appropriate criteria to manage risk while insuring that the development of innovative technologies and processes is encouraged to provide value-added commodities for the consumer.

## 1671 CASE STUDY FOR THE SAFETY ASSESSMENT OF A NUTRITIONALLY IMPROVED FEED/FOOD CROP.

B. G. Hammond, T. Reynolds, G. Hartnell, E. Rice, R. McCoy and K. Glenn. *Product Safety Center, Monsanto Company, St. Louis, MO.*

Corn is deficient in lysine requiring supplementation with this essential amino acid in certain animal diets. When added to diets at nutritional levels, lysine is Generally Recognized As Safe (GRAS) by the FDA and is used as a human food additive when provided at nutrient levels. To increase lysine levels, the coding sequence for the dihydrodipicolinate synthase (cDHDPS) enzyme from *Corynebacterium glutamicum*, was introduced into corn. This enzyme is not feedback inhibited by lysine leading to increased free lysine in grain. Lysine maize, provides an alternative to addition of lysine to animal diets. Lysine maize will be identity preserved for use in poultry and swine diets. This safety assessment focuses on its intended use and inadvertent use should it enter human food. Bioinformatic analyses showed no structural or immunological similarities of cDHDPS to known allergens or toxins. It is structurally and functionally related to similar DHDPS enzymes that are involved in lysine biosynthesis in a wide range of organisms. When incubated *in vitro* with simulated gastric fluid, cDHDPS protein was readily degraded. No adverse effects were observed in mice fed an acute, high dose (800 mg/kg) of cDHDPS protein. An acute oral mouse study is appropriate to assess potential toxicity since most protein toxins act through acute mechanisms. The composition of grain and forage grown at different locations were analyzed as outlined in the OECD consensus document. Targeted metabolites in the lysine synthetic and catabolic pathway were also analyzed. Gain, feed efficiency, and carcass yield of broilers fed diets containing Lysine maize were not different from broilers fed lysine supplemented diets and were superior to broilers fed conventional maize and no added lysine. No meaningful changes in body wt, clinical or microscopic pathology were observed in rats fed Lysine maize for 90 days. Food and feed products derived from Lysine maize are as safe as those derived from conventional corn and produce the intended nutritional benefits.

## 1672 SAFETY ASSESSMENT OF NUTRITIONALLY IMPROVED CROPS THROUGH MODERN BIOTECHNOLOGY.

H. A. Kuiper, G. A. Kleter and E. J. Kok. *RIKILT, Institute of Food Safety, Wageningen University and Research Center, Wageningen, Netherlands.* Sponsor: B. Hammond.

Food crops developed through modern biotechnology, with improved nutritional or health beneficial characteristics demand a rigorous assessment of their safety and nutritional impact. The question has been addressed whether the safety assessment framework as developed for genetically modified (GM) foods with improved input traits, equally applies to these new types of foods (European Network on the Safety Assessment of GM Food Crops, ENTRANSFOOD, Food and Chemical Toxicology 42, 2004, 1043-1202) and the Task Force of the ILSI International Food Biotechnology Committee (Comprehensive Reviews in Food Science and Food Safety 3, 2004, 36-104). In case of nutritionally enhanced food crops, particular attention must be paid to the choice of the appropriate comparator for safety evaluation. In certain cases not the parent crop, but the food component which level is intentionally high in the GM crop, will be the appropriate comparator. Toxicological and nutritional testing of these types of foods in animals should carefully be considered, in particular with respect to optimised diet composition. Moreover the estimation of anticipated intake levels of these types of foods is important, for certain foods systematic post market monitoring may be considered. The possible occurrence and assessment of unintended effects in GM food crops as result of the genetic modification is a key element in the safety assessment and may be of particular relevance for those crops with specific metabolic pathways modified through the insertion of multiple genes. Detection relies basically on a targeted ap-

proach i.e. comparative analysis of crop specific compositional parameters in the GM and non-GM crop. In order to increase the chances of detecting unintended effects, new profiling techniques like transcriptomics, proteomics and metabolomics have the potential to extend the comparative analyses (non-targeted approaches).

## 1673 SAFETY AND NUTRITIONAL ASSESSMENT OF FOODS AND FEEDS NUTRITIONALLY IMPROVED THROUGH BIOTECHNOLOGY.

I. C. Munro. *CANTOX Health Sciences International, Mississauga, ON, Canada.*

A newly emerging class of biotechnology-derived crops is being developed with a focus on improved nutritionally quality. Nutritionally improved foods should be evaluated for their potential impact on human/animal nutrition and health regardless of the technology used to develop them. The evaluation includes assessing both the change in the composition of the individual foods/feeds or ingredients and the impact of the changes on the overall composition of the diets of the projected population(s). The safety assessment begins with a comparative assessment of the new food/feed with an appropriate comparator that has a history of safe use. Compositional analysis is the starting point for this assessment. Current approaches of targeted compositional analysis are recommended for the detection of any alterations. Biosynthetic and catabolic pathways of the newly expressed trait should be compared for the improved crop and the appropriate conventional counterparts. For nutritionally enhanced or altered products, the dietary exposure to nutrient(s) whose content is changed should be estimated and the appropriate set of studies carried out to assess the nutritional quality and the safety of any newly expressed protein(s) and metabolites. Examples of such studies are structure-activity relationships, *in vitro* determinations of digestibility of the proteins and animal feeding studies with the relevant target species. It is concluded that the food safety and diet-health assessment methods currently employed are sufficiently robust to be applied to nutritionally improved foods.

## 1674 VINYL CHLORIDE: LEGACY AND LESSONS LEARNED.

R. O. McClellan. *Toxicology and Human Health Risk Analysis, Albuquerque, NM.*

Vinyl chloride is a major commodity chemical that has been extensively studied relative to its toxicological and carcinogenic properties. Early research conducted by producers and users of vinyl chloride focused on its toxicological properties. The research results were used in a standard safety factor framework to derive threshold limit values for occupational exposure. Long-term rodent inhalation studies were initiated to investigate the potential for chronic toxicity and carcinogenicity. Almost concurrently, carcinogenic responses were observed in the rodent studies and case reports were published on a finding of a rare cancer, hepatic angiomas in workers exposed to high levels of vinyl chloride. More stringent occupational exposure limits were instituted and further research on vinyl chloride initiated. This included epidemiological studies of workers, animal carcinogenicity bioassays and mechanistic investigations. The initial results, while confirming the carcinogenicity of vinyl chloride in humans and rodents, appeared to yield marked differences in carcinogenic potency in humans and rodents. Further research on the metabolic kinetics and molecular dosimetry of vinyl chloride and its metabolites provided a basis for reconciling the original apparent species differences in potency and provided a mechanistic basis for the very specific carcinogenic response, hepatic angiomas. The more stringent exposure standards have been effective in protecting workers. In retrospect, the research conducted on vinyl chloride may be viewed as a success story for how mechanism-based findings can be used to establish appropriate health protective standards. Moreover, the research approach used with vinyl chloride has served as a template for evaluating the toxicity and carcinogenicity of other chemicals.

## 1675 METABOLISM OF VINYL HALIDES AND REACTIONS OF ELECTROPHILIC PRODUCTS.

F. P. Guengerich. *Biochemistry and Center in Molecular Toxicology, Vanderbilt University, Nashville, TN.*

Interest in vinyl chloride (VC) began >35 years ago with reports of unusual liver tumors in some workers. Our own group began studies on the role of metabolism of VC in collaboration with Drs. Gehring and Watanabe of the Dow group. Cytochrome P450 enzymes, particularly 2E1, oxidize VC to an epoxide, which rearranges to 2-chloroacetaldehyde. The epoxide reacts with DNA to form N7-guanyl and etheno adducts, and the haloacetaldehyde preferentially reacts with proteins. These paradigms have been extended, with consideration of roles of acyl

halides as well, to other important vinyl halides including vinylidene chloride, trichloroethylene, and perchloroethylene. Questions still exist about the halide order in the toxicities of vinyl halides. The work with VC stimulated interest in etheno DNA adducts, which have now been identified as products of oxidation of endogenous lipids. These studies provide perspective on the balance of cancer attributable to xenobiotics vs. endogenous processes in risk assessment. (Supported in part by USPHS R01 ES02205, R35 CA44353, R01 ES10546, and P30 ES00267)

## 1676 VINYL CHLORIDE: ESTABLISHING THE ROLE OF DOSIMETRY IN RISK ASSESSMENT.

M. E. Andersen. *CIIT Centers for Health Research, Research Triangle Park, NC.*

Non-linear clearance of drugs such as aspirin and ethanol became well-established in the 1960s. High doses routinely used in long term toxicity testing were expected to cause saturation of clearance pathways, accumulation of test compounds, and toxic consequences. Scientists at Dow Chemical Company, led by Dr. Perry Gehring, began routine evaluations of the metabolism and kinetics of test commodity compounds in the early 1970s and also developed pharmacokinetic (PK) models. The highlight of this body of work was association of the carcinogenic responses of vinyl chloride (VC) with amounts of vinyl chloride (VC) metabolized. Studies with <sup>14</sup>C-VC estimated total amount metabolized and a novel closed atmosphere metabolism chamber permitted assessment of metabolic rate constants. In addition to use of dosimetry to infer the mode of action of VC metabolites, the PK tools developed were subsequently applied by other Dow scientists, their collaborators, and external groups of toxicologists to create more complete physiologically based pharmacokinetic (PBPK) models for inhaled and ingested toxicants that supported dose, dose-route and interspecies extrapolations of kinetics. The expansion of PK and PBPK approaches in dose-response assessment and chemical risk assessment over the past 3 decades owe much to the initial applications of these tools with VC. This talk provides a personal overview of the history of PK modeling in toxicology and dose-response assessment tracing the relationship of the applications of the methods with VC to present more widespread applications of PBPK models with many different types of chemicals, including inhaled organic chemicals, persistent organic pollutants, respiratory tract irritants, metals, and endocrine active compounds.

## 1677 VINYL CHLORIDE: TOXICOLOGICAL AND CARCINOGENIC RESPONSES IN HUMANS AND LABORATORY ANIMALS.

R. O. McClellan. *Toxicology and Human Health Risk Analysis, Albuquerque, NM.*

Vinyl chloride monomer (VCM) is a major commodity chemical. In the 1930s, it was discovered that VCM could be polymerized to polyvinyl VC (PVC), a discovery that led to world-wide production and use of PVC. Using approaches that were contemporary for 1950-1960 the acute toxicity of VC was investigated in multiple species. Results on non-cancer endpoints were used in a traditional safety factor approach to set threshold limit values. In the rapidly developing PVC industry some exposures occurred at levels sufficient to cause scleroderma, e.g. coldness and numbness of the hands and acro-osteolysis of the terminal phalanges. In 1973, an astute physician observed three cases of a rare cancer, hepatic angiosarcoma, and determined that all three patients worked in a PVC production facility as cleaners of polymerization autoclave reactors. Almost concurrently, preliminary results from high level VCM inhalation studies with rats and mice indicated that VCM was carcinogenic. The finding of a carcinogenic response in both workers and laboratory animals immediately triggered extensive new research including clinical and epidemiological studies on VCM workers, carcinogenicity studies in laboratory animals and mechanistic studies. These studies soon confirmed the carcinogenicity of VCM and shifted attention to understanding the site-specificity of the cancers and the nature of the exposure-cancer response relationship. The studies are remarkable in many ways; collaboration of industrial, academic and government scientists, use of state-of-the-art techniques, study of a wide range of exposure concentrations, introduction of kinetic modeling that facilitated a shift from an exposure to a dose metric, attention to inter-species extrapolations and a move from definition of hazard to quantification of cancer risk. Findings in each area will be reviewed to gain insight into lessons learned from evaluating risks of exposure to VCM and how VCM experience has impacted the field of chemical toxicology and risk assessment. Opportunities will be identified for further use of the VCM experience to advance these fields.

## 1678 CONFLICT OF INTEREST.

J. P. Maurissen<sup>2</sup> and S. G. Gilbert<sup>1</sup>. <sup>1</sup>INND, Seattle, WA and <sup>2</sup>Neurotoxicology, Dow Chemical Company, Midland, MI.

The conflict of interest issue has recently been the subject of much attention from the points of view of editorial policy and electoral policy to scientific advisory boards. The perception is that some scientists performing or evaluating research

may be tempted to overlook an adverse effect in a research project (hoping to please the sponsor and to secure future funding from the same source); likewise, other scientists may be tempted to overemphasize the significance of a positive result in their research (to gain access to additional grant support from private or institutional foundations or to favor donations from the general public). A number of scientific journals (including Toxicological Sciences) have recently addressed or reviewed the issue of conflict of interest. Similarly, a number of institutions have also codified their policy to help in the selection of members for scientific advisory boards. A number of questions can be asked around several themes: What is a financial conflict of interest? What about grant renewal, university tenure? What is the importance of non-financial conflicts of interest in the decision process, e.g., number of publications, academic competition, public/professional visibility, satisfaction of accomplishment, loyalty? When does a conflict start and stop being a conflict, apparent or real? Should a scientist with a real conflict of interest be prevented from publishing an editorial or a review paper on the basis of a conflict of interest? What if the conflict is not real but perceived? Should a perceived conflict of interest be enough to disqualify an otherwise competent scientist from election to a panel? What is the role of full disclosure in a successful conflict of interest policy? A group of five panelists (from diverse backgrounds) will present their views concerning the conflict of interest.

## 1679 WHAT IS A "CONFLICT OF INTEREST"?

T. L. Beauchamp. *Kennedy Institute of Ethics, Georgetown University, Washington, DC.* Sponsor: S. Gilbert.

What is a "conflict of interest"? It is too vague to say that it is "A conflict between private interests and official responsibilities of a person in a position of trust." However, it is very difficult to construct a definition that is not at once both too broad (prohibiting too much) and too narrow (allowing too much). Many definitions are also impracticable. A better definition will appeal to the need for impartiality in acting on duties of loyalty to protect certain interests when those interests come into conflict with a personal interest or a duty of loyalty to act in the interests of a different person, group, or institution. No definition will escape the need to construct practical rules of COI. Most institutions now have some rules governing financial COI, but none (or very few) regarding nonfinancial COIs. The following relationships are examples of relationships in large institutions that constitute a conflict of interest when one person is in a position to hire, promote, or advance another person: 1. They have entered into a personal financial relationship with each other. 2. Their relationship is more than professional (regardless of whether the relationship is a positive or a negative one); this includes but is not limited to ...close personal friendship; ...family connection (spouse, domestic partner, parent, son or daughter, cousin to the second degree); ...romantic relationship; ...relationship of trust (lawyer, doctor, trustee, consultant and the like). 3. They have collaborated on a grant or have coauthored or coedited a paper/article/book together. 4. Either has served as a mentor to the other. It is controversial how to implement COI rules and who should determine whether there really is a conflict, by contrast to a perceived conflict. Many believe that it should be left up to each individual person who potentially has a COI. Recusal is often handled in the same manner. But this "personal disclosure" solution is too subjective. Policies so constructed have generally failed to be serious monitors of COI.

## 1680 CONFLICT OF INTEREST IN THE PUBLICATION PROCESS.

L. D. Lehman-McKeeman. *Bristol Myers Squibb Co., Princeton, NJ.*

Conflicts of interest may occur in scientific research in situations where financial or other personal considerations may bias the professional judgment of an author conducting or reporting research or a reviewer involved in the peer review process. Conflicts may be real or perceived, harmful or insignificant. Whereas such conflicts may represent the potential for compromised judgment, they do not necessarily indicate the likelihood that such bias will occur. However, there is increasing sensitivity to the subject of conflict of interest disclosures relative to the publication of peer-reviewed manuscripts, and over the past several years, many biomedical journals including Toxicological Sciences, have adopted policies governing the need for full disclosure of potential conflicts of interest. The International Committee of Medical Journal Editors (ICMJE) has published uniform requirements for manuscripts submitted to biomedical journals, and Toxicological Sciences ascribes in principle to this policy (<http://www.icmje.org/index>). The need for the conflict of interest policy is not driven by the sense that the integrity of toxicological research has been compromised. Rather it is based on the recognition that such disclosures serve to inform readers about any special interests, enabling them to make informed judgments about the potential significance or lack of significance of a disclosure. Similarly, disclosures by reviewers serve to inform the Editor regarding any conflict of interest that could bias their opinions of a manuscript, and reviewers are asked to

disqualify themselves when they believe it is appropriate to do so. For all manuscripts, authors and reviewers are asked to state explicitly whether any conflicts exist. When noted by authors, these disclosures are indicated in the published article. Disclosing conflicts of interest does not ensure a faultless publication process. However, providing such information helps to authors, reviewers and readers to focus on the quality and reliability of the data, and ultimately to collectively benefit the scientific integrity of the journal.

## 1681 ROLE OF GOVERNMENT OVERSIGHT.

*B. A. Schwetz, Office for Human Research Protections, Rockville, MD.*

Conflicts of interest have undermined the trust of the public in research involving animals and humans, whether real or perceived. Many government and nongovernment institutions have developed guidelines to identify and handle competing interests that could compromise the integrity of decisions made by their employees or constituents. Financial conflicts of interest have received more attention than either institutional or professional conflicts of interest, because of available guidelines or perhaps because they are easier to document and quantify, not because they are necessarily more important than institutional or professional conflicts of interest. Many, but not all, institutions have developed internal guidance on conflicts of interest. Federal and state agencies and departments have regulations and guidances that relate to conflicts of interest, primarily for conduct of research involving animals and humans and participation in federal advisory committees. Conflicts of interest are not inherently wrong and are hard to avoid. However, special steps are needed to assure that conflicting and competing interests do not interfere with the responsible conduct of research and other professional and scientific activities.

## 1682 MANAGING CONFLICTS OF INTEREST: DOES DISCLOSURE GO FAR ENOUGH?

*M. Goozner, Cntr for Sciences. in the Public Instrst, Washington, DC. Sponsor: S. Gilbert.*

The laws designed to protect the public in environmental, health, nutrition and related areas recognize that sound regulatory policy depends on sound science. At the same time, businesses subject to regulatory decision making seek to minimize regulatory costs and limit the level of government intervention in the economy. Business interests seek to intervene in the regulatory process by funding scientists whose conclusions are in line with their desired regulatory outcomes. The influence of funding sources on outcomes of scientific research has been well documented. Industry-funded science is more likely to reflect the economic interests of funders. Findings that negatively influence funders' interests are less likely to appear in the literature. Researchers with industry-backing are better positioned in terms of financial support and other benefits to serve on government scientific advisory committees, appear before regulatory bodies and testify before Congress. Gatekeepers of scientific integrity have opted for conflict of interest disclosure policies as the best way to manage these competing interests. Leading scientific journals have adopted disclosure policies. Government agencies are supposed to prohibit conflicts of interest on scientific advisory panels and balance the points of view on committees. Evidence is mounting that disclosure is a failed policy for managing conflicts of interest. Government agencies increasingly waive or overlook conflicts of interest in staffing their advisory committees. Regulatory agencies like the FDA are increasingly funded by industry through user fees and depend on industry-funded science in making regulatory decisions. New strategies are needed to mitigate the effects of scientific conflicts of interest on the regulatory process. Government agencies must adopt new standards of scientific independence for its in-house staff. Congress must provide increased funding for independent scientists to conduct the studies that will determine regulatory decisions. The legal structure must be changed so that decision makers are mandated to rely on independent science when making regulatory decisions.

## 1683 CONFLICT OF INTEREST AND BIAS: A VIEWPOINT FROM INDUSTRY.

*C. Barrow, Dow Chemical Company, Washington, DC.*

In recent years, some groups have questioned the reliability and credibility of public health and environmental research conducted and/or sponsored by industry. Some have even suggested that all industry conducted or funded science be barred from use in weight-of-the-evidence risk assessment decisions and industry scientists be barred from peer review panels. Despite such allegations, industry conducted and funded scientific studies have long been and continue to be recognized by government agencies, many NGOs, and the scientific community at large as necessary and valuable contributions to the understanding of human health and environmental effects of chemical substances. It is clear that in many instances, there is an under-

appreciation of the exceptional measures employed by industry to assure the quality and credibility of scientific studies and data. These include for example: • conducting studies under Good Laboratory Practice (GLP) regulations which entails full availability of the raw, quality-assured data files, • the use of approved national and international protocols and test guidelines, • publication in the peer-reviewed literature, service on editorial boards, and participation on government sponsored peer review boards and committees. Further confidence in the scientific standards and processes of industry generated information is underpinned by the recognition of the EPA that of the various types of data and information employed in risk assessment, GLP derived data will most readily meet the requirements of the Information Quality Act<sup>1</sup>. As appropriately stated by the Society of Toxicology research should be judged on the basis of scientific merit, without regard for the funding source or where the studies are conducted (e.g. academia, government, or industry)<sup>2</sup>. <sup>1</sup>USEPA 260/R-02-008. Guidelines for Ensuring and Maximizing the Quality, Objectivity, Utility, and Integrity of Information Disseminated by the Environmental Protection Agency, October, 2002. <sup>2</sup>Principles for Research Priorities in Toxicology. Adopted by the Society of Toxicology, January 1998.

## 1684 DOSIMETRY AND REPRODUCTIVE/DEVELOPMENTAL STUDY DESIGN AND INTERPRETATION FOR RISK OR SAFETY ASSESSMENT.

*E. Carney<sup>2</sup> and H. A. Barton<sup>1</sup>. <sup>1</sup>USEPA, Research Triangle Park, NC and <sup>2</sup>Toxicology & Environmental Research & Consulting, Dow Chemical Company, Midland, MI.*

Reproductive and developmental toxicity studies in animals are utilized in assessing the potential adverse effects of chemicals and drugs in pregnant women, nursing infants, and children. The results of these studies are extrapolated to humans primarily based on the dose or the exposure in the mother due to the complexity of describing the dose, pharmacokinetics, and tissue dosimetry of chemicals during pregnancy, lactation, and postweaning periods. Transporters are increasingly recognized as a key factor in dosimetry during early life along with development of metabolism and other clearance processes. For pharmaceuticals, measurement of maternal blood levels, and sometimes placental and lactational transfer, are addressed for safety assessment. Classical and physiologically-based pharmacokinetic analyses are increasingly being applied across these life stages of reproduction. Identification of the critical window of sensitivity, and how this critical period and its associated pharmacokinetics correlates with humans, is a significant challenge. This workshop will present approaches to characterizing measures of internal dose in reproductive and developmental toxicity studies, and how this impacts study designs and applications for safety and risk assessment.

## 1685 LIFE-STAGE DEPENDENT DOSIMETRY AND POTENTIAL IMPACTS ON RISK ASSESSMENT.

*H. A. Barton, USEPA, Research Triangle Park, NC.*

Risk assessments for environmental chemicals are typically based on exposures in the mother when effects are observed in reproductive or developmental toxicity studies in animals. Knowledge of the windows of susceptibility and pharmacokinetic changes occurring during development facilitate the use of measures of internal dose in risk assessments. Issues and approaches, including pharmacokinetic modeling, will be described for selected chemicals including perfluorooctanoic acid (PFOA). Evaluation of potential differences in susceptibility to cancer with early life exposures requires distinguishing between differences in exposure and response. For a number of chemicals acting through mutagenic modes of action, specific experimental designs use defined injection doses to the animals at different ages. Therefore, in the absence of adequate pharmacokinetic modeling or experimental data, it is unclear to what extent the differences in tumor incidences reflect differences in exposure or response. Effects were observed in offspring in one-generation studies in rats exposed to perfluorooctanoic acid. One compartment pharmacokinetic analyses were undertaken to describe the average daily area under the curve in blood of PFOA for pregnancy, lactation, post-weaning pups, and male and female adults following gavage dosing. PFOA urinary clearance changes dramatically with age likely due to developmental changes in an organic anion transporter in rat kidney. These estimates were compared to maternal dosing to gain insight into the range of risk estimates that would be obtained depending upon when the critical window occurred. (This abstract does not represent EPA policy.)

## 1686 INCORPORATING DOSIMETRY IN DEVELOPMENTAL TOXICITY ASSESSMENTS: STUDY DESIGN AND DATA INTERPRETATION.

*E. Mylchreest and S. A. Gannon, DuPont Haskell Laboratory, Newark, DE.*

Standard developmental toxicity testing of chemicals generally does not include determination of maternal and developmental dosimetry. Generally, the need for developmental dosimetry data is prompted by the prior observation of developmental

toxicity, and the design of a useful dosimetry study in developing animals is facilitated by prior knowledge of the critical window of exposure during development as well as some knowledge of the toxicokinetics of the chemical. Unfortunately, for most chemicals such information is rarely available, particularly the critical window of exposure and the active metabolite(s) are not known, and selection of sample time points is based on technical feasibility (e.g. whole embryos can be harvested at early time points; placental and fetal blood samples can be obtained at later stages). In the case of perfluorooctanoic acid (PFOA), dosimetry studies were prompted by the observation of a small increase in offspring mortality in the first generation of a two-generation reproduction study. Previous toxicokinetic data indicated that PFOA is not readily metabolized and has a short half-life in adult female rats, but little or no data was available in pregnant or lactating rats. The concentration of PFOA was determined in maternal milk and plasma, placenta, amniotic fluid, and conceptus (embryo, fetus, pup, and fetal and pup plasma) following repeated oral dosing of the dam using 3 graded dose levels during gestation and lactation. PFOA in maternal milk was ten times less than maternal plasma (steady state), which was twice that in fetal plasma near term. Milk levels were generally comparable to pup plasma, which decreased from postnatal day 3 to 7, and were similar thereafter. PFOA was detected in the early embryo, amniotic fluid and placenta. Statements can be made about the relative concentration of PFOA in mother and conceptus but interpretation of the data is challenging due to the lack of a known critical developmental period for induction of offspring mortality.

## 1687 TRANSPORTERS DURING DEVELOPMENT.

C. D. Klaassen. *Pharmacology, U Kansas Med. Ctr, Kansas City, KS.*

Xenobiotics have been thought to distribute into and out of tissues by simple diffusion of the lipid soluble chemicals. However, it has not been clear how xenobiotics could efflux from tissues after they are made much more water soluble by phase I and II biotransformation enzymes. It is now known that there are transport proteins in cell membranes that can transport xenobiotics and their metabolites out of cells. Two important family of transporters that perform these efflux functions are the Multidrug Resistance Proteins (Mdrs) and Multidrug Resistance Associated Proteins (Mrps). These proteins are most abundant in liver and kidney for excreting chemicals, and in brain capillaries where they prevent many chemicals from entering the brain. In addition to these efflux transporters, there are also uptake transporters that aid in the distribution of xenobiotics into cells. Three families of transporters are especially important for uptake: namely Organic Anion Transporters (Oats), Organic Anion Transporting Polypeptides (Oatps), and Organic Cation Transporters (Octs). These transporters are especially predominant in liver and kidney for the elimination of xenobiotics. Some of these transporters are expressed in the placentae similarly to that in liver and kidney, suggesting they might protect the fetus from xenobiotics. The Mdr transporter has been shown as one of those transporters that protect the fetus from xenobiotics.

## 1688 PHARMACEUTICAL PERSPECTIVE ON DOSIMETRY IN REPRODUCTIVE AND DEVELOPMENTAL STUDIES AND THE IMPACT ON DRUG DEVELOPMENT.

G. Pastino. *Drug Metabolism and Pharmacokinetics, Schering Plough Research Institute, Lafayette, NJ.*

Over the past decade, the FDA and others have become increasingly concerned about the safety and efficacy of drugs in the pediatric population. Federal regulations have been developed which mandate pharmaceutical companies to provide information on the safe use of drugs in this population. Exceptions may be made on a case by case basis but only in instances in which children will not be exposed to the drug. Information that may guide the safe and effective use in children can be obtained during pre-clinical drug development through the conduct of reproductive and developmental toxicity studies or studies in juvenile animals. However, these studies generally do not characterize the pharmacokinetic profile and dosimetry. Clinical trials are also conducted in children, the nature and timing of which depends on the specific drug being developed. At a minimum, studies are required to determine the dose that produces similar exposure to that in adults at the recommended dose taking into consideration formulation issues. This is true when the indication, the disease process and the outcome of therapy are similar between adults and children. However, when a drug will be used to treat a disease primarily affecting children, or if the disease is life threatening and there are no other therapeutic options, more extensive safety and efficacy information in children is required. There are several empirical limitations in the conduct of clinical PK studies in children, such as small blood volumes and bioanalytical assays requiring extreme sensitivity. There are also many differences between adults and children in physiological and biochemical processes that may alter the PK profile in children (i.e., absorption, metabolism, protein binding, excretion) all of which need to be considered si-

multaneously in order to accurately assess safety in children. A potentially useful tool for accomplishing this task is the development and appropriate application of physiologically based pharmacokinetic models in juvenile animals and children.

## 1689 PBPK MODELING OF EARLY LIFESTAGES AND ESTIMATION OF DOSIMETRY FOR RISK ASSESSMENT.

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The purpose of dosimetry in human health risk/safety assessment is to provide a quantitative estimate of an exposure concentration or dose in the human population of interest that would be equivalent, from the viewpoint of tissue exposure, to a particular exposure concentration or dose (e.g., NOAEL) derived from toxicity studies. Over time, risk assessment dosimetry has evolved from methods based solely on administered dose or exposure concentration to methods that attempt to define equivalent exposures of a specific target tissue to a chemical or its active metabolite. Recently, there have also been increasing efforts to extend dosimetric methods to early lifestages, focusing on the exposure of the fetus or neonate rather than just the exposure of the mother. This presentation will describe the use of physiologically based pharmacokinetic (PBPK) modeling to provide dosimetric evaluation of gestational, lactational, and neonatal exposures. Specifically, early life PBPK modeling will be discussed from the viewpoint of elucidating the principal factors that must be considered in performing dosimetry for perinatal exposures. Appropriate early life dosimetry is critically dependent on several factors: the lifestage at which the effect of the chemical occurs (gestation, lactation, post-weaning), the kinetic characteristics of the chemical (persistence, bioaccumulation), and the mode of action for the chemical interaction with the target tissue. A categorical default approach for early life dosimetry is proposed, based on equivalent body burden (average tissue concentration) during the window of developmental susceptibility, and using simple compartmental modeling constructs (intake, volume of distribution, clearance). Examples of the approach are provided for both non-persistent (perchlorate) and persistent (Mirex) chemicals.

## 1690 SKIN MODEL SELECTION FOR SAFETY ASSESSMENT OF TOPICAL DRUG PRODUCTS: REGULATORY AND INDUSTRY PERSPECTIVES.

G. G. Qiao. *CVM and CDER, USFDA, Rockville, MD.*

Improper skin model selection in drug R&D and regulatory approval may under- or over-estimate systemic toxicity risk of a topical drug, resulting in significant resource and public health costs. Comparison and careful selection of *in vitro* versus *in vivo*, animal versus human, healthy versus diseased skin models under various exposure conditions during topical drug development and approval are critical. However, our knowledge base in chemical dermal absorption is mainly built on the healthy skin absorption data. For this reason, the regulatory evaluation of drug safety profile has been largely based on healthy skin study data submitted by drug sponsors, especially in the animal drug sector. FDA, per its general guidelines, requires target animal safety profiles to be tested in healthy subjects for veterinary drug products including topical drugs to be applied to diseased skin. Human dermal drug safety testing employs a wider range of animal models for various purposes. The question is whether the healthy skin data can predict what happens in patients with compromised skin and whether our testing methods are reflecting the most recent progresses in this field. Data have suggested diseased or damaged skin can compromise skin barrier function, and thus enhance systemic as well as local toxicity risk. Those issues deserve closer research and regulatory attention. Through this workshop, impact of compromised skin barrier function by skin diseases or by other exposure variables on systemic versus local, short term versus long term risk are addressed from academic research, industrial R&D, and regulatory approval perspectives. Comparative skin histology, skin biology, skin barrier function assessment, dermal absorption, cutaneous drug disposition, and government guidelines for safety testing of human and animal dermal drugs are to be discussed with research data. Inputs from the scientific community on those issues are helpful for future government guideline revisions.

## 1691 COMPARATIVE MODEL SELECTION BETWEEN SPECIES AND ABNORMAL SKIN: MORPHOLOGY, DERMAL DRUG DELIVERY, AND OVERALL BARRIER FUNCTION.

N. A. Monteiro-Riviere. *Center for Chemical Toxicology Research and Pharmacokinetics, North Carolina State University, Raleigh, NC.*

The focus of this presentation is to overview the basic anatomy, biology and barrier functions of human and animal skin in the context of chemical and drug absorption. Several anatomical and physiological factors that can influence the penetra-

tion of a compound demonstrate species and body site differences, making correct selection of the animal model an extremely important component in assessing the penetration of a compound. What animal models may serve as surrogates for humans? The epidermal stratum corneum with its lipid bilayers serves as the rate limiting barrier to absorption for most topically applied chemicals. Skin permeability may be increased or decreased depending on the condition of the skin. Certain skin diseases, as well as vehicle selection, may have an effect on compound penetration. Any disease process that alters the normal anatomical integrity would be expected to modulate chemical percutaneous absorption. Repeated dermal exposure of some compounds can influence the rate of penetration of these compounds through modification of these barrier structures. These factors will be thoroughly discussed in this presentation.

## 1692 SKIN MODEL SELECTION IN TOPICAL DRUG R&D AND REGULATORY APPROVAL: CHALLENGES AND SOLUTIONS IN DRUG SAFETY ASSESSMENT.

G. G. Qiao<sup>1</sup> and A. C. Jacobs<sup>2</sup>. <sup>1</sup>CVM, USFDA, Rockville, MD and <sup>2</sup>CDER, USFDA, Rockville, MD.

Dermal model selection for dermal drug safety testing is complicated by the fact that skin is the application site, the target site, and the port of entry for both systemic activity and toxicity. A careful configuration of a dermal test system regarding its biological, drug-related, and environmental (temperature and relative humidity) variables is essential for successful R&D and regulatory approval of a topical drug. This presentation is to focus on various factors influencing local disposition, systemic absorption, and thus toxicity of a topically applied drug along with supporting research data. Skin pathological condition (disease type/stage and skin pH), skin surface preparation (cleaning, tape stripping, and pre-exposure), dosing site selection (location, site number, and total area), skin barrier function assessment (TEWL and skin hydration), dose formulation and dosage, application procedure (alternative route, occlusion), drug-drug interaction, environmental variables, and dermal data interpretation are among the most relevant factors for assessing local and systemic safety profiles of a drug. Blood AUC may serve as a key parameter in selecting an *in vivo* model if correct extrapolations can be made. For example, if the targeted clinical patients with skin diseases have a risk of larger dermal absorption, the drug may need to be tested for systemic safety in a skin model that gives enhanced dermal absorption (e.g., by removal of the stratum corneum or via subcutaneous injection of the dose when another test model/species is not proper). Similarly, different safety tests require different test models, approaches, and study designs. Gaps between FDA target animal safety guidelines and the latest progress in dermal toxicology research along with potential solutions or alternatives are to be discussed while balancing the R&D and regulatory approval needs.

## 1693 TEST METHODS AND MODELS USED IN THE DEVELOPMENT OF TOPICALLY-APPLIED PRODUCTS.

J. F. Nash. *Central Product Safety, Procter & Gamble Company, Cincinnati, OH.*

A unique aspect of the toxicological evaluation of topically applied products is that the application and target tissue are clearly visible. As such, the health status of the application site, i.e., skin, and the intended use/benefits of the product are important factors when considering human health risk assessment. Preclinical models, *in vitro* or *in vivo*, used to evaluate dermal toxicity, e.g., irritation, utilize ideal skin in combination with exaggerated exposures to the chemical of interest. Beyond such local effects, establishing bioavailability of topically applied compounds is critically important since progression to systemic evaluations is in many cases predicated on the dermal penetration of the chemical. *In vitro* penetration studies using skin explants or more detailed *in vivo* animal pharmacokinetic studies are used to determine absorption into and through the skin. These experiments also utilize non-compromised skin and a range of doses. Later stages of safety testing may include the evaluation of product on targeted lesions or diseased skin. For OTC drug or cosmetic products, human safety evaluations may use subjects with skin disorders or sensitive skin. For example, to establish the human safety of a topically applied facial moisturizer containing salicylic acid, human pharmacokinetic studies were conducted in subjects with normal, acneogenic and photoaged skin. The latter two cohorts were considered to be skin conditions of targeted users of this product. In this example, there was no difference in the systemic bioavailability of salicylic acid among these groups after single or repeated application. Barrier function was uncompromised in these subjects which may account for the similarities among these groups. Thus, there is the need to consider the barrier status of the skin when considering irritation, sensitization, and photo-mediated toxicities. Human safety assessment of topically applied products should include the impact of the health status of skin either empirically, i.e., experimentally, or with the incorporation of conservative safety margins.

## 1694 GUIDANCE AND ANIMAL MODEL SELECTION FOR SAFETY ASSESSMENTS FOR DERMAL DRUG PRODUCT DEVELOPMENT AND APPROVAL.

A. Jacobs<sup>1</sup> and G. Qiao<sup>2</sup>. <sup>1</sup>CDER USFDA, Rockville, MD and <sup>2</sup>CVM USFDA, Rockville, MD.

The International Conference on Harmonization (ICH) Guidelines describe in general when nonclinical studies are needed to support human and animal drug development. These can be followed for general aspects of studies on safety pharmacology, genotoxicity, developmental/reproductive toxicity, and carcinogenicity. However, there are a number of dermal drug-specific issues that need to be addressed, including local tolerance, hypersensitivity, photo effects, and excipients, as well as the appropriate animal model selection. The Center for Drug Evaluation and Research (CDER)/FDA has separate guidances for photosafety testing, immunotoxicity testing, testing of excipients, and juvenile studies. Dermally administered human drugs may be applied to healthy skin, atopic skin, broken skin, psoriatic skin, or ulcerated skin. Although the skin of minipigs best mimics normal human skin, many nonclinical studies for dermal drug products are conducted in other species for a variety of reasons. For example, study of the systemic effects of a dermally applied drug in rats or rabbits may be appropriate for drugs to be applied to the skin of persons with atopic dermatitis. The most appropriate animal model will depend on the endpoint being evaluated (e.g., irritation, sensitization, systemic absorption/toxicity, reproductive/developmental effects, photo effects, or wound-healing effects), the formulation (e.g., patches vs creams vs solution), and the indication (e.g., wound-healing vs atopic dermatitis vs acne). Similarly, the same considerations are widely applicable to animal dermal drug development although a target species is often used since it is simply the best test model and is readily available. However, the impact of skin conditions and exposure variables on systemic and local toxic risk in animal dermal drug development and approval has been largely ignored.

## 1695 CONDUCTING A COMPREHENSIVE TOXICOLOGICAL AND SAFETY EVALUATION OF NANOMATERIALS: CURRENT CHALLENGES AND DATA NEEDS.

M. P. Holsapple<sup>1</sup> and W. H. Farland<sup>2</sup>. <sup>1</sup>HESI, Washington, DC and <sup>2</sup>USEPA, Washington, DC.

Nanomaterials are currently being used in a variety of commercial products including fabrics for clothing, circuitry for electronic components, and delivery systems for pharmaceuticals. In addition, nanotechnology could have a dramatic impact on a number of additional applications such as energy generation and distribution, food processing, and building construction. In light of numerous potential societal benefits of nanotechnology, a systematic approach needs to be developed to conduct a comprehensive toxicological and safety evaluation for nanomaterials. The goal of this Roundtable will be to present the current state-of-the-science associated with a characterization of the safety of nanomaterials. The key points to be addressed will be what kinds of data are needed with an emphasis on testing strategies for exposure assessment and hazard identification. The discussion of exposure assessment will highlight inhalation as a critical route of exposure as well as address the importance of dermal and other routes of exposure. The discussion of hazard identification will balance the need for new approaches as a result of the unique modes of action of nanomaterials with the ability to capitalize on existing methods. This roundtable will also explore how the unique properties associated with nanomaterials should be integrated into the testing strategies, the distinctive environmental and human exposures that result from nanomaterials, and the current challenges with regard to conducting a comprehensive toxicological and safety evaluation of nanomaterials.

## 1696 INHALATION AS A CRITICAL EXPOSURE ROUTE FOR EVALUATING NANOMATERIALS.

T. D. Landry. *Toxicology and Environmental Research and Consulting, The Dow Chemical Company, Midland, MI.*

Discrete particles in the nanoscale size range will predominantly deposit in the respiratory tract by diffusion. Once deposited, nanoparticles (NP, particles < 100nm) may cross biological membranes and access tissues to a substantially greater extent than would occur with larger particles. Inflammation has been observed with some NPs in the respiratory tract, likely reflecting the relatively large surface area of NPs. The accentuated inflammation may reflect physiologic conditions similar to studies in which there was particle overload of (rat) pulmonary clearance. Effects beyond the respiratory tract may also occur, with current investigations directed towards cardiovascular effects. Understanding the exposure and effects of discrete nanoparticles will be an important task for toxicologists. However, with the broad applications of nanomaterials for nanotechnology, a simple focus on discrete nanoparticles

is not adequate. Very high forces draw the NPs to aggregate and agglomerate, markedly affecting their propensity to become airborne, as well as their aerodynamic diameter. Many uses of nanomaterials in composites and structures severely limit or preclude airborne exposure. However, disaggregation, deagglomeration, and dissolution in biological fluids are other considerations that may influence health effects. Although inhalation will be a critical route of exposure in some cases, a full understanding of exposure and fate requires consideration of the technology applications and physical state of the nanomaterials.

#### 1697 DERMAL EXPOSURE ROUTE FOR EVALUATING NANOMATERIALS.

N. A. Monteiro-Riviere, *Center for Chemical Toxicology Research and Pharmacokinetics, North Carolina State University, Raleigh, NC.*

Skin can serve as a primary route of exposure to toxicants including novel nanoparticles (NP). Skin is unique because it is a potential route of occupational and /or environmental exposure to NP and also provides an environment within the avascular epidermis where particles could potentially lodge and not be susceptible to removal by phagocytosis. Can manufactured nanoparticles gain access to the epidermis? Would such particles preferentially locate in the lipids of the stratum corneum after topical exposure? Can keratinocytes exposed to NP elicit an early inflammatory response?

#### 1698 THE VALUE OF EXISTING TESTING STRATEGIES FOR EVALUATING NANOMATERIALS.

J. M. Carter, *Central Product Safety, Procter & Gamble, Cincinnati, OH.*

Information regarding the potential adverse effects of nanomaterials is currently limited. Although engineered nanoparticles have not been systemically tested, a few inhalation and epidemiology studies using ambient ultrafine particles have yielded some results for which preliminary conclusions can be drawn. Additionally, several studies using nano-sized particles have shed some light on the biokinetics and biodistribution of nanomaterials once inhaled or ingested. Pharmaceutical applications have also been a source of information regarding the potential translocation of nanoparticles from the site of exposure to remote areas throughout the body. While much speculation exists regarding the unique hazard of nanomaterials compared to bulk materials of like chemistry, current traditional toxicity testing methods have been used to identify hazards for nanomaterials. Effects from inhalation of micron-sized particles are generally restricted to the lung, or portal of entry, with no systemic distribution. Therefore, traditional inhalation toxicology studies have been typically restricted to studying effects directly related to the lung. Because more is known today regarding the kinetics and distribution of nanoparticles functional endpoints can be expanded beyond the traditional route of entry effect to include systemic effects. Using a combination of short-term mechanistic studies, *in vitro* studies and ultrafine-particle epidemiological studies represent important enhancements to the traditional inhalation toxicity assays. Integrating these traditional tools increase our ability for hazard identification of nanomaterials, a key first step in the risk assessment process.

#### 1699 MODE OF ACTION CONSIDERATIONS IN THE EVALUATION OF SAFETY OF ENGINEERED NANOSCALE MATERIALS.

N. J. Walker, *NIEHS, Research Triangle Park, NC.*

Nanoscale materials can be broadly defined as materials that have at least one critical dimension less than 100nm. While there has been considerable research on ambient ultrafine particles, the characterization of the potential health impact of engineered nanoscale materials that are produced through nanotechnology is an emerging issue of considerable discussion and debate. The diversity of size, aspect, chemical composition, surface chemistry and coating for engineered nanoscale materials raises multiple issues for the evaluation of their potential health impact. Namely (1) what are some of the anticipated modes of action and are there unique physicochemical properties that translate into novel modes of action for these materials? (2) what are the best experimental strategies to address recognized and novel modes of action? (3) can evaluation of a core set of properties or test substances be used for the prediction of effects for a broader set of nanoscale materials? (4) Can key parameters be identified to help ensure the development of safe nanoscale materials?

#### 1700 SUPPORT VECTOR MACHINE ALGORITHM FOR THE PREDICTION OF GENE FUNCTION USING A LARGE CHEMOGENOMIC REFERENCE DATABASE.

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Interpretation of toxicogenomic studies are often hampered by the large proportion of genes of unknown function in the rat genome. While unsupervised clustering of genes across many experimental conditions can be used as a means of inferring gene function through guilt by association, such a method is highly dependent on the variables used and suffers from a lack of predictive power. To more accurately ascribe a function to previously uncharacterized genes, a support vector machine (SVM) algorithm was used. Unlike unsupervised classification methods, the robust and sparse SVM algorithm employed herein identifies a small number of variables which best predict gene function. The variables were derived from over 3700 dose-time point-tissue combinations from rats treated with 600 reference drugs, toxicants and biochemical standards in triplicate short term repeat dose studies. Signatures were derived using training sets consisted of genes with known functions related to oxidative stress, acute phase response, PXR activation, cell cycle as well as other toxicological mechanisms of interest. Iterative split-sample cross validation confirmed that these signatures were capable of positively identifying genes known to play a role in these processes, as well as uniquely identifying other genes and ESTs not previously ascribed with the identified function. In total, we were able to assign a putative gene function to over 1200 uncharacterized genes on the CodeLink RU1 microarray. Application of the gene function signatures to genes representing known drug targets revealed putative new roles for these targets, thus suggesting new uses for small molecule inhibitors of these targets. The methodology could also be extended to identify new drug targets in disease related pathways, and for annotating genes in other organisms. These results illustrate the use of supervised classification algorithms and a large reference database of expression profiles for the functional annotation of gene products to aid drug discovery and development.

#### 1701 A MULTIVARIATE DATA ANALYSIS TECHNIQUE FROM THE SYNTHESIS OF A PRIORI KNOWLEDGE AND EMBEDDED STATISTICAL STRUCTURE.

P. Wilson, *Air Force Research Laboratory, Wright-Patterson AFB, OH.* Sponsor: L. Schlagler.

The application of computer technology and statistical techniques in biological research has become necessary when processing high-dimensionality data sets generated from genomic, proteomic and metabolomic research; usually applying multivariate statistical analysis to reduce the data dimensionality to help with visualization of experimental results or to uncover relationships between experimental parameters and biological response. Previous work in multivariate data analysis used optimization techniques like principle component analysis (PCA) or independent component analysis (ICA), which seek out the statistical dependence structure that optimizes variance or statistical independence, respectively. Many reduction techniques rely largely on a priori assumptions about the underlying statistics of the biological process, often resulting in models with dubious suitability. More recently, a technique called network component analysis (NCA) in biological systems has been offered, which uses expert knowledge of the system's pathways to construct a model for optimization in the least-squares sense to the experimental results. A technique is purposed here that combines the advantages of using the NCA approach, which increases the likelihood of a model's veracity, and the advantages of assuming a general statistical structure to the data, which can elucidate new knowledge about a system's topology, or the connectivity between input parameters and output results. By assuming an underlying Markovian process to the data, the next higher order assumption to statistical independence, additional pathways can be conjectured based on optimizing the entropy.

#### 1702 PROBING ALTERATIONS OF THE MITOCHONDRIAL MEMBRANE PROTEOME IN A MURINE MODEL OF PARKINSON'S DISEASE USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY.

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An important murine model of Parkinson's Disease utilizes the neurotoxicant 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP). The mechanism of MPTP toxicity is thought to involve accumulation of the MPP<sup>+</sup> (1-methyl-4-phenylpyridinium) metabolite into mitochondria. A wide variety of mitochondrial membrane proteins can then be affected by oxidative damage, post-translational modifications, and/or altered production/degradation. Our understanding of this model of neuro-

toxicity requires in-depth analyses of the mitochondrial membrane proteome, but this has been complicated by their extreme hydrophobicity. Consequently, a method was developed to isolate mitochondrial membrane proteins from brain tissue for analysis by multidimensional liquid chromatography-tandem mass spectrometry. Mice (C57BL/6N) were treated with 2 x 10 mg/kg i.p. doses of MPTP two hours apart and were sacrificed 24 hours after the last dose. Mitochondrial fractions were isolated from murine forebrains following homogenization and centrifugation through Percoll gradients. Mitochondrial membranes were enriched using a sodium carbonate procedure before further purification and analysis. Membrane proteome profiles were obtained for both control and treatment groups. Based on the collected MS/MS spectra, 853 proteins (from ~ 9, 500 peptides) were detected in the sample obtained from the MPTP-treated mice whereas 784 proteins (from ~ 6, 500 peptides) were identified in the control. A total of 422 proteins were common to both samples, which included numerous proteins comprising complex I of the respiratory chain and the subunits of complexes II, III, IV, and V ( $F_0F_1$  ATP synthase). Analysis of the proteins unique to the control or the treated samples revealed alterations in the mitochondrial membrane proteome following MPTP administration. Changes in post-translational modifications, including phosphorylation and oxidative damage, are also being conducted.

#### 1703 NOVEL NETWORK ANALYSIS FOR TOXICOLOGY USING KEYMOLNET.

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We developed a comprehensive information platform for toxicological, medical, pharmaceutical and life science research in the post-genome era, named KeyMolnet. Using KeyMolnet, we show new approaches to network analysis for DNA microarray data. Thanks to the rapid progress of technology in recent years, it is now possible to obtain very large quantities of gene expression data at a time. However, difficulties still remain in extracting meaningful information from such large quantities of data and in analyzing the relationship between the data and biological function. We therefore developed an advanced tool, which can generate molecular networks upon demand, and can bind them to physiological events, disease and drug information. Here we show novel approaches to the mechanism analysis of the relationship between the effects of cigarette smoke on human airway epithelial cells and lung cancer, using the public DNA microarray data (GEO accession#GSE994, Proc National Acad Sciences US A. 2004 6;101(27):10143-8., PMID: 15210990) and KeyMolnet. In this analysis, the different molecular mechanisms which occur in the airway epithelial cells of current and ex-smokers are discussed, by comparing the networks generated by up-regulated or down-regulated genes, using our original logical operation function without recourse to cluster analysis. KeyMolnet is suggested to be a powerful tool to enable practical approaches to research into biological and toxicological mechanisms, which in turn contributes to discovery in medical, pharmaceutical and life sciences.

#### 1704 BIOINFORMATICS METHODS FOR LIVER CANCER ANALYSIS USING CROSS SPECIES MAPPING BASED ON RAT GENE EXPRESSION PROFILING.

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Liver cancer is a public health concern in many parts of the world. We used albumin-SV40 transgenic rats, which spontaneously develop hepatic neoplasms within 6-9 months, as a model for the liver cancer study. We performed microarray studies on tumor samples obtained from the transgenic rats. Gene expression profiles were generated from two control, two adenoma and two carcinoma samples with a 10K mouse chip using a dye flip approach. Data from microarray analysis of liver tumors from these animals demonstrated an altered gene expression. Our analysis found that 2223 genes were differentially expressed across the sample sets using an F test with  $P < 0.05$ . A wide range of bioinformatics methods was used to determine the relevance of the findings in rat to human with respect to chromosomal aberration, pathways and functions (Gene Ontology). Using these bioinformatics tools, particularly ArrayTrack that is developed in house and available to the public, we found that genes related to cell cycle control, cell proliferation, apoptosis, transcriptional regulation, and protein metabolism were altered. We also closely examined gene expression in regions of previously identified chromosomal aberrations associated with early hepatic neoplasms in this transgenic rat model using a novel visualization tool. The utility of ArrayTrack was demonstrated in this project for analysis of gene expression data derived from microarray experiment. Analysis indicates that the altered gene expression associated with rat liver tumor development may be useful in the analysis of human liver cancer.

#### 1705 ASSESSMENT OF SPECTRAL INTEGRATION AND NORMALIZATION IN NMR-BASED METABONOMIC ANALYSES.

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Metabonomics involves the quantitation of the dynamic multivariate response of an organism to a pathological event or genetic modification. The analysis of this data involves the use of appropriate multivariate statistical and chemometric techniques to search for patterns. The premise is that changes in this profile of endogenous molecules can be used as a rapid screen for human risk assessment or as a tool to diagnose disease and monitor treatment outcomes. The most common approach in Nuclear Magnetic Resonance (NMR)-based metabonomics is to align the spectra to an internal reference standard, remove regions of endogenous peaks, spectral integration to reduce the number of variables, and normalization. The last two, the size of spectral integration regions and normalization, have not been well studied. We assess the classification accuracy on two distinctly different datasets via principal component analysis (PCA) and a leave-one-out cross-validation approach under an array or spectral integration regions and two normalization schemes. The first dataset consists of urine collected at five time points from 15 young male Wistar-Hannover rats exposed to -naphthylisothiocyanate (ANIT). The second consists of serum collected at two time points from young male C57BL/6 mice with intra-tracheal instillation to porcine pancreatic elastase or saline. This study indicates that independent of the normalization method the classification accuracy is not highly sensitive to the size of the spectral integration region. In addition, for both datasets, it was observed that the data scaled to mean zero and unity variance (auto-scaled) has higher within classification accuracy variability over spectral integration window widths than the data scaled to the total intensity of the spectrum that is mean-centered. (Funded by Battelle BSTI IR&D fund)

#### 1706 THE COMPARATIVE TOXICOGENOMICS DATABASE (CTD).

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Most human diseases involve the interactions between genetic and environmental factors. We are developing the publicly available Comparative Toxicogenomics Database to promote understanding about the effects of environmental chemicals on human health (<http://ctd.mdbi.org>). Our resource supports hypothesis-driven research about the molecular mechanisms of chemical actions by: 1) identifying gene-chemical interactions in diverse organisms through literature curation and integration of toxicologically relevant -omics data; and 2) facilitating cross-species comparative analyses of toxicologically important genes and proteins through the curation of Gene Sets, which group relevant sequences from diverse organisms and provide multiple alignment and phylogenetic analysis results. Gene Sets greatly assist the identification of conserved, functional regions and are providing important insights into the role of these genes in modulating chemical actions. Approximately 85, 000 chemicals are listed in the USEPA Toxic Substances Control Act Chemical Substances Inventory (USEPA 2003); however, the molecular mechanisms underlying their actions are not well understood. The diverse model organisms used in toxicology studies and the increasing quantity of sequence data available from such organisms present new opportunities for structure-function studies that may lead to improved prediction and prevention of toxicity. CTD is cultivating collaborations among toxicologists and other biological resources, encouraging data submission and participation by investigators worldwide that will contribute to the development of CTD, facilitating the integration of diverse, toxicologically relevant data sets, and supporting critical curation of toxicologically important genes and gene-chemical interactions. These features will make CTD an important resource for the toxicology community and help scientists explore the complex relationship between the environment and human health.

#### 1707 WITHIN- AND BETWEEN-ANIMAL VARIATION, AND REFERENCE RANGES OF HEMATOLOGICAL AND SERUM BIOCHEMICAL PARAMETERS IN CYNOMOLGUS MONKEYS.

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[Purpose] Cynomolgus monkeys, one of a number of primates phylogenetically close to humans, are commonly used in animal studies. Basic information obtained from cynomolgus monkeys nourished and maintained under artificial conditions largely contributes to the improvement of an evaluative health condition index, and improves the accuracy of studies in this genus. The purpose of this study is to assess

biological variations and reference ranges of hematological and serum biochemical parameters in cynomolgus monkeys. [Methods] Data from 73 male and 81 female Chinese-bred cynomolgus monkeys aged 3 to 7 years showing no abnormalities during the breeding period were used. After performing suitable transformation, the overall reference range was calculated as mean +/- 1.96SD. To assess within- and between-animal variations, analysis of variance (ANOVA) was applied. A simple method, which applies prior information, was also proposed to estimate individual reference ranges. [Results] Reference ranges of hematological and serum biochemical parameters in cynomolgus monkeys were estimated for males and females separately. Between-animal variances were also evaluated and parameters including MCV, MCH, PT, ALP, T.Chr., and CRNN appeared to show a large between-animal variation. [Conclusion] It is essential to use accurate reference ranges to assess health condition, biological variations, or effects of drugs or endocrine-disrupting chemicals in the study system. For that purpose, we have presented reference ranges for hematological and serum biochemical parameters in cynomolgus monkeys. We also evaluated within- and between-animal variation and found that some parameters show large between-animal variation. Individual reference ranges of these parameters for each animal estimated by the proposed method would make it possible to assess biological variation or effects of drugs or endocrine-disrupting chemicals more accurately.

## 1708

### THEORETICAL TARGET SEQUENCE FOR THE INTERACTION BETWEEN AFLATOXIN B1 AND PROTEINS.

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Aflatoxin B1 (AFB1) is one of the most potent human carcinogens. After CYP-mediated activation to the carcinogenic metabolite AFB1 8, 9-epoxide (AFBO), this reactive compound can form adducts with both proteins and nucleic acids, leading to alterations in protein function and mutations, respectively. The goal of this study was to determine the molecular characteristics that can be considered to predict if a particular peptide sequence might bind to exo-AFBO. Using quantum chemical calculations, the structure of conjugates exo-AFBO-peptide were energetically optimized using the semiempirical method AM1, whereas different molecular properties were calculated at the DFT/B3LYP/631G level. Starting sequences were constructed using cysteine and lysine as covalent bridges and new aminoacids were added once an energetically favorable conjugate was found. Free energies of the reactants and the products, as well as their molecular reactivity descriptors such as global softness, hardness and electronegativity were used to select candidate peptides. Preliminary results have shown that several peptides derived from the sequence MGCES have good tendency to form adducts with AFBO. The conjugate stability increases with the number of intramolecular hydrogen bonds and the geometrical and chemical nature of each peptide, clearly suggesting that the reaction between AFBO and proteins is driven by specific structure-activity relationships. Supported by the University of Cartagena (Cartagena) and Colciencias (Bogota), Colombia. Grant 1107-05-14663.

## 1709

### A DATABASE FOR TRACKING TOXICOGENOMIC SAMPLES AND PROCEDURES WITH GENOMIC, PROTEOMIC AND METABONOMIC COMPONENTS.

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Toxicogenomics generates massive amounts of molecular data in parallel with traditional toxicological endpoints in various formats produced by researchers from different scientific disciplines. Conventional toxicology includes biochemical, histopathological and physiological endpoints, as well as information on treatments, tissues and experimental procedures. The molecular data generated by 'omic technologies are the result of a complex series of experimental and analytical procedures. Thus, data storage and management is essential to toxicogenomics, and requires a more sophisticated system than lab notebooks and electronic spreadsheets. We developed a database for tracking Toxicogenomic Samples and Procedures (TSP) based on the MIAME-Toxicology guidelines and relational database theory. We report here the extension of TSP beyond genomics, to include proteomics and metabolomics components. TSP has a hierarchical structure, capturing information on study design, animal treatments, toxicological endpoints, tissue and biofluid specimens, sample characteristics and quality metrics, and linkage to subsequent toxicogenomics data. The user-friendly interface enables researchers to easily add, edit, save, delete, and navigate different records. TSP is implemented in Microsoft Access software, permitting distribution to any laboratory running Microsoft Office. TSP creates standardized data annotation and entry systems sup-

porting complete reporting and information exchange between researchers from different scientific disciplines. Finally, TSP facilitates exporting data, including corresponding toxicological endpoints and details relevant to study design, into public databases such as CEBS, the Chemical Effects in Biological Systems Knowledgebase. This abstract does not necessarily reflect EPA policy.

## 1710

### INFLUENCE OF PROTEIN ALLERGENS ON DENDRITIC CELL ACTIVATION.

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The induction of immune responses, including allergic responses, by dendritic cells (DC) is dependent not only upon the provision of appropriate antigenic peptides for T lymphocyte recognition, but also upon concomitant delivery of costimulatory signals. We have demonstrated that the major allergens from egg, brazil nut and potato (ovalbumin; OVA, ber e 1 and sol t 1) each induce specific IgE production following systemic administration to BALB/c strain mice. The effects of these proteins on the activation status of cultured mouse (BALB/c) bone marrow derived DC have now been examined. Endogenous endotoxin content of the proteins was 0.53 endotoxin units (EU)/ $\mu$ g (OVA), 0.05EU/ $\mu$ g (ber e 1) and 1.5EU/ $\mu$ g (sol t 1). DC were cultured for 24h with protein (0 to 100 $\mu$ g/ml) prior to assessment of surface marker expression associated with DC maturation (MHC class II, CD40, CD54, CD80 and CD86) by flow cytometry and cytokine content (interleukin [IL]-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-10, IL-12, IL-17 and tumor necrosis factor  $\alpha$ ) of culture supernatants by cytokine microarray. Incubation of DC with either OVA or sol t 1 caused dose dependent increases in membrane marker expression and cytokine secretion, similar effects to those caused by treatment of DC with bacterial lipopolysaccharide (LPS). In contrast, Ber e 1 failed to up-regulate any of the maturation markers examined and did not stimulate cytokine secretion. To evaluate the potential contribution of endogenous endotoxin to DC activation, cells were cultured with OVA or sol t 1 in the presence of polymyxin B (PmB) to neutralize endotoxin. Under conditions where PmB inhibited completely DC maturation and cytokine secretion caused by 100ng LPS (163EU), PmB inhibited only partially DC activation provoked by 100 $\mu$ g OVA (53EU) or 100 $\mu$ g sol t 1 (150EU). These data suggest that while endotoxin contamination may represent an important consideration for the immunogenicity of these proteins *in vivo* and *in vitro*, allergen-specific effects on DC require further investigation.

## 1711

### CUTANEOUS CYTOKINE SECRETION PROFILES INDUCED IN MICE FOLLOWING EXPOSURE TO IRRITANTS OR CHEMICAL ALLERGENS.

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Chemical contact and respiratory allergens induce in mice upon repeated topical exposure divergent immune responses characteristic of T helper (Th1) and Th2 cell activation, respectively. Although, the mechanistic basis for the development of polarized immune responses to these chemicals remains unresolved, we have speculated that the pattern of cytokines induced in the skin upon first exposure to chemical may be important for skewing such responses. Using a skin explant assay we have now compared the cytokine secretion profiles induced following single topical exposure of BALB/c strain mice to the contact allergens dinitrochlorobenzene (DNCB, 1%) and oxazolone (Ox, 0.5%), with the respiratory allergen trimellitic anhydride (TMA, 25%) and the skin irritant sodium lauryl sulfate (SLS, 10%). Groups of mice were exposed on the dorsum of both ears to chemical 2 to 4h prior to preparation of dorsal ear explants for culture for 16h on medium. Analysis of culture supernatants by cytokine array revealed that exposure to DNCB (n=20) or to Ox (n=10) was associated with significant increases in interleukin (IL)-1 $\beta$  release by 4h of treatment (5.4-fold and 6-fold, respectively) compared to treatment with vehicle alone. No significant changes in this cytokine were observed following topical application of TMA (1.2-fold, n=20) or SLS (1.4-fold, n=4). Furthermore, only exposure to DNCB or Ox resulted in early (2h) IL-17 secretion (4.0-fold and 4.8-fold, respectively), which had declined significantly for both chemicals by 4h. In contrast, treatment with TMA promoted a marked early (2h) elevation in IL-10 release (2.8-fold) relative to vehicle, compared with DNCB (0.8-fold), Ox (1.8-fold) or SLS (0.6-fold). Other cytokines such as IL-6, IL-12p40 and IL-12p70 remained largely unchanged. These results suggest that single exposure of mice to irritants or chemical allergens may be associated with the stimulation of a distinct cutaneous cytokine secretion profile and that early production of IL-1 $\beta$  and IL-17 may be indicative of the development of Th1 responses to contact allergens.

## VALIDATION OF A MURINE MODEL OF CHEMICAL-INDUCED ASTHMA IN MICE, USING TRIMELLITIC ANHYDRIDE AND 1-CHLORO-2, 4-DINITROBENZENE.

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Testing the validity of our model of immunologically mediated asthma in mice, developed with toluene diisocyanate, using trimellitic anhydride (TMA), a known respiratory sensitizer and 1-chloro-2, 4-dinitrobenzene (DNCB), a known dermal sensitizer. On day 1, BALB/C mice received the chemical (20  $\mu$ l/ear, TMA 5% and DNCB 0.2%) or vehicle (acetone/olive oil) on each ear. On day 7 they received a dermal boost with the same concentration of the chemical (20  $\mu$ l/ear). On day 10, the mice were intranasally challenged with the chemical (TMA 1% or DNCB 0.02%, 10  $\mu$ l/nostril) or vehicle. Lung function was monitored by whole body plethysmography for 40 min after challenge. Pulmonary inflammation was assessed 24 h after challenge by bronchoalveolar lavage (BAL) and histology. TNF- $\alpha$  and MIP-2 were measured in BAL. Immunological parameters included total IgE serum levels, lymphocyte populations in auricular (AurLN) and superficial cervical lymph nodes (CervLN), IL-4 and IFN- $\gamma$  levels in supernatants of LN cells with or without concanavalin A (ConA) stimulation. Mice sensitized with TMA, boosted with TMA or vehicle and challenged with TMA experienced markedly increased Penh immediately after the intranasal challenge. DNCB treated mice did not show any ventilatory changes after intranasal challenge. 24 h after challenge a neutrophil influx (5-10%) and increase of MIP-2 and TNF- $\alpha$  levels were found in BAL in both TMA and DNCB treated mice. AurLN cells of mice treated with TMA or DNCB showed an increase in CD19+ B-cells. An increased level of IL-4 and IFN- $\gamma$  was found in supernatants of ConA stimulated AurLN and CervLN cells of TMA or DNCB treated mice. Serum total IgE was increased in TMA treated mice only. Both compounds induced a mixed Th1-Th2 response, but only TMA induced a pulmonary immunological response. These results confirm that the model we developed is potentially suitable to specifically screen for pulmonary allergens. DWTC PS/01/43 and FWO-Vlaanderen

## CCL2 KO MICE DEMONSTRATE ENHANCED TH2 RESPONSES FOLLOWING DERMAL SENSITIZATION.

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Host resistance studies have demonstrated the importance of CCR2 (C-C chemokine receptor 2) in Th1 responses and CCL2 (ligand of CCR2) in Th2 responses. These studies were undertaken to evaluate the role of CCR2 and CCL2 in the development of skin sensitization. To begin this evaluation, CCL2 and CCR2 knockout (KO) mice or C57BL/6 wild-type (WT) control mice were dermally exposed to 2% 2, 4-toluene diisocyanate (TDI), 30% alpha-hexylcinnamaldehyde (HCA), or vehicle (acetone). Their response to chemical exposure was evaluated using a modified LLNA (local lymph node assay), analysis of serum IgE, and phenotypic analysis of draining lymph node cells. CCR2 KO mice demonstrated a reduced stimulation index (SI) in the LLNA to 2% TDI, as compared to the WT controls (35.8 & 70.6, respectively) with little difference observed in the response to HCA (SI = 4.5 for CCR2 KO and SE = 5.1 for WT mice). Serum IgE levels after 2% TDI exposure were also significantly lower in CCR2 KO ( $5918 \pm 877$  ng/ml) compared to WT ( $8156 \pm 632$  ng/ml) mice. In contrast, CCL2 KO mice demonstrated an increased SI following exposure to 2% TDI as compared to WT controls (44.8 & 47.4, respectively), as well as a slight increase in proliferation to HCA (7.5 & 5.2, respectively). Moreover, CCL2 KO mice had a significant increase in serum IgE levels after TDI exposure ( $24542 \pm 5651$  ng/ml) when compared to WT treated with TDI ( $9345 \pm 2959$  ng/ml). Consistent with published studies in host resistance, where CCR2 KO mice demonstrated a Th1 defect, CCR2 KO demonstrated a slight decrease in their response to HCA, a Th1 inducing chemical. In contrast to observed effects on host resistance (Th2 defect) in CCL2 KO mice, the CCL2 KO mice demonstrated an enhanced Th2 response following exposure to the dermal sensitizer, TDI.

## DOSE-DEPENDENT INCREASE IN THE PRODUCTION OF NERVE GROWTH FACTOR, NEUROTROPHIN-3, AND NEUROTROPHIN-4 IN A *PENICILLIUM CHRYSOGENUM*-INDUCED ALLERGIC ASTHMA MODEL.

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Increased levels of neurotrophins (nerve growth factor [NGF], brain-derived neurotrophic factor [BDNF], neurotrophin [NT]-3, and/or NT-4) have been associated with asthma as well as in animal models of allergic asthma. In our mouse

model for fungal allergic asthma, repeated pulmonary challenge with *Penicillium chrysogenum* extract (PCE) induced dose-dependent allergic asthma-like responses in mice. The aim of this study was to investigate whether exposures to PCE could increase the production of neurotrophins in bronchoalveolar lavage fluid (BALF) of PCE challenged mice. Mice were exposed to 10, 20, 50, or 70  $\mu$ g of PCE by involuntary aspiration four times over one month. BALF was collected before (Day 0), and at Day 1 and 3 following the final exposure. The levels of NGF, NT-3, and NT-4 were determined by ELISA. Lungs collected at Day 0, 1, and 3 were perfusion-fixed and processed for immunohistochemical examination of NGF production. PCE-exposed mice had dose-dependent increases in NGF, NT-3, and NT-4. A single exposure to PCE did not significantly increase the production of the neurotrophins. Exposures to PCE caused an increase in positive immunohistochemical staining for NGF in epithelial and smooth muscle cells in addition to infiltrated cells such as mononuclear cells and macrophages. Taken together, mice that received multiple exposures to PCE had dose-dependent increases in NGF, NT-3, and NT-4 in BALF. This is the first study to link fungal allergic asthma in an experimental model with enhanced production of neurotrophins in the airways. (Supported by UNC/EPA Cooperative Training Agreement CT829471. This abstract does not reflect EPA policy.)

## CYTOKINE, ANTIBODY AND PULMONARY RESPONSES IN BALB/C MICE FOLLOWING DERMAL EXPOSURE TO SELECTED DIISOCYANATES.

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Exposure to certain low molecular weight chemicals is associated with asthma. A simple test is needed to identify chemicals that pose this hazard. Increases in total serum IgE or Th2 cytokines in draining lymph nodes following dermal exposure have been pursued as means to screen potential asthmagens. Previously, we developed cytokine mRNA profiles using the RNase protection assay for 6 isocyanates, (1% TDI, 2% MDI, 2% HMDI, 2% IDPI, 1% TMI, 1% TMXDI), and for 1% DNCB. The isocyanates could be divided into two groups (high & low responders) based on expression of Th2 cytokines. We hypothesized that dermal exposure to the high responders would result in enhanced pulmonary hyperresponsiveness to methacholine challenge. BALB/c mice received 10 dermal exposures over a period of 4 wks. Treatment groups included 6 isocyanates, DNCB and acetone:olive oil (vehicle). Mice were then challenged with increasing doses of methacholine; responsiveness was assessed using whole body plethysmography (Buxco). Serum, bronchoalveolar lavage fluid (BALF), and lymph nodes were collected for total IgE & IgG subclasses, cell counts, and cytokine profiling. There were no changes in total or differential cell counts for any of the exposure groups compared to vehicle. Significant increases in serum IgE & IgG1 were apparent for all treatment groups compared to vehicle. The cytokine mRNA profile in lymph nodes assessed by real time PCR produced the same pattern of high and low responders seen previously. Mice exposed to HMDI, TMI, and TMXDI (but none of the other treatment groups) showed significant hyperresponsiveness to methacholine. Also, hyperresponsiveness occurred after dermal exposure for only one of the 3 known asthmagens. This data suggests a disconnect between total serum IgE response, cytokine message and hyperresponsiveness. (This abstract does not reflect EPA policy.)

## IN UTERO EXPOSURE TO GENISTEIN (GEN) BY GAVAGE INCREASES IGE AND IGG1 PRODUCTION BY ADULT B6C3F1 MICE FOLLOWING TREATMENT WITH RESPIRATORY ALLERGEN TRIMELLITIC ANHYDRIDE (TMA).

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It has been reported that there is an increase in the use of asthma or allergy drugs in young adults who were fed soy formula during infancy as compared to those who were fed cow milk formula. The objective of the present study was to determine if *in utero* exposure to GEN had any effects on TMA-induced production of total IgE or IgG subclasses by adult B6C3F1 mice. Both male and female mice were exposed to GEN by dosing their dams, e.g. the pregnant C57BL/6 mice ( $\times$  male C3H), with GEN (20 mg/kg) via gavage from day 14 of gestation to post natal day (PND 3). The offspring were weaned at PND 22, and the sensitization to TMA was achieved by treating individual mice with 50  $\mu$ L of 10% TMA on the shaved backs in acetone:olive oil (3:1) at PND 70. Seven days later, the mice were challenged with 25  $\mu$ L of TMA on the dorsal side of both ears. In addition to evaluating the immunoglobulin content in the sera, other immunological responses including the natural killer (NK) cell activity and anti-CD3 antibody-mediated splenocyte proliferation were performed on PND 84. In males, *in utero* exposure to GEN had no effects on the terminal body weight, spleen weight, relative spleen weight, spleen cell

number, NK activity or anti-CD3 antibody-mediated splenocyte proliferation. However, a significant increase in serum levels of IgG1, but not total IgE and IgG, was observed when compared to the vehicle control mice. In females, no effects on the terminal body weight, spleen weight, relative spleen weight, or total spleen cell number were produced. However, a significant increase in serum levels of total IgE, but not IgG1 or total IgG, was observed. Furthermore, an increase in NK cell activity, but not anti-CD3 antibody-mediated splenocyte proliferation, was observed in female mice. Overall, the results demonstrate that GEN modulates the developing immune system in such a way that more IgG1 or IgE is produced upon exposure to TMA in adult mice (Supported by NIEHS R21 ES012286).

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DIFFERENTIAL EXPRESSION OF ARGINASE IN OVALBUMIN (OA) AND TRIMELLITIC ANHYDRIDE (TMA) INDUCED ASTHMA.

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Both TMA, a small molecular weight chemical, and OA, a reference protein allergen, cause occupational asthma. To determine if different mechanisms are responsible for lung eosinophilia in asthma depending on the allergen, Balb/c mice were sensitized with TMA or OA, followed by intratracheal challenge with TMA conjugated to mouse serum albumin (TMA-MSA) or OA, respectively, to elicit the allergic phenotype. MSA challenge was control. 72 hr later, measurement of eosinophil peroxidase in lung demonstrated the same significant increase in eosinophilia with both allergens. RNA was isolated from lung lobes of 6-8 animals in the 4 treatment groups and hybridized with Affymetrix U74Av2 microarrays. Statistical analysis used RMA-normalized, log scale intensities from GeneTraffic. False discovery rates (q-values) were calculated from an overall F test to identify candidate genes with differences in expression for the 4 groups. With a q-value cutoff of 0.1, 855 probe sets were identified as differentially expressed in OA vs TMA induced asthma. 287 genes of the 855 probe sets had a 1.2 fold increase or greater over control for either OA or TMA treated animals. Confirming studies of others, gene expression for arginase 1 was significantly increased in OA induced asthma. However, increased arginase expression was not detected in TMA induced asthma. RT-PCR analysis confirmed the microarray findings. In addition, arginase 1 enzyme activity was significantly increased in OA but not TMA induced asthma. Increased arginase 1 may reduce arginine availability for nitric oxide production in OA, but not in TMA induced asthma. The data suggest that pathways of arginine metabolism and the importance of nitric oxide in asthmatic inflammation may differ in OA and TMA induced asthma. (Support: DAMD 17-02-1-0191 and Supercomputing Inst, University of MN)

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4-HYDROXYNONENAL MODIFICATION OF MOLECULAR CHAPERONES IN A RAT MODEL OF CHRONIC ALCOHOLIC LIVER INJURY.

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Aldehydic lipid peroxidation products formed in excess under conditions of oxidative stress have a documented ability to modify proteins, DNA, and other important biomolecules, likely exacerbating any existing pathological condition. A proteome-wide scan was employed to detect hepatic proteins modified by 4-hydroxyneonenal (4-HNE) in a rat model of chronic alcoholic liver injury. Specifically, in subcellular fractions isolated from the livers of rats fed a combination high fat and ethanol diet, the molecular chaperones Hsp72, Hsp90, and Hsp60 were identified as targets for 4-HNE adduction using two-dimensional electrophoresis, immunoblot, and LC-MS/MS techniques. To test the hypothesis that 4-HNE modification of these heat shock proteins inhibits chaperone-mediated protein refolding, purified recombinant Hsp72, Hsp90, and Hsp60 were treated with 10  $\mu$ M 4-HNE, resulting in a 47%, 68%, and 25% reduction in protein refolding efficiency, respectively. Tryptic digest of 4-HNE-modified Hsp72 and LC-MS/MS peptide analysis demonstrated consistent modification of Cys 267 in the ATPase domain of the protein. The significance of Cys 267 modification was demonstrated using bacterial DnaK which lacks Cys 267 and was completely resistant to inactivation with 10  $\mu$ M 4-HNE. A significant decrease in ATP affinity following modification of Hsp72 with 10  $\mu$ M 4-HNE was demonstrated using ATP-linked agarose beads, and a corresponding decrease in substrate binding was confirmed by co-immunoprecipitation of the Hsp72/luciferase complex. The data presented here demonstrate modification and inhibition of Hsp72, Hsp60, and Hsp90 by micromolar concentrations of 4-HNE, along with a mechanism for Hsp72 inactivation. Inhibition of these chaperones may contribute to the progression of alcoholic liver disease by limiting the ability of a cell to efficiently repair mis-

folded or otherwise damaged proteins. This work was supported by NIH/NIAAA RO1AA09300 and NIH/NIEHS RO1ES09410 (DRP), NIH/NIEHS F32 ES11937 (JAD), and NIH/NIAAA F31 AA014308 (DLC).

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TOXICITY ASSESSMENT OF SILVER NANOPARTICLES (AG 15, 100 NM) IN ALVEOLAR MACROPHAGES.

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The present study was conducted to evaluate the acute toxicity of Ag 15nm and 100 nm nanoparticles in rat alveolar macrophages. For toxicity evaluations, cellular morphology, mitochondrial function (MTT assay), membrane leakage of lactate dehydrogenase (LDH assay), neutral red uptake (NR), reduced glutathione (GSH) levels, reactive oxygen species (ROS), and mitochondrial membrane potential (MMP) were assessed at 24 h under control and exposed conditions. The morphological appearance of control and Ag (15, 100 nm) exposed cells were observed by phase contrast microscope. The morphology of cells exposed to Ag 15 and 100 nm nanoparticles at 15 and 50  $\mu$ g/ml doses displayed cellular shrinkage, and an abnormal size and irregular shape. It was also noted that Ag 15 and 100 nm nanoparticles were surrounded by macrophages, some of these attached to the membrane. The results of biochemical studies showed that mitochondrial function decreased significantly in cells exposed to Ag nanoparticles at dose exposure from 10-50  $\mu$ g/ml. LDH leakage significantly increased in cells exposed to Ag nanoparticles (10-50  $\mu$ g/ml). Further, results demonstrated significant depletion of GSH level, reduced mitochondrial membrane potential, and increases in ROS levels in Ag (15 and 100 nm) exposed cells at 25 and 50  $\mu$ g/ml. A significant depletion of GSH levels appeared to create an overwhelming imbalance between antioxidants and ROS, thus resulting in cellular damage. The results suggest that silver nanoparticles likely to induce oxidative stress in macrophages. The potential activation of an inflammatory response in macrophage cultures will be further investigated by quantifying the levels of cytokines that include TNF- $\alpha$ , MIP-2, and IL-6.

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LIPIDOMIC ANALYSIS OF THE EFFECT OF INHIBITION OF  $Ca^{2+}$ -INDEPENDENT PHOSPHOLIPASE A<sub>2</sub> ON OXIDANT-INDUCED NEURONAL CELL DEATH.

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Recent studies in renal cells have demonstrated that inhibition of  $Ca^{2+}$ -independent phospholipase A<sub>2</sub> (iPLA<sub>2</sub>) increased oxidant-induced cell death. However, no research has examined the effect of iPLA<sub>2</sub> inhibition on oxidant-induced neuronal cell death. Accordingly, we tested the hypothesis that iPLA<sub>2</sub> mediates neuronal cell death induced by oxidants in A172 glioblastomas, a human brain cell line. Reverse transcriptase polymerase chain reactions, immunoblot analysis and activity assays indicated that A172 cells expressed both cytosolic (iPLA<sub>2</sub> $\beta$ ) and microsomal (iPLA<sub>2</sub> $\gamma$ ) isoforms of iPLA<sub>2</sub>. Measurement of annexin V (apoptotic cell marker) and propidium iodide (PI, oncotic cell marker) staining using flow cytometry demonstrated that the oxidants hydrogen peroxide ( $H_2O_2$ ; 0-1000  $\mu$ M) and tert-butylhydroperoxide (TBHP; 0-400  $\mu$ M) induced time- (0-48 hr) and concentration-dependent increases in cell death. Treatment of A172 cells with 2.5  $\mu$ M bromoenol lactone (BEL), a specific iPLA<sub>2</sub> inhibitor, prior to exposure to 500  $\mu$ M  $H_2O_2$  for 12 hr significantly increased oncosis compared to cells exposed to only  $H_2O_2$ . Similarly, treatment of cells with BEL prior to exposure to 200  $\mu$ M TBHP for 12 hr decreased apoptosis and increased oncosis. Analysis of forward and side scatter using flow cytometry was used to verify alterations in the mode of cell death morphologically. We further investigated the hypothesis that iPLA<sub>2</sub> mediates oxidant-induced neuronal cell death by determining the effect of BEL on the phospholipid profile of A172 cells. Common phospholipids expressed in A172 cells included 32:2, 36:3, and 36:4 phosphatidylcholine (PtdCho). Treatment with  $H_2O_2$  alone reduced 32:2 PtdCho 20% compared to untreated cells. BEL alone caused a 10% increase in 36:4 and 36:3 PtdCho but did not alter  $H_2O_2$ -induced decreases in 32:2 PtdCho. These data demonstrate that both microsomal and cytosolic iPLA<sub>2</sub> are expressed in neuronal cell cultures and suggest that iPLA<sub>2</sub> mediates oxidant-induced neuronal cell death, possibly by mechanisms involving phospholipid turnover.

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IDENTIFICATION AND CHARACTERIZATION OF SMALL MOLECULE ACTIVATORS OF THE ANTIOXIDANT RESPONSE ELEMENT.

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We have performed ultra high-throughput chemical screening involving over 1.16 million compounds to identify a number of unique small molecule activators of the antioxidant response element (ARE). The ARE is a transcriptional element respon-

sible for the production of phase II detoxification enzymes that protect cells against oxidative stress. Alternatively, small molecule activators of the ARE may inhibit histone deacetylases and induce caspase activity leading to cell death, both mechanisms having consequences in anticancer chemotherapy. Ligands were identified using an ARE-luciferase reporter construct transiently transfected into IMR-32 human neuroblastoma cells and validated in murine primary neurocortical cultures expressing an ARE-human placental alkaline phosphatase (ARE-hPAP) transgene. Compounds activating the reporters greater than 5 fold were selected for additional study. At 5 and 10 micromolar concentration these compounds protect against acute hydrogen peroxide toxicity in the neuroblastoma and primary cortical cell lines. To validate the ARE activating potential and toxicological effects of the compounds *in vivo*, preparations of the ligands were injected into ARE-hPAP transgenic reporter mice and various tissues were analyzed for hPAP activity. Histochemical analysis of the injected animals also demonstrates the activity. An abbreviated ADMETox profile (bone marrow and hepatotoxicity, cytochrome P450 specificity) further characterizes each compound. These validated compounds serve as lead molecules for explorations intending to identify therapeutically relevant small molecules for use in oxidative stress-related disorders.

## 1722 PI3 KINASE/AKT, A SURVIVAL PATHWAY AGAINST CYP2E1 DEPENDENT TOXICITY.

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AKT deactivation has been proposed as a causal mediator of cell death in several models, including oxidative stress. The objective of this work was to evaluate the possible role of PI3 kinase/AKT as a survival pathway against CYP2E1- and oxidative stress- dependent toxicity. E47 cells (HepG2 cells transfected with human CYP2E1 cDNA) exposed to 25 uM Fe-NTA + 5 uM arachidonic acid (Fe+AA) developed higher toxicity than C34 cells (HepG2 cells transfected with empty plasmid). Toxicity was associated with increased oxidative stress, and activation of calcium-dependent hydrolases calpain and phospholipase A2 (PLA2). LY294002, a specific inhibitor of PI3 kinase, but not LY303511, an inactive analog, increased the toxicity of Fe+AA in E47 cells. LY294002 did not significantly affect PLA2 or calpain activation, CYP2E1 activity, or lipid peroxidation elicited by Fe+AA. Thus, the increased toxicity promoted by LY294002 is downstream of the CYP2E1-dependent oxidant stress and activation of calcium-dependent hydrolases. Fe+AA treatment of E47 cells, but not C34 cells, led to a small decrease in phosphorylation of AKT (-24% at 2h), with no change in total AKT levels. LY294002, but not LY303511, produced a further decrease of phosphorylated AKT in Fe+AA treated E47 cells (-77% at 2h), with no change in total AKT. LY294002 produced a small decrease in phosphorylated AKT in E47 cells not exposed to Fe+AA (-12% at 2h). Transient transfection with active AKT in E47 cells partially inhibited the toxicity by Fe+AA. On the contrary, another chemical PI3 kinase inhibitor, wortmannin, did not affect Fe+AA toxicity in E47 cells. While wortmannin did not affect calpain or CYP2E1 activity, or lipid peroxidation, it significantly inhibited PLA2 activation by Fe+AA in intact cells. Since PLA2 plays such a critical role in the Fe+AA toxicity in E47 cells, use of wortmannin as PI3 kinase inhibitor is complicated in models where PLA2 activation is involved. These results suggest that PI3 kinase/AKT may serve as a survival pathway against CYP2E1 dependent toxicity.

## 1723 INHIBITION OF HUMAN MITOCHONDRIAL ALDEHYDE DEHYDROGENASE BY 4-HYDROXYNON-2-ENAL AND 4-OXONON-2-ENAL.

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Previous studies demonstrated 4-hydroxynon-2-enal (4HNE) to be both a substrate and inhibitor of mitochondrial aldehyde dehydrogenase (ALDH2). Enzyme inactivation by 4HNE is reversible at low  $\mu$ M but could be irreversible at higher concentrations. Structurally analogous to 4HNE is the lipid peroxidation product 4-oxonon-2-enal (4ONE), which has higher reactivity than 4HNE toward protein nucleophiles. Because it is similar in structure to 4HNE but more reactive, 4ONE could be a suicide substrate for ALDH2. The goal of this work was to determine whether 4ONE is a substrate or inhibitor of human ALDH2 (hALDH2) and elucidate the mechanism of inactivation by 4HNE and 4ONE. At  $[4\text{ONE}] \leq 10 \mu\text{M}$ , hALDH2 catalyzed oxidation of 4ONE to 4 oxonon-2-enoic acid (4ONEA) with a yield of 5 mol 4ONEA produced per mol of enzyme (monomer). Synthesized 4ONEA and glutathione reacted with a calculated rate constant 87 fold less than that measured for 4ONE but comparable to that of 4HNE. Analysis of hALDH2 activity toward propanal revealed both 4ONE and 4ONEA to be potent, irreversible inhibitors of the enzyme. Plots of hALDH2 inactivation by 4ONEA were log-linear, and pseudo-first-order rate constants varied linearly with  $[4\text{ONEA}]$ , indicating that experimental  $[4\text{ONEA}] \ll$  dissociation constant. In contrast,

hALDH2 inhibition by 50  $\mu\text{M}$  4HNE was reversible. hALDH2/NAD was incubated with 4HNE, 4ONE and 4ONEA, and mass spectral analysis of tryptic peptides revealed modification of hALDH2 by 4ONE and 4ONEA. In particular, the peptide containing active site Cys 302 was adducted, and results of MS/MS analysis confirmed Cys 302 to be the site of modification. These data indicate that hALDH2 catalyzes oxidation of 4ONE to 4ONEA; however, 4ONEA is a reactive electrophile. Furthermore, both 4ONE and 4ONEA but not 4HNE are potent, irreversible inhibitors that can modify the active site Cys 302.

## 1724 INDUCTION OF OXIDATIVE STRESS IN HUMAN LUNG CELLS BY THE ESTROGEN METABOLITE, 4-METHOXYESTRADIOL.

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Epidemiological studies have demonstrated significantly high incidences of pulmonary dysfunctions and diseases, including lung cancer, in populations exposed to dioxins. Animal studies further showed that lung pathology and cancer could be induced in female rats treated with 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD). In our previous studies, we demonstrated a significant elevation of 4-methoxyestradiol (4-MeOE2), a second stage estradiol (E2) metabolite, together with a suppression on cell growth in TCDD-treated human lung cell (H1355 and BEAS-2B) cultures. As suppression of cell growth may lead to a reduction of cellular repair and regeneration of lung epithelial cells, it becomes important to further explore the role of 4-MeOE2 in pulmonary toxicity. In our present study, we found that 4-MeOE2 induced significant production of ROS in the H1355 cells with an induction of SOD activity in a dose and time dependent manner. At dose of 100nM, we also found that 4-MeOE2 decreased intracellular GSH as well as inhibited the aconitase activity and reduced the ATP level in the cells. Concomitantly, suppression of cell growth was also observed at the same time. Such phenomena (induction of SOD activity, reduction of intracellular GSH, inhibition of aconitase activity, reduction of cellular ATP, and suppression of cell growth) were not observed in cells treated with 2-methoxyestradiol (2-MeOE2), the other second stage E2 metabolite. Our studies provided strong evidence that interaction of TCDD and estrogen (E2) in human lung cells would uniquely elevate a E2 metabolite, 4-MeOE2, which in turn would induce oxidative stress and a series of cellular events leading to an eventual adverse change (suppression of cell growth) in the lung epithelial cells. These findings help to explain and elucidate the high incidences of pulmonary diseases in both humans and animals, especially females, when exposed to dioxins.

## 1725 PROTEOMIC IDENTIFICATION OF NONLETHAL OXIDATIVE INJURY BIOMARKERS.

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Oxidants are byproducts of aerobic metabolism and have been shown to mediate the toxicity of a large number of environmental chemicals. Under most circumstances, the level of oxidants produced inside our body is low and not sufficient to harm cells. Despite this harmlessness, cellular maladaptation to nonlethal doses of oxidants contributes to various diseases including cancer and cardiovascular disease. Detecting cellular injury induced by low dose oxidants becomes important for early diagnosis of these diseases. We have used ESI-LC-MS/MS based proteomics to identify protein factors induced and secreted due to oxidative stress in several cell types. We found that  $\text{H}_2\text{O}_2$  at sublethal doses caused increased secretion of IGFBP-6 and cystatin C from normal human fibroblasts and rat cardiomyocytes. Using doxorubicin to induce oxidative stress *in vivo*, we found increased levels of IGFBP-6 and cystatin C in the plasma of treated mice, suggesting that these two proteins can serve as biomarkers of cellular injury *in vivo*. Proteomic analysis of human fibroblast whole cell lysates detected increased levels of thioredoxin reductase, actin, proteasome alpha2 subunit, proteasome beta2 subunit, tropomyosin 1 and beta-galactoside-binding lectin. cDNA microarray was used to verify our proteomic data and found increased levels of mRNA encoding the proteins listed above. In this study, we have established two biomarkers, IGFBP-6 and cystatin C, that can be used to detect nonlethal doses of oxidative stress injury *in vitro* and *in vivo*. Proteomics of cell lysates combined with cDNA microarray will likely generate additional biomarkers that can potentially serve to diagnose different stages of cellular injury.

## 1726 GR-DEFICIENT MICE (GR1(A1NEU)) ARE SUSCEPTIBLE TO DIQUAT-INDUCED HEPATIC INJURY.

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GR1(a1Neu) mice are genetic knockouts for glutathione reductase (GR) [Rogers, Toxicol Sciences, 2004]. GR catalyzes the reaction of glutathione disulfide (GSSG) to glutathione (GSH) by using the reducing equivalents of cellular NADPH, thus

maintaining the availability of GSH for antioxidant functions. We tested the hypothesis that mice deficient in GR activities would be more susceptible to oxidant injury by administration of the redox-cycling oxidant, diquat. Neu and C3H/HeJ (control) mice were given 0 to 50  $\mu$ mol/kg of diquat and sacrificed between 0.5 and 6 h. Neu mice exhibited hepatic necrosis by histology and elevated plasma ALT activities at doses as low as 5  $\mu$ mol/kg (Neu vs C3H, 429 $\pm$ 26 vs 20 $\pm$ 13 IU/L, at 6 h). Diquat doses of 10 and 50  $\mu$ mol/kg caused substantial mortality in Neu mice by 4 and 2 h, respectively. Elevated plasma ALT activities (140 $\pm$ 69 IU/L) were observed as early as 0.5 h after 7.5  $\mu$ mol/kg of diquat, but C3H mice showed no injury or mortality at doses to 50  $\mu$ mol/kg (19 $\pm$ 6 IU/L) through 6 h. No diquat-dependent changes in hepatic GSH levels were observed in either strain. Basal levels of GSSG are greater in Neu than in C3H mice (138 $\pm$ 30 vs 38 $\pm$ 5  $\mu$ M), and increases in GSSG were only observed in the Neu mice and at the highest doses of diquat (444 $\pm$ 91  $\mu$ M, at 2 h after 50  $\mu$ mol/kg). Histological evaluations indicated moderate hepatic necrosis in diquat-treated Neu mice, and preliminary observations indicate marked renal necrosis. Heterozygous mice (Neu/C3H/HeJ) exhibited half of the hepatic GR activities observed in the wild type C3H/HeJ mice (2.3 $\pm$ 0.9 vs 4.8 $\pm$ 0.9 U/g tissue), but demonstrated no hepatic injury with doses as high as 100  $\mu$ mol/kg (29 $\pm$ 12 IU/L). The markedly greater sensitivity to diquat-induced hepatic injury in the GR-deficient mice reflects the critical contributions that GR makes to the antioxidant defense mechanisms.

**1727**

CAN THE HERSHBERGER ASSAY SPECIFICALLY DETECT AN ANDROGENIC OR ANTI-ANDROGENIC ACTIVITY OF CHEMICAL? -RELATIONSHIP BETWEEN *IN VITRO* AND *IN VIVO* ASSAYS.

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The rodent Hershberger assay was originally developed to detect myotrophic effects of steroids and has been used for the screening of endocrine disrupting chemicals, which function via androgen receptor (AR)-mediated mechanism. Both AR-binding and AR-mediated transactivational assays were also utilized for the simple *in vitro* screening of them or for providing of the mechanism data. In order to evaluate the applicability and performance limitations of these *in vivo* and *in vitro* assays for the mechanism-specific screening of endocrine disruptors, it is very important to analyze the relationships among them. In this study, we performed the comparison between AR-binding or AR-mediated transactivational assays and the rodent Hershberger assay and explored their mechanism-specific performances. Only 33 chemicals of 65 test chemicals showed the same results in both the AR-binding and the Hershberger assays, and the concordance of both assays was 51%. In particular, 29 chemicals of 44 AR-binders (66%) were "negative" in the Hershberger assay. On the other hand, there were only three AR-non-binders, which were judged as "positive" in the Hershberger assay. They were two phthalates and an organo-tin compound. The concordance between the transactivational and the Hershberger assays for the detection of androgenic activity was 85% (55/65 chemicals), while that for the detection of anti-androgenic activity was considerably low (55%) as in the case with the relationship between the AR-binding and the Hershberger assays. To improve such low concordances, a classification of chemicals based on the receptor-binding characteristics of chemicals was studied. Consequently, the concordance between *in vitro* and *in vivo* assays was successfully increased when test chemicals were separated into two categories, ER-binders and ER-non-binders.

**1728**

ABILITY OF THE MALE RAT PUBERTAL ASSAY TO DETECT ENVIRONMENTAL CHEMICALS THAT ALTER THYROID HORMONE HOMEOSTASIS.

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The U.S EPA Endocrine Disruptor Screening Program Tier 1 male pubertal protocol was designed to detect alterations in reproductive development and thyroid function. However, little is known about the efficacy of this protocol as a screen for thyrotocicants with different mechanisms of action. We evaluated three environmental chemicals which reported to alter thyroid hormones: DE-71, thiram, and ammonium perchlorate. DE-71, a polybrominated diphenyl ether mixture which increases the clearance of thyroid hormone, was administered from postnatal day 23 to 53 by gavage to male Wistar rats. DE-71 decreased T4 (3, 30 and 60 mg/kg) and T3 (30 and 60 mg/kg) and increased TSH (30 and 60 mg/kg). The altered homeostasis of thyroid hormone by DE-71 was accompanied by altered histopathology of the thyroid tissue (decreased colloid area, increased follicular cell height) following exposure to 60 mg/kg. Thiram, a dithiocarbamate fungicide reported to inhibit thyroperoxidase activity (T4 to T3) *in vitro*, was administered at doses of 12.5 to 50 mg/kg. Thiram decreased T4 at 50 mg/kg and decreased TSH

at 25 and 50 mg/kg. No differences in T3 or thyroid histology were observed. Thiram also decreased luteinizing hormone at 50 mg/kg and testosterone at all doses. The decreased LH was likely due to the ability of thiram to inhibit norepinephrine synthesis, which is important for normal GnRH function. Perchlorate, an ingredient in rocket fuel, paints, and lubricants and a competitive inhibitor of iodide uptake by the thyroid gland, was administered at 62.5, 125, 250, and 500 mg/kg. Perchlorate decreased T4 and increased TSH at doses of 125 to 500 mg/kg. Thyroid histology was altered at all doses. These results demonstrate that the male pubertal protocol is sensitive to the effects of thyrotocicants that act through a variety of mechanisms. This abstract does not necessarily reflect EPA policy.

**1729**

GENE EXPRESSION PROFILING REVEALS COMMON TARGETS FOR VARIOUS ANDROGEN RECEPTOR ANTAGONISTS IN DEVELOPING FETAL RAT TESTIS.

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*In utero* exposure to androgen receptor (AR) antagonists elicits male reproductive tract deficits in rats similar to those attributed to human testicular dysgenesis syndrome. Although these AR antagonists can compete with androgen at the receptor level *in vitro*, the specific gene and signaling pathway targeted by these compounds during androgen-mediated male reproductive tract development remain to be determined. The present study was performed to examine global gene expression patterns in rat fetal testis following *in utero* exposure to various AR antagonists. Pregnant Sprague-Dawley rats were treated with flutamide (50 mg/kg/day), linuron (50 mg/kg/day), vinclozolin (200 mg/kg/day), p, p'-DDE (100 mg/kg/day) or corn oil vehicle by gavage daily from gestation day (GD) 12 to 19. Dose levels were selected to maximize fetal response with minimal maternal toxicity. Testes were isolated on GD 19, and global changes in gene expression were determined by microarray analysis. Of the approximately 30, 000 genes queried, 90, 142, 81, and 109 genes were significantly altered following exposure to flutamide, linuron, vinclozolin, and p, p'-DDE, respectively. Seventeen genes were commonly altered by all four AR antagonists, with 10 genes up-regulated and 7 genes down-regulated. Changes in mRNA expression of selected common responsive genes were confirmed by real-time RT-PCR. In all treatments but flutamide, there was a decrease in mRNA expression of various genes involved in cholesterol synthesis and steroidogenesis evidenced by both microarray and real-time RT-PCR analysis. However, the testicular testosterone level remained unchanged in all treatments. We conclude that *in utero* exposure to diverse AR antagonists could cause common alterations in certain gene expressions in developing fetal rat testis. Considering the relatively small number of significant genes, low magnitude of alterations in gene expression and lack of pathological effect of AR antagonists, we suspect that fetal testis is not a primary target for AR activity.

**1730**

APPLICATION OF GENE EXPRESSION ANALYSIS OF MANNOSE 6-PHOSPHATE/INSULIN-LIKE GROWTH FACTOR-II RECEPTOR (M6P/IGF2R) IN VENTRAL PROSTATE FOR SCREENING OF CHEMICALS WITH ANDROGEN-MEDIATED ACTIVITIES IN THE RAT HERSHBERGER ASSAY.

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The M6P/IGF2R functions as a cell growth suppressor because of its ability to activate the inhibitory activity of TGF $\beta$  and internalize and degrade extracellular IGF2. Castration leads to the activation of the programmed death of the androgen-dependent prostatic epithelial cells in rat ventral prostate. In order to clarify the potential contribution of M6P/IGF2R of this programmed cell death, steady state levels of mRNA of M6P/IGF2R together with TGF $\beta$  and IGF2 were determined by Real-Time PCR analysis. Expression of M6P/IGF2R mRNA in ventral prostate (VP) and seminal vesicles (SV) was higher than that of liver in intact males. Four days after castration, expression of M6P/IGF2R mRNA was increased in VP and SV but not in liver. Time-course analysis for 10 days demonstrated that weights of VP and SV were significantly decreased 3 and 4 days after castration. Within the first day after castration there was an increase in the levels of mRNA of M6P/IGF2R (8-fold) and TGF $\beta$  (18-fold) in VP, and they continued until the 10th day after castration. SV showed a less than 5-fold increase in M6P/IGF2R and TGF $\beta$  mRNA from 3 days after castration. IGF2 mRNA in VP and SV were increased, but the degree of increase was not consistent. These alterations were attenuated by testosterone propionate (1mg/kg), indicating that expressions of M6P/IGF2R, TGF $\beta$  and IGF2 in VP and SV are under negative androgenic regulation. Liver showed no significant changes in the expression of these genes. Furthermore, the rat Hershberger assay with M6P/IGF2R mRNA analysis detected antiandrogenic effects of flutamide (10mg/kg) and p, p'-DDE (100mg/kg) after at least 4 days of treatment. In contrast, colchicine, a known microtubule disruptor

(0.54 mg/kg) had no affect on the expression of these genes. These findings indicate that M6P/IGF2R expression is a potentially reliable biological endpoint to screen for chemicals with androgen-mediated activity.

### 1731 EFFECTS OF AN ANTIANDROGEN ON PROTEIN AND GENE PROFILES IN ADULT RAT TESTES.

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Exposure to antiandrogens have been shown to perturb the endocrine system in rats and lead to testicular toxicity. However little is known about the profile of proteins and/or gene expression altered in the testis following exposure to antiandrogens. To better understand the molecular changes produced by antiandrogens in the testis, we have used a proteomic approach to analyse testicular tissues from rats exposed to the model compound, flutamide. The antiandrogen flutamide prevents testosterone from binding to the androgen receptor and this process leads to the inhibition of the androgen dependent tissue stimulation (testes, prostate, seminal vesicles..). *In vivo* 28-day toxicity studies were conducted in male Sprague-Dawley rat by gavage with flutamide [0, 6, 30, 150 mg/kg/day]. At the high dose level, flutamide induced a reduction in the mean body weight gain without any change in mean testis weight (absolute and relative). Leydig cell hyperplasia was observed in a dose-related manner and was the only histopathological changes observed following treatment. Control and treated rat testicular protein were analysed by two-dimensional gel electrophoresis (IPG-IEF/SDS-PAGE). Image analysis using PDQuest software allowed the detection of proteins spots differentially expressed following treatment. A number of proteins were identified by MALDI-TOF analysis providing some information on the metabolic pathways which are altered following flutamide treatment (detoxification mechanism, translation, energy metabolism, signaling pathway..). In parallel, qPCR experiments were performed in order to monitor the level of transcript accumulation corresponding to the identified proteins. Further investigations, using these molecular markers, are ongoing to better characterize the molecular changes observed in the testis following antiandrogens treatment.

### 1732 PROTEIN PROFILING OF ANTIANDROGEN EFFECTS IN THE ADULT RAT VENTRAL PROSTATE.

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5-alpha-reductase II is the enzyme which converts testosterone into the more potent androgen, dihydrotestosterone (DHT) which is required for prostate growth and homeostasis. Finasteride, used in therapy of benign prostatic hyperplasia, is an antiandrogenic drug through the selective inhibition of 5-alpha-reductase II. Investigations of antiandrogenic effects in rat ventral prostate of this model compound were approached applying proteomic technologies. Finasteride was daily administered by gavage to young adult Sprague-Dawley rats, at different dose levels (0, 1, 5, 25 and 125 mg/kg per day). After 28 days of treatment, the reproductive tissues including ventral prostates were removed, weighed and examined microscopically. Histopathological changes were observed from 1 mg/kg/day and prostate proteins were separated by two-dimensional gel electrophoresis using isoelectrofocusing (IEF) on immobilized pH gradient strips (pH3-10, pH3-6, pH 5-8, pH7-10) in a first dimension, and SDS-PAGE in a second dimension. Protein patterns of control and treated animals were compared by PD-Quest software analysis and spots were identified by MALDI-ToF mass spectrometry. In the course of this study, several proteins from different cellular compartments were found to be up or down-regulated (chaperones, proteins from mitochondria and cytoskeleton, proteins involved in metabolism and secreted proteins). A similar study is currently under investigation with flutamide (0, 6, 30 and 150 mg/kg per day), an other antiandrogen which prevents testosterone binding to androgen receptor. These investigations are expected to better characterise the molecular changes observed in the prostate following antiandrogen treatment.

### 1733 THE ENDOCRINE PROFILE OF INTACT FEMALE RATS ON THE DAY OF PROESTRUS FOLLOWING EXPOSURE TO ATRAZINE.

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The chlorotriazine herbicide, atrazine, has been shown to disrupt the neuroendocrine control of reproductive function in both the male and female rat. We reported that atrazine caused a dose-dependent suppression of the estrogen-induced

surge of luteinizing hormone (LH) in the ovariectomized female rat. We also found that this herbicide caused an increase in serum estrone and estradiol in the intact male. The purpose of the present study was to determine whether or not the pituitary and gonadal hormones are affected within the same dose range. Therefore, we examined the effect of atrazine on hormone concentrations in the intact proestrous female rat. LE rats with regular 4-day estrous cycles were gavaged daily for 3 days (diestrus I to proestrus) with 0, 6.25, 12.5, 25 and 75 mg/kg of atrazine. On the day of vaginal proestrus (final day of dosing) females were killed at 1200, 1400, 1600, 1800 and 2000 h. At each time point the brain, pituitary, and blood were collected and processed for subsequent assays. Serum prolactin (PRL), LH, progesterone, corticosterone, estradiol, estrone and androstenedione were measured by radioimmunoassay. The peak concentration of LH was suppressed at all doses of atrazine as compared to the controls. Progesterone and androstenedione were increased at 1600 and 1800 h in the 75 mg/kg group. There were no significant differences in estrone, estradiol or corticosterone, although there was an apparent trend for increased levels as the atrazine dose increased. The results of these experiments demonstrate that atrazine suppresses LH at doses as low as 6.25 mg/kg. However, changes in serum steroid hormones were not observed until doses of 75 mg/kg were administered. Further studies are needed to determine the NOEL for the suppression of LH by atrazine and to better evaluate the apparent alterations in steroids. (This abstract does not necessarily reflect EPA policy.)

### 1734 ESTROGENICITY OF MIXTURES OF CHEMICALS ON UTERINE GROWTH OF PREPUBERAL RATS.

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The human diet contains numerous compounds, of which a minority has shown (anti)-estrogenic effects *in vitro* and *in vivo*. The origin of these compounds varies from plant-derived (phytochemicals; PCs) to industrial pollutants and pesticides (xenoestrogens; XEs). It has been postulated that these compounds may act in a non-additive way in combination with endogenous estrogens (e.g. 17 $\beta$ -estradiol=E2). To investigate potential non-additive effects, 2 mixtures were composed that reflected concentrations reported in serum from humans consuming a regular diet. One mixture (PCM) contained coumestrol, genistein, naringenin, (+, -)catechin, (-, -)epicatechin and quercetin the other (XEM) contained, nonyl-, and octylphenol,  $\beta$ -hexachlorocyclohexane, methoxychlor, bisphenol A and dibutylphthalate. Our previous *in vitro* studies, based on cell proliferation and pS2 gene expression in MCF7 cells, demonstrated that observed potency and calculated potency (using estrogen equivalency factors) of combinations of PCM, XEM and E2 did not deviate from the expected additive model. To confirm this additivity in an *in vivo* model, E2, PCM and XEM were tested alone or at various combinations in rat uterotrophic assays. Prepuberal female rats were dosed subcutaneously once every 24 h for 3 days and sacrificed on day 4. The uterine weight served as a measure of estrogenicity. Tested doses for PCs and XEs were within the range reported in plasma of humans consuming a regular diet. ED50 for E2 was found at 1  $\mu$ g/kg bw. ED50s for PCM and XEM were found at doses that are 12 times lower or at least 20 times higher than reported in human plasma respectively. Average uterine weight increase ( $n=5$ /group) of animals dosed with different combinations of PCM or XEM with E2 supported the concept of additivity. Based on our results from uterotrophic assays we conclude that a possible contribution of estrogenic effect caused by PCs from the diet might be more significant than that caused by XEs and that (plant-derived or xeno) estrogenic compounds in human diet could interact with E2 in an additive way.

### 1735 MOLECULAR CHARACTERIZATION OF THE UTEROTROPHIC ASSAY: TEMPORAL GENE EXPRESSION CHANGES IN THE UTERUS FOLLOWING ETHINYLESTRADIOL.

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The uterotrophic assay is undergoing validation as an *in vivo* screen for estrogenicity. In this study, the temporal changes of gene expression were compared to parameters like uterine wet weight and histology. Groups of five juvenile female Wistar rats were subcutaneously dosed with either ethinylestradiol (3 microgram/kg) or corn oil (solvent control) every 24 hours for three consecutive days. Two, four, eight, and 24 hours after the first, four and 24h after the second, and 24 h after the last application, uteri were taken for histopathological examination and for analysis of the expression profiles. Four and eight hours after the first administration, uterine weight increased to about 1.4-fold, then increased further and reached more than 10-fold of the corresponding control values at final necropsy. The earliest detectable uterine change observed by histology was hypertrophy of stromal cells four hours after the first application. Hypertrophy of the luminal and glandular epithelium and of myometrial cells occurred 24 h after the first

application, whereas dilation of the uterine lumen was observed four hours after the second application. At the gene expression level, prominent time-dependent expression patterns were found which could be related to the histopathological observations. Analysis of the approx.1500 significantly deregulated genes revealed a profound remodeling of the uterine tissue including an early upregulation of genes involved in biosynthetic processes, cytoskeletal rearrangement, and vasculogenesis. The expression of cell cycle progression genes increased abruptly 24 h after treatment. Also suggested from the function of some of deregulated genes is an inflammatory response which could explain earlier observations showing inhibition of estrogen-induced increase in uterine weight by antiinflammatory agents. In summary, the analysis of the expression changes allowed further insight into the mechanisms underlying the uterotrophic response in this model.

**1736**

COMPARATIVE GENE EXPRESSION ANALYSIS OF THE MOLECULAR EFFECTS OF SYNTHETIC, PLANT-DERIVED AND PHYSIOLOGICAL ESTROGENS IN THE IMMATURE RODENT UTERUS.

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The xenoestrogen group of endocrine disruptors have the potential to cause reproductive and developmental effects through stimulation or disruption of estrogen receptor signalling. Whilst the exposure of humans to  $\mu\text{g}/\text{kg}$  levels of synthetic environmental estrogens is assumed to be hazardous, exposure to  $\text{mg}/\text{kg}$  levels of phytoestrogens in foods and dietary supplements is frequently proposed to be life enhancing. Given this apparent paradox, an important question is whether estrogens derived from different sources induce their estrogenic effects through similar mechanisms. To address this question, we gave immature mice three daily subcutaneous injections of 17 $\beta$ -estradiol (E2, 2.5  $\mu\text{g}/\text{kg}$ ), the synthetic estrogen diethylstilbestrol (DES, 2  $\mu\text{g}/\text{kg}$ ) or the phytoestrogen genistein (GEN, 50  $\text{mg}/\text{kg}$ ). Control mice received vehicle alone (arachis oil). These doses induced equivalent gravimetric and histologic uterotrophic effects, using the standard 3-day uterotrophic assay protocol. Gene expression profiles of 72h uterine samples, measured using Affymetrix microarrays, revealed that E2, GEN and DES induce essentially identical changes in gene expression. We conclude that, in the rodent uterus, a synthetic estrogen and a phytoestrogen operate via a molecular mechanism analogous to the physiological estrogen, E2. These data demonstrate that the origin of an estrogen - be it physiological, plant-derived or synthetic - plays no role in determining its biological activity in the uterus. This highlights the need for an holistic approach to safety assessment wherein preconceptions of intrinsic hazard are replaced by an objective assessment of likely perturbations of physiological functions caused by combined exposures to xenoestrogens.

**1737**

ESTRADIOL PROTECTS AGAINST ETHANOL-INDUCED BONE LOSS IN FEMALE RATS BY PREVENTING OSTEOCLAST ACTIVATION.

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We hypothesized that protection against ethanol-induced bone loss in pregnancy might be due to increased circulating sex steroids. In the current study, 225 g female Sprague-Dawley rats ( $N = 6/\text{group}$ ) were infused liquid diets with or without isocaloric EtOH substitution of carbohydrate calories using total enteral nutrition at a dose of 13  $\text{g}/\text{kg}/\text{d}$  for 21 d. Additional control and EtOH groups were supplemented with 17 $\beta$ -estradiol (E2, 20  $\mu\text{g}/\text{kg}/\text{d}$  via osmotic minipump) or with E2 + progesterone (P, 75  $\text{mg}/\text{kg}/\text{d}$  s.c. in corn oil vehicle). At the end of the study E2 supplementation increased plasma E2 values from  $3 \pm 1$  to  $66 \pm 12 \text{ pg}/\text{ml}$  ( $p \leq 0.05$ ) and P supplementation increased P values from  $12 \pm 6$  to  $225 \pm 31 \text{ ng}/\text{ml}$  ( $p \leq 0.05$ ) in EtOH treated rats, values similar to or higher than those observed in pregnancy. While E2 supplementation had no effect on tibial bone density in control rats, E2 prevented ethanol-induced bone loss. Total tibial bone density and tibial trabecular bone density were greater in the E2 + EtOH than in the EtOH group ( $p \leq 0.05$ ) as measured by peripheral quantitative computerized tomography both *in vivo* and in isolated tibia. In addition, measurement of plasma collagen type I fragments (RatLapsTM) by ELISA as a marker of osteoclast-mediated bone resorption demonstrated increases with EtOH and suppression in the E2 + EtOH group ( $p \leq 0.05$ ). In contrast both EtOH and E2 reduced plasma osteocalcin, a bone formation marker in an additive fashion ( $p \leq 0.05$ ). The E+P group had no significant effects compared to supplementation with E2 alone. These data suggest E2 protects against ethanol-induced bone loss by preventing ethanol-associated osteoclast activation. (Supported by NIH AA12928 M.R.).

**1738**

ONTOGENY OF P-GLYCOPROTEIN (PGP) IN SELECTED TISSUES OF NEONATAL MALE AND FEMALE CD-1 MICE.

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The mdrla isoform of P-glycoprotein (pgp) is an essential component in capillary endothelial cell, acting as a transmembrane pump to facilitate removal of drugs and toxicants. Using RT-PCR to measure mRNA, and Western immuno-blotting to measure protein, we have assessed development of PGP expression in female CD-1 mouse brain, kidney, liver, and ovaries, and in brain, kidney, liver, testes, and ventral prostate of male mice, on PND 1, 5, 10, 15, 20, and 60. No remarkable gender differences appeared in PGP ontogeny in brain, kidney, and gonads. Expression of both protein and mRNA generally increased from PND 1 to 60, but they were typically more rapid for protein than message. Protein levels at PND 20 were near adult (PND 60) levels, but were only 20-40% of adult levels at PND 1. An exception was expression in liver, already at approximately 70% of adult level at PND 1. Similarly, mRNA expression was 70-100% of adult level by PND 20 in all organs examined, but was only 10% of adult level at PND 1. It is concluded that the ontogeny of PGP mRNA and protein in brain, kidney, gonad and prostate increases from low, but detectable, expression levels at PND 1 to nearly adult levels at PND 20; while the liver appears nearly complete at birth. For uncertain reasons, mRNA levels appear to rise more slowly, relative to adult expression, than do protein levels. The results also suggest, in concordance with our findings in neonatal rats (Yavanhxay et al., 2004, Toxicologist), that the ontogeny of PGP, in the tissues examined, differs from that of human infants and children, which is completely developed from before birth. It calls into serious question the validity of using neonatal rodent models to assess potential toxic effects of natural hormones, pesticides, and environmental endocrine disruptors that interact with the P-glycoprotein pump, because rodent barriers remain far from completely developed at birth.

**1739**

TRANSCRIPT PROFILING OF GENE EXPRESSION CHANGES ASSOCIATED WITH AN INCREASED INCIDENCE OF CANCER AFTER EXPOSURE OF NEONATAL MICE TO XENOESTROGENS.

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Epigenetic changes, including altered DNA methylation status, have recently been implicated in the development of human cancers. It is becoming increasingly apparent that toxicant-induced changes in epigenetic status may also play an important role in some mechanisms of toxicity. The potential role of xenobiotic-induced epigenetic changes in developmental toxicity is exemplified by exposure to diethylstilbestrol (DES), a developmental stage-specific non-genotoxic carcinogen. We have used a rodent experimental model for DES-induced carcinogenesis in order to define the early molecular events, including changes in gene expression, that may be associated with the onset of carcinogenesis. Neonatal female mice were given five daily subcutaneous injections of DES during post-natal days (PND) 1-5 at doses (1 and 1000  $\mu\text{g}/\text{kg}$ ) that have been reported previously to result in a high incidence of uterine tumours after 18 months (31% and 90%, respectively). Arachis oil was used as a vehicle control. Gene expression levels, uterine weights and histological parameters were analysed at PND6, PND17, PND30 and PND50. Gene expression profiling using Affymetrix microarrays revealed that DES-induces a complex pattern of dose-dependent gene expression changes in the uterus. A subset of DES-responsive genes, including lactotransferrin and complement component 3, exhibited persistently altered expression levels throughout the 50 day time course, demonstrating that transient exposure to DES during PND 1-5 results in stable changes in gene expression that may be due to epigenetic phenomena. The mechanistic basis for these persistent changes in gene expression is currently being analysed by measuring the DNA methylation status of CpG island sequences within their promoter regions. These studies provide novel insights into the relationships between epigenetic changes in gene expression and chemically-induced carcinogenesis in a rodent model system.

**1740**

THYROID HORMONE STATUS IN ADULT FEMALE RATS AFTER AN ORAL DOSE OF PERFLUOROOCTANESULFONATE (PFOS).

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Perfluorooctanesulfonate (PFOS), a persistent bioaccumulative acid, is widely distributed in humans and wildlife. Prior studies have observed decreased total and free thyroid hormones (TH) in serum without a compensatory rise in thyrotropin

(TSH). However, we have recently demonstrated that measurement of free thyroxine (FT4) by analog methods in serum containing PFOS is prone to negative bias in that PFOS competes with FT4 for binding. We also observed that hepatic malic enzyme (ME) and  $\alpha$ -glycero-3-phosphate-dehydrogenase ( $\alpha$ G3PD), which are responsive to TH, were either increased (ME) or unchanged ( $\alpha$ G3PD) after treatment with PFOS. To test the hypothesis that PFOS increases free TH availability and the hepatic response to TH, the effects of PFOS on TH status were investigated over a 24-hour time period following a single oral dose of PFOS. Adult female SD rats (5/group) were given 15 mg/kg PFOS or vehicle and sacrificed at 2, 6, and 24 hours post-dose. Sera and liver were harvested and flash frozen immediately. Chemiluminescent (CL) methods were used to measure serum total thyroxine (TT4), total triiodothyronine (TT3), FT4, reverse triiodothyronine (rT3) and TSH. FT4 was also measured by a reference method, equilibrium dialysis RIA (ED-RIA). In addition, translational and transcriptional expressions of ME were evaluated. Compared to controls, TT4, TT3, FT4, and rT3 by CL method were significantly reduced over the 24 hours duration with PFOS. However, FT4 by ED-RIA was significantly increased at 2 (68%) and 6 (90%) hours and returned to control value at 24 hours. TSH activity was comparable to controls at 2 and 24 hours, and was apparently reduced at 6 hours. ME activity was increased by 4, 12, and 43% of control at 2, 6, and 24 hours, respectively. Thus, these results demonstrate that thyroid status was enhanced rather than suppressed following PFOS administration.

**1741**

THE EFFECTS OF POLYCHLORINATED BIPHENYLS ON THYROID HORMONE RECEPTOR MEDIATED ACTION *IN VIVO* AND *IN VITRO*.

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Polychlorinated biphenyls (PCBs) are ubiquitous environmental contaminants routinely found in human and animal tissues. Prenatal exposure to PCBs is associated with neuropsychological deficits, which may be produced by PCBs altering thyroid hormone (TH) action in the brain. In the present study, we first tested whether maternal exposure to a commercial PCB mixture, Aroclor 1254 (A1254), affects TH signaling in the fetal rat brain. Dams were dosed daily with 0, 1, or 4 mg/kg A1254 which significantly reduced their circulating levels of TH, but increased the expression of several TH-responsive genes in the fetal cortex of their offspring, including NSP-A, RC3/Neurogranin, and Oct-1. Additionally, the expression of malic enzyme (ME), a known TH-responsive gene in the liver, was elevated in animals treated with 4 and 8 mg/kg of 6 parent PCB congeners that represent specific classes of PCBs prevalent in A1254, including PCBs 77, 105, 118, 126, 138, and 153. These findings are consistent with a direct action of PCBs on TH receptors (TRs). However, this agonistic effect does not appear to involve interaction with the ligand binding domain of the TR because none of the 6 PCBs utilized in the *in vivo* study competitively bound to rat TRs isolated from hepatic nuclei. Therefore, in order to elucidate the molecular mechanism by which these specific PCB congeners activate TH-responsive gene expression, we are testing each congener independently in an *in vitro* cell culture system. For this congener specific analysis, we are employing H4IE cells to determine the effect PCBs on endogenous ME expression and GH3 cells to examine the effects of PCBs on endogenous growth hormone (GH) expression. Finally, the promoter regions of these TH-responsive genes have been well characterized and contain a TH response element (TRE). Therefore, we are utilizing ME and GH promoters that are linked to a luciferase reporter gene to further establish how PCBs interfere with TR mediated action.

**1742**

COMPARISON OF INHIBITORY EFFECTS OF ETHANOL AND METHYL T-BUTYL ETHER (MTBE) ON RAT LEYDIG CELL TESTOSTERONE PRODUCTION.

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Ethanol (EtOH) is an alternative motor fuel oxygenate proposed to replace MTBE, which is being phased out but persists in the environment. Both are reported to produce some type of testicular toxicity, possibly through inhibition of testosterone synthesis. Is one a more potent steroidogenesis inhibitor? and if so, what are the implications of substituting EtOH for MTBE in motor fuels? Leydig cells from adult Harlan Sprague-Dawley rats isolated using Percoll gradients were exposed for 3 hr to equimolar concentrations of ethanol or MTBE without hCG (baseline) or with hCG stimulation. Total testosterone (T) produced was measured by radioimmunoassay (DPC, Los Angeles). Aminoglutethimide was included routinely as a positive control. When equimolar concentrations of EtOH or MTBE up to 100 mM were compared in the same experiments, both caused similar inhibition of hCG-stimulated cells, whereas only MTBE consistently inhibited baseline T production. Less than 25 mM EtOH had no measurable effect in this system (legal blood alcohol concentration limit is ~17 mM). Different potencies and/or other

dissimilar modes of action of MTBE and EtOH or their metabolites in Leydig cells might be predicted by previously reported effects of each. For example, MTBE (but thus far not EtOH) has been reported to cause Leydig cell tumors in rats, whereas chronic consumption of high doses of EtOH reduces spermatogenesis and fertility and causes testicular atrophy, none of which has yet been reported in rodent reproductive toxicity studies of MTBE. We have also observed in Leydig cell exposure systems that formaldehyde, a metabolite of MTBE, is a more potent T inhibitor than acetaldehyde, an EtOH metabolite. The implications and contributions of these findings to comparative risk assessments will also be presented.

**1743**

CHANGES IN FETAL TESTIS GENE EXPRESSION AND STEROID HORMONE SYNTHESIS INDUCED IN MALE OFFSPRING AFTER MATERNAL TREATMENT WITH DEHP (DI-N-ETHYLHEXYL PHTHALATE).

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Phthalate esters are high production volume, ubiquitous environmental chemicals some of which induce reproductive malformations in rats when administered during sexual differentiation. Recently we have shown that malformations in gubernacular ligament development induced by high doses of DEHP are associated with decreased *insl3* gene expression, a gene critical for proper gubernacular ligament formation. In study one, DEHP (0, 100, 300, 600, or 900 mg/kg/day) was administered orally to Sprague-Dawley dams on gestation days (GD) 8 through 18. On GD18, fetal testes were evaluated for hormone production and changes in gene expression. Results indicate that DEHP induced a dose dependent linear decrease in testosterone production that was significant at 300, 600 and 900 mg/kg. Progesterone production was decreased at 900 mg/kg. *Ins3* gene expression was also decreased in a dose dependent manner. To investigate the ontogeny of gene changes associated with these effects, a second study dosed dams orally with 0 (vehicle only) or 750 mg/kg/day DEHP beginning on GD8. Testes RNA was prepared from fetuses of 3 litters each on GD 16.5, 17, and 18. Microarray analysis was conducted on these samples using Affymetrix gene chips. Preliminary results indicate significant ( $p < 0.05$ ) changes in several genes associated with Leydig cell steroidogenesis and peptide hormone production such as CYP17, StAR and *Ins3* beginning, in some cases, as early as GD 16.5. A more thorough analysis of gene chip results is underway but, thus far, the changes demonstrated are consistent with the malformations previously observed in male offspring after *in utero* exposure to similar doses of DEHP. Disclaimer: This is an abstract of a proposed presentation and does not necessarily reflect EPA policy.

**1744**

MECHANISM OF PHTHALATE-INDUCED DEVELOPMENTAL REPRODUCTIVE TOXICITY: INHIBITION OF TESTOSTERONE AND INSULIN-LIKE 3 HORMONE PRODUCTION BY FETAL LEYDIG CELLS.

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While the phthalate esters (PE) are a diverse class of chemicals, several PEs appear to induce reproductive toxicity via a common mode of action (CMOA). *In utero* DEHP, BBP, and DBP reduce testis testosterone (T) production and *insl3* mRNA levels inducing a syndrome of reproductive malformations unique to PEs. For DEHP, reproductive effects are seen at 11 mg/kg/d and above. Having a CMOA, it is not surprising that combined PE exposures during pregnancy produce dose-additive cumulative toxicity. When PEs are coadministered with an androgen receptor (AR) antagonist, some of the effects also are cumulative. In the pubertal male rat, DEHP, DBP and BBP delay puberty. For DEHP, this delay is associated with lower serum T and reduced T production *ex vivo*. In contrast to their effects on hormone production, the PEs do not appear to be significant AR antagonists *in vitro* or *in vivo*. In a Hershberger assay, a protocol that primarily detects AR antagonists, DEHP, DBP and BBP only cause weak positive to negative responses at a high dosage level (1 g/kg/d) suggesting that AR antagonism is not a major mechanism of PE-induced reproductive toxicity. We suspect that the PEs are altering fetal Leydig cell differentiation, delaying the onset of hormone production during sexual differentiation. The fact that both steroid and peptide hormones are altered *in utero* indicates that mechanism of action does not involve a direct effect on steroidogenic enzymes. In addition, the fact that growth of the liver is induced by DEHP but not DBP at low doses suggests that effects on the liver are not caused by the same CMOA as are effects on the developing male rat reproductive tract. Until the mechanism for PE-induced reproductive toxicity in the rat is fully characterized, uncertainty will exist in the extrapolation of these effects to other mammalian species, including humans. Disclaimer: This is an abstract of a proposed presentation and does not reflect Agency policy.

REPRODUCTIVE SUCCESS AND AROMATASE ACTIVITY IN THE FISH CUNNER (*TAUTOGOLABRUS ADSPERSUS*) EXPOSED TO ATRAZINE OR OCTYLPHENOL IN THE LABORATORY.

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This study was conducted to test the hypothesis that reproduction in fish is altered by exposure to endocrine-disrupting chemicals (EDCs) that modify aromatase activity. Aromatase, a product of the CYP19 gene, is an enzyme complex that catalyzes the conversion of the androgens androstenedione and testosterone to the estrogens estrone and estradiol. Atrazine and octylphenol are EDCs common in aquatic environments, and both have been reported to alter estrogen concentrations in fish or rats. During our laboratory experiments, cunner in spawning condition were monitored daily to determine egg production, egg fertility and egg viability both before and after exposure to atrazine (nominal concentrations 0.1-5 ppm) or octylphenol (nominal concentrations 1-100 ppm). After one week of monitoring pre-exposure reproduction, treatments were delivered by subcutaneous implantation of the test chemical or solvent (controls) in a slow-release matrix of Ethocel® and coconut oil just below the dorsal fin of each fish. Reproduction was monitored for two more weeks, and then fish were euthanized and dissected. Brain, gonad and liver samples were flash-frozen and archived for later analysis. Results of these experiments indicate that atrazine and octylphenol had very little effect on egg production, egg fertility, egg viability, or ability of eggs to hatch. Only fish imprinted with the highest concentration of atrazine (5 ppm) showed a significant reduction in egg production compared to controls. All males, regardless of treatment, produced milt and motile sperm. Preliminary analysis of brains from fish exposed to atrazine showed a significant elevation of aromatase activity in male cunner, and a complete analysis of tissues is now underway. Our results to date suggest short-term exposure to an EDC that modifies aromatase activity does not necessarily translate into an impact on reproductive success of exposed fish once spawning has begun.

AROMATASE INHIBITION: LINKING BIOCHEMICAL MECHANISM OF ACTION TO OUTCOME IN THE SHORT-TERM FATHEAD MINNOW REPRODUCTION ASSAY.

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A 21 d fathead minnow (*Pimephales promelas*) reproduction assay has been proposed as one of five *in vivo* tests to be used for tier 1 screening of endocrine disrupting chemicals (EDCs). Previous studies with the pharmaceutical aromatase inhibitor fadrozole defined a profile of responses consistent with exposure to a potent aromatase inhibitor. In this study fathead minnows were exposed to two fungicides, prochloraz and fenarimol, that were suspected to act as EDCs through inhibition of aromatase activity and/or weak antiandrogenic effects. The purpose was to examine whether weak aromatase inhibitors, with potentially mixed mechanisms of action, would yield a profile of responses consistent with those observed for fadrozole. *In vitro* experiments with fathead minnow brain and ovary homogenates demonstrated that prochloraz could inhibit fathead minnow aromatase activity (IC50s = 7.2-11.4  $\mu$ M). Fenarimol concentrations greater than 5  $\mu$ M caused a significant decrease in aromatase activity *in vitro* but IC50s could not be determined. *In vivo*, both chemicals significantly decreased fecundity and depressed plasma vitellogenin concentrations in females. Additionally, prochloraz caused a concentration dependent decrease in plasma estradiol. Brain aromatase activity was significantly reduced in males exposed to 0.3 mg prochloraz/L, but neither chemical decreased brain aromatase activity in females, *in vivo*. Results of this study help define predictive relationships between *in vitro* biochemical actions of these chemicals and their ecologically relevant *in vivo* effects. *The contents of this abstract do not necessarily reflect official EPA policy.*

EFFECTS OF VARIOUS LACTONE-DERIVATIVES ON AROMATASE (CYP19) ACTIVITY IN H295R HUMAN ADRENOCORTICAL CARCINOMA CELLS AND (ANTI)ANDROGENICITY IN TRANSFECTED LNCAP HUMAN PROSTATE CANCER CELLS.

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Certain lactone-containing secondary plant metabolites display potent biological activities, such as anti-inflammatory or anti-tumor. Aromatase (CYP19) is a key enzyme in the synthesis of estrogens from androgens. Over-expression has been asso-

ciated with increased risk of estrogen-dependent mammary tumors, and aromatase inhibitors are effective in their treatment. Thirteen synthetic lactone-derivatives were evaluated for effects on aromatase activity in H295R cells. Cells were exposed to lactones (0.1-100  $\mu$ M) dissolved in DMSO (0.1% in medium) for 24 h prior to measurement of aromatase activity using the tritiated water-release assay. Also, (anti)androgenic effects of the lactones were assessed in LNCaP cells transfected with human androgen receptor (AR) and an AR-responsive luciferase reporter gene. Three (competitive) inhibitors of aromatase activity were identified (potencies in decreasing order): 3-(3, 4-dimethoxyphenyl)-4-(4-methoxyphenyl)-5H-furan-2-one (TM-7; IC50=1  $\mu$ M; Ki=1.0  $\mu$ M), 3, 4-bis-(3, 4-dimethoxyphenyl)-5H-furan-2-one (TM-8; IC50=2  $\mu$ M; Ki=1.2  $\mu$ M) and 3-(3, 4-dimethoxyphenyl)-4-(3, 4, 5-trimethoxyphenyl)-5H-furan-2-one (TM-9; IC50=3  $\mu$ M; Ki=6.8  $\mu$ M). Concentration-dependent inducers of aromatase (>2fold) were 3, 4-diphenyl-5H-furan-2-one (TM-1), 3-phenyl-4-(4-methylsulfonylphenyl)-5H-furan-2-one (TM-4; Viox), 3-(3, 4-difluorophenyl)-4-(4-methylsulfonylphenyl)-5H-furan-2-one (TM-11) and 3-(2, 4-difluorophenyl)-4-(4-methylsulfonylphenyl)-5H-furan-2-one (TM-13). In transfected LNCaP cells, the three inhibitors TM-7, 8 and 9 appeared to be weakly androgenic below 1  $\mu$ M; they were antiandrogenic above 1  $\mu$ M in the presence of 100 pM dihydrotestosterone. The other lactones showed no consistent pro- or anti-androgenic effects in this system.

IN VITRO EFFECTS OF BROMINATED FLAME RETARDANTS ON THE ADRENOCORTICAL ENZYME CYP17. A NOVEL ENDOCRINE MECHANISM OF ACTION?

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Fire incidents have decreased over the last 20 years partly due to regulations requiring addition of flame retardants (FRs) to materials. The five major BFRs are hexabromocyclododecane (HBCD), tetrabromobisphenol-A (TBBPA) and three commercial mixtures of polybrominated diphenyl ethers (PBDEs) (penta, octa, deca), which are extensively used as FRs. Furthermore, concentrations of PBDEs have been rapidly increasing during the last 10 years in human breast milk and a number of endocrine (*in vitro*) effects have been reported. We used the human adrenocortical carcinoma cell line (H295R) to assess possible effects of some of these BFRs (PBDEs, several of their metabolites (OH-BDEs, MeO-BDEs), TBBPA, HBCD and brominated phenols (BP)) on CYP17 activity. This enzyme catalyzes an important step in the sex steroidogenesis and is responsible for the biosynthesis of dehydroepiandrosterone (DHEA). In order to study this interaction, a novel method was developed. HBCDD, BDE99 and 100 showed significant inhibition of CYP17 activity at the highest concentrations (10  $\mu$ M) used with no signs of cytotoxicity. A strong inhibition of CYP17 activity was found when the cells were treated with BDE47 (6OH-BDE47) causing a concentration dependent decrease of almost 90% at 10  $\mu$ M but with a concurrent decrease in cell viability at the highest concentration. Replacement of the 6OH group by a methoxy group eliminated this cytotoxicity, while still DHEA production was significantly inhibited. Other OH or their methoxy derivatives were used to explain possible structural properties behind this inhibition of CYP 17 and possible associated cytotoxicity. In the case of 4OH-BDE49, CYP17 activity was significantly reduced and replacement of 4OH group by a methoxy group abolished CYP17 inhibition.

ESTROGENIC ACTIVITY OF THE POLYBROMINATED DIPHENYL ETHERS FLAME RETARDANTS.

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Recent studies suggest that polybrominated diphenyl ethers (PBDEs), used as flame-retardants in textiles and electronics, may act as endocrine disruptors. While PBDEs have been found worldwide in air, foodstuff, wildlife and human tissue, the full impact of exposure to these chemicals on tissue development and cancer is not known. The overall goal is to identify the mechanisms by which PBDEs behave as hormones and thereby perturb the cellular physiology of development and cancer. The commercial PBDE mixture DE-71 was incubated with female rat microsomes in a buffer containing NADP<sup>+</sup> and glucose-6-phosphate dehydrogenase (G6PDH) for different time periods (30 min to 24 hours). The pesticide methoxychlor was used as a positive control. The metabolites generated were tested for estrogenic activity using an Estrogen Response Element (ERE) - luciferase gene construct. Other tests in progress include estrogen receptor (ER) binding assay and MAPK phosphorylation assays to determine nongenomic estrogen-like activity. PBDE oxidative metabolites produced *in vitro* during 24 hours incubation induce gene expression

of ERE-luciferase. On average, incubations with NADP<sup>+</sup> increased PBDE estrogenic activity about 6-fold in the ERE-luciferase assay, compared with vehicle control and about 3-fold compared with control PBDE incubations without NADP<sup>+</sup>. Preliminary results from binding assays indicate that neither PBDE nor its 24-hour metabolites bind the ER. Our data confirms findings by Meerts et al. showing that *in vitro* P450 metabolism of PBDEs produces hormonally-active metabolites (2000; *Toxicology Sciences* 56:95) and that such metabolites have estrogenic activity *in vitro* (2001; *Env. Health Pers.* 109(4):399). Ongoing experiments will assess the ability of PBDEs and their metabolites to bind the estrogen receptor and to activate MAPK phosphorylation associated with non-genomic estrogen effects.

**1750** SCREENING ASSAYS FOR ANDROGENIC ACTIVITIES OF XENOBIOTICS.

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The androgen receptor (AR) is a member of the nuclear receptor superfamily and mediates the physiological effects of androgens. The AR binds to specific DNA sequences, so-called androgen response elements (ARE) leading to transcription of androgen-dependent genes. Interference of the AR signalling pathway by xenobiotics may lead to endocrine disruption. The objective of this study was therefore to establish assays in human cells that enable the analysis of androgenic activities of xenobiotics. Since the activity of the AR is modulated by the cellular environment and the AREs of the target genes, we generated reporter plasmids containing either the consensus steroid response element derived from the MMTV promoter (SRE), a human, or a rat ARE. In addition, we cloned the human AR derived from LNCaP cells. We then analysed several well known androgens for their activity in human prostate carcinoma LNCaP cells with endogenous AR as well as in the human HepG2 cells that lack AR. In LNCaP cells, the activities of R1881, testosterone, dihydrotestosterone (DHT) and cyproterone acetate were low on the SRE reporter, whereas no response at all was observed on the human and rat ARE. In contrast, when we transfected AR into HepG2 cells, all androgens tested exerted strong induction on all reporter plasmids tested. For comparison, we also employed the MMTV long-terminal repeat promoter that showed equal responses as the SRE and ARE reporters in HepG2 cells. The androgenic potencies of the compounds tested were comparable in LNCaP and HepG2 cells with the exception of DHT, which showed a 100-fold lower potency in HepG2 cells when compared to LNCaP cells. Since we were unable to obtain high activities in LNCaP cells, we employed another human prostate cell line, RV1 that expresses endogenous AR. In RV1 cells, DHT exerted potencies at sub-nanomolar doses similar to LNCaP cells and displayed high efficiencies that were comparable to the results obtained in HepG2 cells. In conclusion, the potency of DHT depends highly on the cellular environment. Based on these results, RV1 cells are a highly sensitive and appropriate model to screen for androgenic compounds.

**1751** EVALUATION OF POTENTIAL ESTROGENIC AND ANDROGENIC ACTIVITY OF OCTAMETHYLCYCLOTETRAZILOXANE (D4) AND DECAMETHYLCYCLOPENTASILOXANE (D5).

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The estrogenic activity of D4 and D5 was evaluated in ovariectomized Sprague-Dawley (SD) and Fischer 344 (F-344) rats exposed to the highest achievable vapor concentration of D4 (700 ppm) or D5 (160 ppm) via whole body inhalation for 16 hours/day for 3 days. Immediately following exposure animals were euthanized and the effect of test article on uterine weight (wet and blotted) was evaluated. Exposure to D4 resulted in a significant increase in both absolute and relative uterine weights (wet and blotted), and significant increases in luminal and glandular epithelial cell height in both strains of rats. Exposure to D5 did not result in an increase in any of the estrogenic endpoints measured in either strain of rat. Subsequently, D4 and D5 were evaluated for their ability to bind to the estrogen receptor alpha. In a typical competition experiment, D4 (700 ppm) was able to displace ~20% of the 3H-estradiol. Under identical conditions, D5 (160 ppm) did not displace any of the estradiol indicating that this material does not compete for the receptor-binding site. In an *in vitro* luciferase reporter gene assay using MCF-7 cells transiently transfected with a plasmid for estrogen receptor alpha and the luciferase gene, 10μM D4 resulted in an activation of the reporter gene (6-fold increase over baseline controls). D5 did not activate the reporter gene at the same dose range, while 17β-estradiol resulted in the expected increase (35-fold increase at 10 nM dose range). Using *in vitro* and *in vivo* assays, D4 has consistently resulted in a weak estrogenic response, while D5 has not elicited any estrogenic responses. Castrated male F-344 rats were exposed to D4 or D5 for 16 hours/day for 10 days. Typical Hershberger

assay endpoints were evaluated. Neither material resulted in any significant increase in any of the accessory sex organ weights, indicating that neither D4 nor D5 is associated with any androgenic activity. Sponsored by the Silicones Environmental, Health and Safety Council of North America.

**1752**

ESTROGENIC ENDOCRINE DISRUPTOR POTENCY OF ORGANOCHLORINE PESTICIDES DETECTED USING THE LUMI-CELL™ ER BIOASSAY.

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Organochlorine pesticides are found in many ecosystems worldwide, which have resulted in the contamination of the food chain. Endocrine disruptor compounds (EDCs) can have a significant detrimental effect on the endocrine and reproductive systems of both humans and animals. Identification of EDC pesticides requires a relevant bioassay, which can detect these chemicals, and provide a relevant estimate of their endocrine disrupting potency. To detect EDCs, BG-1 cells were stably transfected with an estrogen-responsive luciferase reporter gene plasmid (pGudLuc7ere). The resulting cell line, the LUMI-CELL™ ER bioassay, responds to estrogenic chemicals in a time-, dose dependent- and chemical-specific manner with the induction of luciferase gene expression. Thirteen organochlorine pesticides suspected of possessing estrogenic endocrine disrupting potential were tested. All of the compounds with historical data demonstrating estrogenic activity were shown to possess estrogenic activity. When comparing the estrogenic potency of the pesticides, the order of induction of activity with respect to their EC50 values is: α-Chlordane > Kepone > DDD > pp' DDT > Methoxychlor > Ψ-Chlordane > pp' DDE > Fenarimol > 2, 4, 5-Trichlorophenoxyacetic Acid > Dieldrin > Linuron > Mirex = Vinclozolin. The average minimal effective dose for organochlorine pesticides in animals appears to be 5 ppm or greater. The LUMI-CELL™ ER bioassay is capable detecting organochlorine pesticides at <1 ppm (with a lower limit of detection of <0.1 ppm). This data clearly demonstrates that the LUMI-CELL™ ER high-throughput bioassay system is a fast, reliable, and relatively inexpensive method for detection of environmental EDCs, and could refine, reduce or replace animals in many tests. Meeting requirements mandated by the EPA and ICCVAMs Tier I requirements for EDC detection assays. Supported by NIEHS SBIR grant ES10533-03 and Superfund Basic Research Grant ES04699.

**1753**

SENSITIVE METHOD FOR DETERMINING PESTICIDE ESTROGENIETY AT PICOMOLAR LEVELS USING A SERUM-FREE BG-1 OVARIAN CELL MODEL.

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Most xenobiotic estrogenicity assay methods rely on their direct agonist action on the estrogen receptor (ER). Often, ligand binding assays are used to approximate their ER activation potential. Such methods, however, do have drawbacks since most pesticides activate the ER via a "ligand independent activation" (LIA) by being only weak or non-agonistic with the ER. Therefore, there is a need to develop an assay method that will detect the estrogenic actions of a pesticide regardless of its ER ligand binding ability. Using a serum-free BG-1 ovarian cell model, we investigated the ability of several organochlorine (OC) pesticides to stimulate known estrogenic actions such as increased proliferation, and upregulation of estrogen response element (ERE) dependent proteins. These effects were abolished using ICI 164, 384 (ICI), a pure ER antagonist, thus implicating the requirement of ER for these events. Using these methods, we observed concentration dependent (10pM-100nM) ER mediated cell proliferation in BG-1 cells using the OCs heptachlor epoxide (HE), β-hexachlorohexane (βHCH), and endosulfan I (ENDO). In addition, we were able to observe upregulation of the ERE dependent proteins progesterone receptor and PS2. EMSA studies for ERE binding further supported these OC's ERE activating abilities. Using the same culture conditions, we tested the blocking action of growth factor antibodies for erbB2 (9G6) and insulin-like growth factor (IGF-Ab) receptors and discovered they to inhibit BG-1 proliferation by HE (9G6), βHCH (9G6) and ENDO (IGF-Ab). This experiment confirms the existence of a possible crosstalk between ER and growth factor receptors in OC LIA. In conclusion, we were able to develop a sensitive method for determining both ligand dependent and independent estrogenic activity of selected pesticides. Secondly, we demonstrated the ability of OCs to activate the estrogenic system in BG-1 cells independent of direct ER binding; and third, our data implicates a possible crosstalk component between the ER and growth factor receptor systems for OC LIA.

GENE EXPRESSION PATTERNS OF THE AMPHIBIAN HPT-AXIS IN NORMAL DEVELOPMENT AND AFTER EXPOSURE TO THE MODULATORS METHIMAZOLE, PERCHLORATE AND PROPYLTHIOURACIL.

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In response to the Endocrine Disruptor Screening and Testing Program Advisory Committee recommendations, the USEPA has developed a screening assay capable of detecting the effects of chemicals on the hypothalamus-pituitary-thyroid (HPT) axis in *Xenopus laevis*. The primary endpoints of the screen are developmental rate and thyroid gland histology. However, we are currently developing biochemical (thyroid hormone concentrations), and molecular (gene and protein) measurements to improve diagnostic capability of the assay. In this study, we determined developmental expression patterns of key genes from stages 51 to 66 in the pituitary (thyroid stimulating hormone (TSH) and deiodinases type II and III (DII and DIII)) and thyroid gland (thyroid transcription factor I (TTF I), thyroid peroxidase (TPO), thyroglobulin (TG), and sodium-iodide symporter (NIS)). Measurements were made by quantitative real-time RT-PCR (Q-PCR). Significant patterns of stage-specific gene expression were observed. In the baseline pituitary samples, expression of TSH and DII tracked together peaking at stage 59, while DIII did not peak until stage 62, a pattern which is consistent with a regulatory role in metamorphic climax. Concentrations of methimazole, perchlorate, and propylthiouracil which have been shown to significantly affect metamorphosis and thyroid gland morphology did not result in changes in the expression of TSH, DII, and DIII in the pituitary within 48 h. Additionally, some samples were used to generate amplified RNA probes for use in a focused gene array. The array contains nearly 100 genes of importance to the HPT axis. Results from the two methods for detecting changes in gene expression showed unique advantages: While gene arrays examine many genes in one experiment, the quantitative data from Q-PCR experiments allows more robust statistical comparisons. This abstract does not necessarily reflect EPA policy.

PROTEIN PROFILING OF *XENOPUS LAEVIS* BRAIN CELLS FOLLOWING EXPOSURE TO T4 SYNTHESIS INHIBITORS: POTENTIAL APPLICATION TO THE ASSESSMENT/DIAGNOSIS OF XENOBIOTICS THAT PERTURB THE THYROID PATHWAY.

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Sponsor: J. Nichols.

Development of a cost-effective, non-mammalian screening assay for thyroid axis disrupting chemicals is a goal of the USEPA. To address this need, we are applying a multi-endpoint strategy that combines molecular and *in vivo* methods using the amphibian model, *Xenopus laevis*. As part of this effort, we report the use of a proteomic approach to assess the effects of xenobiotics on the thyroid hormone axis. Differential protein profiling was applied to investigate specific expression patterns produced by a suite of model T4 synthesis inhibitors in pre-metamorphic *Xenopus*. Gel electrophoresis and Mass Spectrometry were used to evaluate the effects of methimazole, perchlorate and propylthiouracil upon protein expression in brain tissue cells. Briefly, proteins were isolated/separated by 2D-PAGE, stained and quantitated by image analysis. Spots showing quantitative differences between control and treated samples were excised robotically and identified by LCMS. Over 20 proteins were found to be differentially regulated by these compounds. To date, we have characterized 4 proteins showing the most consistent/unique changes across chemicals tested. These proteins fell into various functional groups comprising gene expression modulation (hnRNP A1), targeted membrane fusion (SNAP-25), lipid raft formation (flotillin 1), and metabolism (aldolase). Results indicated that SNAP-25, a t-SNARE complex protein, is upregulated by all T4 inhibitors assessed. This finding is consistent with reports of SNAP-25 upregulation in the adenohypophyses of thyroidectomized rats, and downregulation in the adrenal gland by T4. Overall, these data demonstrate the utility of protein profiling to establish characteristic expression patterns which can be used as diagnostic elements for thyroid active chemicals. (This abstract does not necessarily reflect USEPA policy)

PHENOBARBITAL AFFECTS THYROID HISTOLOGY AND LARVAL DEVELOPMENT IN THE AFRICAN CLAWED FROG, *XENOPUS LAEVIS*.

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The USEPA (EPA) has recently proposed an amphibian-based assay intended for screening chemicals abilities to disrupt the thyroid axis. To develop this assay, a number of chemicals have been tested which have well defined mechanism of pitu-

itary-thyroid axis disruption. The objective of the present study was to determine if *Xenopus* is sensitive to chemicals which act by inducing the biotransforming enzyme uridine diphosphate glucuronosyltransferase (UDPGT). In mammals, phenobarbital is known to induce microsomal UDPGTs resulting in increased thyroid hormone glucuronidation and elimination. However, it is not clear if amphibians are sensitive to this mode of thyroid axis disruption. In this study, we exposed stage-54 *Xenopus laevis* tadpoles to a range of phenobarbital concentrations (0, 125, 250, 500, 1000, and 1500 mg/L) via the water. At day 21, we characterized morphology, thyroid gland histology and larval development. Treatment resulted in a concentration dependent increase in mortality and thyroid gland changes, although dramatic effects on developmental rate were not observed. Tadpole mortalities at the experimental concentrations were 0, 0, 2.5, 13, 27, and 45 % respectively over 21 days. Phenobarbital treatment resulted in thyroid hypertrophy, colloid reduction, enlarged irregular follicles, and follicular cell hypertrophy and hyperplasia. These results are consistent with those observed in mammals, thus indicating *Xenopus* tadpoles are sensitive to the thyroid axis disrupting effects of phenobarbital via UDPGT-mediated pathways. Molecular investigation of UDPGT genes is currently underway in our laboratory to further understand the mechanism in *Xenopus* tadpoles. (M. J. Chowdhury is a US National Research Council Associate. This abstract does not necessarily reflect USEPA policy).

SUBLETHAL EFFECTS OF CADMIUM ON DEVELOPMENT IN *RANA PIPIENS* AND *XENOPUS TROPICALIS*.

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While there have been many studies characterizing acute responses in amphibian adults, embryos and larvae to heavy metals such as cadmium the responses of amphibians to chronic exposure to this metals is generally unstudied. Some metals are putative endocrine disruptors and affect sexual development, reproduction, and metamorphosis but the sensitivity of amphibians to these effects are also unstudied. We exposed embryos and tadpoles of northern leopard frogs (control, 0.25, 5.0, 20.0 µg/l, as CdCl<sub>2</sub>; static renewal system) and African clawed frogs (control, 6.25, 25.0, 100.0, 400.0 µg/l, as CdCl<sub>2</sub>; flow-through diluter system) to sublethal doses of cadmium from embryonic stages to complete tail resorption. Survival of embryos (Gosner stage (GS) 17 to 25 for *R. pipiens* and Neiwenkoop and Faber stage (NFS) 29 to 43 for *X. tropicalis*) was > 94% in all treatments for both species. *Rana pipiens* tadpole mortality was positively correlated with dose and was significantly higher in the 20.0 µg/l treatments than all other treatments by week 8 (P<0.05). Tadpole survival was >80% through GS 42, forelimb emergence for all other treatments. Tadpoles exposed to 0.25 and 5.0 µg/l Cd demonstrated increased size by week 7 (P<0.05 and P=0.07 respectively) and were significantly younger at GS 42 (P<0.001 and P<0.05 respectively) relative to control. Swimming speed of tadpoles in 0.25 µg/l treatments was significantly higher than other treatments (p < 0.05). No differences were seen in *X. tropicalis* tadpole survival (>70%) to NFS 65, complete tail resorption. *Xenopus tropicalis* exposed to 6.25, 100.0 and 400.0 µg/l Cd were larger at NFS 65 relative to other treatments. This is the first chronic sublethal Cd study for both *R. pipiens* and *X. tropicalis*. Cadmium was shown to alter growth and development in a native and model amphibian species at ecologically relevant concentrations.

MECHANISMS OF GERMINAL VESICLE BREAKDOWN (GVBD) INHIBITION BY PHENYL-SUBSTITUTED GLYCOL ETHERS IN *XENOPUS OOCYTES IN VITRO*.

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Under normal conditions, *Xenopus* oocytes undergo final maturation which is induced by progesterone via a membrane bound receptor (OMPR) or androgens via a classical intracellular receptor (AR). The progestin/antiprogestin activities of a series of substituted glycol ethers including: ethylene glycol monomethyl ether (EGME), -monoethyl ether (EGEE), -monopropyl ether (EGPE), -monobutyl ether (EGBE), -monophenyl ether (EGPhE), diethylene glycol monomethyl ether (DGME), and triethylene glycol monomethyl ether (TGME) were screened using an *in vitro* *Xenopus* oocyte germinal vesicle breakdown (GVBD) assay. Results suggested that each of the ethylene glycol ethers, but not DGME or TGME were capable of inhibiting GVBD *in vitro* in a concentration dependent manner. Potency, expressed as an IC<sub>25</sub>, ranged from 1.0 µg/L for EGPhE to >10 mg/L for TGME. Results from additional studies suggested that the inhibitory effects of EGME, EGEE, EGPE, and EGBE on GVBD may be mediated primarily through the OMPR, whereas the unanticipated potency of EGPhE is postulated to be a result of bimodal inhibition of both the OMPR and AR pathways. This unexpected potency

of EGPhE based structure activity assessment suggested that additional phenyl-substituted glycol ethers including, EG-monobenzyl ether (EGBeE), EG-diphenyl ether (EGDPhE), and propylene glycol monophenyl ether (PGPhE) should be evaluated in an attempt to establish their relative potencies compared to the EGPhE. The monophenyl-substituted GEs were most capable of inhibiting GVBD producing relative GVBD inhibition potencies: EGPhE > PGPhE > EGME >> EGEE > EGBeE > EGPE >> EGBE >> EGDPhE >> DGME = TGME. These results suggest a remarkable structural specificity of mono-substituted phenyl ethers (EGPhE and PGPhE) to inhibit Xenopus oocyte maturation and warrant additional receptor binding studies.

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NON-ADDITIVE DOSE DEPENDENT GENE EXPRESSION PATTERNS EXHIBITED BY A MIXTURE OF 17-ALPHA ETHYNYLESTRADIOL AND GENISTEIN *IN VIVO*.

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Human populations are generally exposed to subthreshold doses of the phytoestrogen genistein, however there are concerns regarding non-additive interactions with other exogenous and endogenous estrogens. Coadministration of submaximal doses of 17-alpha ethynylestradiol (EE) and genistein (GEN) were studied to identify non-additive gene expression interactions. Immature, ovariectomized mice were gavaged with 3, 10, 30, 100, or 300ug/kg EE; 0.3, 0.9, 3, 9, or 30mg/kg GEN; a 1:100 mixture of EE and GEN (same doses as above), or sesame oil vehicle. Animals were sacrificed 24hrs following administration and uterotrophic effects and hepatic gene expression was examined. All doses of EE and selected doses (3/0.9; 10/3; 30/9; 100/30 ug/kg / mg/kg) EE/GEN induced uterine wet weight; while GEN failed to elicit a significant ( $P = 0.05$ ) uterotrophic response. Custom cDNA microarrays with 13, 824 features representing 8661 unique genes were used to monitor changes in hepatic gene expression from EE, GEN, or mixture treated mice. EE and mixture samples clustered together, while GEN samples clustered separately. Eighty genes exhibited a monotonic response in either EE or GEN treated hepatic samples. Of these, 23 exhibited non-additive interactions as assessed by the single chemical required (SCR) method. Gene functions represented by these 23 genes included steroid metabolism and biosynthesis and response to oxidative species, and are localized to the endoplasmic reticulum and the mitochondria. Results from these studies demonstrate non-additive interactions between EE and GEN that warrant further investigation. Supported by NIH Grants R01 ES11271 and \*T32 ES07255.

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TEMPORAL GENE EXPRESSION ANALYSIS OF MOUSE HEPA-1C1C7 CELLS TREATED WITH 17 $\beta$ -ESTRADIOL BY CDNA MICROARRAY.

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cDNA microarrays, containing 6376 clones representing 4858 unique genes, were used to examine temporal changes in gene expression in the mouse Hepa-1c1c7 hepatoma cell line following treatment with 10 nM 17 $\beta$ -estradiol (E2) or vehicle (DMSO) in serum free media at 1, 2, 4, 8, 12, 24, and 48 hours. MTT and direct cell count assays indicated that cells were viable in serum free media but at a significantly reduce proliferation rate. Model-based t-statistical analysis identified significant changes in the expression of 213 unique genes representing functions associated with lipid metabolism, glucose metabolism and oxidative stress with seven k-means clusters representing the major temporal patterns. Overall gene-specific changes in expression between *in vitro* and *in vivo* models were poor. However, the induction of LDL receptor genes (*Vldlr*, *Sorl1*) and the cholesterol-ester synthesis gene (*Soat1*), and the down-regulation of HDL receptor (*Scarb1*) are in agreement with liver responses in C57BL/6 mice following oral ethynylestradiol exposure and consistent with the reported *in vivo* decreases in serum LDL/HDL ratios elicited by estrogens. Estrogenic regulation of the hepatic liver X receptor (*LXR*), a cholesterol regulator, may also be a contributing factor of activating these events. In contrast, DMSO elicited only 95 early changes in gene expression representing functions associated with cell proliferation, solute homeostasis and xenobiotic metabolism which could be described by two k-means clusters indicating that it is not a significant contributor to the changes observed upon exposure to estrogen. This study shows that although Hepa-1c1c7 cells may not be an appropriate model for overall *in vivo* hepatic effects of estrogen, it may be suitable for more define studies investigating specific mechanisms such as those affecting cholesterol transport and metabolism. Supported by R01 ES11271.

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CONSTITUTIVE ANDROSTANE RECEPTOR (CAR)-DEPENDENT HEPATIC INDUCTION OF UDP-GLUCURONOSYLTRANSFERASES (UGTs) IN WISTAR-KYOTO RATS BY CYP2B INDUCERS.

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UGTs catalyze the glucuronidation of numerous endogenous and xenobiotic compounds. UGT activity is increased by phenobarbital (PB) and other compounds that induce cytochrome P450 2B (CYP2B). Induction of CYP2B by PB is mediated via CAR binding to phenobarbital response element modules (PBREM). The purpose of this study was to determine: 1) CAR dependence of UGT induction by the CYP2B inducers PB, diallyl sulfide (DAS), polychlorinated biphenyl 99 (PCB99), and trans-stilbene oxide (TSO), and 2) whether CYP2B inducers activate PBREM in a CAR-dependent manner *in vivo*. Wistar Kyoto (WK) rats have male-predominant expression of CAR in liver. Thus, a more robust induction of UGTs or activation of PBREM-luciferase reporter activity in male WK rats compared to females would indicate CAR dependence. UGT mRNA levels were examined in livers of male and female WK rats treated with PB, DAS, PCB99 and TSO using the branched DNA assay. UGT1A1, 1A5, and 2B1 mRNA levels were induced in male WK rats by all four CYP2B inducers. However, in female WK rats, induction was either absent or diminished suggesting induction of UGT1A1, 1A5, and 2B1 by CYP2B inducers is CAR-dependent. UGT1A6 mRNA was increased only by TSO, and induction occurred to the same extent in both male and female WK rats, suggesting induction of UGT1A6 by TSO is independent of CAR expression. Hepatic injections of PBREM-luciferase reporter construct into male and female WK rats and subsequent luciferase reporter assays were conducted to examine CAR-dependent activation of PBREM by CYP2B inducers. PB and DAS treatment of male WK rats produced a 20- and 10-fold increase, respectively, in luciferase activity relative to controls; whereas in females, luciferase activity was increased 7 and 3 fold respectively. Together these results indicate that induction of UGT1A1, 1A5, and 2B1 by CYP2B inducers is mediated via CAR. Additionally PB and DAS activate PBREM in a CAR-dependent manner. (Supported by NIH Grants ES-08156 and ES-07079)

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HEPATIC INDUCTION OF MOUSE UDP-GLUCURONOSYLTRANSFERASE (UGT) MNRA EXPRESSION BY PROTOTYPICAL ACTIVATORS OF VARIOUS TRANSCRIPTION PATHWAYS.

D. B. Buckley, J. S. Petrick and C. D. Klaassen. *University of Kansas Medical Center, Kansas City, KS*.

UGTs catalyze the addition of UDP-glucuronic acid to endo- and xenobiotics, enhancing their water solubility and excretion. Many exogenous compounds, such as microsomal enzyme inducers (MEIs), alter gene expression through xenobiotic-responsive transcription factors, namely the aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane X receptor (PXR), peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ), and NF-E2 related factor 2 (Nrf2). These transcription factors regulate xenobiotic-inducible expression of hepatic biotransformation enzymes and transporters. The purpose of this study was to determine the hepatic inducibility of mouse Ugt isoforms by MEIs. Male C57BL/6 mice were treated for four consecutive days with activators of AhR (TCDD, PCB126, and  $\beta$ -naphthoflavone), CAR (TCPOBOP, phenobarbital, and diallyl sulfide), PXR (pregnenolone-16 $\alpha$ -carbonitrile, spironolactone, and dexamethasone), PPAR $\alpha$  (clofibrate, ciprofibrate, and diethylhexylphthalate), and Nrf2 (oltipipraz, ethoxyquin, and butylated hydroxyanisole), respectively. Mouse hepatic Ugt mRNA expression was determined using the branched DNA signal amplification assay. Ugt1a isoforms predominantly expressed in liver were inducible by MEIs. Ugt1a1 mRNA expression was induced by activators of all five transcription factor pathways, Ugt1a5 was induced by Nrf2 activators, Ugt1a6 was induced by AhR ligands, and Ugt1a9 was induced by AhR, CAR, PXR, and PPAR $\alpha$  activators/ligands. Although liver-predominant, mouse Ugt2b isoforms analyzed did not exhibit hepatic mRNA induction by any class of inducer. Results indicate that among the 11 mouse Ugts examined, four isoforms, Ugt1a1, 1a5, 1a6, and 1a9, are inducible in liver. These results suggest that MEIs can work through at least 5 different xenobiotic-responsive transcription factor pathways to induce Ugt1a isoforms in mouse liver. (Supported by NIH Grants ES-08156 and ES-07079)

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INDUCIBILITY OF MOUSE TRANSCRIPTION FACTORS THAT REGULATE HEPATIC METABOLISM AND TRANSPORT.

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Aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), pregnane X receptor (PXR), peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ), and NF-E2 related factor 2 (Nrf2) are transcription factors that coordinately induce expression of biotransformation enzymes and transporters in response to

xenobiotics. Treatment of mice with activators of AhR, CAR, PXR, PPAR $\alpha$ , and Nrf2 induces expression of their associated marker genes: cytochrome P450 (Cyp) 1a1, 2b10, 3a11, 4a14, and NADPH quinone oxidoreductase 1 (Nqo1), respectively. The purpose of this study was to determine whether AhR, CAR, PXR, PPAR $\alpha$ , and Nrf2 activators induce mRNA expression of these transcription factors concomitantly with induction of their corresponding marker genes. Male C57BL/6 mice were treated for four days with activators of AhR (TCDD, PCB126, and  $\beta$ -naphthoflavone), CAR (TCPOBOP, phenobarbital, and diallyl sulfide), PXR (pregnenolone-16 $\alpha$ -carbonitrile, spironolactone, and dexamethasone), PPAR $\alpha$  (clofibrate, ciprofibrate, and diethylhexylphthalate), or Nrf2 (oltipraz, ethoxyquin, and butylated hydroxyanisole). All marker genes were induced by activators of their associated transcription factors. In addition, some marker genes were induced by multiple classes of activator compounds, as 1) Cyp2b10 was induced by CAR, PXR, PPAR $\alpha$  and Nrf2 activators, 2) Cyp3a11 was induced in response to both CAR and PXR activators, and 3) Nqo1 expression was induced by activators of AhR, PPAR $\alpha$  and Nrf2. Of the transcription factors, only PPAR $\alpha$  was induced by more than one of its own ligands. Furthermore, CAR was induced by AhR ligands, and PXR was induced by two of three PPAR $\alpha$  ligands. Overall, the magnitude of transcription factor induction was modest, indicating that their expression is tightly regulated, even in the presence of compounds that markedly induce expression of their marker genes. These data indicate that P450 and Nqo1 induction by xenobiotics occurs with minimal induction of their associated transcription factors. (Supported by NIH Grants ES-09716, ES-09649, ES-08156, and ES-07079)

#### 1764

#### VITAMIN D-INTERACTING PROTEIN 150 (DRIP150) COACTIVATION OF ESTROGEN RECEPTOR $\alpha$ (ER $\alpha$ ) IN ZR-75 BREAST CANCER CELLS IS INDEPENDENT OF LXXLL MOTIFS.

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Vitamin D receptor-interacting protein 150 (DRIP150) has been identified as part of mediator-like complexes that enhance transcriptional activation of the estrogen receptor (ER) and other nuclear receptors (NRs). DRIP150 coactivates ligand-dependent ER $\alpha$ -mediated transactivation in ZR-75 and MDA-MB-231 breast cancer cells transfected with a (luciferase) reporter construct (pERE $\alpha$ ) regulated by three tandem estrogen responsive elements (EREs). Coactivation of ER $\alpha$  by DRIP150 in ZR-75 cells was activation function 2 (AF2)-dependent and required an intact helix 12 that typically interacts with LXXLL motifs (NR-box) in p160 steroid receptor coactivators (SRCs). DRIP150 contains C- and N-terminal NR-boxes (amino acids 1186-1182 and 73-69, respectively), and deletion analysis of DRIP150 showed that regions containing these sequences were not necessary for coactivation of ER $\alpha$ . Analysis of multiple DRIP150 deletion mutants identified a 23 amino acid sequence (811-789) required for coactivation activity. Analysis of the protein crystal structure data base identified two regions at amino acids 789-794 and 795-804 which resembled  $\alpha$ -helical motifs in Lanuguinosa lipase/histamine N-methyl transferase and hepatocyte nuclear factor 1, respectively. Using a squelching assay and specific point mutations into proline residues within each  $\alpha$ -helix, the NZF-SEVRVYN (795-804) region was identified as the critical sequence required for the activity of DRIP150. These results demonstrate that coactivation of ER $\alpha$  by DRIP150 in ZR-75 cells is NR-box independent and requires a novel sequence with putative  $\alpha$ -helical structure. (Supported by NIEHS ES09106 and CA104116)

#### 1765

#### GLUCOCORTICOID RECEPTOR AND STEROID AND XENOBIOTIC RECEPTOR (SXR) REGULATION OF SXR PROMOTER ACTIVITY.

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In recent years, it has been established that steroid and xenobiotic receptor (SXR) or pregnane X receptor (PXR), plays a novel role as a xenosensor by binding a wide spectrum of xenobiotics and altering metabolism by induction of cytochrome P450 enzymes, specifically 3A4. Xenobiotics capable of inducing SXR expression and subsequent CYP3A4 expression can alter metabolic pathways and potentiate drug-drug interactions. Of the three SXR protein isoforms, preliminary experiments demonstrate selective induction of one isoform following glucocorticoid treatment. To study the regulation of SXR gene expression, we cloned a 2kb fragment of the SXR promoter region into the pGL3 luciferase reporter vector (SXR2kb-luc). The SXR2kb-luc construct was transiently transfected into the human hepatoma cell line HepG2, along with human glucocorticoid receptor (hGR) expression vector and treated with the glucocorticoid dexamethasone (Dex). Surprisingly, SXR2kb-luc reporter activity was repressed 40% by hGR alone compared to empty vector controls, and Dex treatment caused an additional ligand-dependent decrease in re-

porter activity. Zhang et al. (1999) demonstrated pregnenolone 16 $\alpha$ -carbonitrile (PCN), a rat SXR ligand, was capable of inducing SXR mRNA expression, suggesting SXR may regulate its own promoter. HepG2 cells were then co-transfected with SXR2kb-luc and either pSG5 empty vector or hSXR expression vector. hSXR expression caused an 60-80% decrease in reporter activity when compared to empty vector controls. When hSXR transfected cells were treated with the hSXR ligand rifampicin, repression of SXR2kb-luc activity was observed to 30% of the vehicle treated controls. These experiments demonstrate significant reporter activity from the binding of endogenous HepG2 cellular proteins to response elements in the SXR2kb promoter. Repression mediated by both GR and SXR suggests a common mechanism may be responsible. Repression of SXR promoter reporter activity, while not the expected outcome, hints at a more complex and dynamic profile of SXR gene expression.

#### 1766

#### HEPATOCYTE RXRALPHA REGULATES THE EXPRESSION OF THE GLUTATHIONE S-TRANSFERASE GENES AND MODULATES ACETAMINOPHEN-GLUTATHIONE CONJUGATION IN MOUSE LIVER.

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Retinoid X receptor alpha (RXR $\alpha$ )-mediated pathways play a critical role in xenobiotic detoxification. Our previous report showed that RXR $\alpha$  modulates acetaminophen (APAP)-induced hepatotoxicity. The present study further elucidates the role of hepatocyte RXR $\alpha$  in detoxification of APAP. Wild type and hepatocyte RXR $\alpha$ -deficient mice were injected with a toxic dosage of APAP (i.p., 500 mg/kg). Liver injury was monitored by measuring serum alanine aminotransferase (ALT) and sorbitol dehydrogenase (SDH) activities and liver histology. Our data demonstrated that hepatocyte RXR $\alpha$ -deficient mice were protected from the hepatotoxic effect of APAP, even though the liver basal glutathione (GSH) levels were significantly lower than wild type mice. To further analyze the mechanism, HPLC analysis of APAP metabolites was performed. Our data revealed a significantly higher amount of APAP-GSH conjugate in the liver and bile of RXR $\alpha$ -deficient mice compared with that of wild type mice. APAP overdose causes accumulation of the toxic electrophile N-acetyl-p-benzoquinone imine (NAPQI), which is detoxified by conjugation with reduced GSH to form the 3-S-glutathionyl conjugate of APAP (APAP-GSH) via the action of glutathione S-transferases (Gsts). Therefore, northern blot analyses were performed to measure the gene expression of Gst family members in APAP-treated and untreated mice for both genotypes. The mRNA levels of seven Gst family members were up-regulated in RXR $\alpha$ -deficient mice, whereas Gstpi was down-regulated. After APAP treatment, Gst theta3 was up-regulated, while Gst alpha1 and 3 were down-regulated in RXR $\alpha$ -deficient mice. It is likely that the alteration of the gene expression profile of Gst family in the liver of RXR $\alpha$ -deficient mice enhances APAP-GSH conjugation, which in turn contributes to the protection of RXR $\alpha$ -deficient mice from APAP-induced hepatotoxicity.

#### 1767

#### P38 MITOGEN-ACTIVATED PROTEIN KINASE IS INVOLVED IN CONSTITUTIVE ANDROSTANE RECEPTOR SIGNALING TO REGULATE CYP2B INDUCTION BY XENOBIOTICS.

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The constitutive androstan receptor (CAR) is a member of the nuclear receptor (NR) superfamily. CAR activation causes induction of genes encoding xenobiotic and steroid-metabolizing enzymes CYP2B. In contrast to classic endocrine NRs that show very selective ligand binding, CAR displays relatively low affinities for its ligands. CAR also differs from most other NRs in having a strong constitutive activity in the absence of ligand. Like NRs, the mitogen-activated protein kinase (MAPK) signaling pathways play critical roles in regulating gene expression and control diverse cellular responses to internal and external stimuli. MAPK pathways have been implicated in numerous NR-regulated functions since the NRs and their transcriptional coregulators are often among the downstream substrates of the kinases. In this study, we investigated the role of MAPK in CAR-mediated CYP2B gene induction by examining the three main MAPK pathways, ERK, p38, and JNK. Real time RT-PCR analyses of CD-1 mouse primary hepatocytes showed that while blocking the ERK and JNK MAPK pathways did not have significant effects on the induction of CYP2B10, inhibition of the p38 MAPK pathway not only reduced endogenous CYP2B10 gene expression, but also minimized the induction of CYP2B10 by TCPOBOP, a potent CAR activator. Examination of kinase activation showed that TCPOBOP treatment increased the level of phosphorylated p38 in mouse primary hepatocytes. Also, transient transfection assays in HepG2 cells demonstrated that inhibition of the p38 MAPK pathway repressed CAR transactiva-

vation, but that blocking the ERK and JNK pathways did not inhibit CAR activation. These results indicate that p38 MAPK activity is required for CAR-mediated induction of the CYP2B gene in response to xenobiotics.

## 1768

### EVIDENCE FOR CAR-DEPENDENT HEPATOCELLULAR PROLIFERATION IN TCPOBOP-TREATED CONSTITUTIVE ANDROSTANE RECEPTOR KNOCKOUT (CAR<sup>-/-</sup>) MICE.

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TCPOBOP (1, 4-bis[2-(3, 5-dichloropyridoxyl)] benzene), a potent agonist of CAR, is a mitogen and liver tumor promoter in mice. In this study, male and female wildtype (C57BL/6) and CAR<sup>-/-</sup> mice were treated with TCPOBOP (3 mg/kg) for 4 days, and hepatic gene expression was determined with Affymetrix murine genome U74Av2 microarrays. Data were analyzed with Rosetta Resolver (statistical significance of  $p \leq 0.01$ ). A total of 232 hepatic transcripts were altered by TCPOBOP in a sex-independent, CAR-dependent manner. As expected there was no evidence of CYP2B induction in CAR<sup>-/-</sup> mice, whereas Cyp2b10 increased 100- and 25-fold, in male and female WT mice, respectively. TCPOBOP treatment increased liver weight 2-fold and caused marked hepatic hyperplasia and hypertrophy in WT mice, but was without effect in CAR<sup>-/-</sup> mice. The hyperplastic response in WT mice was associated with increased expression of Ki-67 and PCNA. Furthermore, cyclins A2, B1 and B2 were increased at least 4-fold in both sexes along with induction of cyclin dependent kinase 1 (8-fold), suggesting increased mitotic activity, with particular effects in S-phase and the G2 to M transition. Induction of genes controlling cytokinesis (Ect2, 8-fold) and nucleotide metabolism (ribonucleotide reductase M2, 5-fold; thymidine kinase, 2.5-fold) was evident. In opposition to changes associated with increased cell cycle kinetics, there was a marked increase in expression of genes that stimulate terminal differentiation, growth suppression and apoptosis including growth arrest and DNA-damage inducible (Gadd) genes, particularly Gadd45 $\alpha$  (15-fold), Gadd45 $\beta$  (25-fold) and growth-differentiation factor-15 (4-fold). These results indicate that TCPOBOP treatment alters expression of genes involved in the regulation of cell cycle control and DNA synthesis, with the balance skewed to favor cell proliferation. As no similar changes were observed in CAR<sup>-/-</sup> mice, the results also indicate that TCPOBOP-induced alterations in hepatic cell cycle regulation are CAR-dependent.

## 1769

### EFFECTS OF 2, 2', 4, 4, ' -TETRABROMODIPHENYL ETHER ON CAR AND PXR REGULATED GENE EXPRESSION IN WEANLING FEMALE RATS.

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The polybrominated diphenyl ether (PBDEs) mixture, DE-71 causes endocrine disruption by altering thyroid function via the induction of hepatic uridinediphosphate-glucuronosyltransferases, (UGTs). DE-71 also induces ethoxresorufin-O-deethylase (EROD), and pentoxyresorufin-O-deethylase (PROD). These studies suggest that DE-71 is a mixed inducer of Ah receptor (AhR), pregnane X receptor (PXR) and constitutive androstane receptor (CAR) regulated genes. There are no definitive conclusions on the ability of PBDE-47, a major congener in DE-71, to act as a CAR/PXR agonist. This study examined the ability of PBDE-47 and DE-71 to activate genes in the AhR, CAR, and PXR pathways by quantitative real-time PCR. Female Long-Evans rats (28 day old) were orally administered 3, 10, 30, or 100 mg PBDE-47/kg/day or 30 mg DE-71/kg/day for 4 days. Animals were sacrificed 24 hours after the final dose; livers were collected and stored at -80 degrees Celsius until analyzed. PBDE-47 caused a dose-dependent increase in PROD activity starting at 10mg/kg/day, and only a slight increase in EROD activity at the highest dose (100mg/kg/day). Neither PBDE-47 nor DE-71 affected CAR, PXR, RXR, Mrp2, Oatp2, or UGT2B mRNA expression. DE-71 significantly increased AhR, CYP2B1, CYP2B2, UGT1A6, and UGT1A7 mRNA levels, while significantly decreasing Mdr1A, and UGT1A1 mRNA levels. PBDE-47 significantly increased AhR mRNA at 100mg/kg/day. Significant increases were also seen in CYP2B1 and 2B2 mRNA at 30 and 100mg/kg/day PBDE-47. DE-71 induced CYP1A1 over 90-fold, while PBDE 47 only induced CYP1A1 12 fold at the highest dose tested. CYP2B1 was induced 350-fold by DE-71 and 1100-fold by 100 mg PBDE-47/kg/day. The data indicate that PBDE-47 and DE-71 alter AhR and CAR/PXR regulated genes, although PBDE-47 is more efficacious as a CAR/PXR activator than DE-71. The AhR activated genes suggests that PBDE-47 may be a weak AhR agonist or contains minor dioxin-like contaminants. (This abstract does not necessarily reflect USEPA policy).

## 1770

### TRANSCRIPTIONAL ACTIVATION OF CREATINE KINASE GENE EXPRESSION BY INSULIN-LIKE GROWTH FACTOR (IGF-I).

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Previous studies from this laboratory revealed that IGF-I increased cathepsin D mRNA level and reporter gene activities in breast cancer cells transfected with a plasmid containing chloramphenicol acetyltransferase (CAT) reporter gene linked to a cathepsin D gene promoter. This study investigated the mechanism of IGF-I regulation of creatine kinase B (CKB) gene expression in breast cancer cells. IGF-I increased CAT activities (4- to 8-fold) in cells transfected with the constructs containing CKB promoters -2897 to +5, -1434 to +5, -1201 to +5, -528 to +5, -228 to +5, and -195 to +5. CKB promoter sequence -568 to -523 contains two Sp1 binding sites and one estrogen responsive element (ERE), and previous studies showed that both the Sp1 binding sites and ERE were involved in CKB induction by estrogen in breast cells. Indeed, IGF-I induced CAT activities in cells transfected with reporter constructs containing CKB promoter sequence -568 to -523, and mutation of either Sp1 binding sites or ERE did not decrease the CAT activity. However, mutation or truncation of both the Sp1 binding sites and ERE abolished the IGF-I-induced CAT activities. In MDA-MB-231 breast cancer cells, cotransfection with wild-type ER restored the IGF-I-induced CAT activities in cells transfected with the constructs containing CKB promoter sequence -568 to -523, but not with the ER containing a mutation of serine at 118. The IGF-I-induced CAT activities were decreased in MCF-7 cells transfected with reporter constructs containing CKB promoter -568 to -523 and treated with PD 98059, a MAPK inhibitor. In addition, cotransfection of dominant negative ras or MAPK expression plasmids abolished the IGF-I-induced CAT activities in MCF-7 cells transfected with reporter constructs containing CKB promoter -568 to -523. These results suggested IGF-I activated CKB gene expression through activation of ER via the ras-MAPK pathway. (Supported by ES09106 and ES09253)

## 1771

### ANALYSIS OF GENE EXPRESSION DURING UTERINE INDUCTION AND REGRESSION IN IMMATURE, OVARIECTOMIZED RATS FOLLOWING TREATMENT WITH ETHYNODIOL ESTRADIOL.

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Changes in uterine physiology, cell type morphology and gene expression were evaluated in a comprehensive time course study after oral administration of 100  $\mu$ g/kg bw ethynodiol estradiol (EE) to immature, ovariectomized SD rats. Animals were dosed once or once daily on 3 consecutive days and uteri were harvested 2, 4, 8, 12, 18, 24, 72, 84, 96, 120, 144, or 168 hrs after treatment. Animals in the histological study received an i.p. injection of 5-bromo-2-deoxyuridine (BrdU, 50  $\mu$ g/kg bw) 2 hours prior to sacrifice. Uterine wet weight, a classical marker of estrogen exposure, was maximally induced ~6-fold at 72 hrs. Global changes in gene expression were examined using custom rat cDNA microarrays consisting of 8, 567 clones representing 5, 818 unique genes. 1, 755 genes exhibited a significant change at one or more time points as determined using a model-based t-statistic/Empirical Bayes approach. In order to phenotypically link these changes in gene expression to the physiological alterations observed in the uterotrophic response, complementary histological and morphometric assessment of uterine mid-horn sections stained with eosin and hematoxylin were performed. Additional histological slides were stained with anti-BrdU for assessments of proliferation. Pathological findings included indications of edema, eosinophil infiltration, hypertrophy, hyperplasia, angiogenesis and apoptosis consistent with the temporal changes in gene expression at time points preceding or overlapping these phenotypic markers. These assessments provide baseline data characterizing estrogen elicited responses in the rodent uterus, as a comparator for future estrogenic endocrine disruptor studies. Funding provided by NIH grant: ES011271 and ES07255-16

## 1772

### EFFECTS OF ESTROGEN ON IMMATURE, OVARIECTOMIZED MICE: A MULTI-APPROACH, TISSUE-BY-TISSUE COMPARISON.

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17 $\alpha$ -Ethynodiol estradiol (EE) elicited gene expression and histological changes in the uterus, mammary gland, liver, femur and kidney were examined in immature, ovariectomized C57BL/6 mice. Mice were gavaged with 100 $\mu$ g/kg EE and tissues

were harvested after 2, 4, 8, 12, 18, and 24 hrs or at 72 hours following three consecutive 24 hr doses. cDNA microarrays containing 13361 clones (7810 unique, annotated genes) were used to compare gene expression profiles of EE treated animals to time-matched, vehicle controls. Active gene lists were compiled for each tissue using a model-based t-value/empirical Bayes method based upon filtering by P1(t) values. Clustering of responses indicate that mammary and bone exhibit comparable changes in expression with kidney displaying the most divergent responses. Comparisons of active gene lists indicate that EE regulates common biological processes (i.e. proliferation and xenobiotic metabolism), in addition to tissue-specific responses (i.e. lipid metabolism in the liver). Genes associated with proliferation were highly represented in all tissues examined but showed tissue specificity suggesting a lack of a common proliferative mechanism elicited by EE. The temporal expression of growth factors, transcription factors and cell cycle regulators was consistent with the 7.5-fold increase in uterine blotted weight at 72 hrs, and the concomitant increases in uterine luminal epithelial cell height and BrdU incorporation. These comprehensive studies measuring multiple endpoints allow for the phenotypic anchoring of the observed gene expression changes and facilitate distinguishing adaptive responses from changes associated with a physiological effect. Results from these studies indicate that EE does not elicit a common, tissue-independent expression profile. Supported by NIH grants ES 011271 and T32 ES07255.

**1773**

REGULATION OF TRANSCRIPTIONAL ACTIVATION OF LARGEMOUTH BASS ESTROGEN RECEPTORS ALPHA, BETA 1 AND BETA 2 BY ESTRADIOL AND THE CONTAMINANTS NONYLPHENOL AND METHOXYCHLOR IN HEPG2 CELLS.

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The purpose of this study was to measure the activation of the three estrogen receptor (ER) isotypes found in the largemouth bass (*Micropterus salmoides*) (LMB) by 17 $\beta$ -estradiol (E2), nonylphenol (NP) and methoxychlor (MXC). NP is a deterrent that has been found to be present in the environment and has been shown to act as a disruptor of the estrogen receptor mediated signaling pathway by virtue that it up regulates vitellogenin in largemouth bass *in vivo*. MXC is a less environmentally persistent analog of DDT that has also been shown to act as an estrogen. Using transient transfection assays in human HepG2 cells we find that each receptor type is responsive to the endogenous ligand E2 in a dose-dependent manner. When cells were treated with E2, ER $\alpha$  activity becomes distinguishable from control at 0.1  $\mu$ M and continues rising through 1.0  $\mu$ M, while ER $\beta$ 2 and  $\beta$ 1 begin to rise between 0.05 and 0.075  $\mu$ M, but reach a maximal response at 0.5  $\mu$ M. For NP, both ER $\alpha$  and ER $\beta$ 2 were activated by 5  $\mu$ M and continue to rise through 10  $\mu$ M. However, higher concentrations of NP are cytotoxic. No response was seen for ER $\beta$ 1 upon treatment with NP. Preliminary data for MXC show that as with human ER $\alpha$ , the LMB ER $\alpha$  is activated between 0.5 and 1.0  $\mu$ M MXC. The similarity of the E2 dose response curves obtained for ERs  $\beta$ 1 and  $\beta$ 2 supports the view that the two subtypes are recent duplicates in teleosts, as has been suggested in the literature. The lack of responsiveness to NP for ER $\beta$ 1 indicates that this receptor isotype may have its own function and suggests that *in vivo* responses to NP is through activation of either ER $\alpha$  or ER $\beta$ 2.

**1774**

ESTROGEN INFLUENCES THE GROWTH OF CELLS THROUGH MODULATING THE INTRACELLULAR REDOX STATE.

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The intracellular redox state is characterized by a balance between oxidant production and reducing equivalents which allow reactive oxygen intermediates (ROI) to function as second messengers. The primary effect of ROI signaling molecules is the reversible oxidation of proteins that cause a change in protein function resulting in signal transduction. Growth factors rapidly stimulate the production of low to intermediate levels of intracellular ROI which signal cell proliferation. Therefore, we investigated whether estrogen mediated changes of cellular redox state influenced the growth of cells. The data presented here lead to the following major findings: 1) physiological concentrations (100 pg/ml) of estrogen rapidly modulate cellular redox state and 2) estrogen-induced phosphorylation and binding of transcription factors (i.e. AP-1), and cell cycle gene expression (i.e. cyclin D1) are modulated by cellular redox state. Our results suggest that a nongenomic pathway(s) is involved in the support of estrogen-dependent growth. The biological significance of our findings from this study provide the basis for the development of novel antioxidant-based drugs or gene therapy targeted for the prevention and treatment of estrogen-dependent cancer.

**1775**

THE EFFECT OF POLYCHLORINATED BIPHENYLS (PCBs) ON THYROID HORMONE RECEPTOR (TR) AND CO-REGULATOR INTERACTIONS ON DNA.

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PCBs may exert adverse effects, especially in the developing brain, by interfering with thyroid hormone (TH) signaling. Previous studies have clearly documented that PCB exposure can reduce circulating levels of TH. Thus, one hypothesis is that PCBs can exert adverse effects by causing hypothyroxinemia. However, we find that developmental exposure to PCBs can cause TH-like effects on the expression of TH-responsive genes in the developing brain. Thus, there is a very clear difference between the effect of PCB exposure on circulating levels of TH, and the effect of PCB exposure on TH signaling. While the simplest explanation for this observation is that PCB congeners or PCB metabolites bind to the TR and activate gene expression, we have failed to identify such congeners or metabolites in extensive studies. To determine the extent to which PCBs directly affect TH signaling through its receptor, we have begun to characterize the ability of PCB exposure to increase TH-responsive genes in liver, heart, and *in vitro*. In the heart, we find that PCB exposure does not affect the expression of MHC- $\alpha$ , MHC- $\beta$ , or SERCA-2 (all TH-responsive genes), despite causing a significant reduction in serum TH. Thus, the effect of PCB exposure has different effects on TR-mediated gene expression in different tissues. To further investigate this issue, we are using the Chromatin Immunoprecipitation (ChIP) assay. This approach will allow us to test whether PCB exposure influences the ability of TR to bind to DNA regulatory elements of TR-regulated genes as well as to determine whether TR associates with different coregulators (NCoR or SRC-1).

**1776**

GENETIC TOXICOLOGY AND BONE MARROW CYTOTOXICITY STUDIES OF THE CORTICOTROPIN-RELEASING HORMONE (CRH) RECEPTOR ANTAGONIST, ANTALARMIN.

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Antalarmin (AA; N-butyl-N-ethyl-[2, 5, 6-trimethyl-7-(2, 4, 6)-trimethylphenyl]-7H-pyrido[2, 3-d]pyrimidin-4-yl amine) is a CRH type 1 receptor antagonist being developed as a treatment for stress-related pathologies, including depression and anxiety. Genetic toxicology studies were conducted to identify mutagenic or clastogenic effects of AA, and a multi-species bone marrow cytotoxicity study was performed to investigate possible species specificity in AA toxicity. AA was not mutagenic in bacterial mutagenesis assays conducted using Ames tester strains TA 98, TA 100, TA 102, TA 1535, and TA 1537; Ames assays were conducted both with and without metabolic activation. AA was also negative in the L5178Y TK $+$ -mammalian cell mutagenesis (mouse lymphoma) assay conducted both with and without metabolic activation. Micronucleus evaluations demonstrated that AA was not clastogenic in bone marrow cells harvested from CD rats (6/sex/group) receiving oral exposure to AA at doses of up to 1000 mg/kg/day for 14 days. These data demonstrate that AA is not genotoxic in standard preclinical genetic toxicology bioassays. *In vitro* cytotoxicity studies were performed using CFU-GM neutrophil progenitors harvested from rat, dog, and human bone marrow to determine whether the rat or the dog more closely predicts human bone marrow responses to AA. AA was cytotoxic to canine bone marrow cells; by contrast, both rat and human bone marrow cells were insensitive to AA cytotoxicity. Because significant bone marrow toxicity was observed in subchronic toxicity studies in dogs but not in rats, these *in vitro* data suggest that the bone marrow cytotoxicity of AA in the dog may not be a good predictor of bone marrow toxicity in humans. The rat appears to be the more appropriate species for prediction of human bone marrow responses to this drug. (N01-CM-87102)

**1777**

SUBCHRONIC ORAL TOXICITY OF THE CORTICOTROPIN-RELEASING HORMONE (CRH) RECEPTOR ANTAGONIST, ANTALARMIN, IN DOGS.

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Antalarmin (AA; N-butyl-N-ethyl-[2, 5, 6-trimethyl-7-(2, 4, 6)-trimethylphenyl]-7H-pyrido[2, 3-d]pyrimidin-4-yl amine) is a CRH type 1 receptor antagonist being developed as a treatment for stress-related pathologies, including depression

and anxiety. In a 14-day range-finding oral toxicity study, AA doses of 0, 10, 50, 100, and 200 mg/kg/day were evaluated in beagle dogs (1/sex/group). AA doses of 50, 100, and 200 mg/kg/day induced dose-related decreases in body weight and food consumption; these effects were associated with repeated emesis and duodenal inflammation. AA doses  $\geq$ 50 mg/kg/day induced bone marrow and lymphoid suppression, thymic atrophy, hepatic toxicity (hepatocyte degeneration; cholestasis), and renal toxicity (cortical cell necrosis). At 10 mg/kg/day, AA toxicity was limited to decreased WBC and reticulocyte counts, lymphoid depletion, and minimal hepatic changes. In a 90-day oral toxicity study in beagle dogs (5/sex/group), AA doses of 0, 4, 8, and 16 mg/kg/day had no effect on survival, body weight, food consumption, electrocardiographic measures, or a Functional Observational Battery. The bone marrow and lymphoid system were primary sites of AA toxicity: at 16 mg/kg/day, AA induced significant bone marrow and lymphoid depletion, with only minimal liver toxicity (hepatocyte degeneration and cholestasis). All AA doses decreased reticulocyte counts. No evidence of renal toxicity was present in any dose group. After a 30-day recovery period, hematologic and microscopic findings had normalized. The Maximum Tolerated Dose (MTD) for AA in dogs is  $>$ 200 mg/kg/day ( $>$  4000 mg/m<sup>2</sup>/day). Based on the bone marrow and lymphoid toxicity observed at 16 mg/kg/day, the No Observable Adverse Effect Level (NOAEL) for AA in dogs is 8 mg/kg/day (160 mg/m<sup>2</sup>/day). (N01-CM-87102)

## 1778

### SUBCHRONIC ORAL TOXICITY OF THE CORTICOTROPIN-RELEASING HORMONE (CRH) RECEPTOR ANTAGONIST, ANTALARMIN, IN RATS.

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Antalarmin (AA; N-butyl-N-ethyl-[2, 5, 6-trimethyl-7-(2, 4, 6-trimethylphenyl)-7H-pyrrolo[2, 3-d]pyrimidin-4-yl] amine) is a CRH type 1 receptor antagonist being developed as a treatment for stress-related pathologies, including depression and anxiety. AA doses of 0, 25, 50, 100, 250, 500, and 1000 mg/kg/day were evaluated in a 14-day range-finding oral toxicity study in CD rats (5/sex/group). AA doses of 500 and 1000 mg/kg/day induced mortality, and thus clearly exceeded the Maximum Tolerated Dose (MTD); clinical pathology and histopathologic evaluation of tissues identified hepatic and renal toxicity at these doses. By contrast, no limiting toxicity was seen in rats receiving AA at doses of  $\leq$ 250 mg/kg/day for 14 days. Plasma AA peaked at 2 hours, and remained detectable at 24 hours; plasma AA levels did not increase with increasing duration of exposure. In a 90-day oral toxicity study in CD rats (20/sex/group), AA doses of 0, 30, 100, and 300 mg/kg/day had no effects on survival, body weight, food intake, clinical signs, or a Functional Observational Battery. Clinical pathology assays and histopathologic evaluation of tissues identified the liver and kidney as primary targets of AA toxicity; no evidence of bone marrow toxicity was observed. At 300 mg/kg/day, modest increases in GGT were correlated with hepatocellular necrosis, chronic inflammation, and bile duct hyperplasia and fibrosis. Renal pathology at 100 and 300 mg/kg/day included inner cortical necrosis, inflammation, hypertrophy, and nephrophy. After a 30-day recovery period, clinical chemistry and most microscopic changes had normalized; however, minimal to mild hepatic fibrosis and minimal to mild renal cortical inflammation remained. The MTD for AA in rats is 300 mg/kg/day (1800 mg/m<sup>2</sup>/day). The No Observable Adverse Effect Level (NOAEL) for AA in rats is 30 mg/kg/day (180 mg/m<sup>2</sup>/day). (N01-CM-87102)

## 1779

### CYPROHEPTADINE AND CYCLIZINE INHIBIT INSULIN SECRETION IN RIN5F CELLS BY INHIBITING CALCIUM CHANNELS.

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Cyproheptadine and cyclizine induce vacuolation of  $\beta$ -cells in rat pancreas. Vacuolation by cyproheptadine is associated with loss of insulin while that by cyclizine is accompanied by insulin secretion. The purpose of this study is to characterize the effect of these compounds on insulin in the pancreatic cell line RIN5F and to test the hypothesis that calcium channels mediate the effect on secreted insulin. Cells were treated with compound (10nM to 10 $\mu$ M) for 16h and insulin was measured by ELISA. Cyproheptadine caused a dose-dependent decrease in secreted and intracellular insulin with a maximal inhibition of 80-90% at 10 $\mu$ M. Inhibition of intracellular insulin was due to decreased insulin2 mRNA. Treatment with cyclizine led to a biphasic increase in secreted insulin of 20% at low doses followed by a 30% decrease at 10 $\mu$ M; inhibition of intracellular insulin by cyclizine, however, was dose-dependent with a maximal inhibition of 80% at 10 $\mu$ M. KCl was used to activate calcium channels and nimodipine, a calcium channel antagonist, was used as a positive control. Cyproheptadine inhibited KCl-mediated calcium entry into

cells with a maximal inhibition of 100% at 10 $\mu$ M. Cyclizine enhanced KCl-mediated calcium entry at low doses followed by a 50% inhibition at 10 $\mu$ M. The magnitude of inhibition of calcium entry by both compounds corresponded well with their effect on insulin secretion. Cyproheptadine (IC50;1.6 $\mu$ M) was as potent as nimodipine (IC50;0.7 $\mu$ M) in its inhibition of KCl-induced calcium entry, suggesting that it may be a calcium channel antagonist. Nimodipine, like cyproheptadine, also mediated a dose-dependent decrease in secreted insulin, further arguing for the role of calcium channels in insulin secretion. In summary, cyproheptadine and cyclizine inhibit both secreted and intracellular insulin and inhibition of secreted insulin may be due to inhibition of calcium channels.

## 1780

### REPEAT DOSE TOXICITY STUDIES IN RATS AND DOGS WITH TH0318, A DPP IV-RESISTANT ANALOG OF NATURAL GLP-1.

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TH0318 is a modified natural GLP-1 analog, having a hydroxybenzoyl moiety conjugated to the N-terminal end of the natural GLP-1 sequence, that is under development for the treatment of Type 2 Diabetes Mellitus. The repeat dose toxicity of TH0318 was evaluated in 14-day repeat-dose studies in rats (10-15/sex/group) given twice daily (3 to 4 hours apart) subcutaneous doses of 0, 0.5, 2 or 8 mg/kg bid (0, 1, 4 or 16 mg/kg/day) and in dogs (3-5/sex/group) given twice daily (3 to 4 hours apart) subcutaneous doses of 0, 0.1, 0.4 or 1.2 mg/kg bid (0, 0.2, 0.8 or 2.4 mg/kg/day). There was no evidence of systemic target organ toxicity in either species. Pharmacodynamic effects of TH0318 included reductions in body weight gain/ slight body weight loss and/or reductions in food intake at 4 mg/kg/day and greater in rats and at 0.8 mg/kg/day and greater in dogs. In both species, a local irritant reaction at the injection site was attributed primarily to the vehicle (pH 3.9) and showed partial reversibility following a 14-day off-dose period. Plasma toxicokinetic data (C<sub>max</sub> and AUC) demonstrated that animals were exposed to TH0318 and related C-terminal immunoreactive peptides in a dose-related manner; values on Day 14 were higher than Day 1. The no-observed-adverse-effect-level for systemic toxicity was 16 and 2.4 mg/kg/day in rats and dogs, respectively, representing 266- to 516-fold the maximum anticipated human dose. These results, together with those obtained from a battery of safety pharmacology and genetic toxicology studies, indicate that TH0318 has an appropriate toxicological profile in animals for the conduct of clinical safety evaluations in humans.

## 1781

### CARCINOGENICITY EVALUATION OF ATAZANAVIR IN MICE.

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The tumorigenic potential of atazanavir (ATV) was evaluated in a 2-year oral carcinogenicity study in CD-1 mice at doses of 20, 40, and 80 mg/kg/d in males and 40, 120, and 360 mg/kg/d in females. At the end of treatment, the incidence of hepatocellular adenomas was increased in females at 360 mg/kg/d; no other drug-related tumors were observed. As ATV was previously shown to cause hepatocellular necrosis in mice at high doses and was not genotoxic (weight of evidence), a 3-month timecourse study was conducted to investigate whether the increase in liver adenomas was a result of increased hepatocellular proliferation secondary to cytotoxicity and/or decreased apoptosis. In this study, ATV doses of 40 and 80 mg/kg/d in males and 120 and 360 mg/kg/d in females were administered for 2 weeks and 1 and 3 months. In males, generally minimal hepatic effects occurred at multiple timepoints, principally at 80 mg/kg/d, and included increased ALT and liver weights, hepatocellular hypertrophy, and low incidences of hepatocellular vacuolation and single-cell necrosis (high dose only). There was no stimulation of hepatocellular proliferation as measured by increased 5-bromo-2'-deoxyuridine (BrdU) nuclear labeling or inhibition of apoptosis as measured by decreased terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) in high-dose males. Hepatotoxicity was more pronounced in females, particularly at 360 mg/kg/d, and characterized by minimal to marked increases in ALT and/or AST and liver weights, hepatocellular vacuolation, and high incidences of single-cell hepatic necrosis at all time points. In females, hepatotoxicity was accompanied by dose-related increases (3-15X) in BrdU labeling; TUNEL was also increased (2-7X). In conclusion, these data indicate that the increase in hepatocellular adenomas in ATV-treated female mice was likely due to increased hepatocellular proliferation secondary to cytotoxicity. These results are consistent with a well-recognized nongenotoxic mechanism of tumor formation in rodents.

## MULTIPLE DOSE TOXICITY STUDY OF IMEXON IN FISCHER 344 RATS.

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This study was conducted to determine the target organ toxicity and reversibility of imexon, a potential anticancer agent, in Fischer 344 rats treated intravenously through the tail vein daily for 5 consecutive days in weeks 1 and 3. Target dose levels studied were 0, 50, 100 and 150 mg/kg/day. No mortality occurred. Dehydration, slight diarrhea, dark material around eyes, and tail lesions were seen during the dosing periods in a dose-dependent manner. Body weight gains and food consumption in all dose groups were lower compared to the control group by the end of weeks 1 and 3. Imexon-induced anemia and leukocytopenia were seen in all test article-treated groups, and neither anemia nor leukocytopenia were completely reversed after a two week recovery period (day 37). This was possibly due to myelosuppression caused by this cytotoxic anticancer agent, which suppressed all hematopoietic cell lines (except platelets). The increases in total protein levels and significant decreases in serum alkaline phosphatase (ALKP) levels in the 100 and 150 mg/kg/day dose groups were seen in males and females. These changes were completely reversed on day 37. The organ weights of liver, lungs/bronchi and spleen were dose-dependently decreased and spleen weights remained lower on day 37. Histopathologic lesions were found on day 22 in the following target organs: kidney, spleen and thymus in both males and females, and vagina and uterus in females in all test article-treated groups. The severity and/or incidence of the majority of lesions were dose-dependent and most of these changes were resolved on day 37, except for the vagina and uterus lesions which were seen on day 37 at all doses including 50 mg/kg/day. Under the conditions of this study, the no-observed-adverse-effect-level (NOAEL) of imexon was not attained. Sponsored by NCI Contract N01-CM-87103.

## SEQUENCE DEPENDENT ANTAGONISM BETWEEN RALOXIFENE, 5-FLUOROURACIL AND PEMETREXED.

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Raloxifene (RAL), a selective estrogen receptor modulator (SERM) approved for the prevention of osteoporosis and Pemetrexed (MTA) approved for the treatment of mesothelioma are currently under investigation for their antineoplastic effects in breast cancer. In the current study, RAL reduced the cytotoxic effects of high dose MTA in human MCF-7 breast cancer cells *in vitro*. When RAL is given 24 hours prior to a nontoxic dose of 5-fluorouracil (5-FU) and a high dose of MTA, the cytotoxic effects of MTA are antagonized. Antagonism is not observed when cells are exposed to a priming and non-toxic dose of 5-FU and high dose MTA 24 hours prior to RAL. The growth rates of MCF-7 cells administered 10µM MTA, 1µM 5-FU, and 10µM RAL alone were 65.0%± 5.42%, 86.0% ± 10.62%, and 68.3% ± 2.88% of the control, respectively. The combinations of RAL 24 hr prior to 5-FU 2hr prior to MTA and 5-FU 2hr prior to MTA 24 hr prior to RAL exhibited growth rates of 87.6% ± 1.51% and 65.0% ± 0.94% of control respectively. RAL arrests cells in the G1 phase of the cell cycle, thereby preventing the progression of cells to the S phase where 5-FU and MTA exert cytotoxicity therefore, these studies suggest that a) the interactions between RAL, 5-FU and MTA are sequence dependent and b) RAL antagonizes the cytotoxicity of MTA. Acknowledgements- This study was supported by grant RCMI-NIH 5G12RR03048 and Eli Lilly.

## UTILIZATION OF MULTIPLE ENDPOINTS TO INVESTIGATE DIFFERENTIAL TOXICITY PRODUCED BY TOPOISOMERASE II INHIBITORS, DOXORUBICIN AND ETOPOSIDE.

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Utilization of paradigm compounds can be a powerful tool to investigate differential toxicities. Doxorubicin (Dox) is a well-studied anthracycline antibiotic topoisomerase II inhibitor used to treat certain kinds of cancer. Doxorubicin is associated with cardiotoxicity and this effect is observed in multiple species including human. Etoposide (Eto), similar to Dox, inhibits topoisomerase II. However, Eto is not linked to cardiotoxicity. Therefore, Dox and Eto were used as comparator compounds to identify potential differential toxicities through the analysis of multiple toxicity endpoints. Male S-D rats (-300-350 g) were treated, i.v., with up to 5 doses of either Dox (0.5, 5.0 mg/kg) or Eto, (0.5, 5.0 mg/kg) over a two-week period. Rats treated with Dox at 5.0 mg/kg demonstrated substantial body weight loss and dosing was therefore discontinued. The remaining treatment groups continued on

study and appeared clinically similar to controls. Initial results indicated that a number of parameters were affected by the treatments. White cell counts were significantly reduced in animals that received either low or high dose Dox treatment. Conversely, only the high dose of Eto induced changes in white cell counts. Assessment of individual white cell populations indicated that lymphocytes and basophils were the primary targets of both compounds. Other white cells including neutrophils, eosinophils, and monocytes were resistant to treatments. Thymus to body weight ratios were significantly altered by the high dose of Dox and Eto. Other organ weights, including the heart, were similar to controls. Histological changes in high dose groups included marked bone marrow depletion (Dox and Eto), marginal zone depletion in spleen (Dox only) and marked increases in hepatocyte mitotic figures (Eto only). In addition to the above parameters, cytokine and gene expression changes for certain target tissues were assessed.

## ORAL TOXICITY STUDY OF A RETINOIC ACID ANALOGUE, 9-CIS-UAB-30, IN RATS.

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9-cis-UAB-30 is a conformationally constrained nuclear retinoid X receptor selective agonist with cancer chemopreventive activity. The toxicity of 9-cis-UAB-30 (0, 3, 15 and 100 mg/kg/day) was studied in CD<sup>®</sup> rats following 28 days of daily oral gavage administration. Plasma drug levels in week 4 were found to increase with the increasing dose. There were no drug-related mortalities, clinical signs of toxicity, or changes in body weight gains, food consumption or ophthalmic parameters in any dose group. Dose-dependent hepatomegaly was seen most significantly in the 100 mg/kg/day dose group females. Significant increases in serum aspartate aminotransferase (36.5% in females) and alkaline phosphatase levels (25.9 and 29.8% in males and females, respectively) were seen in the 100 mg/kg/day dose group. Moderate hypoalbuminemia and hyperglobulinemia resulted in decreased A/G ratio in this dose group females. Histopathology revealed hepatocellular change consistent with hepatic glycogen deposition. Liver appeared as a target tissue. 9-cis-UAB-30 exhibited a small, but statistically significant procoagulant effect as evidenced by decreases in prothrombin time (PT) in the 100 mg/kg/day dose group females (10%) and males (4.9%), and in the 15 mg/kg/day dose group females (3.1%) and males (4.3%). Elevated plasma fibrinogen levels were seen, significantly in the 100 mg/kg/day dose group males. Mild eosinopenia (males) and neutrophilia (females) were the only hematological parameters altered at 100 mg/kg/day. 9-cis-UAB-30 did not exhibit hypertriglyceridemia, a common undesirable effect of retinoids. Under the conditions of this study, the NOAEL for 9-cis-UAB-30 was 3 mg/kg/day primarily due to a small procoagulant effect of uncertain clinical significance. (Supported by NCI Contract No. N01-CN-15119).

## CHANGES IN THE METHYLATION STATUS OF GC-RICH REGIONS OF DNA DURING THE PROMOTION STAGE OF SKIN TUMORIGENESIS.

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Aberrant DNA methylation, an epigenetic regulator of gene expression, plays a key role in carcinogenesis. In general, the level of methylation and the level of transcription are related inversely. Thus, altered methylation can affect phenotype. Using the SENCAR two-stage mouse skin tumorigenesis model, progressive changes in methylation both gene-specific and in GC-rich regions can be evaluated in a time-dependent fashion during the promotion stage. Mouse skin was initiated with 7, 12-dimethylbenz[ $\alpha$ ]anthracene (DMBA) and then promoted thrice weekly with 27mg of cigarette smoke condensate (CSC) for 4 or 8 wks plus 4 or 8 wk recovery groups. DNA was isolated and GC-rich methylation was assessed using a newly developed, quantitative assay that permits simultaneous analysis of multiple sites of methylation across the genome. This involves the use of methylation sensitive restriction digestion and arbitrarily primed PCR with electrophoretic separation of PCR products. Additionally the methylation of the promoter region of *Ha-ras*, an oncogene frequently implicated in skin tumorigenesis, was assessed using the enzymatic regional methylation assay (ERMA) and bisulfite sequencing. No change in methylation was observed in the 5' promoter region of *Ha-ras*. In GC-rich regions, promotion for 4 wks yielded 21 sites of changed methylation, 20 of these were hypermethylation (HyperMe). The HyperMe decreased to 19 and 13 following a 4 or 8 wk recovery period, respectively. Promotion for 8 wks resulted in 40 total sites of changed methylation, 38 of these were HyperMe and this number decreased to 8 following an 8 wk recovery period. Reversibility is a hallmark of tumor promotion. These data support our hypothesis that progressive, nonrandom changes in methylation are involved in tumor promotion.

DIETHANOLAMINE (DEA) AND PHENOBARBITAL (PB) PRODUCE AN ALTERED PATTERN OF METHYLATION IN GC-RICH REGIONS OF DNA IN B6C3F1 MOUSE HEPATOCYTES SIMILAR TO THAT RESULTING FROM CHOLINE DEFICIENCY.

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DNA methylation (5-methylcytosine content of DNA) is an epigenetic mechanism regulating transcription. Disruption of normal methylation patterns can facilitate the aberrant gene expression underlying carcinogenesis. DEA, a nongenotoxic alkylamine, produces liver tumors in mice. Studies suggest DEA inhibits choline uptake and causes biochemical changes consistent with choline deficiency. Rodents fed methyl-deficient diets exhibit altered methylation of hepatic DNA and an increase in liver tumors, e.g., choline deficiency causes liver tumors in B6C3F1 mice. We hypothesize that DEA-induced choline deficiency leads to altered methylation patterns which may facilitate tumorigenesis. B6C3F1 mouse hepatocytes in primary culture were grown in the presence of 4.5mM DEA, 3mM PB or choline-deficient media for 48 hrs. PB, a nongenotoxic rodent liver carcinogen known to alter methylation in mouse liver, was included as a positive control. Global, average, DNA methylation was assessed using the SSI methylase assay which evaluates methylation at CpG sites. The methylation status of GC-rich regions of DNA, often associated with promoter regions, was assessed via methylation sensitive restriction digestion and arbitrarily primed PCR in which PCR products were separated and detected using an electrophoretic method. Global methylation status was not affected. DEA, PB, and choline-deficient treatment resulted in 54, 63, and 54 total sites of change in GC rich regions, respectively, and the majority were hypomethylations. Specific DEA and PB sites of change were very similar to those exhibited by choline deficiency, i.e., 71% of the total alterations, and 84% of the hypomethylations, were identical to those resulting from choline deficiency. These data indicate altered DNA methylation is an epigenetic mechanism involved in DEA-induced mouse liver tumorigenesis.

DNA HYPMETHYLATION INDUCED BY DRINKING WATER DISINFECTION BY-PRODUCTS IN MOUSE AND RAT KIDNEY.

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Haloacetic acids including dichloroacetic acid (DCA), trichloroacetic acid (TCA) and dibromoacetic acid (DBA), and trihalomethanes including chloroform and bromo-dichloromethane (BDCM) are major organic disinfection by-products (DBPs) found in finished drinking water after chlorination treatment. In male mice and rats, chloroform and BDCM when administered by oral gavage are nephrocarcinogens. In N-methyl-N-nitrosourea-initiated male mice, TCA promoted kidney tumors while DCA promoted kidney tumors only when co-administered with chloroform in drinking water. DBA has been shown to share biochemical and molecular activities in common with DCA and/or TCA. DNA hypomethylation has been proposed as a mechanism for non-genotoxic carcinogens. In this study, we determined the ability of these DBPs to cause hypomethylation of DNA and the c-myc protooncogene in mouse and rat kidney. Male and female B6C3F1 mice were administered 0, 0.3, 3.0 g/L DCA or 0, 0.4, 4.0 g/L TCA concurrently with 0, 0.1, 0.3, 1.0 and 1.6 g/L chloroform in drinking water. In male but not female mice, DCA, TCA and to a less extent chloroform decreased the methylation of DNA and the c-myc gene in the kidney. Co-administering chloroform increased only DCA but not TCA-induced hypomethylation of the gene. Male B6C3F1 mice and Fischer 344 rats were administered DBA at concentrations of 0, 1000 and 2000 mg/L in drinking water, and BDCM at dose levels of 0, 50 and 100 mg/kg by gavage and at concentrations of 0, 350 and 700 mg/L in drinking water for up to 28 days. Both DBA and BDCM caused a time- and dose-dependent hypomethylation of renal DNA in mice and rats. Hypomethylation of the c-myc gene was observed in mice after 7 days of DBA-exposure. In summary, the ability of the disinfection by-products to cause DNA hypomethylation correlated with their carcinogenic activity in mouse and rat kidney. (Supported by USEPA STAR grant R82808301 and NIEHS grant R03 ES10537)

LOSS OF HISTONE ACETYLATION BY NICKEL: AN EPIGENETIC MECHANISM OF NICKEL CARCINOGENESIS.

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Although nickel is a potent human and animal carcinogen and occupational exposure to nickel could cause lung and nasal cancers, it is generally not mutagenic in most classical bacterial or mammalian cell assays. We have previously shown that

carcinogenic nickel exhibited its epigenetic effects on silencing expression of a transgenic gpt gene by enhancing DNA methylation and chromatin condensation, as well as by decreasing histone H3 and H4 acetylation on this transgene. In addition, nickel has been shown to cause a global loss of histone H4 acetylation both in yeast and mammalian cells. Histone acetylation occurs at the lysine residues most often located within the N-terminal tails of core histones including H2A, H2B, H3, and H4. The balance of histone acetylation and deacetylation, is regulated by histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzyme activities, and is critical in regulating the dynamics of chromatin and gene transcription. In general, hyperacetylated histones are mostly associated with active genomic regions, whereas hypoacetylation mainly results in gene repression and silencing. Recent studies have shown that the balance of histone acetylation-deacetylation favors hypoacetylation in tumor cells and histone hypoacetylation plays an important role in gene silencing and carcinogenesis. In this study, we show that carcinogenic nickel decreased not only global acetylation of histone H4, but also that of H2A, H2B, and H3. By using our gpt transgene system, we are able to show that the acetylation of H2A and H2B mirror that of H3 and H4 since the acetylation of H2A and H2B was also lost in the nickel-silenced gpt cell clones. Besides its inhibitory effects on HAT activity, nickel may decrease histone acetylation by depleting the cellular acetyl Co-A, the substrate of the protein acetylation in the cell. Our new findings add further information contributing to nickel carcinogenesis and support to the view that nickel is an epigenetic carcinogen. Moreover, they provide insight for histone code hypothesis.

SEXUAL DIMORPHISM IN DNA METHYLATION IN THE LIVER OF HEXACHLOROBENZENE-TREATED RAT : A POSSIBLE MECHANISM FOR TUMOR PROMOTION.

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Hexachlorobenzene (HCB), a widespread environmental contaminant, causes gender-specific liver tumor promotion in the rat where only females develop liver cancer. We have previously shown that in female but not male rat livers HCB causes significant decreases compared to controls in : i) the total number of expressed genes as measured by microarray analysis, ii) the mRNA and protein levels of the liver gap junction subunits, connexin 26 and connexin 32 (Cx32), which are considered to be tumor suppressive, and iii) gap junctional intercellular communication (GJIC). Modulation of gene expression is the hallmark of epigenetic carcinogens such as HCB. DNA methylation exerts mechanistic control of gene expression. This study aimed to assess the effects of HCB on DNA methylation as a mechanism responsible for the reduction of global, and more specifically Cx32, gene expression. Global DNA methylation was assessed using arbitrarily primed PCR ; total DNA was extracted (N=4), amplified by PCR and digested by RsaI and HpaII. HCB induced DNA hypermethylation of HpaII restriction site. Local DNA methylation on the Cx32 promoter was measured using methylation specific-PCR (MSP) of bisulfite-treated DNA (N=4). Results showed a 2.5-fold increase ( $p<0.05$ ) in the methylation of the Cx32 promoter in HCB-treated female rat livers compared to controls. Moreover, results showed a marked sexual dimorphism in DNA methylation, with males having lower levels ( $p<0.05$ ) compared to control females, which correlated with the gender-difference in Cx32 mRNA levels. Overall, data suggest that by inducing aberrations in global methylation, and more specifically in local hypermethylation of the Cx32 promoter, HCB acts as a tumor promoter by modifying gene expression only in female rat livers. This work brings a better understanding of the modulation of DNA methylation in the reduction of gene expression by HCB in rat livers and strongly supports its role in rat hepatocarcinogenesis.

LOSS OF BRG1 AND LUNG CANCER PATHOGENESIS.

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The SWI/SNF chromatin remodeling complex uses the energy of ATP to alter nucleosomes to make DNA accessible to transcription machinery. Aberrations in chromatin remodeling can lead to development of cancer, such as loss of the SWI/SNF complex member BAF47 in rhabdoid tumors. BRG1, one of the catalytic subunits in human SWI/SNF complexes, is lost in ~30% of NSCLC cell lines. LOH surrounding the BRG1 loci at 19p13.2, has been shown in human lung tumor tissue. Loss of BRG1/BRM expression was found in ~10% of human primary tumors and correlated with poor prognosis. Similarly, loss of E-cadherin (a metastasis suppressor and cellular adhesion molecule) expression was found in ~10% of NSCLC and correlated with dedifferentiation and decreased survival. E-cadherin is commonly silenced by promoter hypermethylation in NSCLC. We have performed chromatin immunoprecipitation to show BRG1 is at the promoter

of E-cadherin in MCF7 cells that express high levels of E-cadherin. BRG1/BRM deficient cells examined by western blot were found to have significantly reduced levels of E-cadherin protein expression. Transfection of BRG1 into BRG1/BRM deficient cells induces reexpression of E-cadherin protein in a similar manner as treatment with 5-azacytidine. Therefore, loss of BRG1 expression may promote aberrant methylation of the E-cadherin promoter and subsequent silencing of the gene. We are currently screening H522 cells, a lung cancer cell line deficient for BRG1/BRM, for expression of other markers of lung cancer such as; p16 and FHIT. We will then check if transfection of BRG1, and treatment with 5-azacytidine are able to reexpress those markers. Bisulfite sequencing will be performed to see if those markers promoters are demethylated upon transfection of BRG1, and treatment with 5-azacytidine. We will also do chromatin immunoprecipitation to see if BRG1 is present at those promoters. These experiments should help establish a role for BRG1 in maintaining methylation patterns of critical genes in normal lung tissue.

## 1792 SECONDARY GENETIC ALTERATIONS IN LUNG TUMORS POSSESSING A PRIMARY KI-RAS MUTATION.

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Mutation of the *Ki-ras* gene is an early event in spontaneous and chemically-induced murine lung tumors. We previously described a mouse lung cancer model whereby the mutant human *Ki-ras*<sup>G12C</sup> allele is expressed specifically in the lung in a doxycycline (DOX)-inducible manner. Mice treated with DOX developed hyperplastic lesions as early as 12 days and adenomas were first visible by 3 months. Adenomas were present up to 12 months with relatively no tumor progression. To identify secondary genetic alterations, lung adenomas from transgenic mice induced by 9 and 12 months of DOX treatment were analyzed for alterations in expression of *p16Ink4a*, *retinoblastoma* (*Rb*), and cyclin D1 by real-time PCR. Adenomas isolated from mice treated for 9 or 12 months with DOX exhibited decreased expression of *p16Ink4a* in 7/22 (32%) and 1/6 (17%) of the adenomas, respectively, and displayed reduced expression of *Rb* in 21/22 (95%) and 6/6 (100%) of the adenomas, respectively. No alterations in expression of cyclin D1 were observed. Analysis of lung adenomas for mutations in *p16Ink4a* by SSCP demonstrated that all the tumors (33) analyzed thus far exhibited the wild type sequence. The *Ki-ras*<sup>G12C</sup> mice display a relatively benign tumor phenotype, exhibiting mostly hyperplasias and adenomas, with few tumors progressing to carcinomas. These data suggest that alterations in *p16Ink4a* and *Rb* play an important role in the early stages of lung tumorigenesis. (Supported by NCI grant CA91909).

## 1793 COMPARISON OF EXPRESSION PROFILES OF ENU-INDUCED FORESTOMACH TUMORS AND URETHANE-INDUCED LUNG TUMORS IN RASH2 MICE.

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Transgenic mice carrying a human prototype c-Ha-ras gene with its own promoter region (rasH2 mice) have been widely used in many 6-month short-term carcinogenicity tests, and have been recognized to be susceptible to genotoxic carcinogens. To clarify the mechanisms underlying the enhancement of carcinogenesis in rasH2 mice, we performed microarray analyses on chemically induced forestomach and lung tumors, and compared their gene expression profiles. Animals were given a single intraperitoneal injection of 120 mg/kg N-ethyl-N-nitrosourea (ENU) or 5-time intraperitoneal injections of 1000 mg/kg urethane at 2-day intervals. Three ENU-induced forestomach squamous cell carcinomas obtained at week 20 and 3 urethane-induced lung adenomas obtained at week 10 were used for the gene expression analysis. Compared with normal forestomach or lung tissue from non-treated mice, we selected up- and down-regulated genes in each tumor. In addition, we analyzed mRNA expression of human c-Ha-ras gene and some molecules involved in the Ras-MAPK pathway in these tumors. In microarray analysis, many genes related to cell cycle and transcriptions were up-regulated in both ENU- and urethane-induced tumors. Furthermore, the genes for extracellular matrix component, cytokines and chemokines were particularly up-regulated in ENU-induced tumors. RT-PCR analysis confirmed the overexpression of human c-Ha-ras mRNA in both tumors. The up-regulation of mouse endogenous ras genes was observed in only ENU-induced tumors. These results suggest that overexpression of transgene is involved in the enhanced carcinogenesis of both ENU- and urethane-induced tumors. Up-regulation of many genes for extracellular matrix component, cytokines and chemokines observed in ENU-induced tumors are considered to reflect tumor progression.

## 1794

### CYTOCHROME P4502E1 (CYP2E1) MEDIATED METABOLISM IS A PRE-REQUISITE FOR THE PATHOGENESIS OF URETHANE-INDUCED GENOTOXICITY AND CARCINOGENICITY.

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Urethane exposure induces a multiplicity of tumor types in numerous animal species. A by-product of fermentation, human exposure to urethane occurs through the consumption of alcoholic beverages and fermented foods. Studies conducted in this laboratory demonstrated that CYP2E1, CYPs other than CYP2E1, and esterase to be responsible for 96.4%, 3.2%, and 0.4% of urethane metabolism, respectively. Current work was designed to address the hypothesis that CYP2E1-mediated activation is necessary for the development of urethane-induced genotoxicity and carcinogenicity. Therefore, CYP2E1-null and wild-type mice were administered 0, 1, 10, or 100 mg urethane/kg daily (5 days/week) for 6 consecutive weeks. At the end of dosing, half of the animals were euthanized to assess mutagenesis (micronuclei (MN)) and markers indicative of cell proliferation (Ki-67). The remaining animals were kept without exposure for 7 months. A significant dose-dependent increase in MN-polychromatic (PCE) and normochromatic (NCE) erythrocytes in wild-type mice was detected. In contrast, urethane did not induce an increase in MN-NCE at any dose level in CYP2E1-null mice; while a small but significant increase was observed in MN-PCE at the highest administered dose. Ki-67-labeling indices were also significantly higher in the liver and the lungs of wild-type mice given 100mg urethane/kg vs. control and CYP2E1-null mice treated similarly. At the end of the 7-month recovery, significant increases in the incidences of tumors were observed in the liver, lung, and Harderian gland of wild-type mice. However, a significant inhibition in the incidence of urethane-induced tumors was observed in all 3 organs as well in the number of lung tumors/lung of CYP2E1-null vs. wild-type mice. In conclusion, this data proved that CYP-mediated bioactivation is a pre-requisite for the development of urethane-induced genotoxicity and carcinogenicity.

## 1795

### EFFECTS OF AFB<sub>1</sub> ON EXPRESSION OF P53 AND MDM2 AND ON APOPTOSIS IN HUMAN LUNG CELLS.

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Aflatoxin B<sub>1</sub> is a potent dietary hepatocarcinogen, and inhalation of AFB<sub>1</sub>-laden grain dusts has been postulated to be a lung cancer risk. Because mutations and altered expression of the tumor suppresser gene p53 have been observed in liver tumors associated with dietary AFB<sub>1</sub> exposure, we examined the effects of this mycotoxin on the expression of p53 and MDM2 in human bronchial epithelial cells (BEAS-2B) transfected with cDNA for cytochromes P450 (CYP) 1A2 (B1A2) or CYP 3A4 (B3A4), two of the isozymes responsible for AFB<sub>1</sub> activation in the liver and possibly the lung. Untreated B1A2 and B3A4 cells constitutively expressed p53, while AFB<sub>1</sub> exposure (0.015–15  $\mu$ M over 30 minutes) caused a concentration-dependent decline in p53 expression over time in B1A2 cells, and to a lesser extent, in B3A4 cells. The AFB<sub>1</sub>-mediated decrease in p53 continued for at least 12 hr after exposure to 1.5  $\mu$ M AFB<sub>1</sub>. Mirroring the decrease in p53 expression was a concentration-dependent increase in the expression of the 76 kDa MDM2 isoform in B1A2 cells. While AFB<sub>1</sub> did not induce DNA laddering, cleavage of procaspase-3 was detected after 4 hours of AFB<sub>1</sub> treatment as well as with the protein kinase inhibitor staurosporine. These data demonstrate that low, environmentally-relevant AFB<sub>1</sub> concentrations alter the expression of p53 and MDM2 as well as trigger apoptotic events in human lung cells. This study also supports the hypothesis that as in human liver, CYP 1A2 in human lung cells appears to play a dominant role in the molecular activation of AFB<sub>1</sub>.

## 1796

### NOVEL LAVENDAMYCINS AND AB RING SYSTEM ANALOGUES: *IN VITRO* CYTOTOXICITY, APOPTOSIS INDUCTION AND METABOLISM BY NAD(P)H:QUINONE OXIDOREDUCTASE 1 (NQO1).

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Lavendamycins and their AB ring system analogues can be bioactivated by cancer cells that overexpress NAD(P)H:quinone oxidoreductase 1 (NQO1) to exhibit selective toxicity. We examined the metabolism of these compounds by recombinant human NQO1 and their cytotoxicity to human colon adenocarcinoma cells with either no detectable NQO1 activity (BE-WT) or high NQO1 activity (BE-NQ). Metabolism was monitored by a spectrophotometric assay that quantifies cytochrome c reduction at 550 nm and toxicity was determined by MTT and clono-

genic assays. The correlation between the chemosensitivity results of the two assays was evaluated for 3 lavendamycin analogues. There was excellent linear correlation between the IC50 values of the two assays for the lavendamycins for BE-WT ( $r=0.999$ ,  $P=0.03$ ), BE-NQ ( $r=0.999$ ,  $P=0.025$ ) and both cell lines ( $r=0.990$ ,  $P=0.0001$ ). Of the 13 lavendamycins examined for cytotoxicity, the best substrates for NQO1 also showed selective toxicity toward BE-NQ cells. However, neither the AB ring system analogues that were also good substrates nor lavendamycin poor substrates showed selective toxicity. Although both lavendamycins and AB ring system analogues can be bioreductively activated by NQO1, only lavendamycins have the beta-carboline moiety that can act as part of a molecular cavity with potential metal binding sites. Therefore, the lack of selective toxicity of the AB ring system analogues might be due to their inability to utilize metals to bind DNA or act as catalysts for the production of reactive oxygen species. The role of NQO1 in lavendamycin-induced apoptosis was also examined. The concentration required to induce apoptosis was 5-20 fold less in BE-NQ cells compared to BE-WT cells for the best substrate suggesting a selective induction of apoptosis in the NQO1-rich BE-NQ cell line. Supported by NIH grant R15 CA78232.

**1797**

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ASSAY FOR N-GLUCURONIDATION 4-AMINOBIPHENYL IN MICE LIVER MICROSOMES AND EXPRESSED HUMAN SUPERSOMES.

M. H. Al-Zoughoul, *Environmental Health, University of Cincinnati, Cincinnati, OH*. A new high performance liquid chromatography (HPLC) method has been developed to determine the N-glucuronidation activity of mice liver microsomes and expressed human supersomes toward the carcinogenic 4-Aminobiphenyl (4-ABP). The HPLC method uses a chemically synthesized 4-ABP-N-Glucuronide (4-ABP-G) as a standard to determine the amount of glucuronide formed. 4-ABP-G was synthesized by chemical reaction between D-gucuronic acid and 4-ABP and the product formed was confirmed by mass spectrometry (MS) to be that of 4-ABP-G. This was used as reference to build the standard curve. The method was specific and sensitive. The calibration curve for the 4-ABP-G was linear from 25-500 pmol/200ml. The intra- and inter-day precision and accuracy for 4-ABP-G were 4% and 7% respectively. The lower detection limit was 10 pmol/200ml. The intra- and inter-day precision of the UDP-glucuronosyltransferase (UGT) activity in mice liver microsomes and expressed human UGT1A4 and UGT1A9 were less than 3 and 8 % respectively. The method proved to be very sensitive and it enabled us to determine the kinetics of UGTs without the need to use the radioactive [ $^{14}C$ ]-UDPGA, which is expensive and difficult to handle. We used this method to determine the kinetics of recombinant human UGT1A4 and UGT 1A9 for 4-ABP. The  $K_m$  and  $V_{max}$  of UGT1A4 were 321nM and 133/min/mg protein and 120nM and 145 pmol/min/mg protein respectively for UGT1A9. Therefore, this method is applicable to determine the glucuronidation status towards carcinogenic arylamines. Glucuronidation of arylamines is an important step in bioactivation and distribution of the active metabolites to the target organ(s). The method could be also applicable to study kinetics of nitrogen-containing drugs for evaluation of drug interactions and side effects using small amounts of biological samples.

**1798**

URINARY BLADDER ENDPOINTS IN WORKERS AND RATS EXPOSED TO PERFLUOROOCTANESULFONYL FLUORIDE (POSF) AND RELATED COMPOUNDS.

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Workers with potential high exposure to POSF-related compounds, including perfluorooctanesulfonate (PFOS), had 3 bladder cancer deaths vs. 0.2 expected (SMR 12.8; 95% CI 2.6 — 37.4). These compounds have not shown genotoxicity, and toxicology studies (including cancer bioassays) with PFOS and related sulfonamides have not shown bladder effects, but repeat-exposure data was not available for POSF. To further study the association of exposure to bladder cancer, we investigated potential bladder effects of POSF in rats and bladder cancer incidence in workers. Rats were exposed by inhalation to 0, 30, 100, or 300 ppm POSF 6 hrs/day, 5 days/week for 4 (0 & 300 ppm) or 13 weeks. Complete urinalysis was done on overnight urines. SEM/X-ray element analysis for crystals/calculi and immediate pH were conducted on fresh morning urines. Sagittal sections of urinary bladder were taken for SEM analysis and assessment of cell proliferation by proliferating cell nuclear antigen (3 sections,  $10^3$  cells/section). Results: There were no treatment-related findings with respect to urinalysis parameters, including pH and solids, nor changes of the urothelium, including no exposure-related increase in cell proliferation. Epidemiology: Because of high survivorship, living members ( $N = 1895$ ) of the study were contacted by mail to answer a questionnaire about bladder cancer, and 74% participated. Validation occurred by medical record or death certificate for those original members deceased ( $N = 188$ ). Incidence of bladder cancer was compared to expected based on NCI SEER data. Results: 11 bladder cancers were identified. The Standardized Incidence Ratio (SIR) for the entire cohort was 1.3 (95% CI 0.6 — 2.3). The SIR for ever working and working for more than one

year in a high exposed job were 1.7 (95% CI 0.6 — 3.8) and 1.1 (95% CI 0.2 — 3.3), respectively. These studies do not support the hypothesis that the excess risk of bladder cancer initially reported was due to exposure to POSF-related materials or PFOS.

**1799**

DNA DAMAGE AND CYTOGENETIC EFFECTS OF OCHRATOXIN A IN RATS *IN VIVO*.

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Ochratoxin A (OTA) is a potent nephrotoxin and renal carcinogen in rats, but the mechanism of OTA induced tumor formation is unknown. OTA has been shown to be negative in most genetic toxicology test *in vitro*. However, the potential of OTA to induce genotoxic effects has not been investigated in male rats, the most sensitive species for OTA induced tumor formation. In this study male F344 rats were repeatedly administered OTA (0, 0.25, 0.5, 1.0 and 2.0 mg/kg b.w.) or the non-chlorinated analogue ochratoxin B (OTB; 2.0 mg/kg b.w.) for 2 weeks and DNA breakage was analyzed in target and non-target tissues using the comet assay. DNA strand breaks were evident in liver, kidney and spleen, but a similar degree of DNA damage was observed in rats treated with OTB, despite the lower toxicity of OTB. Moreover, the presence of DNA damage did not correlate with histopathological alterations which were evident in the kidney, but not in the liver, suggesting that additional events may be required for renal tumor formation by OTA. In liver and kidney, the extent of DNA damage was further enhanced in the presence of Fpg-glycosylase, which is known to convert oxidative DNA damage into strand breaks. In contrast, no spots indicative of DNA adducts were observed in kidney DNA extracted from OTA treated animals by 32P-postlabeling analysis. A small, but not significant increase in the incidence of chromosomal aberrations was observed in splenocytes from rats treated with OTA *in vivo* and subsequently cultured *in vitro* to express chromosomal damage. These aberrations are not typically caused by chemical carcinogens which form covalent DNA adducts and together with the lack of evidence for formation of DNA adducts as assessed by postlabeling, these data suggest that OTA may cause genetic damage in target and non-target tissues independent of direct interaction with DNA.

**1800**

$\alpha_{2u}$ -GLOBULIN AND RENAL TUMORS IN NATIONAL TOXICOLOGY PROGRAM (NTP) STUDIES.

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Chemical-induced tumors in the kidney tubules of male rats in association with  $\alpha_{2u}$ -globulin nephropathy are not considered predictive of risk to humans by the International Agency for Research on Cancer and some US regulatory agencies. Essential criteria have been outlined to establish the role of  $\alpha_{2u}$ -globulin nephropathy in renal carcinogenesis. Criteria were based on a hypothesized mechanism of action that involves sustained tubular cell proliferation and consequent development of neoplastic lesions resulting from  $\alpha_{2u}$ -induced nephropathy. A review of NTP studies revealed inconsistencies with this proposed mechanism, including, in some cases, far weaker kidney tumor responses than expected, based on the extent of  $\alpha_{2u}$ -globulin nephropathy. Recent NTP studies with Decalin, propylene glycol mono-*t*-butyl ether and Stoddard solvent IIC included extensive evaluations of  $\alpha_{2u}$ -related nephropathy, and were thus used in assessing the linkage between key renal events in prechronic studies with tumors in 2-year studies. This review revealed an apparent association of tumor responses with renal  $\alpha_{2u}$ -globulin concentrations and granular casts, and to a lesser extent with linear mineralization and tubular hyperplasia. However, tumor responses did not agree with hyaline droplet accumulation or cell labeling indices, albeit evaluations at single time points may not represent cell proliferation variations over time. These results suggest that  $\alpha_{2u}$ -globulin nephropathy may be necessary, but not sufficient to induce the development of tumors, with  $\alpha_{2u}$ -associated cell damage playing a more central role in renal tumor development than cell proliferation responses.

**1801**

THE TSC-2 TUMOR SUPPRESSOR GENE MODULATES MULTIPLE CELL PROLIFERATION PATHWAYS.

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The tuberous sclerosis-2 (Tsc-2) tumor suppressor gene, which encodes the protein tuberin, is a renal tumor suppressor gene. The Eker rat, derived from the Long-Evans strain, provides an excellent model for studying renal carcinogenesis. A germline insertion in the Tsc-2 gene, predisposes these rats to the development of 2, 3, 5-tris-(glutathione-S-yl)hydroquinone (TGHQ) induced renal tumors. A single

treatment of primary renal epithelial cells derived from Eker rats with TGHQ transformed these cells into colonies (QT-RREs) that exhibit aneuploidy, anchorage-independent growth *in vitro* and tumorigenicity in nude mice. The remaining functional allele of the Tsc-2 locus in QT-RRE cell lines is lost during TGHQ transformation, resulting in a loss of tuberin expression. To investigate the role of tuberin in tumor suppression we restored tuberin expression by transient transfection of the Tsc-2 gene into the QT-RRE cells. Utilizing 2D gel electrophoresis coupled with MALDI-TOF peptide mass mapping and post source decay, we compared the protein expression of the Tsc-2 null and Tsc-2 positive QT-RRE cells. Both peroxiredoxin 1 and peroxiredoxin 3 showed an increase (~2 fold) in protein expression. Elevated expression of peroxiredoxins can decrease cellular proliferation by altering H2O2 homeostasis. Several proteasome subunits, which negatively regulate cyclin D1, was also elevated (~3 fold) revealing another proliferation pathway affected by tuberin. A substantial decrease in procathepsin expression (~4.5 fold) also occurred in Tsc-2 transfected QT-RRE cells. Cathepsin D is a lysosomal acid proteinase, which participates in the malignant progression of various cancers, including breast, gastric, and ovarian cancers. In combination, the results reveal that tuberin exerts its tumor suppressor effects by modulating several pathways involved in the regulation of cell proliferation. (GM39338, ES06694, ES07784)

**1802**

GLOBAL EXPRESSION PROFILING OF MALE RAT KIDNEY: CO-ADMINISTRATION OF A SPECIFIC ESTROGEN RECEPTOR (ER) ANTAGONIST INHIBITS A DYNAMIC 17B-ESTRADIOL (E2) RESPONSE.

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Estrogens have been shown to induce renal tumors in carcinogenicity and initiation-promotion studies as well as in renal tumor animal models such as the Syrian hamster. For example, estrogen administration to Syrian hamsters induces an 80-100% incidence of renal carcinomas in males. The mechanisms responsible for tumor formation are unknown, but likely involve ER-mediated mitogenesis, leading to the increased cell turnover via altered growth factor and oncogene expression. To gain further insight into the potential mechanism of ER-mediated carcinogenesis in the kidney, we have used gene expression profiling to identify altered cell growth and regulation pathways. As previously reported (Endocrinology 144:701, 2003), the kidney is indeed transcriptionally responsive to 17b-estradiol (E2), with only the uterus and pituitary gland showing a greater response among 13 tissues profiled. In the current study, male rats were dosed daily for 15 days with 20 mg/kg E2, 5 or 10 mg/kg ICI-182, 780 (ICI) or a combination of 20 mg/kg E2 and 5 or 10 mg/kg ICI. Renal expression profiles were generated using Affymetrix GeneChips and accessory sex glands (ASG) weights were measured to confirm the biological response to estrogen. The relative ASG weight decreased by 70% in animals dosed with E2, and returned to control levels in animals that were co-administered E2 and ICI, confirming the role of the ER in this response. Consistent with the ASG effects, there was also a robust transcriptional response in kidneys from the E2-treated animals (1, 221 differentially regulated with fold change greater than 1.5x at  $p < 0.5$ ) that was almost completely attenuated by co-administration with ICI, again demonstrating the role of the ER in mediating the expression changes. Genes involved in cell cycling, growth hormone signaling and cell proliferation were modulated by E2, consistent with a non-genotoxic mechanism of renal tumor formation.

**1803**

APOPTOSIS, SENESCENCE AND CYTODIFFERENTIATION IN CARCINOGENIC MODELING.

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The hazard function power parameter (k) in failure-time-distribution Weibull models applied to bioassay data, likely represents the number of critical events in multistage-carcinogenesis. We modeled data from experiments in tumor-suppressor-gene-p53-knockout mice (p53<sup>+/-</sup>, p53<sup>-/-</sup>) and found an estimated reduction in critical carcinogenic events to 5 & 4 compared to 6 steps in the wild type controls. This reduction in mechanistic events (steps) occurs in both malignant lymphomas and soft-tissue-sarcomas in the experimental knockout mice. A further reduction in the number of critical events was found in colonies of quadruple knockouts p53<sup>+/-</sup>p27<sup>+/-</sup> compared to p53<sup>+/+</sup>p27<sup>+/+</sup>, p53<sup>+/-</sup>p27<sup>+/+</sup>. These latter results indicate that the number of stages predicted by Weibull and gamma-distribution-gamma- accumulation models show a preemptive loss in steps

controlling cell-cycle progression in these carcinogenesis. Using a similar computer modeling approach, we also estimated the number of steps of rhabdo- myosarcomagenesis in INK4a/ARF<sup>+/+</sup>, INK4a/ARF<sup>+/+</sup> with or without HGF/SF transgene. We found a further reduction of 1-2 steps in cancer development instigated by the loss of this cytodifferentiation-autocrine loop in addition to the cell-cycle control. A reduction in steps apparent from our computation approach was also found in bioassay data from the SMAD3<sup>+/</sup>, p27<sup>+/</sup> colonies in contrast to SMAD3<sup>+/</sup>, p27<sup>+/</sup>; APCMin<sup>+/</sup>, p27<sup>+/</sup>; APCMin<sup>+/</sup>, p27<sup>+/</sup> mice. This leads to the interpretation that in carcinogenesis control of apoptosis and senescence (Cell-Cycle-arrest) likely plays a more important role than autocrine-cytodifferentiation. Disclaimer: The opinions and conclusions expressed in this paper are those of the authors and do not necessarily reflect those of their affiliated institutions.

**1804**

NUMBER OF CRITICAL CELL-CYCLE-EVENTS IN CANCER DEVELOPMENT OF P53-P27 KO MICE.

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The power parameter k of the hazard function in Weibull model, failure-distribution models likely represents the number of critical events in multistage-carcinogenesis. We have modeled data from experiments in tumor suppressor gene-p53-knockout mice (p53<sup>+/</sup>, p53<sup>-/-</sup>) colonies with contrasting results to the wild type. Our statistical results estimate a reduction in specific carcinogenic events from 6 in the wild type to either 5 or 4 in the knockout mice. The reduction in mechanistic events (steps) was found in both malignant lymphomas and soft-tissue-sarcomas comparing p53<sup>+/</sup> and p53<sup>-/-</sup> colonies to wild type controls. The genetic load of these animals plays apparently a critical load in determining the number of steps. When the computation was expanded to investigate the effects of p27Kip1 and p27Cip1 in regulating cell-cycle-progression and DNA-damage-repair response with p53, the latencies of tumor development in various experiments in p53<sup>+/</sup>p27<sup>+/</sup>, p53<sup>+/</sup>p27<sup>+/</sup>, p53<sup>-/-</sup>p27<sup>+/</sup> and p53<sup>+/</sup>p27<sup>+/</sup> combinations in mixed (C57BL/6J/C3H/NIH) and uniformed (C57BL/6J) animals were respectively shortened. These latter results indicate that the number of events predicted by Weibull and gamma-distribution-gamma-accumulation models show that genetic mechanisms in these respective carcinogenesis were further reduced by 2 steps. Disclaimer: Opinions and conclusions in this paper are the authors and do not necessarily reflect their affiliated institutions.

**1805**

ARNT, A PUTATIVE COACTIVATOR OF C-MYC/MAX SIGNALING PATHWAY, INTERACTS WITH C-MYC.

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ARNT (aryl hydrocarbon receptor nuclear translocator) is the DNA binding partner of the AHR (aryl hydrocarbon receptor) that together are thought to mediate most of the toxic and carcinogenic actions of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD). By binding to different partners, ARNT is involved in many signaling pathways and plays corresponding roles in embryonic development, the hypoxia response and development of the central nervous system. To delineate the putative role of ARNT in modulating the oncogenic pathway of c-Myc/Max, we performed transient transfection assays using a reporter gene regulated by p53 promoter and c-Myc and ARNT expression plasmids. We discovered ARNT is able to enhance c-Myc induction of the p53 reporter gene and that this effect is independent of DNA binding by ARNT. In addition, we have used yeast two hybrid and mammalian two hybrid assays and found that ARNT and c-Myc are able to directly interact with each other both in yeast and mammalian cells. Confocal imaging indicates that the dominant colocalization of ARNT and c-Myc occurs in the cell nucleus. Our data indicates that ARNT is capable of enhancing p53 promoter activity by modulating the c-Myc/Max pathway and that this effect is mediated by a direct interaction between ARNT and c-Myc. These data also support the idea that TCDD, via activation of AHR, may sequester ARNT from its endogenous role in p53 gene induction and accelerate cell cycle progression and/or inhibition of apoptosis.

**1806**

A POTENTIAL ROLE FOR C-MYB, BAX AND THE ARYL HYDROCARBON RECEPTOR IN BENZENE-INITIATED TOXICITY.

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Benzene is a leukemogen found in agents such as cigarette smoke, industrial solvents, and gasoline. Chronic occupational exposure to benzene has been correlated with oxidative stress and acute myelogenous leukemia. Childhood acute leukemia

has been associated with highly variable expression of the proapoptotic factor bax, which is inhibited by phosphorylation in the presence of reactive oxygen species (ROS) and is involved in controlling cell death particularly in hematopoietic cells. The oncogene c-myb is important in hematopoiesis and its over-expression has been implicated in the pathogenesis of myelogenous leukemias. Our laboratory has demonstrated that chicken erythroblast HD3 cells show an increase in c-Myb activity and an increase in ROS when exposed to the benzene metabolites catechol, benzoquinone, and hydroquinone. Activation of the aryl hydrocarbon receptor (AhR) by environmental toxicants has been associated with carcinogenesis. Interestingly, benzene-initiated hematotoxicity is absent in mice lacking the AhR suggesting an imperative role for this receptor in benzene toxicities. Using mouse hepatoma wild type and AhR null cells we investigated the role of this receptor on metabolite initiated increases in c-Myb activity and bax phosphorylation. Our results illustrated that both cell types showed increases in c-Myb activity when exposed to varying concentrations of catechol and hydroquinone for 24 hr. Interestingly, protein expression of c-Myb was increased after catechol exposure in AhR null cells while decreased in wild type cells. Our results also demonstrated that wild type cells exposed to catechol had decreased phosphorylated bax protein expression and increased dephosphorylated bax protein expression. In contrast, AhR null cells showed an increase in phosphorylated bax and no change in dephosphorylated bax. These results suggest a role for bax and the AhR in the toxicity initiated by benzene metabolites. Support: CIHR

**1807**

THE ESTROGENIC ACTIVITIES OF ARYL HYDROCARBON RECEPTOR AGONISTS ARE DUE TO DIRECT ACTIVATION OF ESTROGEN RECEPTOR  $\alpha$

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A recent study reported that aryl hydrocarbon receptor (AhR) agonists activated estrogen receptor (ER)-mediated signaling through a mechanism in which the ligand-activated AhR complex acts as a coregulator of ER action. This study investigates the Ah- and estrogen-responsiveness of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), 3-methylcholanthrene (3-MC), and 3, 3', 4, 4', 5-pentachlorobiphenyl (PCB) in MCF-7 cells. All three AhR agonists induced transactivation (up to 20-fold) in cells transfected with a construct (pDRE<sub>3</sub>) containing 3 tandem dioxin-responsive elements (DREs), and small inhibitory RNA for the AhR (iAhR) decreased this response, whereas small inhibitor RNA for ER $\alpha$  (iER $\alpha$ ) had no effect. In a parallel study, 17 $\beta$ -estradiol (E2), PCB and MC (but not TCDD) induced transactivation (3- to 5-fold) in MCF-7 cells transfected with a construct (pERE<sub>3</sub>) containing 3 tandem estrogen response elements (EREs). E2-, 3-MC- and PCB-induced transactivation was significantly decreased in cells cotransfected with pERE<sub>3</sub> and iER $\alpha$ , whereas iAhR had no effect on ligand-induced transactivation. These results suggest that the estrogenic activity of 3-MC and PCB was AhR-independent and due to a direct effect on ER $\alpha$ ; this was also confirmed by showing that the antiestrogen ICI 182, 780 also blocked PCB- and 3-MC-induced activity. These data coupled with results in AhR-negative breast cancer cells show that PCB and 3-MC directly activate ER $\alpha$  independent of AhR expression. (Supported by NIEHS ES09106 and ES04176)

**1808**

INHIBITION OF ESTROGEN RECEPTOR-NEGATIVE BREAST CANCER CELL GROWTH BY ARYL HYDROCARBON RECEPTOR AGONISTS

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MDA-MB-453 and BT-474 breast cancer cell lines overexpress epidermal growth factor receptor 2 (ErbB2) and represent *in vitro* models for later stage hormone-resistant breast cancer. Treatment of these cell lines with aryl hydrocarbon receptor (AhR) agonists such as 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), diindolylmethane (DIM), and 6-methyl-1, 3, 8-trichlorodibenzofuran (MCDF) inhibit growth of both cell lines with IC<sub>50</sub> values < 10 nM, 2  $\mu$ M and 1  $\mu$ M, respectively. Similar results were obtained for phosphatidylinositol-3-kinase (PI3-K) and mitogen-activated protein kinase (MAPK) inhibitors LY294002 and U0126, respectively, and these compounds also inhibited constitutively-active kinase activities in these cell lines. In contrast, TCDD, MCDF and DIM did not significantly block kinase activities despite their inhibition of cell proliferation. We also investigated the effects of TCDD, DIM and 6-MCDF on expression of critical cell cycle regulatory proteins (cyclin D1, p27 and p21) and on apoptosis. These responses were not

significantly affected by AhR agonists. In contrast, treatment of MDA-MB-453 cells with 10 nM TCDD, 10  $\mu$ M DIM or 2  $\mu$ M 6-MCDF resulted in an 8-, 4.5- and 6-fold increase in HES-1 protein expression in MDA-MB-453 cells. HES-1 protein has previously been identified as an inhibitory transcription factor that is induced by TCDD in ER-positive T47D breast cancer cells. Induction of HES-1 by AhR agonists in MDA-MB-231 suggests that this protein may play a role in the AhR-responsive antimitogenic activity of AhR agonists in ER-negative breast cancer cell lines. (Supported by NIEHS ES09106 and ES04176)

**1809**

CONSEQUENCES OF INTERACTION OF THE EQUINE ESTROGEN METABOLITE, 4-HYDROXYEQUILENIN, WITH ESTROGEN RECEPTOR  $\alpha$

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4-Hydroxyequilenin (4-OHEN) is a redox-active phase I metabolite of several equine estrogens present in Premarin, the most widely prescribed estrogen replacement formulation in North America. It has been shown that 4-OHEN was more toxic and induced considerably more DNA damage in ER $\alpha$  positive breast cancer cells compared to an ER $\alpha$  negative cell line. Coadministration of the antiestrogen 4-hydroxytamoxifen diminishes this differential DNA damage implying that 4-OHEN-mediated oxidative DNA damage could be ER-dependent. It has been shown that 4-OHEN has a very poor binding affinity and weak estrogenic activity in Ishikawa cells using the alkaline phosphatase induction assay. However, it has never been investigated if 4-OHEN is truly estrogenic and interacts with ER leading to ER-dependent DNA damage in other established cell-based assays. In the present study, the ability of 4-OHEN to interact with ER was investigated using the estrogen-responsive element (ERE)-luciferase activity assays in ER-positive MCF-7 cells. Our data showed that 4-OHEN is able to activate ERE-luciferase activity and 4-OHEN-induced luciferase activity was blocked in the presence of a pure antiestrogen. In order to understand the mechanism of 4-OHEN-mediated ER/ERE transactivation, it was investigated if aryl hydrocarbon receptor (AhR) could play a role since 4-OHEN can interact with AhR which then can act as a coactivator to induce ER $\alpha$ -mediated gene transcription. Knockout of AhR mRNA did not affect 4-OHEN-mediated ER/ERE transactivation, implying that AhR is not required for such gene transcription and 4-OHEN may directly interact with ER. In summary, 4-OHEN could act as an estrogen agonist and it may directly interact with the ER. Our data imply that 4-OHEN may be genotoxic as a chemical carcinogen but also as a hormone which could induce cell proliferation-induced mutations.

**1810**

G2/M BLOCK OF PRIMARY MAMMARY EPITHELIAL CELLS FROM PRE-MENOPAUSAL WOMEN IN RESPONSE TO GENOTOXIC CARCINOGEN EXPOSURE

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The mammary tissues are targets for many lipophilic environmental chemicals, including organochlorines, polycyclic aromatic hydrocarbons (PAH) and arylamines. Studies were designed to compare the effect of B[a]P and TCDD, prototypic PAHs, on the primary mammary epithelial cells (HMEC) from pre-menopausal women. The response of HMEC to these chemicals exposure on cellular proliferation and differentiation was evaluated using fluorescence-activated cell sorter (FACS) analysis, immunoblotting and immuno-cytological staining coupled-fluorescent microscopy. Exposure to TCDD for 20 hours has no significant effect on the cell cycle distribution. The treatment with B[a]P has resulted in enhanced BrdU incorporation by an average of 7-fold over control. Exposure to B[a]P for 20 hours caused a block in G2/M phase, resulting in cells accumulation in this phase at an average 13 percentage higher than untreated control cells. While no change is observed in the fraction of cells in the S phase, an average of 13 % was depleted from G1/Go phase, indicating the cells ability to transit through S-phase. Western immunoblotting analysis showed that these cells failed to activate P53 and its downstream target in response to the high dose (10  $\mu$ M) of B[a]P. These cells failed to undergo apoptosis and responded by increasing their mitotic activities, as evidenced by the enhanced PCNA staining. Due to their role in bioactivation of B[a]P to the ultimate genotoxic metabolites, CYP1A1 and CYP1B1 expression and induction by B[a]P and TCDD was examined in these cells. The data obtained from real time RT-PCR showed CYP1A1 mRNA to be induced by 100-300-fold with TCDD and about 50-150 folds by B[a]P. The data point to an anomalous cellular response of mammary epithelial cells from these subjects to genotoxic carcinogen. Funded by U54-CA91408-03 and DAMD17-02-01-0483.

CHANGES IN ESTROGEN METABOLISM IN CATECHOL-O-METHYLTRANSFERASE (COMT) DEFICIENT MICE ARE ASSOCIATED WITH INCREASED DEVELOPMENT AND ALTERED GENE EXPRESSION IN THE MOUSE MAMMARY GLAND.

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Estradiol (E2) has two catechol estrogen (CE) metabolites that are inactivated by COMT, a phase II biotransformation enzyme. Studies in our lab demonstrated that a low activity polymorphism for COMT is associated with increased risk for breast cancer and that COMT inhibition leads to increased oxidative DNA damage in MCF-7 cells. Additional studies show that mammary gland cells from COMT knockout (KO) mice metabolize E2 to CEs, but do not produce methoxy estrogen metabolites, which are thought to be anti-mitogenic. Additionally, preliminary experiments show that serum E2 levels (pg/mL) are higher ( $p \leq 0.04$ ) in the KO mice ( $42.1 \pm 13.4$ ;  $n=5$ ) than in their wild-type (WT) counterparts ( $7.0 \pm 1.9$ ;  $n=3$ ). CEs bind to the estrogen receptor, are estrogenic, and are precursors of estrogen quinones that cause DNA damage. Thus, we hypothesized that mammary gland development in the KO mice would be affected by the observed alterations estrogen metabolism. To test this hypothesis, mammary glands were excised from 4 week old KO and WT mice and subjected to whole mount examination of the epithelial tree. Ductal branching was quantified by counting terminal end buds and RNA was isolated for microarray analysis of gene expression. Our results show that the KO mice have ( $p \leq 0.02$ ) more terminal end buds per gland ( $34.30 \pm 5.030$ ;  $n=3$ ) than WTs ( $19.8 \pm 6.9$ ;  $n=5$ ), suggesting greater development. Microarray analysis shows that 15 genes were significantly up-regulated in the KOs (fold change range: 1.5-6.8;  $p \leq 0.05$ ). The up-regulated genes have been associated with cell signaling, growth/proliferation and cancer, suggesting that the alterations in estrogen metabolism might result in changes in gene expression in the mammary gland and may contribute to altered development. (Supported by NIH grants R01 CA77550, T32 ES07141, and P30 ES03819).

INVESTIGATION OF OCTAMETHYLCYCLOTETRAZILOXANE (D4) AND DECAMETHYLCYCLOPENTASILOXANE (D5) AS DOPAMINE D2-RECEPTOR AGONISTS.

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Oncogenicity studies of D4 and D5 have shown uterine adenomas and adenocarcinomas, respectively. Binding of dopamine receptor agonists to dopamine D2-receptors on pituitary lactotrophs inhibits the process of prolactin secretion. In the rat, decreased circulating levels of prolactin induces a decrease in circulating progesterone and consequently, an elevated estrogen:progesterone ratio. The neoplastic effects of dopamine receptor agonists on the uterus have been attributed to this apparent estrogen dominance. Experiments were conducted to evaluate the potential for D4 and D5 to modulate pituitary prolactin secretion as dopamine D2-receptor agonists. Utilizing an *in vitro* cell line, derived from a rat pituitary tumor (MMQ, ATCC #: CRL-10609), 10  $\mu$ M D4 and D5 were each shown to decrease maitotoxin-induced prolactin release by 55% without affecting viability. An *in vivo* model was then used to assess serum prolactin levels in reserpine-treated female Fischer 344 rats following 6-h vapor inhalation exposure to 700ppm D4 or 160ppm D5. In this model, serum prolactin levels were decreased 88% by 700ppm D4 and 50% by 160ppm D5 relative to reserpine control. Pretreatment with sulpiride, a dopamine receptor antagonist, blocked the effect of D4 and D5 suggesting that these cyclic siloxanes were acting on the pituitary as dopamine D2-receptor agonists *in vivo*. These results and the known species differences in reproductive physiology provide support for a potential mode of action that is not relevant humans. (This work was sponsored by the Silicones Environmental, Health and Safety Council of North America)

TRANSPLACENTAL AND POSTNATAL EXPOSURE OF AIDS DRUGS ZIDOVUDINE (AZT) AND LAMIVUDINE (3TC) IN C3B6F<sub>1</sub> TRP53(+/−) TRANSGENIC MICE.

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AZT/3TC antiviral drug combinations are given during pregnancy to reduce maternal-fetal HIV transmission. AZT is genotoxic in fetal mice and monkeys and carcinogenic in mice. We assessed a new C3B6F<sub>1</sub> trp53(+/−) p53 haplodeficient transgenic mouse model to be used for cancer bioassays. These mice, produced by

mating *Taconic C57BL6(N12)trp53(−/−)* males and C3H females, possibly have similar tumor profiles to B6C3F<sub>1</sub> mice. Haplodeficient C3B6F<sub>1</sub> trp53(+/−) and wild-type C3B6F<sub>1</sub> trp53(+/+) mice were dosed with 0, 40, 80, 160 mg/kg AZT or 160 mg/kg AZT combined with 100 mg/kg 3TC, by gavage in aqueous methylcelulose/polysorbate 80 (2/0.1%), transplacentally from GD12 to GD18 then postnatally from PND 1 to PND 28. P53 deficient mice are susceptible to fetal and neonatal mortality particularly when exposed to genotoxic compounds *in utero*. In a previous study using perinatal C57BL6(N5)trp53(+/−) mice dosed with 200 mg/kg AZT, we obtained relatively low pup survival in both control and treated groups (75 & 17% respectively at PND-28). In contrast, C3B6F<sub>1</sub> trp53(+/−) were more robust. Survival in these pups was >95% and >85% for the control and dosed groups respectively and was greater for haplodeficient than for wild type mice. The AZT and AZT/3TC treatment produced only small (<10%) reductions in body weight gain, but mice from the AZT/3TC dose groups showed increased *hprt* mutation frequency when evaluated at PND 28. In adult humans hepatic AZT glucuronidation by UDP-glucuronosyltransferase (UGT) is a major detoxification pathway. In contrast, liver from C3B6F<sub>1</sub> trp53(+/−) and C3H mice expressed very low levels UGT activity towards AZT (0.2–1.5 pmol/min/mg microsomal protein; human and rhesus monkey liver activities ranged between 1.5–2.5 nmol/min/mg microsomal protein), and only low levels of AZT-glucuronide were detected in serum from AZT treated mice. Since human neonates also express low levels of hepatic UGT activity, the C3B6F<sub>1</sub> trp53(+/−) mouse may be a good model for evaluating risk of perinatal AZT exposure.

EFFECT OF A COMPLEX MIXTURE FROM COAL TAR - SRM 1597 ON THE METABOLIC ACTIVATION OF CARCINOGENIC POLYCYCLIC AROMATIC HYDROCARBONS IN CHINESE HAMSTER V79 CELLS.

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A complex mixture of polycyclic aromatic hydrocarbons (PAH) extracted from coal tar, Standard Reference Material (SRM) 1597, has been shown to initiate tumor formation in mouse initiation-promotion assays in our laboratory (*Carcinogenesis*, 2001, 22(7) 1077-1086). To understand the role of SRM 1597 on PAH activation and DNA adduct formation, we examined the effects of SRM 1597 on the metabolic activation to DNA-binding derivatives of two carcinogenic PAH, benzo[*a*]pyrene (BP) and dibenzo[*a, h*]pyrene (DBP). Chinese hamster V79 cells expressing either human cytochrome P450 (CYP) 1A1 or CYP1B1 were used to determine the PAH-DNA adduct levels on exposure to SRM 1597, BP, DBP or co-treatments of SRM 1597 and BP or DBP. SRM 1597 inhibited BP-DNA adduct formation through the entire exposure time in the human CYP1A1 expressing cells. On the contrary, V79 cells expressing human CYP1B1 were unable to metabolize SRM 1597 or co-treatments of SRM 1597 and BP or DBP to DNA-binding metabolites. However, both CYP1A1 and CYP1B1 expressing cells metabolized BP and DBP to their respective DNA-binding metabolites. In order to assess the ability of SRM 1597 to inhibit the activity of CYP enzymes, ethoxresorufin *O*-deethylase assay was performed using microsomes isolated from V79 cells expressing CYP1A1 and CYP1B1. Competitive inhibition of resorufin product formation on exposure to SRM 1597 was observed in microsomes from V79 cells expressing both human CYP1A1 and CYP1B1. The results from this study suggests that the relative risk that PAH mixtures pose to humans may be dependent on the ability of the complex mixture to competitively inhibit the metabolic activation of carcinogenic PAH by the induced CYP enzymes and decrease DNA binding metabolites. Supported by NIH grant CA28825.

COMPETING ROLES OF ALDO-KETO REDUCTASE 1A1 AND CYP1A1/CYP1B1 IN THE METABOLIC ACTIVATION OF (+/-)-BENZO(A)PYRENE-7, 8-DIOL IN HUMAN BRONCHOALVEOLAR CELLS: INFLUENCE OF CYP INDUCTION.

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Human aldo-keto reductases (AKR1A1, AKR1C1-AKR1C4) catalyze a novel pathway of (+/-)-Benzo[*a*]pyrene-7, 8-diol (BP-7, 8-diol) activation which leads to BP-7, 8-dione that differs from the cytochrome P450 (CYP1A1/CYP1B1) pathway which leads to anti-benzo[*a*]pyrene-diol epoxide (anti-BPDE). The roles of AKR1A1 vs. CYP1A1/CYP1B1 in the activation of BP-7, 8-diol were equivalent in bronchoalveolar cell extracts containing 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD)-induced CYP1A1/CYP1B1 and stably expressed AKR1A1, and were dependent on the redox-state (NAD<sup>+</sup> : NADPH). This study compares the roles of

AKR1A1 and CYP1A1/CYP1B1 in the activation of BP-7, 8-diol in intact human bronchoalveolar H358 cells manipulated to express both pathways. The proposed metabolites formed in cell cultures via AKR1A1 and CYP1A1/CYP1B1 were identified by LC/MS and quantitatively measured by HPLC coupled to in-line radioactive detection using [<sup>3</sup>H]-(+/-)-BP-7, 8-diol. In TCDD-treated H358 cells (CYP1A1/CYP1B1 expression), BP-tetrols derived from anti-BPDE increased over 3-12 h to a constant level. In H358 AKR1A1-transfected, formation of BP-7, 8-diol was elevated for 3-12 h and significantly decreased after 24 h indicating that subsequent events consumed BP-7, 8-diol (e.g., metabolism or covalent modification of macromolecules). Unexpectedly, BP-tetrols were detected in AKR1A1 transfecteds even though they do not constitutively express CYP1A1/CYP1B1. The AKR1A1 product BP-7, 8-diol can trans-activate the aryl hydrocarbon receptor which could account for the CYP1A1/CYP1B1 metabolites. In H358 AKR1A1 transfecteds treated with TCDD, the formation of BP-tetrols was further elevated while levels of BP-7, 8-diol decreased due to the competing role of TCDD-induced CYPs. AKR1A1 thus plays an important role in PAH carcinogenesis due to the formation of the reactive and redox-active BP-7, 8-diol which induces the CYP pathway [Supported by PO1-CA92537 awarded to TMP].

sum of As species) in all samples, except maternal livers. In maternal livers, iAs represented 26 to 28% of sum of As species. Notably, iAs concentrations were lowest in fetal tissues. Highest concentrations of MAs were found in maternal liver, lung, and blood. No TMA<sub>3</sub>O was detected. These data suggest that in utero exposures to iAs result in accumulation of DMAs in fetal tissues. High levels of DMAs in fetal tissues may contribute to the aberrant patterns of gene expression that underlie the carcinogenic effects of in utero exposure to iAs. (This abstract does not necessarily reflect EPA policy.)

## 1816

### COMPARISON OF GENE EXPRESSION IN KIDNEY AND URINARY BLADDER FROM RATS TREATED WITH DIMETHYLArsinic ACID.

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Arsenic is widespread in the environment and a human carcinogen. A major metabolite of inorganic arsenic (iAs) in most species, including humans, is dimethylarsinic acid (DMA). Arsenic in the form of DMA is also used as a pesticide. Unlike iAs, DMA induces urinary bladder tumors in rats. DMA is believed to induce bladder cancer through a combination of target cell cytotoxicity, oxidative stress, and DNA damage. We propose that these effects are specific to the cancer target cell and not a general feature of DMA exposure. The present study compares the gene expression associated with DMA exposure in kidney to a previous examination of the rat transitional epithelium lining the urinary bladder. Female F344 rats were treated with 1, 4, 40, or 100 ppm DMA in the drinking water for 4 weeks. The rats were euthanized and kidneys were taken for histology and RNA extraction for 2-dye custom spotted microarray analysis with 4.5K known rat genes. The array signal intensity were subtracted from background, normalized, and analyzed using a one-way ANOVA model with a p value of 0.01 in GeneSpring. There was no significant renal histopathology whereas the urinary bladder had dose-dependent cytotoxicity. Microarray analysis of the transitional epithelia showed that DMA treatment altered the expression of apoptosis, cell cycle regulation, proliferation, signal transduction and oxidative stress response genes. The major functional categories affected in the kidney were genes that control metabolism, particularly lipid metabolism. Differential gene expression profiles between cancer target and non-target tissues can be used to suggest events in target tissues associated with DMA-induced carcinogenesis. [This abstract does not necessarily reflect EPA policy.]

## 1817

### SPECIATION OF ARSENIC IN THE MATERNAL AND FETAL MOUSE TISSUES FOLLOWING GESTATIONAL EXPOSURE TO ARSENITE.

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Earlier work found that *in utero* exposure of mice to inorganic arsenic (iAs) produces tumors during adulthood. To help define the biokinetics of arsenic (As) after *in utero* exposure, pregnant C3H mice were given carcinogenic doses of 42.5 or 85 ppm As (as sodium arsenite) in the drinking water from day 8 to 18 of gestation. Total As (TAs) and As species were analyzed in the blood, liver, lung, and placenta collected from dams and in the blood, liver, and lung collected from fetuses at day 18 of gestation. TAs was determined by hydride generation-atomic fluorescence spectrometry. Speciation analysis was carried out by hydride generation-atomic absorption spectrometry, using a liquid nitrogen cold trap for separation of arsines. The latter method simultaneously determined iAs, methyl-As (MAs), dimethyl-As (DMAs), and trimethylarsine oxide (TMA<sub>3</sub>O). TAs levels were generally higher in the maternal tissues than in fetal tissues at either exposure level. For both dams and fetuses, the highest concentrations of TAs were found in the liver and lung. DMAs was the predominant species in maternal and fetal blood and tissues. DMAs represented 46 to 78 % and 85 to 97% of sum of As species in tissues of dams and fetuses, respectively. In contrast, iAs was a minor species (0 to 12% of

## 1818

### UPTAKE OF INORGANIC AND ORGANIC DERIVATIVES OF ARSENIC AND ASSOCIATION WITH GENOTOXIC EFFECTS IN MAMMALIAN CELLS *IN VITRO*.

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<sup>2</sup>Institute of Environmental Chemistry, University of Duisburg-Essen, Essen, Germany and <sup>3</sup>Institute of Physiological Chemistry, University of Duisburg-Essen, Essen, Germany. Sponsor: E. Nelson.

The aim of our study was the elucidation of the uptake capabilities of different cell types in relation to the toxic effects of inorganic arsenic and its methylated metabolites. Chinese hamster ovary cells (CHO-9), human hepatoma cells (Hep G2) and primary rat hepatocytes (pRH) were exposed to arsenate [i-As(V)], arsenite [i-As(III)], monomethylarsinic acid [MMA(V)], monomethylarsonous acid [MMA(III)], dimethylarsinic acid [DMA(V)], dimethylarsinous acid [DMA(III)], trimethylarsen oxide [TMAO(V)] for 1 h. The chemicals were applied at different concentrations (0.1 microM to 10 mM) and cellular uptake was measured by ICP-MS. Genotoxic effects were assessed in CHO-9 cells by micronucleus (MN) assay, chromosome aberrations (CA) and sister chromatid exchanges (SCE). Thiobarbituric acid-reactive substances (TBARS) were determined as indication for the formation of reactive oxygen species. Our results show that pRH are most active in cellular uptake of metal(lloid)s followed by Hep G2 and CHO cells. Also, the highest generation of products of lipid peroxidation was found in pRH compared to Hep G2 and CHO-9. MMA(III) and DMA(III) induced genotoxic effects even more pronounced than i-As(V) or i-As(III). A significant increase in the number of MN, CA and SCE was found in CHO cells for DMA(III), MMA(III), i-As(III) and i-As(V). Altogether, we have shown that arsenic compounds in the trivalent oxidation state are better membrane permeable than the pentavalent species. The release of free radicals caused by organo-arsenicals might contribute to the damage of liver cells. This cell damage is dependent upon the uptake capabilities and the cellular extrusion mechanism.

## 1819

### GENE EXPRESSION ANALYSIS TO IDENTIFY INTERSPECIES CONCORDANCE OF THE MECHANISMS OF ARSENIC-INDUCED BLADDER CANCER.

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Arsenic is a human carcinogen that induces urinary bladder cancer. Several mechanisms have been proposed for arsenic-induced cancer. Although inorganic arsenic (iAs) does not induce tumors in adult rodents, dimethylarsinic acid (DMA), a major metabolite of iAs, is a rat bladder carcinogen. DMA causes a dose-dependent increase in toxicity and induces modulation of genes that control apoptosis, cell proliferation, and the response to oxidative stress in transitional epithelium of the rat urinary bladder. For extrapolation of rat data to human, normal human transitional epithelial cells (Urotsa) were exposed to DMA at 2µM, 20µM, 200µM or 8000µM. Total RNA was isolated and microarray analysis was conducted using the Affymetrix Human U133 Plus 2.0 chips. One way ANOVA identified 1728 genes (p<0.05) out of the 36, 967 known genes on the chip. Global expression changes were further characterized using Expression Analysis Systematic Explorer (EASE). In addition to confirming previously described processes, EASE identified additional pathways including phosphorylation, ion transport, protein synthesis and catabolism that were modulated after DMA exposure. Further analysis using Ingenuity Pathway Analysis suggest that altered ERK/MAPK signaling (ERK1/2, CMYC), apoptosis (CASP3, PARP), Wnt-β signaling (WNT, AKT), cell cycle regulation (CYCLIN D, CDKN2A), and PI3K/AKT signaling (FRAP1, HSP90) all play a role in DMA induced cellular toxicity. These pathways are consistent with our previous *in vivo* studies. This study demonstrated that an *in vitro* human cell system predicted the same key pathways involved in DMA-induced toxicity as was identified after *in vivo* exposure in rats. [This abstract does not necessarily reflect EPA policy.]

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR  $\beta$  (PPAR $\beta$ ) SUPPRESSES ANGIOGENESIS.

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This laboratory has demonstrated that peroxisome proliferator-activated receptor- $\beta$  (PPAR $\beta$ ) functions to attenuate cell proliferation and skin carcinogenesis as PPAR $\beta$ -null mice exhibit increased epidermal hyperplasia and skin tumor formation compared to wild-type mice. Further, preliminary analysis suggests that the activation of PPAR $\beta$  with GW0742, a specific PPAR $\beta$  ligand, can significantly inhibit skin carcinogenesis and keratinocyte cell proliferation in wild-type mice. An additional carcinogenic process that may be regulated by PPAR $\beta$  is tumor angiogenesis, since preliminary data demonstrates dysregulated angiogenic signaling in the absence of PPAR $\beta$  expression. We hypothesized that PPAR $\beta$ -null mice would display higher levels of angiogenesis due to increased hypoxia-inducible factor-1 (HIF-1) activity leading to increased expression of vascular endothelial growth factor (VEGF). Here we demonstrate with an *ex vivo* angiogenesis assay that PPAR $\beta$ -null mice exhibit increased microvessel formation compared to wild-type mice, indicating that PPAR $\beta$ -null are more sensitive to angiogenic stimuli. The activation of PPAR $\beta$  with GW0742 appears to limit the angiogenic response in wild-type mouse aortic cultures but not in similarly treated aortic cultures from PPAR $\beta$ -null mice. Further, we show that VEGF mRNA is more highly expressed in PPAR $\beta$ -null mice, and the expression of VEGF can be suppressed in wild-type mice upon treatment with GW0742. These data suggest that PPAR $\beta$  may function to suppress tumor angiogenesis through a mechanism that involves HIF-1, and that PPAR $\beta$  may represent a useful molecular target to inhibit tumor angiogenesis. Supported by the National Cancer Institute (CA89607).

IMMUNOHISTOCHEMICAL ANALYSIS OF EXOCRINE PANCREATIC TOXICITY INDUCED BY 2, 3, 7, 8-TETRACHLORODIBENZO-*P*-DIOXIN IN FEMALE HARLAN SPRAGUE-DAWLEY RATS.

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In previous studies of dioxin and dioxin-like compounds conducted for 2 years by the National Toxicology Program using female Harlan Sprague-Dawley rats, a rare occurrence of acinar cell adenomas and carcinomas in the pancreas was noted. Acinar cytoplasmic vacuolation, atrophy, inflammation, and arteritis developed at high incidence. In this investigation, we sought to identify the mechanism involved in the early stages of acinar cell lesions. The pancreas was examined immunohistochemically and/or morphometrically from animals treated for 14 and 31 weeks with 100 ng TCDD/kg or corn oil vehicle. Cytochrome P450 (CYP) 1A1 and aryl hydrocarbon receptor (AhR) were evaluated to assess a direct effect of TCDD on the pancreas. Pancreatic CCK-A receptor (CCKAR) and cholecystokinin 8 (CCK) in the duodenum were evaluated, since CCKAR null mice exhibit increased pancreatic atrophy. Amylase expression was examined to visualize acinar structural changes that could affect enzymatic production. Proliferative activity was measured using PCNA staining. Overexpression of CYP1A1 was found in 31-week-treated animals in vacuolated acinar cells that showed decreases in AhR, CCKAR, and amylase expressions with statistical significance. CCKAR expression was increased significantly in non-vacuolated acinar cells. No significant change in CCK expression was noted in the duodenum. H&E analysis revealed that apoptotic activity in acinar cells was increased in 14- and 31-week-treated animals. An increase in proliferation occurred in acinar cells of the 31-week-treated animals only. Our findings indicate that pancreatic acinar cells are responsive to TCDD and that dioxin-induced acinar cell lesions might be related to a direct effect of TCDD on the pancreas. The decrease in CCKAR expression and the increase in CYP1A1 in vacuolated acinar cells may be involved in the pathogenesis of pancreatic acinar lesions.

## INCREASED MITOCHONDRIAL GLUTATHIONE CONTENT ENHANCES APOPTOSIS IN NRK-52E CELLS INDUCED BY THE DNA ALKYLATING AGENT CISPLATIN.

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Previous work showed that increases in mitochondrial glutathione (GSH) content in NRK-52E cells by stable transfection with the cDNA for either of two GSH transporters from rat kidney mitochondria, the dicarboxylate carrier (DCC) or the 2-oxoglutarate carrier (OGC), protected the cells from apoptosis induced by either an oxidant (tert-butyl hydroperoxide) or a mitochondrial toxicant that is both an

oxidant and an alkylating agent (S-(1, 2-dichlorovinyl)-L-cysteine). To determine whether increases in mitochondrial GSH content can also protect NRK-52E cells from DNA alkylating agents, changes in cellular morphology and apoptosis induced by exposure to cisplatin were assessed in both wild-type NRK-52E cells (NRK-52E-WT) and NRK-52E cells stably transfected with the cDNA for the OGC (NRK-52E-OGC). NRK-52E-WT cells incubated for up to 24 hr with 0, 10, 50, 100, or 200  $\mu$ M cisplatin exhibited time- and concentration-dependent changes in cellular morphology consistent with progressive cell death. Photomicrographs showed progressive vacuolization and detachment of cells from the culture surface, indicating cell injury and death. Analysis of cell cycle by propidium iodide staining and flow cytometry showed time- and concentration-dependent increases in apoptosis, with maximal apoptosis (33-58%) occurring with 50-100  $\mu$ M cisplatin at 24-hr incubation times. Contrary to expectations, NRK-52E-OGC cells exhibited increased sensitivity to cisplatin as compared to wild-type cells, as judged by apoptosis and morphology. The fraction of NRK-52E-OGC cells exposed to cisplatin undergoing apoptosis was markedly increased (>90% in cells incubated for 8 hr with 50  $\mu$ M cisplatin). These results suggest that rather than protect the cells from injury induced by cisplatin, increased levels of mitochondrial GSH either increases delivery of cisplatin to cellular alkylation targets or enhances metabolism of cisplatin to a reactive species that induces cell injury. (Supported by NIH Grant DK40725.)

## CISPLATIN REDUCES VIABILITY, BUT NOT HEAT SHOCK PROTEIN LEVELS IN RAT KIDNEY CELLS.

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Kidney toxicity is a major treatment-limiting side effect of a number of anti-cancer agents, including cisplatin. Unfortunately, cisplatin-induced kidney damage is often detected only when patients are in the early stages of renal failure, and intervention is unable to stop the progression to failure. In part, the inability to detect cisplatin-induced kidney damage stems from a lack of biomarkers of early kidney toxicity. Thus, this work tested the hypothesis that changes in the levels of selected heat shock proteins (HSP60 and HSP70) could serve as early indicators of cisplatin-induced kidney toxicity. To test this hypothesis, normal rat kidney (NRK) cells (20,000 cells/well) were cultured in media containing cisplatin ( $10^{-7}$  to  $10^{-3}$  M). After culture, cells were subjected to alamar blue assays to measure viability and they were subjected to western blot analyses for HSP60 and HSP70. The results indicate that cisplatin reduces the viability of NRK cells in a dose-dependent manner. The viability of cells treated with low doses of cisplatin ( $10^{-7}$  to  $10^{-5}$ ) was similar to controls (n = 5). In contrast, the viability of cells treated with high doses of cisplatin ( $5 \times 10^{-5}$  to  $10^{-3}$ ) was significantly reduced compared to controls (viability for  $5 \times 10^{-5}$  =  $68.6 \pm 9.9$ ;  $10^{-4}$  =  $45.6 \pm 4.3$ ;  $5 \times 10^{-4}$  =  $28.4 \pm 4.0$ ; and  $10^{-3}$  =  $1.2 \pm 0.7$  % control; n = 5; p < 0.0001). Despite the ability of cisplatin to reduce cell viability, cisplatin did not affect the levels of HSP60 or HSP70. Western blot analysis indicated that control and cisplatin-treated cells had similar levels of HSP60 (control =  $0.6 \pm 0.1$ ;  $10^{-5}$  =  $0.5 \pm 0.1$ ; and  $5 \times 10^{-5}$  =  $0.5 \pm 0.1$  densitometric units; n = 3; p = 0.8). Collectively, these data indicate that cisplatin reduces the viability of NRK cells in a dose-dependent manner, but it does not affect the levels of selected HSPs. These data suggest that selected HSPs may not be good candidate biomarkers for cisplatin-induced kidney damage. Supported by NIH CA097421.

## TCDD-INDUCED GENE EXPRESSION CHANGES IN DEVELOPING ZEBRAFISH HEARTS.

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Exposure to 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD) causes developmental cardiovascular toxicity in various animal models, including zebrafish embryos, by activating the aryl hydrocarbon receptor (AHR) transcription factor. This results in altered gene expression patterns in tissues that likely mediate toxicity. Exposure of zebrafish embryos to TCDD results in smaller heart size, reduced peripheral blood flow, pericardial edema, abnormal heart morphology, and cardiac dysfunction. To identify changes in gene expression in the hearts of embryos that are direct responses to TCDD exposure microarray analysis was performed on heart tissue extracted from zebrafish embryos exposed to 1 ng/ml TCDD or DMSO at 72 hpf (hours post fertilization) for 1 h. Total RNA was extracted from hearts mechanically removed from treated embryos at 74 hpf (2 h post dosing) and 76 hpf (4 h post dosing) for analysis on Affymetrix zebrafish arrays. The TCDD-induced upregulation of P4501A indicates that the AHR pathway is activated in the heart and

demonstrates that such gene expression changes can be detected as early as 2-4 h post dosing. We also identified altered expression of genes involved in cell cycle regulation, proliferation, differentiation, cell signaling, cell adhesion, and cell communication. These changes are consistent with the effects on cardiac morphology and function observed later than 76 hpf. At 4 h post dosing, TCDD-treated embryos show no signs of reduced peripheral blood flow or pericardial edema indicating that the observed changes in heart gene expression precede these later occurring effects. This suggests that TCDD-induced cardiovascular toxicity, at least in part, is due to TCDD activation of AHR in the heart. Identification of AHR regulated genes in the heart is the first step in elucidating the mechanism of TCDD-induced cardiac toxicity in zebrafish embryos. (UW Sea Grant).

## 1825 CHARACTERIZATION OF TCDD CARDIOTOXICITY IN EMBRYONIC ZEBRAFISH.

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2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD) has been shown to act as an aryl hydrocarbon receptor (AHR) agonist, and the activation of the AHR pathway has been demonstrated to mediate the toxic effects of TCDD. In the zebrafish model of developmental toxicity, the following endpoints of dioxin toxicity have been reported: reduction in blood flow, edema, craniofacial malformations, growth retardation, reduced heart rate, anemia, impaired bladder inflation and mortality. In this study we characterize the effects of TCDD on the developing zebrafish heart and assess the role of the AHR pathway in mediating the TCDD cardiotoxicity using the morpholino knockdown technology. Zebrafish embryos exposed to TCDD shortly after fertilization exhibit altered cardiac looping at 72 hours post fertilization (hpf), with the atrium positioned posterior to the ventricle in a stretched, thinned heart tube. In contrast, the looped control heart is S-shaped, with the two chambers largely overlapping, when observed laterally. In addition, the TCDD-treated hearts become smaller, as their total tissue volume assessed using stereological software on serial heart sections is significantly reduced at 72 and 96 hpf in comparison to control. To further investigate this effect, a strain of transgenic zebrafish, which expresses red fluorescent protein specifically in the nuclei of cardiac myocytes has been used to count individual myocytes in control and treated hearts. The TCDD-exposed hearts contain a significantly lower number of cardiomyocytes at all inspected time points: 48, 72 and 96 hpf. The detection of this toxic effect on myocardium at 48 hpf indicates that the heart, and specifically the myocardium can be one of the earliest targets of TCDD toxicity in zebrafish. In addition to the effects on heart morphology and size, we found that dioxin had a striking effect on heart function, affecting cardiac contractility. Specifically, it leads to a complete lack of ventricular contraction observable as early as 96 hpf. The described cardiotoxic effects of TCDD were found to be mediated by the AHR pathway. (UW Sea Grant).

## 1826 STUDIES TO IDENTIFY GENE CHANGES IN THE HEART DURING RECOVERY FROM HEART TOXICITY INDUCED BY BIS(2-CHLOROETHOXY)METHANE.

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Cardiotoxicity is induced in F344/N rats after dermal administration of bis(2-chloroethoxy)methane (BCEM) ((Dunnick et al. 2004a; Dunnick et al. 2004b)). BCEM heart damage occurs by day 2 in all three regions of the heart (atrium, ventricle, interventricular septum) and is characterized by myofiber vacuolation, necrosis, mononuclear-cell infiltration, and atrial thrombosis. Ultrastructural analysis reveals that the primary site of damage occurs in the mitochondrion. Damaged mitochondria have disrupted cristae and loss of membrane structure. Ultrastructural analysis also reveals distention of the myocardial sarcoplasmic reticulum. By day 5, even though dosing is continued, the heart toxic lesions began to resolve, and by study-day 16 the heart appeared normal. Microarray gene analysis was used to test the hypothesis that genes changes at onset of toxicity (day 2) would differ from genes changes during recovery (day 5). RNA was extracted from the whole male rat hearts at day 2 and day 5, and analyzed using Agilent rat oligonucleotide microarray. The significant gene changes (vs. control) at day 2 included: 240 up regulated genes; 101 down regulated genes. The significant gene changes at day 5 included: 113 up regulated genes; 102 down regulated genes. Gene changes at day 2 were different from those at day 5, with the exception of 27 down regulated genes that were common on day 2 and 5. We hypothesize that specific gene changes enable the heart to recover from chemical-induced toxicity.

## 1827

### INCREASED GENE EXPRESSION AND LEVELS OF ANTIOXIDANTS AND PHASE 2 ENZYMES IN CARDIAC CELLS TREATED WITH 3H-1, 2-DITHIOLE-3-THIONE: PROTECTION AGAINST OXIDANT AND ELECTROPHILE-INDUCED CELL INJURY.

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Oxidative and electrophilic species are critically involved in various cardiac disorders. Accordingly, administration of exogenous antioxidants has been shown to be protective against oxidative cardiac injury. However, whether induction of endogenous antioxidative and phase 2 defenses by chemical inducers in cardiomyocytes also affords protection against oxidative and electrophilic injury has not been carefully investigated. Using cultured rat cardiomyocytes, we have studied the induction of the above cellular defenses by 3H-1, 2-dithiole-3-thione (D3T), and the protective effects of the D3T-upregulated cellular defenses on oxidant and electrophile-induced cell injury. Incubation of cardiomyocytes with D3T resulted in a marked induction of a scope of key cellular antioxidants and phase 2 enzymes. These included catalase, glutathione (GSH), GSH peroxidase, GSSG reductase, GSH S-transferase, NAD(P)H:quinone oxidoreductase-1, aldoe reductase, and heme oxygenase-1. D3T treatment also resulted in increased mRNA expression of the above antioxidative and phase 2 genes. Notably, all of the three major isoforms of GST genes (A1, P1 and M1) in cardiomyocytes were upregulated by D3T. D3T pretreatment of cardiomyocytes led to a marked protection against the toxicity induced by H<sub>2</sub>O<sub>2</sub>, xanthine oxidase (XO)/xanthine, and 4-hydroxyxynonenal (HNE), as determined by MTT reduction assay and electron microscopic studies. D3T pretreatment also caused a great reduction in intracellular accumulation of ROS, and HNE-protein adduct formation in cardiomyocytes following incubation with H<sub>2</sub>O<sub>2</sub>, XO/xanthine, and HNE, respectively. Taken together, this study demonstrates that a battery of endogenous antioxidants and phase 2 proteins in cardiomyocytes can be upregulated by D3T via increased gene expression, and that the chemically-upregulated cellular defenses are accompanied by increased resistance to oxidative and electrophilic cell injury (supported by HL71190).

## 1828

### DIFFERENTIAL GENE EXPRESSION IN MICRODISSECTED MESENTERIC VASCULAR ELEMENTS OF RATS 1-HOUR FOLLOWING FENOLDOPAM (SKF-82526) ADMINISTRATION.

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Vascular toxicity observed in pre-clinical toxicology species presents a hurdle for drug development. Assessing clinical relevance of these findings has been hampered by a limited understanding of the molecular and biochemical mechanisms leading to development of drug-induced vascular injury. To investigate early events in the pathogenesis of drug-induced vascular injury, Fenoldopam, a vasoactive DA1 agonist, was given to rats at a dose known to induce mesenteric arterial damage 12 hours following treatment. GeneChip expression analysis of selectively enriched regions of mesenteric arteries [endothelium (E) or smooth muscle (SM)], isolated from OCT-embedded frozen sections of mesentery using laser capture microdissection, were used to evaluate differential gene expression in vehicle control rats and rats killed 1 hour after being given 100mg/kg Fenoldopam. Comparative analysis of E or SM enriched samples between control and treated rats revealed a number of regulated transcripts. Real-Time RT-PCR and/or in situ hybridization and immunohistochemistry were used to confirm select subsets of these transcripts. Bioinformatic analysis identified multiple signaling pathways involved in vasoregulation (dilation/resistance), hemorrhage, extracellular matrix remodeling, cell adhesion, cell growth, metabolism, stress response and inflammation/ immunoregulation. Alterations in gene expression patterns of transcripts in these pathways has provided useful insight into the potential pathogenic mechanism involved in the early evolution of morphologic lesions that eventually lead to intramural hemorrhage and medial damage seen in the mesenteric vasculature 12 hours following administration of Fenoldopam.

## 1829

### GENOMIC ANALYSIS OF THE MECHANISM OF CYCLOPHOSPHAMIDE-INDUCED CARDIOTOXICITY IN RATS: COMPARISON WITH HUMAN CASES OF MYOCARDITIS.

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Myocarditis, defined as an inflammation of the heart muscle, is caused by various infections, but may also result from exposure to chemotherapeutics like cyclophosphamide. Clinical prediction of myocarditis is difficult and detection can occur too

late to prevent the development of cardiomyopathy. Therefore, an understanding of the mechanism of cardiotoxicity of agents such as cyclophosphamide would provide a basis upon which to drive development of enhanced detection with the ultimate goal of prophylaxis. The present study assessed alterations in gene expression using Affymetrix RG\_U34A GeneChip® arrays in the left ventricle of rats following treatment with cyclophosphamide and compared them with those findings observed in the left ventricle of histopathologically-confirmed cases of human myocarditis samples from Gene Logic's BioExpress® database using Affymetrix HG\_U133A GeneChip® arrays. Rats were dosed once via IP injection with either 20mg/kg or 200mg/kg cyclophosphamide and sacrificed 6, 24, 48 or 192 hours post-dosing. Induction in the expression of complement pathway components, cytokines and apoptosis-associated genes occurred in both rat (200mg/kg dose, 192 hours-post-treatment) and human samples. A significant difference between the rat study and human samples was noted, however, in that the rat cyclophosphamide study exhibited significant induction in the expression of antioxidant defense genes at the 200mg/kg dose, 192 hour- timepoint, while there was a significant repression in the human myocarditis samples in genes such as heme oxygenase and glutaredoxin 2. This suggests a reduction or reduced protective capacity of this mechanism in the human condition but not in the rat. In conclusion, these results provide preliminary mechanistic data of the initiation of inflammatory and apoptotic events in rats following cyclophosphamide treatment and in human myocarditis samples with noted differences in the regulation of antioxidant responsive genes.

**1830**

A RAT HEART GENE-EXPRESSION SIGNATURE PREDICTIVE OF HERG POTASSIUM CHANNEL BINDING.

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Life-threatening *torsade de pointes* arrhythmias, linked to HERG (human ether-a-go-go-related gene) potassium channel inhibition, have resulted in the withdrawal of several drugs, including terfenadine, astemizole, and cisapride, from the US market in recent years. Cardiac safety related to  $I_{Kr}$   $K^+$  channels is a major concern for pharmaceutical companies as regulatory agencies have a heightened awareness of cardiac safety pharmacology for new molecular entities. Early identification of off-target HERG channel inhibitory effects has therefore become a high priority in drug development programs. We used a commercially available *in vitro* HERG channel binding assay (MDS Pharmacology), which measures competitive binding affinity relative to the radioligand [ $^3$ H]-astemizole, to classify 167 compounds by their potency into low and high-potential HERG channel inhibitors. Based on their affinity for HERG binding in the *in vitro* assay, the compounds were subsequently segregated into positive and negative classes to form a training set for use in a robust and sparse support vector machine (SVM) algorithm. High-affinity HERG channel binding compounds ( $IC_{50} < 0.5 \mu M$ ) were used as the positive class for the training set and low affinity binding (% inhibition at  $10 \mu M < 10$ ) as the negative class. The SVM generates a Drug Signature™ predictive of HERG channel binding using gene expression in left ventricles isolated from male Sprague-Dawley rats treated for up to 5 days with compounds at a maximum tolerated dose. Based on 60:40 split-sample cross validation on the training set, the signature performance was estimated to have a true positive rate of 76% and true negative rate of 98%. The use of biomarkers predictive of cardiac pathological endpoints from short-term exposure animal models will help to reduce time and cost and improve the success rate of drug development.

**1831**

AHR AND ARNT SPECIFICALLY BIND PUTATIVE DIOXIN RESPONSE ELEMENTS (DREs) UPSTREAM OF BETA-ADRENERGIC RECEPTOR GENES.

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2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin (TCDD) causes cardiovascular toxicity in laboratory animals, including decreased ventricular contractility, decreased myocyte proliferation, increased cardiac apoptosis and inhibition of coronary vasculogenesis. Beta-adrenergic receptor ( $\beta$ -AR) signaling plays an important role in each of these processes. We hypothesize that TCDD exposure directly increases  $\beta_1$ -AR gene expression followed by a maladaptive decrease in  $\beta$ -AR signaling. We have detected a 30% increase in cardiac  $\beta_1$ -AR mRNA in embryonic day 10 (D10) chicks exposed to 0.3 pmol TCDD/g egg prior to incubation (D0) and have observed a reduction in the ability of TCDD-exposed embryonic chicks to increase heart rate in response to isoproterenol, a  $\beta$ -AR agonist. TCDD exposure, however, does not decrease the ability of the chick heart to respond to pharmacological agents effecting downstream events of the  $\beta$ -AR. This suggests that TCDD is mediating its effects at the level of the receptor. The existence of 5, 3 and 2 putative dioxin response elements (DREs) in the 5' enhancer region of the human, rat and mouse  $\beta_1$ -AR genes, respectively, raises the possibility that transcription of the  $\beta_1$ -AR gene is directly regulated by the aryl hydrocarbon receptor (AhR) and its dimerization partner the

AhR nuclear translocator (ARNT). In support of this hypothesis, we detect specific binding of  $\beta_1$ -AR DREs to AhR and ARNT in HeLa cell and cardiac tissue extracts by electrophoretic mobility shift analysis (EMSA). However, this binding is not dependent on the presence of AhR ligand. Current EMSA studies in our laboratory are determining the ability of *in vitro* expressed human and mouse AhR and ARNT to bind  $\beta_1$ -AR DREs in the presence and absence of exogenously applied AhR ligand. Transient transfection experiments using a luciferase reporter gene under the control of  $\beta_1$ -AR promoter will determine whether DRE binding is sufficient to transactivate gene expression, indicating the functionality of  $\beta_1$ -AR DREs. Supported by NIH R15 ES011806.

**1832**

GLUTATHIONE-S-TRANSFERASE A4-4 MODULATES iNOS EXPRESSION THROUGH NF- $\kappa$ B TRANSLOCATION.

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4-Hydroxy-2-nonenal (4-HNE) is known to induce cell apoptosis through activation of c-Jun N-terminal kinases and the caspase cascade pathway. Our recent work in endothelium showed that overexpression of glutathione S-transferases (GSTs) in endothelium protects against oxidative damage from aldehydes such as 4-HNE. But the role of GSTs for NF- $\kappa$ B activation and related inducible nitric oxide synthesis (iNOS) remain unknown. In this study, we tested the effect of 4-HNE on iNOS expression and the NF- $\kappa$ B pathway to further elucidate the protective effect of GSTs. A stable transfection of mouse pancreatic islet endothelial cells (MS1) with cDNA of mGSTA4-4, an  $\alpha$ -class GST with highest activity toward 4-HNE, was established. Transfected cells demonstrated significantly higher GSTs enzyme activity and expressed significantly increased resistance to the cytotoxicity of 4-hydroxy-2-nonenal (4-HNE) ( $p < 0.05$ ). mGSTA4-transfected cells showed significant upregulation of iNOS in comparison with vector-transfected cells when treated with 4-HNE. The inhibition of I $\kappa$ B kinase (IKK) by 4-HNE was reversed by mGSTA4 transfection. These studies indicate an important role for GSTs in phosphorylation of I $\kappa$ B $\alpha$  causing the translocation of NF- $\kappa$ B into nucleus. Over expression of mGSTA4-4 in endothelial cells may play a key role in modulating blood vessel tone by upregulation of iNOS, NF- $\kappa$ B. Thus, GSTA4-4 is likely to be an important defensive mechanism against oxidants that act as atherogens. (Supported by NIH grant HL65416.)

**1833**

CHEMICAL INDUCTION OF ANTIOXIDATIVE AND PHASE 2 DEFENSES IN CARDIOVASCULAR TISSUES *IN VIVO*: INVOLVEMENT OF NRF2 SIGNALING AND IMPLICATIONS FOR CARDIOVASCULAR PROTECTION.

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Accumulating evidence supports a causal role for oxidative and electrophilic stress in cardiovascular diseases. In this context, chemical induction of endogenous antioxidative and phase 2 defenses in cardiovascular tissues may represent a novel strategy for protecting against oxidative cardiovascular injury. However, our understanding of the chemical inducibility of antioxidative and phase 2 enzymes, and the underlying signaling mechanisms in cardiovascular tissues *in vivo* is limited. This study was undertaken to investigate the inducibility of cardiovascular antioxidants and phase 2 enzymes by 3H-1, 2-dithiole-3-thione (D3T), and the signaling role of Nrf2 in the regulation of the above cellular defenses in animals. A number of endogenous antioxidants and phase 2 proteins were induced in both cardiac tissue and vasculature in mice and rats after oral administration of D3T. These include superoxide dismutase, catalase, glutathione, glutathione reductase, glutathione S-transferase, and/or NAD(P)H:quinone oxidoreductase-1. The basal expression and chemical inducibility of the above cellular defenses varied between myocardium and vasculature. Nrf2-knock out mice were used to explore the signaling pathways involved in the regulation of the above cardiovascular enzymes. We observed that both the constitutive expression and the chemical inducibility of the cardiovascular antioxidants and phase 2 enzymes were reduced in Nrf2-null mice as compared with those in Nrf2(+/-) mice, indicating a critical role for Nrf2 signaling in the regulation of cardiovascular antioxidant and phase 2 gene expression *in vivo*. Studies are currently under way to determine the protective role of endogenous antioxidants and phase 2 enzymes in animal models of oxidative cardiovascular disorders, including doxorubicin-induced cardiomyopathy and myocardial ischemia-reperfusion injury (supported by HL71190).

**1834**

UPREGULATION OF ENDOGENOUS ANTIOXIDANTS AND PHASE 2 PROTEINS BY RESVERATROL IN CULTURED AORTIC SMOOTH MUSCLE CELLS LEADS TO CYTOPROTECTION AGAINST OXIDATIVE AND ELECTROPHILIC STRESS.

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Resveratrol is known to be protective against oxidative cardiovascular disorders. However, the underlying mechanisms remain unclear. This study was undertaken to determine if resveratrol could upregulate endogenous antioxidants and phase 2 enzymes in cultured aortic smooth muscle cells (ASMCs), and if such increased cellular defenses could provide protection against oxidative and electrophilic vascular cell injury. Incubation of rat ASMCs with resveratrol at low micromolar concentrations resulted in a significant induction of a scope of cellular antioxidants and phase 2 proteins in a concentration- and time-dependent fashion. These cellular defenses include superoxide dismutase, catalase, glutathione (GSH), glutathione reductase, GSH S-transferase (GST), NAD(P)H:quinone oxidoreductase-1 (NOQ1), and heme oxygenase-1 (HO1). Notably, induction of catalase, GST, NOQ1, and HO-1 was most remarkable among the above resveratrol-inducible antioxidants and phase 2 enzymes. Moreover, resveratrol treatment also dramatically increased the mRNA expression of the above cellular antioxidative and phase 2 genes. Pretreatment of ASMCs with resveratrol afforded a significant protection against xanthine oxidase (XO)/xanthine- or 4-hydroxy-2-nonenal-induced cytotoxicity, as assessed by MTT reduction assay and morphological changes. Resveratrol pretreatment also led to a marked reduction in intracellular accumulation of ROS in ASMCs after incubation with XO/xanthine. Taken together, this study demonstrates for the first time that a scope of endogenous antioxidants and phase 2 enzymes in cultured ASMCs can be potently induced by resveratrol at low micromolar concentrations, and that such elevated cellular defenses are accompanied by a markedly increased resistance to oxidant- or electrophile-mediated vascular cell injury. The results of this study have revealed a new mechanism, which may contribute to the cardiovascular protective effects of resveratrol (supported by HL71190).

**1835**

DEXRAZOXAN INHIBITS ALTERATIONS IN GLYCOLYTIC AND OXIDATIVE CARDIAC METABOLISM INDUCED BY DOXORUBICIN.

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Doxorubicin (DOX) is widely prescribed for the treatment of assorted cancers. However, its success is limited by the development of a cumulative and irreversible mitochondrial cardiomyopathy. The mechanism is believed to involve generation of reactive oxygen species (ROS) leading to oxidative tissue injury which in some cases can be avoided by co-administering selected antioxidant agents, the most promising of which is dextrazoxan. In this investigation, we explored the cardioprotective potential of dextrazoxan by monitoring its effects on metabolic flux through glycolytic and oxidative pathways using <sup>13</sup>C NMR isotopomer analysis. Rats were treated with 6 weekly injections of either: I) saline (control group); II) DOX (2 mg/Kg, s.c.); iii) dextrazoxan (DEX) (25 mg/Kg, i.p.); or iv) DOX + DEX. Hearts were subsequently excised and perfused by the Langendorff method with Krebs-Hensleit bicarbonate buffer supplemented with 5.5 mM glucose, 1.2 mM [3-<sup>13</sup>C]lactate, 0.09 mM [3-<sup>13</sup>C]pyruvate, 0.12 mM [1, 3-<sup>13</sup>C]acetooacetate, 0.05 mM [1, 3-<sup>13</sup>C]13<sup>13</sup>C long-chain fatty-acids. <sup>1</sup>H NMR spectra of lactate in the perfusate provides an estimate of glycolytic flux. DOX caused a 40% increase in glycolytic flux, which was prevented by co-administering DEX, which by itself had no effect on glycolytic rates relative to controls. A <sup>13</sup>C NMR isotopomer analysis of glutamate revealed that DOX treatment caused a 30% reduction in long-chain fatty-acid oxidation and a concomitant increase in glucose oxidation. Long-chain fatty acid oxidation capacity was restored by DEX co-administration. In summary, long-term treatment with DOX alters the metabolic status of cardiac tissue in favor of glycolysis over fatty acid oxidation. Reversal by DEX implies an important role for ROS in modulating this shift in metabolic regulation caused by DOX. (Supported by NIH HL58016 and POCTI/CBO/38611/01).

**1836**

METALLOTHIONEIN PROTECTION FROM PROTEIN NITRATION CAUSED BY LPS/TNF-ALPHA-DERIVED INTRACELLULAR PEROXYNITRITE.

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Our previous study showed that metallothionein (MT) prevented the damage of protein and DNA caused by peroxynitrite in an *in vitro* cell-free system. In STZ-induced diabetic mouse hearts, protein nitration, shown by 3-nitrotyrosine (3-NT), significantly increased as compared to control, which was significantly inhibited in MT transgenic (MT-TG) mouse. In order to test whether MT can also prevent protein nitration caused by intracellular peroxynitrite, a primary culture of neonatal mouse cardiomyocytes was exposed to LPS (100 $\mu$ g/ml) and TNF- $\alpha$  (50 $\mu$ g/ml). 3-NT significantly increased along with a significant increase in cell death in wild-type (WT) cardiomyocytes, detected by lactate dehydrogenase (LDH) release.

However, both 3-NT and LDH release were significantly prevented in LPS/TNF- $\alpha$ -treated MT-TG cardiomyocytes. Furthermore, the 3-NT and LDH release were also prevented by incubation of superoxide mimic (MnTTPyP) or peroxynitrite scavenger (urate) for 24 hours in the LPS/TNF- $\alpha$ -treated WT cardiomyocytes. These results indicate that MT can intracellularly prevent peroxynitrite-caused protein nitration, as it did *in vitro* cell-free system, and protein nitration caused by peroxynitrite is a direct cause of the cytotoxicity. (supported, in part, by ADA, Philip Morris USA Inc. and NIH)

**1837**

THE ROLE OF ENDOTHELIN-1 AND REACTIVE OXYGEN SPECIES IN CARDIAC HYPERTROPHY OBSERVED IN ARYL HYDROCARBON (AHR) NULL MICE.

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AhR is a ligand-activated transcription factor that mediates the toxicity of environmental pollutants, however the regulation and function of AhR are still largely unknown. Functional inactivation of AhR (AhR null mice) results in profound effects on the cardiovascular system. Previously, we reported AhR null mice exhibit cardiac hypertrophy associated with increased plasma endothelin-1 (ET-1) levels. Increases in both heart-to-body weight (HW/BW) ratios and ET-1 (plasma and tissue) were found to be age-progressive. Therefore, we tested the hypothesis that the observed hypertrophy results from an increase in ET-1 expression and downstream pathways regulated through the ETA receptor. Furthermore, we investigated whether reactive oxygen species (ROS) were associated with cardiac hypertrophy observed in AhR null mice. To determine if the age-progressive increase in ET-1 was associated with cardiac effects, we measured mRNA expression of -myosin heavy chain (MHC) and atrial natriuretic factor (ANF). Results revealed the hypertrophy marker genes are significantly increased in 2 mo AhR null mice (5.4- and 3.9-fold, respectively), compared to C57Bl6, with further elevations seen at age 5 mo. To examine the role of ET-1 in cardiac hypertrophy, 2 mo AhR null and C57Bl6 mice were treated with ETA receptor antagonist BQ-123, delivered via osmotic minipumps (60 days). BQ-123-treated AhR null mice showed reduced HW/BW ratios, compared to untreated AhR null mice (treated: 0.492; untreated: 0.552; C57Bl6 controls: 0.478 grams) and decreased LV posterior wall thickness. BQ-123 treatment also resulted in a significant decrease in expression of cardiac hypertrophy marker genes -MHC and ANF, and also cardiac fibrosis in AhR null mice. Furthermore, TBARS analysis showed decreased levels of cardiac ROS in BQ-123- treated AhR null mice. Such findings indicate that cardiac hypertrophy in AhR null mice is mediated by ET-1 and ROS. Supported by ES10433 and ES12072 to MKW, USEPA U91621501 to AKL.

**1838**

PCB-INDUCED INFLAMMATORY RESPONSE IN CAVEOLIN-1 DEFICIENT MICE.

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Caveolae are membrane microdomains enriched in cholesterol, sphingomyelin and numerous proteins involved in cellular signaling. They are especially abundant in endothelial cells, and the presence of caveolin-1 protein, a marker and major functional component of caveolae, has been shown to be a protective factor in the development of atherosclerosis. Polychlorinated biphenyls (PCBs) are persistent lipophilic environmental contaminants, that can contribute to the development of atherosclerosis by inducing inflammatory reactions and subsequent endothelial cell dysfunction. We evaluated the potential role of caveolae in preventing PCB-mediated pro-atherogenic events. Caveolin-1 (Cav-1) deficient mice were treated with PCB 77 (ip injection, 170  $\mu$ M/kg) for 24 hours. Total plasma cholesterol was higher in Cav-1-/- mice compared to wild type animals. PCB treatment significantly decreased plasma cholesterol in Cav-1 deficient mice. The expression of selected genes was quantified by real time PCR. With or without PCB treatment, aortas of Cav-1-/- mice had lower mRNA levels of vascular adhesion molecule-1 (VCAM-1), compared with wild type mice. On the other hand, gene expression of CYP 1A1 in liver was comparable in both Cav-1 deficient and wild type mice upon PCB 77 treatment. Peroxisome proliferator activated receptor  $\gamma$  (PPAR- $\gamma$ ) is partially located in caveolae and has been reported to modulate caveolin-1 protein expression. Pretreatment with the PPAR- $\gamma$  agonist thiazolidinedione (25  $\mu$ M) for 6 hours resulted in protection of endothelial cells against PCB 77 (3.4  $\mu$ M) toxicity, in terms of decreased DNA-binding activity of both NF- $\kappa$ B and STAT-3. Our data provide evidence that the endothelial inflammatory response induced by coplanar PCBs may be mediated in part through signaling pathways associated with caveolin-1. (Supported in part by grants from NIEHS/NIH (ES 07380)

PREVENTION OF DIABETES-ENHANCED LPS CARDIOTOXICITY IN CARDIAC-SPECIFIC METALLOTHIONEIN-OVEREXPRESSING TRANSGENIC MICE.

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Previous studies have shown that diabetes significantly enhances LPS-induced cardio-toxicity along with a significant increase in inflammatory cytokines and oxidative damage. Metallothionein (MT) functions as a potent antioxidant in the heart to protect against oxidative injury induced by various stresses including diabetes. In the present study, we examined whether MT can prevent diabetes-sensitized LPS cardiotoxicity using a cardiac-specific MT-overexpressing (MT-TG) mouse model. Type 1 diabetes was induced by streptozotocin (STZ) in the MT-TG and the wild-type (WT) control mice. Two months after STZ treatment, diabetic mice and non-diabetic mice were administrated with a single dose of LPS (2mg/kg) and sacrificed 1, 4.5 and 24 h after the treatment for examination of cardiac toxicity using serum markers creatinine kinase (CPK) and troponin T along with measurements of serum and cardiac pro- or anti-inflammation cytokines including tumor necrosis factor-alpha (TNF- $\alpha$ ), IL-6 and IL-10, by an ELISA method. Results showed that in the WT mice, diabetes significantly enhanced LPS-induced cardiotoxicity, as shown by significant increases in serum CPK and troponin T, and also enhanced the serum and cardiac IL-6 production. However, the diabetes-sensitized LPS cardiotoxicity was significantly prevented in the MT-TG diabetic mice although the IL-6 remained up-regulated in the MT-TG diabetic mice. These results suggest that the enhanced LPS cardiotoxicity in the WT diabetic mice may be associated with the oxidative damage derived from the enhanced production of IL-6 and MT prevents oxidative damage leading to the prevention of cardiac toxicity without affecting IL-6 production. (Supported in part by grants from ADA, Philip Morris USA, Inc. and NIH)

GENETIC BACKGROUND-DEPENDANT VARIATION IN EGFR-RELATED CARDIAC DYSFUNCTION.

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The epidermal growth factor receptor (EGFR/ErbB) family regulates diverse cellular functions such as growth, differentiation, cell motility and survival. Overexpression and/or aberrant signaling by ERBB receptors, especially EGFR and ERBB2, occurs frequently in human cancer, thus targeted therapies have been developed to inhibit ERBB signaling. Clinical trials have revealed major similarities between patient toxicity and phenotypes in mice with reduced ERBB receptor function. Some patients receiving the anti-ERBB2 drug Herceptin/Tarceva develop life-threatening dilated cardiomyopathies; this cardiotoxicity is recapitulated in mice with cardiac-specific ERBB2 ablation. As over-expression of the closely related EGFR occurs in 30 % of human primary tumors, EGFR inhibitors are being tested in ongoing clinical trials. To date there have been no reports of cardiotoxicity, but recent pre-clinical data suggests a subset of patients may also experience cardiotoxicity. Recently, aortic valve hyperplasia and stenosis was described in mice homozygous for the Egfr wa2 hypomorphic mutation. We have detected additional heart defects arising from reduced EGFR activity that vary with genetic background. Histological examination of the hearts of adult C57Bl/6J(B6) Egfr wa2/wa2 mice reveals severely enlarged, fibrotic hearts with hypertrophied cardiomyocytes. Echocardiography describes a dilated left ventricular chamber, impaired systolic function and thickened chamber walls. In addition, hearts of B6 Egfr wa2/wa2 mice show significant expression changes in classic markers for cardiac hypertrophy. By contrast, inbred 129S1 and hybrid B6.129 Egfr wa2/wa2 mice display none of these defects, despite having enlarged aortic valves. Moreover, wild-type B6 mice chronically exposed to the EGFR inhibitor EKB-569 have altered cardiac function and enlarged cardiomyocytes. These results imply that a subset of patients on anti-EGFR therapy may be at risk for cardiotoxicity. Identification of 129S1 modifiers may reveal new targets for the treatment of cardiac hypertrophy.

EFFECTS OF ARSENIC ON ENDOTHELIAL CELL ACTIVATION AND ACETYLATED-LDL UPTAKE BY MACROPHAGES.

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Cardiovascular disease (CVD) is an important cause of morbidity and mortality in the developed world. Arsenic is widely dispersed in the environment, and epidemiological studies have linked chronic arsenic exposure to a range of CVDs including

atherosclerosis, ischemic heart disease, peripheral vascular disease and hypertension. Atherosclerosis is an inflammatory disease, characterized by release of cytokines and chemokines and activation of endothelial cells. We used a combination of ELISA, flow cytometry and Western blot analysis to study endothelial cell activation in human aortic endothelial cells (HAECS). Our results showed that arsenic decreased expression of VE-cadherin, a protein that is responsible for maintaining tight junctions between the endothelial cells. This could lead to increased permeability of the endothelial barrier. We also found that arsenic modulated the expression of vascular cell adhesion molecule (VCAM-1) and interleukin-6 (IL-6) at concentrations as low as 1 uM at various time points up to 48 h. These events could lead to an increase in transmigration of monocytes across the endothelium. The monocytes are differentiated into macrophages within the intima of the blood vessel and play a key role in formation of foam cells. Using flow cytometry, we assessed the effects of arsenic exposure on uptake of acetylated LDL (Ac-LDL) by macrophages. Differentiated THP-1 cells were treated with arsenic-conditioned medium from HAECS and mouse macrophage cells (RAW264.7) were treated with serum from atherosclerotic apoE-/-LDLR-/- mice that had been exposed to arsenic in drinking water for 18 weeks. Arsenic treatment led to increased Ac-LDL uptake in both cases suggesting that endothelial activation by arsenic could facilitate foam cell formation. (Supported by NIH grant P20 RR17670).

EXAMINATION OF THE VASCULAR INJURY IN SPRAGUE-DAWLEY (SD) RATS INDUCED BY THE PHOSPHODIESTERASE (PDE) IV INHIBITOR SCH 534385: A COMPARISON WITH SCH 351591.

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Our previous study showed that the PDE IV inhibitor SCH 351591 induced vascular injury in SD rats and suggested that a panel of serum proteins could potentially serve as biomarkers for detecting and monitoring vascular lesions (Toxicologist 78: 375, 2004). The present study was begun to determine whether the structurally distinct PDE IV inhibitor SCH 534385 would induce similar vascular injury and, if so, whether these same biomarkers would again be observed. SD rats were given SCH 534385 (20 or 40 mg/kg/day for 3 days) by gavage. Microscopic evaluation of tissues obtained 24 h after the final dose revealed dose-dependent vascular injury. Arterial hemorrhage and necrosis, periarterial inflammation, and microvascular injury (fibrin insudation and fibrin exudation) were found in the mesentery, pancreas, kidney, liver and small intestine. Activation of immune system cells (endothelial cells, mast cells, and macrophages) and proliferation of fibroblasts were also noted at sites of inflammation. Lymphocyte numbers were markedly decreased in splenic and thymic cortex. In peripheral blood, granulocytes were elevated and lymphocytes decreased. SCH 534385 induced increases in the serum proteins alpha-1-acid glycoprotein, haptoglobin, GRO/CINC-1 (homolog of human interleukin-8), vascular endothelial growth factor, and tissue inhibitor of metalloproteinase-1. No significant changes were found in C-reactive protein. The pathological changes in the splanchnic vasculature, elevations in serum proteins, and alterations in leukocyte numbers in rats treated with this compound were similar to those seen in rats given SCH 351591. The data show that two structurally different PDE IV inhibitors, SCH 351591 and SCH 534385, induced similar vascular injury and comparable changes in biomarkers. Studies are underway to further characterize the etiology of vascular damage induced by PDE IV inhibitors and the clinical utility of serum biomarkers.

SMOOTH MUSCLE CELL MARKERS OF FENOLDOPAM INDUCED VASCULAR INJURY.

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Vascular lesions in the rat occur as a result of toxic insult or spontaneous disease. The mechanism (s) is unknown. Vascular smooth muscle cells (SMC) appear to be the primary target because damage to endothelial cells (EC) is not apparent. Research activities have focused on characterization of changes in protein expression and/or enzymes specific to SMC as lesions develop. Fenoldopam (FP, 6-chloro-7-8-dihydroxy-1-[ $\rho$ -hydroxyphenyl]-2, 3, 4, 5-tetrahydro- [H]-3-benzazepine) consistently induces vascular lesions in mesenteric arteries of the rat and is therefore a useful tool to study these events. In an investigative study, immunohistochemistry (IHC) was utilized as one endpoint to identify and characterize some of these proteins and/or enzymes. Proteins evaluated include von Willebrand factor (vWF), Smooth muscle  $\alpha$  actin (SMA), caveolin-1, (Cav-1) and the enzyme extracellular superoxide dismutase (ecSOD). Normal rat mesenteric artery SMC express Cav-1, SMA and ecSOD, while EC express vWF and Cav-1. Administration of

fenoldopam caused segmental arterial damage characterized by medial necrosis, hemorrhage and edema. The SMC layer of damaged arteries had a marked increase in vWF expression, and a significant loss of Cav-1, SMA and ecSOD. EC of damaged arteries expressed vWF comparable to controls and uninjured arteries while immunoreactivity for Cav-1 was lacking in these cells. These findings support previous observations that SMC are the likely primary targets of fenoldopam induced vascular injury and evaluation of serum analytes for Cav-1 and SMA should be further investigated as potential diagnostic markers. Additionally, serum vWF may not be useful as a reporter of progressive injury because of increased vascular permeability and leakage into the surrounding tissues. Furthermore, the reduction in SMC ecSOD suggests a role for free radicals in the mechanism of fenoldopam induced vascular injury.

#### 1844

#### ACROLEIN-INDUCED DYSLIPIDEMIA IN MICE: A RISK FACTOR OF CARDIOVASCULAR DISEASE.

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Acrolein is a highly reactive, ubiquitous aldehyde pollutant of industrial and automobile exhausts, tobacco smoke, and foods. Acrolein inhalation adversely affects the cardiovascular system while the effects of oral acrolein exposure on cardiovascular risk factors are less studied. Using C57Bl/6 and ApoE-null male mice (7-16 wks old) as models of normal and at-risk human populations, we determined the effects of acute oral acrolein exposure on plasma lipoprotein levels and endothelial function. Acrolein exposure in mice (0.1-5 mg/kg) within the range of expected daily human intake induced significant dose- and time-dependent alterations of plasma lipoprotein levels and electrophoretic mobility (EPM) on agarose gels. Oral acrolein (5 mg/kg) significantly increased total plasma cholesterol (mg/dl, control=64.4; treated=115.3, n=10, 9), phospholipids (mg/dl, control=132.5; treated=230.7, n=10, 7), and triglycerides (mg/dl, control=67.5; treated=382.1, n=10, 9) at 24h, which were positively correlated with plasma lipoprotein EPM changes and unaltered by pretreatment with the glucocorticoid receptor antagonist RU486 (25 mg/kg). Plasma ultracentrifugation and FPLC fractionation revealed significant alterations in VLDL and HDL EPM and VLDL lipid levels of acrolein-treated mice compared to controls. Plasma EPM changes were significantly altered at 12h and 24h but not at 1h, 4h, or 48h post-acrolein exposure and preceded plasma lipid level changes. Acrolein exposure significantly increased liver weight/body weight ratio, decreased plasma albumin and glucose, but did not alter plasma liver enzyme activity levels. Additionally oral acrolein significantly decreased acetylcholine-induced relaxation in isolated aorta. Oral acrolein caused dramatic VLDL-associated dyslipidemia and decreased endothelial function perhaps due to direct lipoprotein modification(s) and hyperlipidemia, and thus, may contribute to increased cardiovascular disease risk. This work supported by NIEHS PPG 1 PO1 ES11860.

#### 1845

#### ATTENUATION OF HYPEROXIA-INDUCED RETINOPATHIES IN NEONATAL RATS BY RETINOIC ACID.

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Supplemental oxygen administration, which is routinely encountered in the treatment of premature infants having respiratory distress, contributes to the development of retinopathy of prematurity (ROP). Retinal capillary damage, caused by hyperoxic exposures, often leads to retinal vascularization and proliferative retinopathy. Several studies suggest that vascular endothelial growth factor (VEGF) plays an important role in abnormal vessel proliferation in ROP patients. In this study, we tested the hypothesis that neonatal exposure of rats to a combination of all-trans retinoic acid (RA) and hyperoxia would alleviate oxygen-mediated retinopathy, and that modulation of VEGF expression contributes to the retinoprotective effects of RA. Newborn Fisher rats were maintained in room air or exposed to hyperoxia ([gt] than 95% O<sub>2</sub>) for 7 days. Some animals were treated i.p. with RA (0.5 mg/kg) or vehicle (saline), once daily for 5 days. Animals were sacrificed 1 or 30 days after termination of hyperoxia, and retinopathy was assessed by histological analysis of the retina. VEGF mRNA expression was studied by *in situ* hybridization and RT-PCR, and protein expression by Western blotting. Exposure of animals to hyperoxia alone for 7 days showed reduced number of retinal vessels, many of which were constricted, at 1 day compared to those breathing room air, and there were no significant differences in the retinal pathology of animals that were given RA + hyperoxia. At the 30 day time point, the oxygen-exposed animals displayed formation of new retinal vessels, whereas animals given RA + hyperoxia showed significantly lesser extent of neovascularization. At the 1 day as well as the 30 d time points, hyperoxia significantly upregulated retinal VEGF mRNA and protein expression, compared to those given RA + hyperoxia. In conclusion, the results of our study

support the hypothesis that RA protects animals from oxygen-induced retinopathy, and that downregulation of VEGF expression contributes to the retinoprotective effects of RA. (Supported in part by NIH grant KO8 HL04333 to XC.)

#### 1846

#### THE SUSCEPTIBILITY OF INBRED MOUSE STRAINS TO HYPEROXIA-INDUCED LUNG INJURY.

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To evaluate the effect of genetic background on susceptibility to hyperoxia-induced lung injury, male mice (6-8 wks) from nine genetically diverse strains (129/SvImJ, A/J, BALB/cJ, BTBR T+/-tf/tf, C3H/HeJ, C57BL/6J, Cast/Ei, DBA/2J, and FVB/NJ) were exposed to >99% O<sub>2</sub> or room air for 72 hours. Inflammation in the airways and alterations of lung permeability, total bronchoalveolar lavage protein concentrations and lung wet-to-dry ratios, determined that there was a range of susceptibility among the mouse strains. The susceptible strains, BALB/cJ and CAST/Ei, were the only strains to have infiltration of neutrophils in the airways and were of six strains, including A/J, BTBR T+/-tf/tf, C3H/HeJ, and DBA/2J, to have significantly increased IL-6 levels in the airways. In addition, the BALB/cJ and CAST/Ei strains were among five strains, including BTBR T+/-tf/tf, C57BL/6J and 129/SvIm strains, which had the greatest alterations of lung permeability. In contrast, of the resistant strains, DBA/2J, FVB/NJ, A/J and C3H/HeJ, the DBA/2J strain was the only strain not to have significant alterations of lung permeability compared to air-exposed controls. Furthermore, total superoxide dismutase (SOD) and manganese-SOD (Mn-SOD) activity was measured and correlated to the amount of injury for each strain. The BALB/cJ strain was the only strain that had significantly increased total SOD activity. No strains had significantly increased Mn-SOD activity compared to the air-exposed controls; however, the amount of activity correlated to the permeability of the lungs following the hyperoxic exposure. The differences of lung injuries among the inbred mouse strains after hyperoxia challenge suggest a genetic basis for the lung response to oxygen stress. Research sponsored by NIH awards: ES07498, ES09607, HL62628, HL66611, HL66604, and HL62641.

#### 1847

#### OXIDATION OF PURINE NUCLEOSIDES AND NUCLEOTIDES WITH DIMETHYLDIOXIRANE: STRUCTURAL CHARACTERIZATION OF REACTION PRODUCTS.

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Oxidative damage to DNA bases has been linked to adverse outcomes including genotoxicity and ageing. Reactive oxygen species such as hydroxyl radicals, superoxide anion and singlet oxygen have been implicated as causative agents of oxidative damage. We investigated the spectrum of products generated from oxidation of purine nucleosides and nucleotides with the powerful chemical oxidant dimethyldioxirane (DMDO). DMDO acts by epoxidation rather than by radical chemistry, and we wished to determine whether both these routes yielded similar oxidation products. DMDO was generated from the action of ozone on acetone; oxidation reactions were carried out at 0 °C. Products were characterized by HPLC, by proton and multidimensional heteronuclear NMR, and by electrospray MS in the positive ion mode. Epoxidation at the C4-C5 double bond followed by solvolysis represented the major product fraction. Thus in aqueous solution 2'-deoxy-4, 5-dihydroxyguanosine was formed from 2'-deoxyguanosine, at molar ratios of DMDO-to-nucleoside ranging from 1:1 to 5:1. With the nucleoside in excess over DMDO little oxidation was observed. 2'-Deoxy-8-oxoguanosine was similarly oxidised to 2'-deoxy-4, 5-dihydroxy-8-oxoguanosine. In methanolic solution the analogous product 2'-deoxy-4-hydroxy-5-methoxy-8-oxoguanosine was formed, confirming the involvement of solvent. The latter was further verified by conducting oxidations in perdeuterated methanol. Oxidation of 2'-deoxyguanosine-5'-phosphate yielded 2'-deoxy-4, 5-dihydroxyguanosine-5'-phosphate. Thus oxidation by a mechanism analogous to the mixed-function oxidases generates products distinct from those arising from radical chemistry. The oxidized nucleotide was more susceptible to deglycosylation than was the nucleoside, raising questions about the stability of such a lesion in DNA and its role in generation of abasic sites.

#### 1848

#### PI3-KINASE MEDIATED INDUCTION OF NRF-2 PROTEIN BY OXIDANTS IN CARDIOMYOCYTES.

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Oxidative stress plays an important role in many diseases including heart disease. The levels of antioxidant and detoxification enzymes in the cell can be critical in determining how cells respond to oxidative insult. Previous studies from our lab

found that sublethal doses of  $H_2O_2$  induce the expression of a battery of antioxidant and detoxification enzymes in primary cultured neonatal rat cardiomyocytes (CMCs). Using a promoter-reporter chimerical construct transfected into CMCs, we found that  $H_2O_2$  activates the Antioxidant Response Element (ARE), a cis-element present in the promoters of many antioxidant and detoxification-related genes. Activation of the ARE is regulated by the transcription factor NF-E2-related factor 2 (Nrf-2), which also contains an ARE in its promoter and may undergo positive feedback when the ARE is activated.  $H_2O_2$  induces the expression of Nrf-2 mRNA and protein in CMCs. Nrf-2 protein induction is detectable within 1-2 hrs after  $H_2O_2$  treatment, a time frame that precedes the activation of the ARE promoter (4 hrs). Using pharmacological inhibitors and dominant negative forms of signaling molecules, we found that the Phosphoinositol-3 Kinase (PI3K) pathway regulates the induction of Nrf-2 without affecting its nuclear translocation. Chemical inhibitors of PI3K, LY294002 and Wortmannin, blocked Nrf-2 elevation at the protein level and prevented  $H_2O_2$  from activating the ARE. Dominant negative Nrf-2 failed to inhibit the induction of Nrf-2, arguing against Nrf-2/ARE mediated positive feedback after oxidative stress. Taken together,  $H_2O_2$  induces Nrf2 protein elevation via a PI3K mediated mechanism in CMCs and such induction does not involve Nrf2-mediated positive feedback.

**1849**

**HYPERGLYCEMIA-INDUCED OXIDATIVE STRESS ACTIVATES THE HEXOSAMINE PATHWAY AND INHIBITS MITOCHONDRIAL BIOGENESIS.**

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High glucose conditions routinely observed in diabetes have been shown to cause a variety of pathological changes. Hyperglycemia is known to induce the generation of ROS, and is implicated in the cellular oxidative stress that is believed to be responsible for the observed "glucose toxicity". However, little is known about the action of this nutrient on the expression of transcription factors in different cell types, especially those involved in mtDNA replication and transcription. We hypothesized that high glucose concentrations stimulate the generation of ROS, decreasing NRF-1 and Tfam transcription and, as a consequence, lead to a decrease in mtDNA copy number. Furthermore, the associated decrease in mitochondrial oxidative phosphorylation may cause a compensatory stimulation of the hexosamine pathway. HepG2/C3A cells were cultured in the presence of 30 mM glucose for 7 days, while cells cultured in the presence of 5 mM glucose served as controls. After 7 days exposure to high glucose, HepG2C3A cells showed a 3.6-fold increase in ROS generation and a 25% decrease in mtDNA, which was accompanied by a 16% decrease in both Tfam and NRF-1 transcripts, which regulate mtDNA replication and transcription. The expression of plasminogen activator inhibitor-1 (PAI-1), which plays a pivotal modulatory role in regulating the activity of the hexosamine pathway, was increased 60% in hyperglycemic conditions. These results indicate that high glucose concentrations regulate mtDNA copy number by modulating the expression and perhaps transcriptional activity of Tfam and NRK-1, suggesting that the decrease of mtDNA content and inhibition of mitochondrial metabolism may be pathogenic hallmarks in the altered metabolic status associated with diabetes. (Supported in-part by HL58016 and HL 72175).

**1850**

**HYDROQINONE, p-BENZOQUINONE, BUT NOT BENZENE OR PHENOL CAUSE AN INCREASE IN REACTIVE OXYGEN SPECIES IN HD3 CELLS.**

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Exposure to the environmental toxicant benzene, a cyclic organic compound, has been proposed to lead to carcinogenesis. While the mechanism behind benzene mediated leukemogenesis remains unknown, it is generally accepted that benzene exerts its toxicity after being metabolized by cytochrome P450s. The c-Myb oncoprotein is important in blood cell production, as mice deficient in this protein die on the 15th day of gestation. This gene has been found to be overexpressed in a number of leukemic cell lines and other cancers. We hypothesize that benzene may act upon c-Myb as a potential mechanism of leukemogenesis and that oxidative stress from benzene's metabolites regulate c-Myb activity. Previously, we have shown that the benzene metabolites catechol, hydroquinone, and benzoquinone cause increases in c-Myb activity and in phosphorylated c-Myb protein expression in chicken erythroblast HD3 cells after 24 hours of exposure. However, these changes were not observed in cells exposed to benzene or phenol. Our objective was to evaluate the role of reactive oxygen species (ROS) by exposing HD3 cells to 300  $\mu$ M benzene, 300  $\mu$ M phenol, 30 or 300  $\mu$ M catechol, 50  $\mu$ M hydroquinone and 50  $\mu$ M p-benzoquinone for up to 24 hours. Exposure to 50  $\mu$ M hydroquinone or p-benzo-

quinone resulted in a significant increase in cells with higher ROS at 24 hours as detected by 5-(and-6)-chloromethyl-2', 7'- dichlorodihydrofluorescein diacetate and flow cytometry. Furthermore, addition of 10  $\mu$ M phenol did not further increase the presence of ROS in cells exposed to 50  $\mu$ M of hydroquinone. These results support our hypothesis that benzene mediates aberrant c-Myb activity through ROS.

**1851**

**MEASUREMENT OF OXIDATIVE STRESS USING KINETIC HIGH CONTENT CELL-BASED ASSAYS.**

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We have developed a high content bioassay that enables fully automated measurement of oxidative stress in individual cells in microplate format. The bioassay involves measurement of individual cell responses that are automatically extracted from high-resolution digital images captured using automated kinetic fluorescence imaging microscopy. The bioassay is based on detecting the conversion of non-fluorescent dihydroethidium (DHE) to fluorescent ethidium by reactive oxygen species (ROS). The assay involves simultaneous addition of DHE and test compounds to cells followed by acquisition of image data at regular intervals. The formation of ethidium is quantified as the accumulation of fluorescence of the ethidium:DNA complex in individual cell nuclei. Nuclear morphology was simultaneously monitored to determine when compound treatment causes irreversible cellular damage. Multiple concentrations of known oxidative stress inducers, such as tacrine and rotenone, were tested in the same experiment. Cumulative ROS production and nuclear condensation were automatically quantified at each time point using the Target Activation BioApplication on the Cellomics KineticScan® HCS Reader. Rates of ROS generation were also derived from the first 30 min. for each experimental condition tested to (a) enable measurement of the initial linear rate of ROS production and (b) eliminate effects of any eventual degradation of cellular metabolism/viability. Dose-dependent oxidative stress was observed for tacrine and rotenone. In addition, analysis of individual cell responses showed varying patterns of ROS generation and nuclear condensation. The assay was found to perform well using multiple relevant cell types, including HepG2 cells (human liver carcinoma) and primary rat hepatocytes. In conclusion, this bioassay is a valuable screening tool for conducting rapid quantitative assessment of oxidative stress on a large scale, involving testing of several experimental variables, including drug concentrations, cell types and time course.

**1852**

**ANALYSIS OF THE ROLE OF THE ANTIOXIDANT RESPONSE ELEMENT IN THE PROGRESSION OF AMYOTROPHIC LATERAL SCLEROSIS.**

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Oxidative stress plays a prominent role in a number of neurodegenerative diseases including amyotrophic lateral sclerosis (ALS). ALS is characterized by the progressive loss of motor neurons in the brainstem and spinal cord. The antioxidant response element (ARE) and its transcription factor, NF-E2 related factor 2 (Nrf2), play a pivotal role in the activation of detoxification pathways in multiple cellular systems. Interestingly, the development of ALS pathogenesis in the prototypical mouse model, superoxide dismutase with a G93A mutation (SOD<sup>G93A</sup>), revealed a time-dependent activation of the ARE using an hPAP reporter mouse (ARE-hPAP mice) in the spinal cord, as well as some key areas of the brain, brainstem, and cortex. SOD<sup>G93A</sup> transgenic mice exhibit ALS symptoms including muscle atrophy, motor neuron loss and eventual hindlimb paralysis at 120 days. SOD<sup>G93A</sup>/ARE-hPAP mice displayed increased ARE activation, as measured by defined hPAP enzymatic activity, temporally with disease progression. We have used explant spinal cord cultures to further investigate this mechanism of ARE activation *in vitro*. Spinal cord slices are cultured from postnatal day eight ARE-hPAP reporter mice and treated with known ARE activators following eight days *in vitro*. Spinal cord cultures from ARE-hPAP mice were treated with adenovirus (Ad-Nrf2) to detect if viral infection of Ad-Nrf2 would stimulate the ARE. Ad-Nrf2 infection has previously been shown to activate the ARE and is neuroprotective both *in vitro* and *in vivo*. Preliminary data shows significantly increased histological staining for hPAP activity in Ad-Nrf2 vs. GFP or PBS treated cultures. Ex-vivo spinal cord cultures represent a novel approach to exploring the mechanisms of ALS-related ARE activation and the resultant protective mechanisms.

**1853**

**ORAL GLUTATHIONE RAPIDLY ELEVATES GLUTATHIONE LEVELS IN LUNG EPITHELIAL LINING FLUID AND LAVAGED CELLS.**

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The lung adapts to oxidative stress by upregulation of antioxidants such as glutathione (GSH), uric acid and ascorbic acid. GSH plays an important role in protecting the lung from oxidative stress generated by endogenous and exogenous oxi-

dants. Previous studies in our laboratory have shown that following primary lung infection, levels of GSH in the epithelial lining fluid (ELF) are elevated. The levels of GSH in the ELF are low in a number of lung diseases including cystic fibrosis, acute respiratory distress syndrome, HIV infection and lung transplantation. These low ELF GSH levels have been proposed to play a role in the lung pathophysiology associated with these conditions and may sensitize this population to environmental air pollutants. The purpose of this study was to determine whether oral GSH treatment could increase ELF GSH levels in mice. Mice were given a bolus oral dose of GSH (300mg/kg). Mice were sacrificed at various time points and blood and bronchoalveolar lavage fluid was collected. Serum and lavage fluid as well as cells obtained from lavage fluid were measured for GSH levels by HPLC with electrochemical detection at 15, 30, 60, 100 and 240 min post dose. The concentration of GSH in the ELF was estimated by using the urea dilution method. GSH levels steadily increased and peaked at 60 minutes in the ELF. The GSH peak level was three-fold higher as compared to the control animals. GSH levels within lavaged cells peaked at 60 min and decreased at 100 and 240 min, but remained elevated as compared to controls. This data suggests that oral GSH treatment can increase ELF GSH level and could be used as therapeutic approach to protect the lung from toxic effect of oxidants generated by various lung disease processes and environmental exposures. (Supported by: Cystic Fibrosis Foundation grant and NIH grant HL075523).

## 1854

### SELECTIVE ENHANCEMENT OF HYPEROXIC LUNG INJURY BY AUROTHIOPROGLUCOSE IN GLUTATHIONE-DEFICIENT MICE SUGGESTS CROSS-TALK BETWEEN THIOREDOXIN AND GLUTATHIONE SYSTEMS.

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Reduction of glutathione disulfide (GSSG) to glutathione (GSH) by glutathione reductase (GR) is an essential function of GSH-dependent antioxidant defense mechanisms, but GR-deficient (Neu) mice are not markedly more susceptible to hyperoxic lung injury. Greater thioredoxin (Trx) and thioredoxin-2 (trx-2) levels in the livers of Neu mice suggest compensation by Trx-dependent mechanisms. To test the hypothesis that the Neu mice are dependent on Trx/thioredoxin reductase (TrxR) mechanisms for protection against hyperoxic lung injury, 0 or 25 mg/kg aurothioglucose (ATG) were administered IP to six week-old Neu and C3H/HeJ mice, which were exposed to >95% O<sub>2</sub> for 96 h or maintained in room air. Lung injury was assessed by right lung weight to body weight ratios. Data were log transformed and analyzed by three-way ANOVA, with differences noted at p<0.05. In Neu, but not in C3H/HeJ mice, mortality was observed at ATG doses greater than 25 mg/kg. Exposure of Neu and C3H/HeJ mice to >95% O<sub>2</sub> for 96 h caused lung injury. Pretreatment with ATG exacerbated hyperoxic lung injury in Neu mice (11.9±2.5 vs 6.8±1.3 g right lung/kg body, n=3, p<0.01; saline/room air 3.8±0.3), whereas ATG pretreatment did not enhance lung injury in C3H/HeJ mice. Enhanced hyperoxic lung injury in Neu mice by administration of ATG suggests functionally important cross-talk between the GSH and Trx systems in the GR-deficient animals. Whether direct increases or decreases in expression of Trx/TrxR-dependent mechanisms can be employed to increase or decrease resistance to oxidant stresses or problems arising from diseases or environmental exposures remains to be determined.

## 1855

### PHENOTYPIC ANCHORING OF ACETAMINOPHEN-INDUCED OXIDATIVE STRESS AND GENE EXPRESSION PROFILES IN RAT LIVER.

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Toxicogenomics is an emerging field providing the ability to define in greater detail the underlying molecular events preceding and accompanying toxicity. The incorporation of this new technology requires careful validation and altered gene expression patterns should be corroborated with conventional indices of toxicity in a dose and time dependent manner. Acetaminophen (APAP) is a therapeutic drug with documented liver toxicity that causes similar metabolic and toxic responses in rodents and humans. APAP overdose-associated toxicity in liver is an important clinical problem and a significant contributor to acute liver failure. Recent gene expression profiles for APAP have demonstrated an oxidative stress signature beginning at sub-toxic doses that we hypothesized can be phenotypically anchored to conventional biomarkers of oxidative stress. Liver tissue was obtained from same experimental animals used to generate micro-array data and histopathology where male rats were given a single dose of APAP at sub-toxic (50 and 150) and toxic (1, 500 and 2, 000 mg/kg) doses and sacrificed at 6, 24, and 48 hrs. Oxidative stress was as-

sessed by measuring expression of base excision repair genes (BER), quantifying oxidative lesions in genomic DNA, and evaluating lipid peroxidation in liver. Toxic doses of APAP significantly induced gene expression levels of several BER genes including PCNA, PARP, APE, and Ogg1. Although induction of BER genes occurred with toxic doses of APAP, significant increases in abasic sites over controls was not observed for any doses examined. In summary, the increased expression of BER genes was found to correlate with toxic doses of APAP, the onset of hepatic necrosis, and an increase in activity of alanine aminotransferase. Conversely, the presence of an oxidative stress signature at sub-toxic doses must be interpreted carefully without further experimental corroboration.

## 1856

### NEAR-INFRARED LIGHT TREATMENT IN A CELLULAR MODEL OF MITOCHONDRIAL DYSFUNCTION.

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Mitochondrial dysfunction and oxidative damage to biological molecules in the retina and optic nerve is known to contribute to the pathogenesis of Lebers hereditary optic neuropathy (complex I dysfunction) and has been postulated to contribute to retinal aging and age related retinal diseases. Photobiomodulation by red to near-infrared (630 to 1000 nm) light has been shown to stimulate mitochondrial energy production, accelerate wound healing and promote and cellular survival following a mitochondrial insult. The present studies were undertaken to test the hypothesis that: near-infrared (NIR) light emitting diode (LED) photobiomodulation will stimulate mitochondrial function, attenuate oxidative stress and improve cell survival in a cellular model of complex I dysfunction. 661W cells, a photoreceptor-derived cell line, were plated at a density of 150, 000 cells/cm<sup>2</sup> in 96 well plates and incubated at 37 °C in DMEM supplemented with 5% FBS. At 72 h, cultures were treated with the complex I inhibitor, rotenone, (1-250 micromolar) for 24 hr. Duplicate cultures were pretreated with (24 and 48 h after plating) with 670nm LED light (power intensity of 28 mW/cm<sup>2</sup> and fluence of 4 joules/cm<sup>2</sup>) exposed to rotenone (72 h) and given one post-670 nm LED treatment, one h after the addition of rotenone. Rotenone produced a concentration-dependent increase in reactive oxygen species (ROS) generation and cell death. 670 nm LED treatment produced a significant attenuation in ROS production with little observable change in cell death. These studies support the hypothesis NIR photobiomodulation decreases ROS production in an *in vitro* model of an important clinical disease. This may lead to the development of NIR-LED as a innovative, non-invasive, therapeutic approach for the treatment of retinal diseases. (Supported by Fight for Sight Student Fellowship SF04041 and DARPA N66001-04-1-8923)

## 1857

### TOLL-LIKE RECEPTOR 4 (TLR4) CONTRIBUTES TO OZONE-INDUCED INCREASES IN COSTIMULATORY MOLECULE (CD86) EXPRESSION IN MICE.

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Rationale: Ambient ground level ozone (O<sub>3</sub>) induces airway inflammation and may modulate adaptive immune responses in the lung. T-cell activation is at least partially dependent on macrophage-induced upregulation of co-stimulatory molecules such as CD86. Previous work demonstrated that the innate immune receptor TLR4 modulates pulmonary inflammatory responses to O<sub>3</sub>. We hypothesized that CD86 expression is upregulated in response to O<sub>3</sub> and depends on TLR signaling. Methods: We exposed O<sub>3</sub> resistant, TLR4 dysfunctional C3H/HeJ (HeJ) mice and O<sub>3</sub> susceptible, TLR4 sufficient C3H/HeOuJ (OuJ) mice to 0.3 ppm O<sub>3</sub> or air for up to 72 hrs. Mean total protein concentration (a marker of lung hyperpermeability) and numbers of polymorphonuclear leukocytes (PMNs) were determined in bronchoalveolar lavage fluid (BALF). Surface CD86 expression was assessed via flow cytometry on macrophages recovered from BALF and confirmed in whole lung homogenates using Western blot analysis. Results: Mean total protein concentration, numbers of PMNs, and total cells increased significantly in HeJ and OuJ mice after 48 and 72 hr O<sub>3</sub>, and were significantly greater in OuJ compared to HeJ mice. CD86 expression was significantly elevated in OuJ compared to HeJ mice at baseline and after O<sub>3</sub>. Conclusion: This study demonstrates that macrophage-specific CD86 expression increases in response to 0.3 ppm O<sub>3</sub>. TLR4 may partially mediate O<sub>3</sub>-induced adaptive immune responses through upregulation of CD86.

## 1858

### ARSENIC TRIOXIDE INDUCED OXIDATIVE STRESS IN SPRAGUE-DAWLEY RATS.

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Arsenic is a known human carcinogen and induces a variety of human diseases in addition to cancers of the lung, skin, bladder, kidneys and liver. The mechanism(s) by which arsenic induces cancer, however remains poorly understood. Recent studies have pointed out that arsenic toxicity is associated with the formation of reactive

oxygen species, which has a role in the pathogenesis of arsenic-induced diseases. The aim of the present study was to investigate the effect of arsenic trioxide on the lipid peroxidation changes in the plasma samples of Sprague-Dawley rats. Four groups of six male rats each weighing approximately  $50 \pm 2$  g, were injected intraperitoneally, once a day for five days with doses of 5, 10, 15, 20 mg/kg body weight (BW) of arsenic trioxide dissolved in distilled water. A control group was also made of six animals injected with distilled water without chemical. Following anaesthetization, blood specimens were immediately collected in tubes containing EDTA as an anticoagulant, and the concentration of malondialdehyde (MDA), a convenient index of lipid peroxidation in plasma samples was determined using colorimetric assay from Calbiochem. Concentration of MDA was significantly ( $p < 0.05$ ) higher in the treated groups when compared with the control group, indicating a gradual increase in lipid peroxidation with increasing doses of arsenic. Our results demonstrate that arsenic trioxide has a potential of causing oxidative stress, a biomarker of cellular injury, in the plasma samples of Sprague-Dawley rats. Keywords: arsenic, reactive oxygen species, oxidative stress, malondialdehyde (MDA), lipid peroxidation, Sprague-Dawley rats.

1859

EFFECT OF ACETAMINOPHEN ON CYTOSOLIC AND MITOCHONDRIAL GLUTATHIONE IN THE LIVERS OF WILD-TYPE, GCLM-HETEROZYGOUS, AND GCLM-NULL MICE.

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Acetaminophen (APAP) is metabolized by CYP450s to N-acetyl-p-benzoquinoneimine, a reactive molecule, which is detoxified via glutathione (GSH) conjugation. APAP overdose can cause GSH depletion, which is correlated with the degree of observed APAP-induced liver injury. Mitochondrial GSH (mtGSH) status is hypothesized to play a pivotal role in this process. GSH synthesis is in part controlled by glutamate cysteine ligase (GCL), a heterodimer of catalytic (GCLC) and modulatory (GCLM) subunits. In order to investigate the hypothesis that mtGSH is preferentially maintained under conditions of chronic GSH depletion and acute oxidative stress, we utilized our GCLM-null mouse model. GCLM  $+/+$ ,  $+/$ -, and  $-/-$  mice ( $n=7$  ea.) were fasted for 12-hours followed by i.p. injection of saline or APAP (300 mg/kg). Mice were euthanized 6 hours post-treatment and hepatic mitochondria isolated. Analysis of mean cytosolic GSH (ctGSH) (nmol GSH/mg wet liver) in control mice demonstrated  $-/-$  mice to contain 18% of  $+/+$  mice. Mean ctGSH was observed to decrease by 52 and 60% upon APAP treatment of  $+/+$  and  $-/-$  mice, respectively. Mean mtGSH in control  $-/-$  mice was not statistically different from  $+/+$  mice. A decrease in mean mtGSH upon APAP treatment was only observed in  $-/-$  mice (68% decrease). However, as a percent of combined (ct+mt) GSH, mtGSH of  $-/-$  mice was observed to be 67 and 200% greater than mtGSH of APAP-treated and control  $+/+$  mice, respectively. APAP treatment increased the mean percentage of GSH in mitochondria of  $+/+$  mice by 55%. Measures of  $-/-$  mice were not statistically different from  $+/+$  mice. These results demonstrate that under conditions of chronic GSH depletion, as occurs in  $-/-$  mice, or acute oxidative stress, as induced by APAP, mtGSH is not depleted to the same degree as ctGSH. These results support the hypothesis that mtGSH is preferentially maintained even as ctGSH is being depleted. Supported by NIH grants 1P42ES04696, 1R01ES10849, 1T32ES07032, and 1P30ES07033.

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ANTIOXIDANT GENE EXPRESSION LEVELS IN BRAIN AREAS THAT ARE TARGETS OF 1, 3-DNB MEDIATED NEUROTOXICITY.

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1, 3 Dinitrobenzene(1, 3-DNB), a chemical entity used in the production of dyes, plastics and explosives, is a neurotoxicant in rats affecting cerebellar function and targeting distinct brain-stem nuclei. Our lab has established that in the presence of the dinitrobenzene isomers, electron transfer through Nitric Oxide Synthase (NOS) may result in the simultaneous production of both nitric oxide and superoxide anion radical to produce peroxynitrite. It is postulated that 1, 3 DNB mediated free radical formation and the oxidative stress results in increased energy metabolism and cellular changes in redox and antioxidant status. We are investigating the possible changes in gene expression levels of important antioxidant enzymes, Cu/Zn superoxide dismutase (SOD1) and glutathione peroxidase (GSH-Px) in neuronal NOS localized areas of brain. In preliminary experiments, rats were dosed with 1, 3-DNB (10 mg/kg in corn oil i.p. every 12h \* 3). The experimental design consisted of 5 groups of rats which differed by 1) receiving 1, 3-DNB in corn oil or only corn oil and, 2) the time of brain removal after final dose of 1, 3-DNB (0, 6, 12, and 24 hr). Brain samples consisting of the lower half of the cerebellum plus the

pons and medulla were removed and RNA was isolated and standardized spectrophotometrically as a function of protein concentration. RTPCR was performed wherein 2.5 mg of total RNA was reverse-transcribed into cDNA and PCR reaction was performed followed by separation, visualization and densitometric analysis. They showed that following 1, 3-DNB, GSH-Px was increased by around 35% at both 12 hr and 24 hr but SOD1 levels showed little to no increase (10%) at 24 hrs. These data show that there are specific differences in the regulation of at least these two antioxidant genes by 1, 3-DNB. Experiments are now underway to define the detailed time-course of gene expression following 1, 3-DNB as well as the expression and regulation of other genes involved in oxidative stress like MnSOD and P53

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ENHANCED RENAL TOXICITY OF FERRIC NITRILOTRIACETATE IN NRF2 DEFICIENT MICE.

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Intracellular defense systems against oxidative stress play a key role in the prevention of diseases related with aging and carcinogenesis. Knock-out mice for Nrf2, a transcriptional factor to antioxidant-response genes, including phase2 metabolizing enzymes, show high sensitivity to oxidative stress, offering an useful animal model for understanding such cellular responses. In order to clarify a possible role of Nrf2 in oxidative stress occurring in the kidney, expression of renal toxicity induced by ferric nitrilotriacetate (Fe-NTA), a known renal carcinogen for rodents involving oxidative stress in the carcinogenesis, was investigated using three genotypes. Male Nrf2 (+/+, +/- or -/-) mice received single injection of 3 or 6 mg iron/kg of Fe-NTA, and killed at 1, 2, 4 and 24 hours after the treatment for collecting kidney samples. Histopathological examination revealed that renal lesions such as pyknosis or fragmentation of nuclei, and degeneration or desquamation of proximal convoluted tubular cells were expressed earlier in the -/- mice than +/+. Lipid peroxidation (TBARS) and DNA oxidation (8-oxodeoxyguanosine), major parameters of oxidative stress, were significantly and dose-dependently increased in the kidney of Fe-NTA-treated groups and showed some tendency to be more severe in the -/- mice. Total glutathione (GSH) and  $\gamma$ -glutamylcysteine synthetase activity were both reduced constitutionally in the -/- mice. Moreover, the Fe-NTA treatment depleted GSH more severe in the -/- mice than the +/+. Thus, it is suggested that Nrf2-mediated GSH-regulation system plays an important role in the cellular defense against iron-induced oxidative stress in the kidney.

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THE BIO-NANO INTERFACE: EXAMINING THE INTERACTIONS BETWEEN WATER SOLUBLE FULLERENES AND BIOLOGICAL MEMBRANES.

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The cytotoxicity of water-soluble fullerene species is a sensitive function of their surface derivatization. In three different human cell lines, dermal, liver, and brain, the lethal dose of fullerene changed over 7 orders of magnitude with relatively minor alterations in fullerene structure. An aggregated form of C<sub>60</sub>, the least derivatized of the four materials, was substantially more toxic than highly soluble derivatives such as C<sub>3</sub>, Na<sup>+2-3</sup>[C<sub>60</sub>O<sub>7.9</sub>(OH)<sub>12-15</sub>]<sup>(2-3)</sup>, and C<sub>60</sub>(OH)<sub>24</sub>. Oxidative damage to the cell membranes was observed in all cases where fullerene exposure led to cell death. We show that under ambient conditions in water fullerenes can generate superoxide anions, and postulate these oxygen radicals are responsible for membrane damage. We have also utilized Atomic Force Microscopy (AFM) to examine the interaction of the aggregated form of C<sub>60</sub> with artificial membranes. The AFM images show that the C<sub>60</sub> aggregate selectively binds only to the membrane. This work demonstrates both a strategy for enhancing the toxicity of fullerenes for certain applications such as cancer therapeutics or bactericides, as well as a remediation for the possible unwarranted biological effects of pristine fullerenes.

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INVOLVEMENT OF REACTIVE OXYGEN FORMATION IN THE 3-HYDROXY-3-METHYLGLUTARYL-COA REDUCTASE INHIBITOR-INDUCED SKELETAL MUSCLE CELL DEATH.

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Statins (3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors) are potent cholesterol lowering agents, which may cause myopathy and in rare cases even rhabdomyolysis in man. The mechanisms leading to rhabdomyolysis are not yet

known. There is indirect evidence that reactive oxygen species (ROS) might play a role in muscle cell necrosis. The purpose of this study was to determine whether ROS is formed and contributes to cell death in human skeletal muscle primary culture (hSkMCs) in response to statin treatment. Thus, hSkMCs were incubated with atorvastatin (ATV), simvastatin (SIM) or pravastatin (PRA) at different concentrations and for varying time. ROS formation, caspase-3 activation (apoptosis), cellular ATP contents and LDH-leakage (surrogate for necrotic cell damage) were measured. All statins, except PRA caused: 1) increased ROS formation (after 30 min of incubation), 2) caspase-3 activation (after 2 hours), 3) increased LDH-leakage (after incubation times of 4, 24 or 48 hours) and 4) increased cellular ATP contents after 1 and 2 hours, which dropped below control levels after 24 or 48 hours of incubation. In general SIM was more potent than ATV, having effects at lower concentration and after shorter incubation times. In the presence of the antioxidants N-acetyl cystein (NAC), dithiothreitol (DTT) and DL- $\alpha$ -tocopherol-polyethylene-glycol-1000-succinate (TPGS), statin-induced caspase-3 activity as well as increased LDH-leakages were inhibited. Inhibition of LDH-leakage was also achieved by co-incubation with the caspase-3 inhibitor AC-DEVD-CHO. The present data confirm that oxidative stress is an early event following the treatment of hSkMCs with statins. The statin-induced ROS formation might contribute to the apoptotic and necrotic cell death in hSkMCs.

**1864**

SURFACE ACTIVITY OF ERIONITE SAMPLES FROM DIFFERENT LOCALITIES.

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Erionite is an aluminosilicate with a structure that consists of silicon (Si) tetrahedrally coordinated to oxygen atoms. The tetrahedra are arranged into six-membered rings which in turn form channels or cages through which cation migration takes place. The Si atoms are occasionally replaced by the Al atoms in the framework, giving rise to a negative charge on the mineral, which is balanced by positive counter-ions contained in the cages. Counter-ions normally associated with erionite are Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup> and Mg<sup>2+</sup>, but other cations such as iron also exist naturally in trace amounts. Iron has been reported to act as a source of radicals in erionite fibers, but the nature and the mechanism of the generating iron surface sites are still not clear and most reports present only limited mineral characterization of samples. Erionite is more carcinogenic than crocidolite and amosite asbestos fibers, in both laboratory animals and humans. Six erionite fiber samples obtained from different localities were studied. Mossbauer Spectroscopy (MS) analysis has shown that these samples contained different proportions of iron in either ferric or ferrous oxidation states, or both. Photoelectron Spectroscopy (XPS) confirmed these observations. Scanning Electron Microscopy (SEM) has indicated that not all fibers in a particular sample contained iron. The ability of these erionite samples to peroxidise lipids was also tested and it was found that these samples could initiate the peroxidation of lipids to various extents. Treatment of these samples with desferrioxamine could decrease but not completely abolish this peroxidation ability. These results elucidate the bioavailability of iron in these erionite samples which in turn may determine their toxicity and carcinogenicity.

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IDENTIFICATION OF NF- $\kappa$ B MODIFICATIONS GENERATED BY CIGARETTE SMOKE ALDEHYDES.

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Cigarette smoke is associated with an increased incidence and severity of respiratory tract infections, which appears to be due to profound suppression of T cell responses in smokers' lungs. Our lab has identified several immunosuppressive compounds in the vapor phase of cigarette smoke that block T cell cytokine production. Acrolein and crotonaldehyde are  $\alpha$ ,  $\beta$ -unsaturated aldehydes that inhibit cytokine production by covalently binding to and inactivating the p50 subunit of NF- $\kappa$ B. Pretreatment of purified p50 with 1  $\mu$ M acrolein blocked its ability to bind an NF- $\kappa$ B target sequence. The saturated aldehydes (acetaldehyde, butyraldehyde, and propionaldehyde) had no effect. Crotonaldehyde also inhibited binding, but at much higher doses (1 mM). Mass spectrometry analysis of purified p50 treated with acrolein, crotonaldehyde or the saturated aldehydes revealed that several amino acids were covalently modified. Acrolein bound to Cys61, Lys146 and Lys274, amino acids critical for DNA binding. Additionally, acrolein was found conjugated to Cys272 and His306, which are involved in p50/p65 heterodimer formation. Crotonaldehyde also reacted with Cys61, but did not bind any other

amino acid in the DNA-binding domain of p50. No modification of p50 by the saturated aldehydes was detected even though present in high levels in cigarette smoke. These observations suggest that acrolein inhibits NF- $\kappa$ B activity by reacting with cysteines, lysines and histidines which prevents DNA binding and heterodimer formation.

**1866**

2, 3, 7, 8-TETRACHLORODIBENZO-P-DIOXIN (TCDD) INDUCES THE EXPRESSION OF FAS AND FAS LIGAND THROUGH DISTINCT PATHWAYS INVOLVING DRE AND NF-KAPPAB MOTIFS ON THE PROMOTERS.

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We have shown previously that TCDD induces apoptosis in immune cells through upregulation and recruitment of Fas and Fas ligand (FasL). In the present study, we examined the regulation of Fas and FasL promoters following TCDD exposure. To this end, we cloned murine Fas and FasL promoters into pGL3 vector that contained luciferase gene. EL4 cells transiently expressing Fas or FasL promoter were treated with 100nM TCDD and luciferase assays were performed 18 hrs post treatment. We observed 15-20 and 6-10 fold induction of promoter activity for Fas and FasL respectively following TCDD exposure. However, the induction of luciferase was significantly reduced (1-2 folds) when alpha-naphthoflavone, an AhR antagonist was used in the culture together with TCDD, demonstrating that the regulation of Fas and FasL promoters by TCDD may be mediated by TCDD-AhR interaction. To further understand the regulation of Fas and FasL promoters by TCDD and participation of AhR, we generated various clones of Fas promoter containing either dioxin response elements (DRE) or nuclear factor kappaB (NF- $\kappa$ B) binding motifs as well as clones that contained NF- $\kappa$ B sites of FasL promoter and used them for transfection. We also generated mutations in DRE and NF- $\kappa$ B regions present in Fas and FasL promoters. TCDD treatment of EL4 cells transiently expressing DRE or NF- $\kappa$ B regions of Fas promoter demonstrated varying levels of luciferase expression (5-7 folds). We observed 2-3 fold luciferase induction when NF- $\kappa$ B1 or NF- $\kappa$ B2 of FasL promoter was used for transfection. Use of DRE or NF- $\kappa$ B mutants demonstrated similar results. Together, our data demonstrate that TCDD-AhR interactions may regulate Fas promoter through DRE and NF- $\kappa$ B binding sites and FasL promoter, only through NF- $\kappa$ B motifs. (This work was supported in part by NIH grants R01AI053703, R01ES09098, R01 AI058300, R01DA016545 and R01HL058641).

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2, 3, 7, 8-TETRACHLORODIBENZO-P-DIOXIN (TCDD) DISRUPTS THE NORMAL DAILY RHYTHMS OF HEMATOPOIETIC PRECURSORS.

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Hematopoietic stem cells (HSC) and progenitor cells are responsible for the daily production of all blood cells through an ordered process of proliferation and differentiation. These processes are controlled by both extrinsic and intrinsic factors including a myriad of transcription factors. One possible member of this network is the aryl hydrocarbon receptor (AhR), a member of the bHLH-PAS transcription factor family and the mediator of TCDD toxicity. Previous studies indicated that *in vivo* exposure to TCDD decreased the ability of HSC to rescue irradiated mice. Interestingly, the numbers and function of these hematopoietic precursors are regulated in a circadian manner. Because many of the bHLH-PAS transcription factors are involved in the regulation of circadian physiology, we investigated whether disruption of normal AhR signaling following exposure to TCDD could alter the circadian rhythm of these cells. Six week old female C57BL/6 mice were given a single oral exposure to TCDD, and bone marrow was isolated and immunolabeled five days later. Flow cytometry revealed shifts in both the phase and magnitude of the daily cycle of HSCs, common myeloid progenitors, granulocyte-macrophage progenitors, and megakaryocyte-erythrocyte progenitors. In addition, the daily rhythm of functionally defined high proliferative potential-colony forming cells (HPP-CFC) was also shifted. Dual staining with 7-AAD and Pyronin Y revealed an alteration of the ratio of quiescent to cycling HSC following TCDD treatment. When HPP-CFC were exposed to TCDD *in vitro*, their ability to respond to assay conditions was either inhibited or enhanced in a maturation phase- and dose-dependent manner. It therefore appears that while TCDD may directly impact the proliferative capacity of hematopoietic precursors *in vitro*, the net effect following an *in vivo* exposure is more complex due to additional levels of regulation. This research was supported by NIH Grant ES04862, Training Grant ES07026, and Center Grant ES01247.

INTERFERON-GAMMA REVERSES TCDD-MEDIATED SUPPRESSION OF THE IGM ANTIBODY RESPONSE AND ATTENUATES CYP1A1 INDUCTION.

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2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a potent suppressor of the primary immunoglobulin(M) response. The molecular mechanism is dependent on the aryl hydrocarbon receptor and involves a disruption of B cell differentiation into plasma cells. We have previously reported that direct addition of interferon (IFN) $\gamma$  to splenocyte cultures attenuated TCDD-mediated suppression of the anti-sRBC IgM antibody forming cells response (AFC). The objective of the present study was to further characterize the effect of IFN $\gamma$  on mRNA levels for the Ig  $\mu$  heavy chain (IgH), kappa light chain (Igk) and J chain (Igl) as well as determine whether the ability to antagonize suppression by TCDD of the anti-sRBC IgM AFC response was shared by other forms of IFN. Direct addition of TCDD (30 nM) to spleen cell cultures produced a significant suppression (58%) in the anti-sRBC IgM response and a decrease in mRNA levels for IgH (37%), Igk (36%) and IgI (68%), when compared to vehicle control. However, in splenocytes co-treated with TCDD (30 nM) and IFN $\gamma$  (100 Units/ml), complete reversal of both the TCDD-mediated suppression on the anti-sRBC IgM AFC response and inhibition of mRNA levels for IgH, Igk and IgI was observed. Likewise, TCDD-mediated induction of CYP1A1 mRNA levels was diminished (50%) by IFN $\gamma$ . Time of addition studies revealed that IFN $\gamma$  had to be present in culture within the first 24 h after antigen sensitization to attenuate the TCDD-mediated suppression of the AFC response. No attenuation of the TCDD-mediated suppression of the anti-sRBC IgM AFC response was observed with IFN $\alpha$  or IFN $\beta$ . These results suggest that signaling induced through the IFN $\gamma$  receptor interfere with the AhR signaling cascade. (Supported in part by NIH ES02520, P42 ES04911, and University of Ulsan, Korea).

MAP KINASES, BUT NOT CALCIUM OR REACTIVE OXYGEN SPECIES, ARE INVOLVED IN PPAR $\gamma$  AGONIST-INDUCED PRO/PRE-B CELL APOPTOSIS.

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Peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) is expressed highly in the immune system and influences multiple aspects of immune function. We have shown that the high-affinity PPAR $\gamma$  ligand GW7845 activates the stress kinase (p38 MAPK and JNK) pathway and that this pathway contributes to GW7845-induced apoptosis in pro/pre-B cells. Here, we have extended our studies to investigate kinase activation by other PPAR $\gamma$  agonists, to examine the mechanism of p38 MAPK activation, and to determine how p38 MAPK interfaces with the caspase cascade. Treatment of a non-transformed murine pro/pre-B cell line (BU-11) with the synthetic PPAR $\gamma$  agonist GW7845 (40  $\mu$ M) or the endogenous PPAR $\gamma$  agonist 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>) (10  $\mu$ M), strongly induce p38 MAPK phosphorylation and p38 MAPK kinase activity. Pre-treatment with the p38 MAPK inhibitor PD169316 (5  $\mu$ M) prevented PPAR $\gamma$  agonist-induced apoptosis and reduced p38 MAPK phosphorylation, JNK phosphorylation, NF- $\kappa$ B-DNA binding, cytochrome c release and caspase-3 activation. However, the calcium chelator BAPTA and the antioxidant ebselen had minimal effects on kinase activation or caspase activation. These data support the conclusion that PPAR $\gamma$  agonists induce early B cell apoptosis through a PPAR $\gamma$ - and p38 MAPK-dependent signaling cascade. This research was supported by: RO1-ES06086, P01-ES11624

CASPASE-8 IS NOT THE MOST PROXIMAL CASPASE INVOLVED IN DMBA-INDUCED BONE MARROW B CELL APOPTOSIS.

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Environmental PAHs are both carcinogenic and immunosuppressive. We have shown that 7, 12-dimethylbenz[a]anthracene (DMBA) induces a loss of bone marrow cellularity *in vivo* and bone marrow B cell apoptosis *in vitro*. Here we examine the caspase pathway activated during DMBA-induced apoptosis in primary bone marrow pro-B cells and in a non-transformed pro/pre-B cell line (BU-11). Significant apoptosis was induced within 10 hours in both primary pro-B cells and in the BU-11 cell line when cultured with bone marrow stromal cells and exposed to 1  $\mu$ M DMBA. Apoptosis was accompanied by the activation of caspase-3 and was suppressed by a caspase-3 inhibitor. DMBA treatment also activated caspase-8, although several results indicated that caspase-8 was neither the most proximal caspase activated nor was its activation dependent on TNF receptors or on Fas: 1)

DMBA did not induce TNF- $\alpha$ , TNF- $\beta$ , or LT- $\beta$  mRNA and/or protein expression in bone marrow stromal cells, 2) DMBA-induced apoptosis was not mitigated in BU-11 cells cultured with TNF- $\alpha^{-/-}$  bone marrow stromal cells, 3) the level of primary pro-B cell apoptosis was not affected by deletion of TNF- $\alpha$ , TNFR1/R2, or Fas in the B cells. In contrast, caspase-8 activation appeared to be caspase-3-dependent since it occurred only after caspase-3 activation and was suppressed by a caspase-3-specific inhibitor. Results presented here demonstrate that the BU-11 system is an accurate model of DMBA-induced primary pro-B cell apoptosis and that caspase-8 is likely activated by a more proximal caspase (e.g. caspase-3) and not by a TNFR family member. This work was supported by RO1-ES06086 and P01-ES11624.

THE ROLE OF PAH METABOLISM IN AHR-DEPENDENT INHIBITION OF HUMAN B CELL PROLIFERATION.

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Polycyclic aromatic hydrocarbons (PAHs), ubiquitous environmental hydrocarbons, are immunosuppressive and carcinogenic AhR ligands. Previous studies indicated that activation of human B cells with surrogate T cell signals, CD40 ligand (CD40L) and IL-4, profoundly up-regulates AhR expression and activity. This led to the hypothesis that such activated B cells would be more sensitive to environmental AhR agonists, potentially through increased activity of the AhR-dependent CYP1A1 enzyme and agonist metabolism. To test this directly, CD40L / IL-4-activated human B cells were treated with AhR ligands that are either readily metabolizable (B[a]P) or relatively non-metabolizable (TCDD), and then evaluated for CYP1A1 induction and changes in cell function. Treatment with either AhR ligand significantly increased CYP1A1 expression. However, we found that only B[a]P significantly reduced the proliferation of activated B cells, whereas TCDD did not. Thus, AhR activation per se is not entirely sufficient for the suppression of B cell growth, instead, AhR-induced CYP1A1-mediated metabolism of the agonist B[a]P is also responsible for growth suppression. This hypothesis was supported by subsequent observations: 1) 1-PP, a CYP1A1 inhibitor, completely protected CD40L IL-4-activated B cells from B[a]P-induced growth suppression; 2) direct application of the B[a]P metabolites B[a]P-7, 8-dihydrodiol or BPDE potently inhibited B cell proliferation; 3) growth inhibition induced by the B[a]P-7, 8-dihydrodiol, which is metabolized into BPDE by CYP1A1, was inhibited by 1-PP while 1-PP had no effect on BPDE-induced growth inhibition. These data suggest that expansion of activated B cell clones are particularly sensitive to metabolizable AhR ligands, such as B[a]P, and that this sensitivity is mediated by the AhR-induced CYP1A1-mediated generation of reactive end products, such as BPDE, that are both carcinogenic and immunosuppressive. (Supported by RO1-ES06086 and P01-ES11624.)

DISTINCT EFFECTS OF TGF-B1 ON THE SURVIVAL AND DIVISION OF CD4+ AND CD8+ T CELLS: A ROLE FOR T CELL INTRINSIC SMAD3.

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TGF-b1 is critical for maintaining T cell immune homeostasis as illustrated by multi-organ inflammation in mice deficient in TGF-b1 signaling, yet the cellular targets and molecular details remain poorly understood. TGF-b1 signals through transmembrane receptor serine/threonine kinases to activate multiple intracellular effector molecules, including the cytosolic signaling transducers of the Smad protein family. Using a loss-of-function model to define the role of TGF-b1-induced Smad3 signaling in the regulation of CD4+ and CD8+ T cell expansion, we show found that TGF-b1 inhibits the entry of CD4+ and CD8+ T cells into the cell cycle as well as their progression through subsequent rounds of divisions. However, Smad3 was essential for TGF-b1 to inhibit TCR-induced division of CD4+ but not CD8+ T cells. Not all CD8+ T cell responses to TGF-b1 are Smad3-independent as demonstrated by an inability of TGF-b1 to suppress IL-2 production in both Smad3-/- CD4+ and CD8+ T cells. Neutralization of IL-2 signaling did not alter the nonresponsiveness of Smad3-/- CD4+ T cells to TGF-b1-induced growth arrest. The Smad3-dependent effects of TGF-b1 were all T cell intrinsic as they were reproduced in purified CD4+ and CD8+ T cells stimulated with plate-bound anti-CD3 + anti-CD28. We also found that Smad3-/- CD8+ T cells were more susceptible to TCR-induced apoptosis and exogenous TGF-b1 partially rescued this CD8+ selective death through a Smad3-independent pathway. Collectively, these findings establish for the first time that TGF-b1 discriminatory regulates CD4+ and CD8+ T cell expansion by signaling through distinct intracellular pathways.

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EXPOSURE TO TCDD AUGMENTS CD25 EXPRESSION ON RECENTLY ACTIVATED T CELLS.

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TCDD is a potent immunosuppressive chemical that mediates its immunotoxic effects through binding to the AhR, a ligand-activated transcription factor that is expressed in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Previous studies have shown that the distal promoter of the *interleukin (IL)-2* gene has three AhR-response elements (ARE) and that IL-2 production may be enhanced by TCDD in activated T cells. Because IL-2 mediates both survival and death signals in activated T cells, changes in the IL-2 signaling pathway by TCDD may play an important role in its immunotoxicity. IL-2 signaling in T cells is regulated by the expression of the high-affinity IL-2 receptor, CD25. CD25 itself is dose-dependently upregulated by IL-2. To determine if TCDD exposure alters expression of CD25, we used flow cytometry to measure CD25 on CD4<sup>+</sup> and CD8<sup>+</sup> donor T cells during the first five days of an acute graft-versus-host response. F1 host mice were treated with vehicle or TCDD one day prior to donor T cell injection. TCDD exposure significantly increased the percentage of CD4<sup>+</sup> and CD8<sup>+</sup> donor T cells that expressed CD25, with a maximum increase observed on day 2. Using CFSE to track cell division, the increased percentage of CD25<sup>+</sup> T cells was apparent by the second cell division and was maintained through four divisions. The median expression level of CD25 also increased with each round of cell division in TCDD-treated mice. When donor T cells were obtained from AhR<sup>-/-</sup> mice, TCDD did not alter CD25 expression on CD4<sup>+</sup> T cells whereas a small increase was still observed in CD8<sup>+</sup> T cells. When CD4<sup>+</sup> and CD8<sup>+</sup> donor cells from AhR<sup>+/+</sup> and AhR<sup>-/-</sup> mice were purified and recombined, the induction of CD25 expression on CD4<sup>+</sup> T cells was driven by the AhR in CD4<sup>+</sup> T cells. These results are consistent with an ARE-mediated increase in IL-2 production in CD4<sup>+</sup> T cells acting in an autocrine or paracrine fashion to enhance CD25 expression on CD4<sup>+</sup> T cells. AhR-mediated regulation of CD25 expression on the CD8<sup>+</sup> T cells was not clearly delineated. This work was supported by NIH grant P01ES00040.

**1874**

Δ<sup>9</sup>-TETRAHYDROCANNABINOL (Δ<sup>9</sup>-THC) ELICITS A CALCIUM ELEVATION IN T CELLS THROUGH THE TRPC CHANNELS.

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Cannabinoid compounds have been widely reported to alter immune function. Previous studies from this laboratory have shown T cells to be a sensitive target to cannabinoid treatment. The objective of the present studies was to examine the effect of Δ<sup>9</sup>-THC, the primary psychoactive constituent in *Cannabis sativa*, on the regulation of intracellular calcium ([Ca<sup>2+</sup>]<sub>i</sub>) in HPB-ALL cells, a human CB2 expressing T cell line. Δ<sup>9</sup>-THC (10-12.5 μM) induced a robust rise in [Ca<sup>2+</sup>]<sub>i</sub> in resting HPB-ALL cells, which was antagonized upon pretreatment with the CB2 receptor antagonist, SR144528. Studies performed in the absence of extracellular calcium revealed that the Δ<sup>9</sup>-THC-mediated elevation in [Ca<sup>2+</sup>]<sub>i</sub> was due to influx of extracellular calcium. Furthermore, pretreatment with thapsigargin to deplete intracellular calcium pools did not abrogate the Δ<sup>9</sup>-THC-mediated elevation in [Ca<sup>2+</sup>]<sub>i</sub>, indicating that Δ<sup>9</sup>-THC-mediated rise in [Ca<sup>2+</sup>]<sub>i</sub> was independent of calcium stores. Pretreatment of cells with inhibitors of calcium channels revealed that the Δ<sup>9</sup>-THC-mediated elevation in [Ca<sup>2+</sup>]<sub>i</sub> was more sensitive to the receptor-operated calcium channel blocker, SKF96365, than to the store-operated calcium channel blocker, LaCl<sub>3</sub>. Recently, several members of the TRPC (transient receptor potential canonical) subfamily of cation channels have been implicated in the formation of store- and/or receptor-operated calcium channels. RT-PCR analysis for the TRPC channel subfamily demonstrated that HPB-ALL cells express TRPC1, a channel gated by diacylglycerol (DAG). Treatment of HPB-ALL cells with OAG, a DAG analog, led to a rise in [Ca<sup>2+</sup>]<sub>i</sub>. Moreover, when cells were sequentially treated with OAG followed by Δ<sup>9</sup>-THC, Δ<sup>9</sup>-THC failed to elicit a further rise in [Ca<sup>2+</sup>]<sub>i</sub>. Together these data suggest that Δ<sup>9</sup>-THC elicits a cannabinoid receptor-dependent elevation in [Ca<sup>2+</sup>]<sub>i</sub> in resting T cells independently of calcium store depletion, putatively through the OAG-gated TRPC1 channels. (Supported by NIH grants DA07908 and DA016828)

**1875**

SODIUM ARSENITE DECREASES PROLIFERATION IN PHA-STIMULATED LYMPHOCYTES, BY INHIBITING INTERLEUKIN-2 EXPRESSION AND SECRETION.

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Evidence from human and animal models have shown that arsenic (As) induces suppression of T lymphocytes. We have previously shown that sodium arsenite inhibited phytohemagglutinin (PHA)-induced proliferation in murine T lympho-

cytes (Goytia-Acevedo et al., 2003) probably by a mechanism mediated by high intracellular Ca<sup>2+</sup> [Ca<sup>2+</sup>]<sub>i</sub> level. On the other hand, it has been shown that sodium arsenite reduces secretion of IL-2 in human lymphocytes and the expression of its receptor (Yu et al., 1998); however the effect of arsenite on IL-2 gene transcription has not been evaluated. Thus, we evaluated, the effect of sodium arsenite on IL-2 mRNA expression by RT-PCR, and IL-2 secretion by ELISA in PHA-stimulated spleen T lymphocytes obtained from female C57BL/6 mice. T lymphocyte activation was measured through CD69 expression by cytofluorometry, and ERK1/2 phosphorylation was determined by immunoblotting. Sodium arsenite (1 and 10 μM) significantly inhibited IL-2 mRNA expression and IL-2 secretion in a concentration-dependent manner in PHA-stimulated lymphocytes (24 h). Interestingly, in PHA-stimulated lymphocytes (4 h), sodium arsenite (1 and 10 μM) significantly inhibited (75%) CD69 expression. These results were in agreement with our previous data showing that sodium arsenite (1 and 10 μM) inhibited PHA-induced ERK1/2 phosphorylation in a concentration dependent manner (Conde-Moo et al., 2003). Our results suggest that sodium arsenite modulate IL-2 gene expression probably by interfering with ERK1/2 signaling, and this may be related, at least partially, to the inhibition of PHA-induced proliferation of T lymphocytes. Further research on the relationships between As-induced [Ca<sup>2+</sup>]<sub>i</sub> imbalance and the ability of As to modulate IL-2 gene expression is needed. This work was partially supported by the Mexican Council for Science and Technology (Conacyt 34508-M).

**1876**

MECHANISMS OF 4-HYDROXYNONENAL-HNE (LIPID HYDROPEROXIDE) INDUCED APOPTOTIC DEATH IN CD4<sup>+</sup> T LYMPHOCYTES: RELEVANCE TO HIV AND HCV PATHOGENESIS.

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During the process of lipid peroxidation, several reactive low-molecular weight products are formed, including reactive aldehydes as 4-hydroxynonenal (HNE). 4-HNE, one of the major aldehydic products of the peroxidation of membrane ω-6 polyunsaturated fatty acids, has been suggested to contribute to oxidant stress mediated cell injury. 4-HNE is associated with multiple pathophysiological conditions causing immune dysfunction, such as HIV and HCV infection. CD4<sup>+</sup> T lymphocytes are the central regulators of both cell-mediated and humoral immune responses and loss of their survival can affect multiple immune functions. Since both lipid peroxidation and loss of CD4<sup>+</sup> T lymphocytes are observed during the clinical progression of HIV and HCV infection, the present work was undertaken to examine the effects of 4-HNE on CD4<sup>+</sup> T cell survival. The data obtained showed that exposure of Jurkat CD4<sup>+</sup> T cells to 4-HNE induces significant loss of survival due to the induction of apoptotic cell death. Further investigation showed that 4-HNE down-regulates Akt kinase activation, and results in enhanced FasL expression (mRNA and protein). In turn enhanced FasL expression led to the induction of the death initiating signaling complex (DISC) as documented by the correspondent decrease in c-FLIPs protein and caspase-8 activation. These data identify the pathogenic molecular mechanism(s) induced by 4-HNE in CD4<sup>+</sup> T cells and are consistent with a potential role of lipid peroxidation-derived products in programmed cell death. Importantly, this work has begun to elucidate the potential role of lipid peroxidation (4-HNE) in the pathogenesis of immunosuppressive disorders involving CD4<sup>+</sup> T cell depletion such as HIV and HCV infection and can lead to the development of the much needed anti-oxidant therapies.

**1877**

MECHANISMS OF LEAD-INDUCED IMMUNOTOXICITY: THE ROLE OF IL-2 IN ALLO-ENHANCEMENT.

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Despite great efforts to remove lead (Pb) from the environment it continues to be implicated in various adverse health effects. Pb is a documented immunotoxicant and, T cell-mediated processes are believed to be particularly sensitive to it. Using an allogeneic mixed lymphocyte culture system, which measures T cell proliferation in response to peptide/MHC complexes, we have observed marked enhancement (approximately 3 fold) of T cell proliferation in the presence of Pb. Since interleukin 2 (IL-2) is an autocrine growth factor that drives T cell proliferation, we sought to determine whether an increase in IL-2 production was responsible for this allo-enhancement. The kinetics (days 1-6) of IL-2 production in control versus Pb-treated allo-MLC were measured by bioassay. These experiments revealed that the initial production of IL-2 was not affected by Pb; however, IL-2 levels declined more rapidly in the Pb-exposed splenocyte cultures compared to control. This more rapid decline in IL-2 levels raised the issue of whether subpopulations of cells were utilizing IL-2 and if this utilization of IL-2 served as the basis of increased proliferation. To address this issue, splenocytes and CD4<sup>+</sup> T cells set up in allo-MLCs were cultured in the presence and absence of Pb as well as with neutralizing levels of anti-IL-2. Anti-IL-2 added on day 0 markedly suppressed T cell proliferation in both

control and Pb-treated allo-MLC. Anti-IL-2 added on day 3 partially attenuated, but did not ablate, the Pb-induced enhancement of allo-reactive T cell proliferation; suggesting that allo-enhancement is both IL-2 dependent and independent. Due to the fact that Pb did not cause an increase in IL-2 levels and that IL-2 dependent proliferation is not the major basis of allo-enhancement, another cytokine IL-4, was considered. Pb allo-enhancement was not affected by anti-IL-4. Thus, allo-enhancement due to Pb exposure is not a result of increased IL-2 production or the work of a common Th2 cytokine.

**1878 ENFUVIRTIDE: *IN VITRO* STUDIES OF PUTATIVE IMMUNOTOXIC EFFECTS RELATED TO ACTIVATION OF N-FORMYL PEPTIDE RECEPTORS.**

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*In vitro* studies suggest enfuvirtide (ENF) may suppress monocyte IL-12 secretion by activating N-formyl-peptide receptors (FPRs). If seen *in vivo*, this effect could increase the susceptibility of ENF-treated HIV patients to opportunistic infections. This series of studies examined potential FPR-related immunomodulatory effects of ENF *in vitro*. Methods: To assess the effect of ENF on cytokine secretion by human monocytes and dendritic cells (DCs), purified cell fractions were incubated with varying concentrations of ENF, N-formyl-methionyl-leucyl-phenylalanine (fMLP; + control) or TPI (neg. control). Cells were stimulated with IFN- $\gamma$  + fixed *S. aureus* or LPS. After 24 hrs, culture supernatants were assessed for IL-12 p70 and IL-10 levels. Human neutrophils were pre-treated with ENF, TPI or GM-CSF (+ control), or untreated, before activation with LPS or LPS + IFNg. Levels of IL-6, IL-8, IL-10 & TNF $\alpha$  in the supernatant were measured using a human inflammation CBA assay. Similar or analogous cytokine assessments were performed on mice and rat neutrophils. To assess DC maturation, immature DCs were cultured with fMLP or ENF then stimulated into maturation with LPS or TNF $\alpha$ . The expression of FPRs and surface markers characteristic of mature DCs was quantified using flow cytometry. Results: ENF did not significantly affect secretion of IL-12 p70 or IL-10 by FPR-positive human monocytes. Similar results were obtained for mature, FPR-negative DCs. However, monocyte IL-12 secretion declined when an artificial pH drop was induced in the with acetic acid, a solvent for ENF. ENF did not appreciably affect secretion of IL-8, IL-6 or TNF $\alpha$  by human neutrophils, or cytokines from mouse or rat neutrophils. ENF did not induce up-regulation of DC surface markers, CD83 or co-stimulatory molecules, suggesting that ENF does not interfere with DC maturation. Conclusion: This study suggests that ENF is unlikely to exert an immunosuppressive effect on the key cells of the innate or adaptive immune system.

**1879 INFLAMMATORY RESPONSES IN MOUSE RAW264.7 MACROPHAGES CAN BE ALTERED BY INTERACTIONS BETWEEN INDOOR AIR MICROBES IN SIMULTANEOUS EXPOSURE.**

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Indoor air exposure in moisture-damaged buildings consists of a complex mixture of fluctuating concentrations of various microbial species and other indoor air pollutants. Adverse health effects in such environments are usually associated with inflammatory response. To elucidate the influence of microbial interactions on inflammatory responses, the effects of simultaneous exposure with modified proportions of actinomycete *Streptomyces californicus* and fungus *Stachybotrys chartarum* were studied in mouse RAW264.7 macrophages. Cells were co-exposed for 24 hours to the total dose of  $3 \times 10^5$  spores/ml in five different proportions (*Str. californicus*:*S. chartarum* 10:1; 5:1; 1:1; 1:5; 1:10). In addition, the macrophages were exposed to the spores of these microbes separately at the respective doses. The production of cytokines (MIP2, IL-6 and TNF $\alpha$ ) was measured immunochemically, and nitric oxide (NO) was assayed by the Griess method. When the co-exposure contained more of the fungal (*S. chartarum*) than the bacterial (*Str. californicus*) spores, even more than 3-fold synergistic increase in all the measured cytokine concentrations was detected compared to the sum response caused by these microbial spores separately. When the co-exposure contained an equal number (1:1) or more (10:1) of bacterial than fungal spores, synergistic increase was only seen in the MIP2 concentrations (60 % and 30 % increase respectively). Instead, the antagonistic NO production was detected when co-exposure contained more bacterial than fungal spores (5:1) compared to the sum response induced by the microbial spores separately (40 % decrease). The present results revealed that mutual proportion of fungal and bacterial spores in simultaneous exposure affect the nature of their interactions leading to increased or suppressed production of inflammatory mediators in RAW264.7 macrophages.

**1880**

**SIMULTANEOUS EXPOSURE TO MOLDY HOUSE MICROBES *STREPTOMYCES CALIFORNICUS* AND *STACHYBOTrys CHARTARUM* CAUSE SYNERGISTIC INFLAMMATORY RESPONSES IN HUMAN WHOLE BLOOD CELL CULTURE.**

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Microbial exposure in moisture damaged building consists of a complex mixture of compounds, including bacteria, fungi and their metabolites. Synergistic interactions between moldy house microbes *Streptomyces californicus* and *Stachybotrys chartarum* have been recently shown in mouse macrophages *in vitro*. In this study, we measured production of tumor necrosis factor alpha (TNF- $\alpha$ ), interferon-gamma (IFN- $\gamma$ ) and interleukin-6 (IL-6) in human whole blood cell culture after 24 hour exposure to spores of *S. chartarum* and two microbial toxins (vomitoxin and valinomycin) alone and together with spores of *Str. californicus*. Lipopolysaccharide (LPS) was used as a positive control. Data are reported as mean $\pm$ SD pg/10<sup>6</sup> leukocytes. Spores of *Str. californicus* ( $5 \times 10^4$  spores/ml) were alone capable of inducing production of IFN- $\gamma$  ( $160 \pm 100$ ), TNF- $\alpha$  ( $500 \pm 250$ ) and IL-6 ( $2100 \pm 600$ ). Co-exposure to *Str. californicus* ( $5 \times 10^4$  spores/ml) together with *S. chartarum* ( $10^5$  spores/ml) caused a synergistic (1.7-fold) increase in the production of IL-6. Also LPS (0.1 mg/ml) alone triggered TNF- $\alpha$  and IL-6 responses in whole blood cell culture ( $1400 \pm 400$  and  $6100 \pm 1200$ , respectively) and simultaneous exposure with valinomycin (10 ng/ml and 50 ng/ml) caused a slight increase in the production of these cytokines. In conclusion, moldy house microbe *Str. californicus* evoked inflammatory responses in human blood cells *ex vivo* and these responses were potentiated by *S. chartarum*. Current results are in line with *in vitro* findings and support the view that the synergistic interactions between different exposure agents may explain the health complaints already at the relatively low microbial concentrations in moisture damaged buildings.

**1881**

**THE BIOLOGICAL ACTIVITY OF GRAM-POSITIVE BACTERIA *STREPTOMYCES CALIFORNICUS* CANNOT BE COMPLETELY INHIBITED WITH LPS -INHIBITOR PMX.**

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The microbial characteristics of airborne particulate matter (PM) are poorly known. Studies on microbial components have mainly concentrated on the microbial cell wall fragment lipopolysaccharide (LPS). However, components of other microbes in PM such as gram-positive bacteria *Streptomyces californicus* may have equally potent effects. Thus, if the activity of the biological fraction of the PM is evaluated only by using LPS inhibitors such as Polymyxin B (PMX), the remaining effects may be erroneously interpreted to originate from inorganic fraction of the PM while it can actually be the biological fraction other than LPS causing the effects. To test this hypothesis, we studied the effects of LPS inhibitor PMX on the production of inflammatory mediators induced by spores of gram-negative bacteria *Pseudomonas fluorescens* ( $10^5$  microbes/ml) and gram-positive bacteria *Streptomyces californicus* ( $10^6$  microbes/ml). Mouse macrophages (RAW264.7) were exposed to either of these microbes with increasing doses (0, 5-20  $\mu$ g/ml) of PMX. Inflammatory mediators were measured from the cell culture medium; cytokines (TNF $\alpha$  and IL-6) immunochemically and nitric oxide (NO) by Griess-method. Viability of the cells was determined by using the MTT-test. Exposure to the spores of *P. fluorescens* increased markedly the production of all inflammatory mediators in mouse cells. PMX inhibited dose-dependently the production of these markers, e.g. the production of IL-6 decreased  $93 \pm 4$  % from  $2200 \pm 240$  pg/ml. The spores of *S. californicus* increased the production of IL-6, TNF $\alpha$  and NO as well, but PMX was not able to inhibit totally the production of these mediators; the production of IL-6 decreased  $61 \pm 9$  % from  $1400 \pm 270$  pg/ml. Altogether, these data indicate that immunotoxic effect induced by *Streptomyces californicus* in mouse macrophages cannot be completely blocked by PMX. This should be taken into account when considering the role of biologically active components of PM in immunotoxic activity.

**1882**

***IN VITRO* AND *IN VIVO* IMMUNOLOGIC RESPONSES BY POLYACRYLAMIDE NANOPARTICLES TO MH-S CELLS AND RATS.**

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Nano-scale technologies are currently being employed by a variety of industries and incorporated into pharmaceuticals, electronics and cosmetics. The development of nanoparticles for enhanced drug delivery offers the potential for increased speci-

ficiency of currently used pharmaceuticals, while decreasing the total dose required. Polyacrylamide (PAA) nanoparticles are being developed for enhanced MR imaging, drug delivery, photodynamic therapy, and real time analysis of intracellular measurements at the level of a single cell. Evaluation of histopathologic changes and clinical chemistry provides strong evidence that PAA nanoparticles are well tolerated by rats at intravenous doses up to 500 mg/kg; while pharmacokinetic data implicates the reticular endothelial systems in the clearance of this material. To further investigate the cellular (macrophage) responses of a nano-scale polymer that has been accumulated by an immune cell, the mouse alveolar macrophage cell line, MH-S, was used to study the effect on cellular responses such as; viability, morphology, and the induction of cytokines and chemokines. Furthermore, tissue recruitment of antigen presenting cells (dendritic) was evaluated in liver and spleen. In culture, data suggests that the PAA nanoparticles are well tolerated by the MH-S cells. Scanning electron micrographs show changes in the morphology of MH-S cells at 24h and 48h with significant rounding and retraction of phagopodia suggesting effects on cellular adherence properties. Measurements of secreted TNF- $\alpha$  and MIP-1- $\alpha$  by MH-S were not detectable at significant levels by ELISA. Finally, fluorescently labeled nanoparticles were detected in hepatic but not splenic lymph nodes and positive staining for the dendritic cell marker, OX-62 was detected in liver but not spleen 14 days after exposure to PAA nanoparticles. These data suggest that PAA nanoparticles are targets for phagocytosis by macrophages and may elicit inflammatory responses *in vivo* and *in vitro*.

### 1883

#### IMPACT OF EXPOSURE TO JET A JET FUEL ON CYTOKINE PRODUCTION IN RAT LUNG MACROPHAGES.

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A variety of adverse health effects have been reported in military and civilian fuel handlers which have been attributed to exposure to jet fuel mixed atmospheres. Many of the reported effects are similar to those produced by endotoxin (LPS). Both commercial and military fuel stores have been shown to be contaminated by LPS in addition to other microbial products. The nature and degree of contamination may be influenced by a variety of factors, including the age of the jet fuel, as lengthy storage conditions may allow for microbial growth to occur with a concomitant increase in LPS levels. In a previous study, we had utilized aged JP-8 jet fuel. Exposure of rat lung macrophages to this aged JP-8 led to a significant increase in production of the pro-inflammatory cytokines IL-6 and TNF- $\alpha$ , compared to the control group. Filtration of the JP-8 prior to exposure resulted in a reduction in the levels of these cytokines, but the reduction was not statistically significant. Since the length of time the JP-8 was allowed to age may not accurately reflect what typically occurs under real world conditions, we carried out another set of exposures, but with fresh jet fuel. In place of JP-8, we utilized Jet A, which differs from JP-8 in that it lacks the additive packages present in JP-8. In this study, we exposed cultured rat lung macrophages to samples of Jet A, samples of Jet A that had been filtered to remove microbial contamination including LPS, and filtered Jet A which had been spiked with a known quantity of LPS. We evaluated the spent media for levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  cytokines in the spent media. Our preliminary findings have been similar to what was observed with the JP-8 exposures, with a significant increase in levels of the pro-inflammatory cytokines IL-6 and TNF- $\alpha$ , in all three JP-A groups, as compared to the control group. Filtration of the Jet A appears to have resulted in a small reduction in levels of these cytokines which was not statistically significant.

### 1884

#### MACROPHAGES ARE THE PROXIMAL TARGET CELL IN LEAD-INDUCED IMMUNOTOXICITY.

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Lead (Pb) is a persistent environmental contaminant and a known immunotoxin. Although Pb is immunosuppressive *in vivo*, many indices of immune function *in vitro* are, paradoxically, enhanced by Pb. In particular, CD4 $^{+}$  T cell proliferation in mixed lymphocyte culture (MLC) is markedly enhanced. The goal of this study was to identify Pb's target cell in MLC in which BALB/c-derived CD4 $^{+}$  T cells responded to allo-antigen presenting cells (APC). We found that Pb allo-enhancement was abrogated when APCs were paraformaldehyde-fixed prior to use as stimulators in MLC. Also, CD4 $^{+}$  T cell proliferation in response to the T cell mitogen, concanavalin A, was not enhanced with Pb treatment. These data ruled out a direct effect of Pb on CD4 $^{+}$  T cells. We next sought to identify which APC type mediated Pb allo-enhancement. Using magnetic bead separation and adherence properties of 3 APC types, B cells, macrophages, and dendritic cells (DC) were used, alone, or in combination as stimulators in MLC. Allogeneic B cells, alone, were not able to stimulate enhanced proliferation in alloreactive CD4 $^{+}$  T cells with Pb treatment. Pb allo-enhancement was restored when CD11c-enriched, syngeneic splenocytes were added to these cultures. Of the CD11c-enriched splenocytes, the adherent fraction

appeared to be most critical for Pb allo-enhancement suggesting that DC or macrophages were the proximal target of Pb. Based on differential adherence properties between macrophages and DC, we suspect that the macrophage is more responsible for the allo-enhancing effects of Pb. The results of these experiments suggest a scenario wherein enhanced cross-priming of syngeneic macrophages with allogeneic B cells is the underlying bases of Pb allo-enhancement. These findings implicate the macrophage as a key cellular target in Pb immunotoxicity, and begin to define the mechanism(s) whereby Pb exerts deleterious effects on T cell function. Although speculative, we envision that this scenario may have implications in transplantation immunology.

### 1885

#### WOOD DUST-INDUCED PULMONARY INFLAMMATION IN MICE.

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In addition to nasal and sino-nasal adenocarcinomas, wood dust exposure can induce nonmalignant, mainly respiratory diseases such as asthma and chronic bronchitis. In the present study, we have elucidated the mechanisms by which exposure to wood dust induces inflammatory responses in murine lungs. BALB/c mice were exposed to intranasally administered birch or oak dusts twice a week for three weeks. TiO<sub>2</sub> and LPS were used as controls. Inflammatory cell infiltration was characterized from bronchoalveolar lavage (BAL) fluid. Cytokine, chemokine, and chemokine receptor mRNA expression in lung tissue was assessed by real time PCR. Bronchial responsiveness to methacholine was determined by whole body plethysmography. Intranasal installation of birch or oak dusts elicits in mice influx of inflammatory cells to the lungs. Lymphocytes dominated after oak dust exposure, whereas eosinophil infiltration was higher after birch dust exposure. Infiltration of inflammatory cells was associated with an increase in the mRNA levels of several cytokines, chemokines, and chemokine receptors in lung tissue. Oak dust appeared to be a more potent inducer of these inflammatory mediators than birch dust. Methacholine induced bronchial responsiveness was not altered significantly after wood dust installation. Our findings suggest that exposure to wood dust may significantly influence development of inflammatory process in the airways by modulating the expression of proinflammatory cytokines and chemokines. Supported by the EU 5th Framework Programme Grant QLK4-2000-00573.

### 1886

#### ATRAZINE-INDUCED INHIBITION OF NK-CELL MEDIATED CYTOTOXICITY.

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Atrazine (ATR) is a chlorinated triazine compound used extensively as an herbicide in the United States. As a result of this extensive use ATR is the most commonly encountered water contaminant in the United States. ATR exposure models, both *in vivo* and *in vitro*, have demonstrated multiple forms of immune suppression. Our preliminary data, in addition to work published by others, has shown that ATR exposure inhibits lymphocyte mediated cell lysis. An ATR-induced reduction of cytotoxic activity has been observed in both human natural killer cells and mouse CD8 $^{+}$  lymphocytes. Earlier published studies failed to identify a mechanism for ATR toxicity or subcellular target(s) of ATR exposure. Therefore, the goal of our study was to determine the mechanism of ATR-induced inhibition of cell mediated lysis by better characterizing the toxicological phenotype of ATR-exposed lymphocytes. Human peripheral blood lymphocytes were cultured *in vitro* with concentrations of ATR ranging from 30 to 0.03 $\mu$ M. No change in viability at any of the assayed concentrations was measurable. As others have described, a reduction in NK cell specific lysis was observed. However, we have extended these previous studies as described below. a) We noted a reduced T cell IL-2 production as result of allogeneic cell stimulation. IL-2 is potent modulator of NK cell function. b) A reduction in NK cell specific lysis in ATR containing cultures which had not been stimulated to produce IL-2 was apparent. c) The ability of NK cells to stably bind the NK-specific K562 target cells in the absence of *in vitro* IL-2 stimulation was also found to be reduced. This data suggests that ATR directly decreases the lytic potential of human NK cells. We hypothesize that ATR-exposed NK cells have reduced levels of lytic proteins. We plan to further test this hypothesis by assessing expression levels of these proteins. Supported by NIEHS and NIOSH grants.

### 1887

#### ALTERATIONS IN CYTOKINE PRODUCTION IN THE PRESENCE OF THE PESTICIDES, BENTAZON, ISOXAFLUTOLE AND TERBUFUS.

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Millions of pounds of pesticides are applied annually around the world to protect crops, lawns and gardens from unwanted weeds and insects. As a result of their widespread use pesticides are common contaminants of water supplies. Even

though extensive screening of pesticides for potential health risks are performed prior to their release for use by the general public in many cases the immunotoxic potential has not been fully evaluated. We have chosen to evaluate three pesticides, bentazon, isoxaflutole and terbufus for their immunotoxicity. Bentazon, a post-emergence herbicide, is used to control broadleaf weeds in soybean, corn, peanuts and rice fields and is a common contaminant of water supplies. Isoxaflutole, a pre-emergence herbicide, is usually applied with atrazine to corn and sugarcane fields. Terbufus, an organophosphate insecticide, is used on corn, sugar beets, grain, sorghum, soybean, wheat, rice and cotton fields. Although terbufus is tightly regulated, it too is found in the water supply. Cytokines are important mediators of the immune response. Thus, they can be used to evaluate the immunotoxic potential of chemicals. To determine if these three herbicides have the potential to be immunotoxic, we assayed their effect on interleukin-2 (IL-2) secretion by the human T cell lymphoma Jurkat. All three pesticides inhibited IL-2 secretion by phorbol ester and calcium ionophore stimulated Jurkat cells. Bentazon and isoxaflutole decreased IL-2 secretion by the Jurkat cells at a concentration of 100  $\mu$ M. However, lower doses of bentazon (5  $\mu$ M) actually increased IL-2 secretion by Jurkat cells. When Jurkat cells were exposed to terbufus (5-100  $\mu$ M), a decrease in IL-2 secretion was also observed. Together these data suggest that bentazon, isoxaflutole and terbufus appears to be immunotoxic. Supported by grant P20-RR16477 from the National Center for Research Resources award to WV Biomedical Research Infrastructure Network.

## 1888

### THE FUNGICIDES

#### ETHYLENEBISDITHIOTOCARBAMATES INHIBIT NF-KB ACTIVATION AND CYTOKINE PRODUCTION IN THE HUMAN PROMYELOCYTIC CELL LINE THP-1.

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Literature data indicate that ethylenebisdithiocarbamates may have immunomodulatory effects. The purpose of this work was to characterize the molecular mechanism of action of ethylenebisdithiocarbamates underlying their inhibitory effect on lipopolysaccharide (LPS)-induced TNF-alpha and IL-8 release. The human promyelocytic cell line THP-1 was used throughout the study. Cells were treated with increasing concentrations of mancozeb, zineb and ziram different times. TNF-alpha and IL-8 release was evaluated following LPS stimulation by specific sandwich ELISA. Cell viability was assessed by measuring lactate dehydrogenase leakage. All tested fungicides, at non cytotoxic concentrations, induced a dose and time related inhibition of LPS-induced TNF-alpha and IL-8 release. It has been shown that LPS triggers multiple signaling molecules, such as protein-tyrosine kinase, protein kinase C, Ras, Raf-1, I<sub>K</sub>B kinase, MEK, mitogen-activated protein kinases, etc. Subsequently, the signals further transduce to downstream pathway and activate numerous transcription factors, including AP-1, NF-<sub>k</sub>B and ATF-2. This in turn induces a large amount of genes encoded for inflammatory mediators and cytokines. There are several indications that reactive oxygen species may act as a cellular secondary messenger and it has been demonstrated that H<sub>2</sub>O<sub>2</sub> can activate NF-<sub>k</sub>B, which in turn regulates the expression of many immune and inflammatory molecules, including TNF-alpha. In order to define the molecular target of ethylenebisdithiocarbamates, their effect on LPS-induced NF-<sub>k</sub>B activation were evaluated by Western blot analysis. We demonstrated that all three fungicides, interfering with ROS generation, directly inhibit NF-<sub>k</sub>B activation, preventing I-<sub>k</sub>B degradation and NF-<sub>k</sub>B p65 and p50 nuclear translocation, this in turn results in decreased cytokine production in monocytes.

## 1889

### ANALYSIS OF THE PROTEOMIC CHANGES INDUCED BY PROPANIL ON A MACROPHAGE CELL LINE.

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DCPA (3, 4-dichloropropionaniline) is a post-emergent herbicide used extensively to control weeds in rice. Previous studies have demonstrated that treatment of the IC-21 macrophage cell line with DCPA resulted in changes of critical macrophage functions, including a decrease in cytokine production, and in the ability to phagocytize and kill bacteria. To understand the mechanism involved in the propanil induced changes, a proteomics analysis was undertaken. For this study, IC-21 cells were exposed to 100  $\mu$ M propanil or ethanol (vehicle) and stimulated with lipopolysaccharide. After 30 min of exposure, cytosolic fraction was isolated and analyzed by two-dimensional gel electrophoresis. Differential statistical analysis identified 12 spots that were all decreased in the propanil exposed cells compared to vehicle. Mass spectrometry analysis was used to identify the proteins, which was later confirmed by other methods. One of these proteins, Sorting Nexin 6 (SNX6)

interacts with the TGF-beta family of receptor serine-threonine kinases and associates with early endosomes during intracellular receptor trafficking. Another protein, H<sub>+</sub>-ATPase functions as a cytoplasmic pH-regulator promoting acidification of intracellular compartments in macrophages during the respiratory burst. A third protein, fibroblast growth factor regulated-1 (FR-1) protein induced by FGF-1 stimulation is important for enhancement of phagocytosis and plays a role in promoting inflammation. Confirmation of other significantly changed proteins and linking their identity to the alterations in macrophage function is currently in progress. Together the data from toxicoproteomics analysis provides an additional evidence of the mechanism by which propanil suppresses macrophage functions. Supported by the USPHS-NIEHS and NCI.

## 1890

### ALTERED ACTIVATION OF T CELLS BY SILICA-EXPOSED ANTIGEN-PRESENTING CELLS.

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The classic immune response consists of vital cross-talk between an antigen presenting cell (APC) and a T cell (TC). Antigen is incorporated in the MHC class II molecule and expressed on the surface of the APC for TC stimulation. The resulting TC response includes upregulation of cytokine production, surface marker and receptor expression, and cell proliferation. Previous work has shown that crystalline silica appears to increase the APC activity of macrophages, as measured by TC cytokine production, specifically interferon (IFN)-gamma, interleukin (IL)-13. In an effort to determine the nature of this increase, additional elements of the APC-TC interaction were assayed including macrophage cytokines, TC proliferation, TC early activation markers, and intracellular signaling molecules. While TC proliferation experiments correlated with the lymphocyte cytokine production. Macrophage cytokines, including TNF-alpha, IL-6 and IL-12, significantly decreased following silica exposure. In addition, surface expression levels of the early TC activation markers CD25, CD44, CD62L and CD69 were found to lag behind both saline- and TiO<sub>2</sub>-treated controls. These results indicate that activation of the TC by silica-exposed APC is altered such that the normal rules of activation are unable to be applied. Our present observations regarding the effects of crystalline silica and an atypical immune activation could explain the resulting chronic inflammation and fibrosis associated with silicosis. This work is supported by NIH grants ES 04804 and RR-017670

## 1891

### ROLE OF NEUTROPHILS IN 1-NITRONAPHTHALENE-INDUCED PULMONARY INJURY AND REPAIR.

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Acute pulmonary injury is often associated with a neutrophilic inflammatory response. Neutrophils can enhance injury through release of oxidative agents and phagocytosis, however neutrophils also promote repair through the clearance of injured and necrotic cells. 1-Nitronaphthalene (1NN), a ubiquitous environmental pollutant, is a pulmonary cytotoxicant that has been directly associated with an inflammatory response in the rat lung. The purpose of this study was to characterize the role of neutrophils in acute injury and repair from 1-nitronaphthalene in the adult rat lung. Rats received an IP injection of an anti-neutrophil serum or serum carrier prior to administration of either 50 or 75 mg/kg 1NN. This anti-neutrophil serum reduced circulating neutrophils from 14% to 0% by 14 hours post treatment. In contrast, rats given the carrier serum had an elevation of neutrophils from 15% to 54%. The anti-neutrophil serum also inhibited neutrophils from epithelial transmigration with only 0.13% found in BAL fluid of neutrophil-deficient rats and 12% found in neutrophil-sufficient rats. Lungs were evaluated at 24 hrs or 4 days post 1NN by high resolution light microscopy. 1NN induced acute injury at 24 hrs was similar between the neutrophil-sufficient and neutrophil-depleted rats and included areas of extensive vacuolated and necrotic cells and denudation of the basement membrane. At 4 days post injury, both neutrophil-sufficient and neutrophil-depleted rats had similar signs of repair including thickened epithelium, proliferation of ciliated and nonciliated cells and very few vacuolated or necrotic cells. There was no difference in epithelial thickness between neutrophil-depleted rats at 24 hrs and 4 days. However, neutrophil sufficient rats had a thicker epithelium at 4 days compared to 24 hrs suggesting repair of the epithelium. We conclude that neutrophils do not play a significant role in acute pulmonary epithelial 1NN injury. However, it does appear that neutrophils promote repair from acute epithelial injury. NIH ES004311, ES06700, ES04699

## 1892

### PARTICULATE MATTER-INDUCED AIRWAY INFLAMMATION CORRELATES WITH IMMUNE ADJUVANT POTENTIAL.

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Rationale: Epidemiologic studies suggest a causal relationship between particulate air pollution and the increase in allergic airway disease. Animal inhalation toxicity studies showed that ultrafine particles are more potent than fine particles in induc-

ing local airway inflammation and immunotoxicity studies suggest that particles have adjuvant activity. The aim of this study was to investigate to what extend fine and ultrafine particles cause local airway inflammation and if the severity of inflammation correlates to the adjuvant potential. Methods: Balb/c mice were exposed intranasally to ovalbumin (OVA) with or without fine and ultrafine Carbon Black (CB) and Titanium dioxide (TiO<sub>2</sub>) particles. Local airway responses were assessed by studying LDH, total protein, TNF- $\alpha$ , and numbers of inflammatory cells in bronchoalveolar lavage (BAL) fluid. To study the adjuvant activity of the particles, antigen – specific cytokine production by cells of the mediastinal lymph nodes, OVA – specific serum IgE levels and allergic airway inflammation after antigen challenge were studied. Results: Ultrafine CB and TiO<sub>2</sub> particles induced severe airway inflammation and cause allergic sensitization against OVA. Fine particles of both CB and TiO<sub>2</sub> were less toxic and induced only moderate inflammation and allergic sensitization. Conclusions: These data indicate that ultrafine particles are more toxic and induce a stronger inflammatory response than fine particles, and these ultrafine particles have a stronger adjuvant activity. Together data suggest a direct link between airway inflammation and adjuvant activity.

**1893**

IMMUNE RESPONSE OF HUMAN LYMPHOBLASTOID AND BRONCHIAL EPITHELIAL CELL LINES TO ORGANIC COMPOUNDS FROM FINE PARTICLES (PM2.5) IN PUERTO RICO

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Fine atmospheric particles (PM2.5) were sampled in an urban, industrialized area (Guaynabo) and in a rural reference site (Fajardo) in Puerto Rico. Sampling of fine air particles was performed for periods of 72 hrs from Nov/2000 to Sept/2001 using RAAS 2.5-400 Andersen Instrument air sampler. Composite samples were established by pooling them for each month and performing soxhlet extraction for further analyses. Organic extracts (Hex and Acetone) from composite samples collected during a year (2000-2001) were re-dissolved in DMSO and used to evaluate the effect of organic extracts from stations described above on the immune-response (17 different human cytokines) of two human cell types. After exposure to the various extracts, the release of these cytokines by human lymphoblastoid (CEMss PBMC) and bronchial epithelial (BEAS-2B) cell lines were assessed using Bio-Plex Cytokine Array System (BioRad). Results from these experiments revealed two interesting findings. Both organic extracts from Guaynabo and Fajardo exert a significant increase on the release of GM-CSF by human lymphoblastoid cell line. Relative increase in MCP-1 was also observed for these extracts, which indicates the need for monocytes recruitment. This research provides the tools for studying the effects of specific organic components in air particulate matter and elucidating their role on the development of respiratory diseases such as Asthma in Puerto Rico.

**1894**

MURINE PULMONARY MACROPHAGE EXPRESSION AND PRODUCTION OF TNF $\alpha$  AND MIP-2 AFTER EXPOSURE TO DIESEL EXHAUST PARTICLES (DEP) AND EXTRACTS.

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DEPs constitute an important fraction of particulate air pollution and have been shown to cause inflammation of the airways. The aim of this study was to investigate the inflammatory cytokine response of alveolar macrophages exposed to DEP and DEP-extracts. A murine alveolar macrophage cell line was incubated for 4 hours with varying doses of DEPs or DEP-extract (10, 50, or 150  $\mu$ g/ml) as well as vehicle control and endotoxin (0.2, 1, or 5  $\mu$ g/ml). After exposure, biochemical markers of cytotoxicity (lactate dehydrogenase (LDH), ATP production, and calcein) were measured. Pro-inflammatory cytokine expression and production of TNF $\alpha$  and MIP-2 were measured by real time PCR and ELISA, respectively. Expression and production of TNF $\alpha$  and MIP-2 were greatly increased in LPS-exposed macrophages indicating the ability of the cells to make these cytokines. Overall, DEPs and their extracts showed no cytotoxicity by the ATP and calcein assays although LDH levels were increased in culture supernates. TNF $\alpha$  expression and production was not affected by exposure to any of the DEPs or their extracts. MIP-2 production was greatly reduced after exposure to the highest concentration of any of the DEPs. In contrast, MIP-2 mRNA expression was increased in a dose dependent fashion compared to controls suggesting a disconnect between genetic induction and protein synthesis. These results confirm recent reports that *in vitro* particle exposures increase gene expression of cytokines but reduce the level of protein measured by immunoassay, either via quenching antigenic moieties or by in-

terfering with the assay. This phenomenon should be considered while conducting *in vitro* experiments involving exposure to particulate matter. (Supported by UNC Cooperative Training Agreement CT829472. This abstract does not reflect EPA policy.)

**1895**

IN-VITRO INFLAMMATORY AND CYTOTOXIC RESPONSES TO URBAN AIR FINE AND ULTRAFINE PARTICULATE SAMPLES COLLECTED DURING CONTRASTING POLLUTION SITUATIONS IN SIX EUROPEAN CITIES (PAMCHAR).

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Ambient air particulate (PM) samples collected in six European cities in selected seasons were studied: Duisburg (autumn), Prague (winter), Amsterdam (winter), Helsinki (spring), Barcelona (spring) and Athens (summer). Fine (PM<sub>2.5,0.2</sub>) and ultrafine (PM<sub>0.2</sub>) particles were collected using an optimized high volume cascade impactor (HVCI). The particles were extracted with methanol and dried; the PM<sub>0.2</sub> suspension was also filtered to remove glass fiber filter fragments. Mouse macrophages (RAW 264.7) were exposed in a dose-related manner (15, 50, 150 and 300  $\mu$ g/ml) to the PM samples for 24 h. Thereafter, nitric oxide (NO) production was measured by the Griess method, production of cytokines (TNF $\alpha$ , IL-6, and MIP-2) immunochemically (ELISA) and cytotoxicity by using the MTT test. Both PM<sub>2.5,0.2</sub> and PM<sub>0.2</sub> induced mostly similar, dose-dependent NO production and cytotoxicity. The differences in cytotoxic potency between the PM samples from six cities were large; the cell viability after exposure to the largest mass dose was 32%-61% with PM<sub>2.5,0.2</sub> and 26%-77% with PM<sub>0.2</sub>. Correspondingly, the largest NO production ranged from 2.5 to 5.5  $\mu$ M with PM<sub>0.2</sub> and from 2.5 to 4.1  $\mu$ M with PM<sub>2.5,0.2</sub>. The differences in cytokine production between the two PM size classes were large. PM<sub>0.2</sub> induced only minor TNF $\alpha$  and no IL-6 production and, thereby, there were no differences between the six city samples. In contrast, PM<sub>2.5,0.2</sub> induced in most cases large IL-6 and TNF $\alpha$  productions with remarkable differences between the six city samples. In conclusion, there were clear differences between the six city PM samples with regard to their potency to induce cytotoxicity and production of inflammatory mediators in mouse macrophages. PM size class had a large effect on cytokine production but not on the induced NO production or cytotoxicity. Grants: EC-FP5 contract QLK4-CT-2001-00423 and The Academy of Finland and TEKES.

**1896**

EXPRESSION AND KINETICS OF TOLL-LIKE RECEPTORS AND INFLAMMATORY CYTOKINE SECRETION IN MURINE ALVEOLAR MACROPHAGES.

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Toll-like receptors (TLR) play a major role in pathogen recognition of the innate immune response. To date ten TLR family members have been identified, and recognize different pathogen-associated molecular patterns (PAMPs). All of these TLRs stimulate the immune system and can result in the activation of Nfk $\beta$ 1 and upregulation of immunomodulatory cytokines. The purpose of this study was to evaluate the mRNA expression and protein levels of TLR2 and TLR4 following microbial stimulation and relate their signaling effects to pro-inflammatory cytokine production. Murine alveolar macrophages (cell line MH-S) were incubated with either ethanol killed *Streptococci* sp (gram positive) or *Pseudomonas* sp (gram negative) bacteria, or media alone. Cells were harvested at 0, 2, 6, 12, and 24 hours post stimulation and cellular mRNA was assessed by real time PCR analysis for expression of TLR2 and TLR4. Culture supernatants were quantified by ELISA for levels of IL-1 $\beta$ , TNF- $\alpha$ , IL-6, and MIP-2. A significant increase was seen in the mRNA levels of TLR2 and TLR4 with both bacterial species. TLR2 message was upregulated more quickly (2 hours) with the streptococci and this effect persisted for longer (through 24 hours) than the pseudomonas. TLR4 signal was also up-regulated by both bacteria but the initial signal was much higher in cells incubated with streptococci. Cytokine analysis indicated that IL-1 $\beta$  production peaked after 2 hours of incubation while TNF- $\alpha$ , IL-6 and MIP-2 levels were highest after 6 hours of incubation. In contrast to the TLR signaling, the pseudomonas bacteria had increased cytokine output compared to the streptococci at 2, 6, and 12 hours. Taken together the results suggest that the strength of TLR signaling does not coincide with the level of inflammatory cytokine production *in vitro*, and suggests that other PAMPs function through alternative signaling mechanisms. (Funded by EPA CT829470. This abstract does not reflect EPA policy).

## IMPROVED RAT MODELS FOR PREDICTIVE TOXICOLOGY.

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The belated discovery of drug toxicity after successful preclinical trials plagues the pharmaceutical industry. We believe that a major reason for this failure is that researchers often neglect the role genetics plays in predicting drug toxicity in model systems. Current rodent models typically lack genetic diversity, have variable phenotypic responses, or cannot be reliably produced. To address this, PharmGenix rat panels (each comprised of six F1 strains) were created using a novel combinatorial breeding strategy. These PharmGenix rats are the first genetically diverse system that can faithfully recapitulate a heterozygous test population in a controlled fashion. PharmGenix panels capture over 80% of the genetic diversity found in the rat genome and enable drug responses to different genome backgrounds to be tested simultaneously. PharmGenix rats were tested for their sensitivity to the toxic effects of gentamicin, methapyrilene, and tacrine. Following drug treatment, biomarkers in the urine and blood were analyzed and organ pathology was assessed. Gentamicin caused elevations in BUN and creatinine in two of the PharmGenix strains. F344 rats also responded to gentamicin, but it was only the differential response exhibited by the PharmGenix panel that revealed that genetic components underlie the toxic response. After methapyrilene treatment, most of the strains exhibited elevations in ALT and AST. However, several of the PharmGenix hybrids were more sensitive than either CD-IGS or F344 in detecting toxicity. Tacrine led to elevated ALT and AST levels in three of the PharmGenix strains but not in the industry-standard CD-IGS or F344 strains. Abnormal clinical chemistries were accompanied by histopathological changes in the kidney (gentamicin) and liver (methapyrilene). These results underscore the need to consider the influence of genetic components when evaluating new drugs in preclinical studies. Researchers who discover a differential toxic response among PharmGenix strains will use this information as a starting point for drug optimization, drug rescue, and mechanistic studies.

## IN VIVO HEPATIC CELL PROLIFERATION IS AN EARLY, SENSITIVE MARKER OF HEPATOTOXICITY.

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Advances in stable isotope/ mass spectroscopic technology now make rapid, high throughput, quantitative determination of cell proliferation possible. We have applied this technology to the evaluation of hepatotoxicity of drugs. The underlying principle of this approach is that liver cells regenerate rapidly, replacing cells lost through apoptosis or necrosis. This regenerative response can be used as a biomarker of cell death *in vivo*. The effects of hepatotoxins (carbon tetrachloride (CCl<sub>4</sub>)) and griseofulvin (gris)) and non-toxic agent (tamoxifen (tam)) on hepatic cell proliferation were measured using the recently described heavy water (<sup>2</sup>H<sub>2</sub>O) labeling technique. A quantitative dose response to CCl<sub>4</sub> at doses from 0.05 to 0.3 ml/kg was seen in mice. Maximal hepatic cell proliferation (8%/day) was achieved at 0.3ml/kg and was not increased by higher doses. Necrosis was histologically evident in only 20% of the livers at doses below 0.3 ml/kg. In contrast there was a significant increase in cell proliferation in 100% of livers > 0.05 ml/kg. BrdU incorporation correlated with <sup>2</sup>H<sub>2</sub>O results (although with higher s.d.). Dose and strain effects on the response to griseofulvin were also evaluated. 5 days of dietary gris produced a linear dose response from 0.1% to 0.5% (w/w diet) (all doses we significantly different from control p<0.1). The reported NOEL for gris is 0.5%. BALB/c mice are reportedly more resistant than C57Bl/6 to gris induced hepatotoxicity. At 14 days of gris treatment cell proliferation was significantly higher in C57Bl/6 mice (67%) compared to BALB/c (53%) (p<0.05). Finally, safe, doses of tam were given to rats. One or three weeks of tam (15 mg/kg/day) showed no increase in hepatic cell proliferation. In conclusion, the measurement of hepatic cell proliferation is a rapid, sensitive and quantitative marker of liver cell death that is scalable to high throughput assessment of hepatotoxicity.

## INVESTIGATIONS OF THE EFFECTS ON PROTEIN EXPRESSION INDUCED BY HEPATOTOXICANTS ON PRIMARY CULTURED RAT HEPATOCYTES.

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Hepatotoxicity is one of the major concerns, and we have to observe that carefully on drug development. It would be really valuable to select drug candidates at the stage of screening with regard to hepatotoxicity, without large-scale toxicity studies.

Biomarkers will enable us to detect the potentials for liver damage prior to the appearance of visible changes such as morphology. Therefore, novel biomarkers for hepatotoxicity, which are available at screening stage, are strongly needed. We investigated the effects on protein expressions in the primary rat hepatocytes of well-known liver toxicants, acetaminophen (APAP), amiodarone (AD) and tetracycline (TC), using proteomic approaches. Rat hepatocytes cultures were exposed to APAP (10mM), AD (50uM) and TC (500uM) for 6 and 24 hours. Toxicity was determined by lactate dehydrogenase leakage, mitochondrial respiration ability and morphological observations. Proteins in the rat hepatocytes were precipitated with trichloroacetic acid (TCA), and then analyzed by two-dimensional gel electrophoresis (2DGE). The results of gel-to-gel comparisons between control and treated group revealed that 73, 111 and 70 spots were affected by APAP, AD and TC treatments for 24 hours, respectively. Identifications of differentially expressed protein by means of Q-ToF mass spectrometry indicated that several proteins that were involved in energy metabolism and signal transduction, such as glutathione S-transferase (GSTs), apolipoprotein A-IV and senescence marker protein-30 (SMP-30, Regucalcin) were altered. We concluded that the proteins identified in present study have a potential to be biomarker candidates for hepatotoxicity, and altered protein expression patterns also allow us to consider hepatotoxicity with a mechanism base.

## PROTEOMIC ANALYSIS OF LIVER AND SERUM FROM LIPOSACCHARIDE TREATED RATS DURING ACUTE INFLAMMATION.

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Lipopolysaccharide (LPS), induces an acute systemic endotoxemia and widespread inflammatory response after i.p. exposure with release of cytokines, chemokines and bioactive peptides. The purpose of this proteomic study was to determine changes in liver and serum proteins in male, Sprague-Dawley rats to gain insight into the pathophysiology of acute systemic inflammation after LPS 5 mg/kg, i.p. Proteomic analysis was by two dimensional (2D) gel protein separation, image analysis and mass spectrometry (MS) identification of proteins. The acute inflammatory phase of LPS showed TNF $\alpha$  and IL6 ELISA increases. Liver homogenates were analyzed by 2D gels and mass spectrometry (MS) at 2 and 6 hr in both liver and serum. Proteomic analysis of liver after 2 hr LPS revealed 152 protein features were changed at p<0.005 and at 6 hr there were 192 features altered. A large increase in liver carbamoyl-phosphate expression (20 to 60 fold) was consistent with nitric oxide formation produced by LPS-induced inflammation. Other liver proteins induced were SAM synthetase isozymes (15 to 40 fold), ATP synthase isozymes, Hsp 70 and 90 isoforms and others proteins that supported acute phase protein production. Serum was analyzed after immunoaffinity removal of abundant proteins such as albumin, immunoglobulins and others. Remaining serum proteins were then analyzed by gradient elution into five fractions that were dialyzed and analyzed by 2D-MS. In serum, complement C3 isoforms were consistently reduced by LPS treatment while Apo-A1 precursor was consistently increased and alpha-inhibitor H4 heavy chain (liver protein) increased at 2 hr but fell at 6 hr after LPS treatment. These observations from proteomic analysis suggest an interplay among liver protein and serum proteins changes involved acute inflammation and production of acute phase proteins during acute exposure to LPS.

## EVALUATION OF THE BACKGROUND DATA OBTAINED IN THE TOXICOGENOMICS PROJECT IN JAPAN.

H. Nitta, T. Miyazaki, H. Totsuka, T. Miyagishima, T. Urushidani and T. Nagao. *Toxicogenomics Project, National Institute of Health Sciences, Tokyo, Japan*. Sponsor: [T. Inoue](#).

National Institute of Health Sciences and 17 pharmaceutical companies have started a five-year project, the Toxicogenomics Project in Japan (TGPJ) in 2002. The objective of TGPJ is to construct a large-scale database of over 150 drugs on rats and develop a system which will forecast the toxicity of new chemical in the early stage of drug development. In TGPJ, 0.5 w/v% methylcellulose solution is usually employed as vehicle for oral administration of test drugs to 5 animals. Gene expression profiles in liver (and additionally in kidney for some cases) are evaluated in 3 out of 5 animals for each group by Affymetrix GeneChip. Autopsy is performed 3, 6, 9, 24 hours after single administration, and 3, 7, 14, 28 days after repeated administration. The gene expression data are being accumulated in the database together with the traditional toxicology data. In order to evaluate drug effects using microarray data, we generally employ the fold change of the value compared to the control. Therefore, our investigation has been especially focused on the variation of control data obtained in TGPJ. The treatment of animals has been done in 4 different contract research organizations, and gene expression profiling in liver

was performed by using RAE230A and RAE230 2.0 GeneChip. We evaluated vehicle control data from the first 28 compounds (672 chips). Perchip normalization was applied for data analysis. Within the 3 control data in the same group, scatter plotting of each pair constantly showed a good correlation. It was noteworthy that inter-organization variations of the data were negligible. We also confirmed that many of the circadian genes, such as albumin D site-binding protein, period 1, etc., showed reproducible and typical expression patterns in the single dose study. Moreover, many of the genes, which are reported to be age-dependently up- or down-regulated, showed reproducible changes in the repeated dose study. These results suggest that the data being accumulated in our database are well-qualified and reproducible.

## 1902

### THIOACETAMIDE AND METHAPYRILENE SHOWED A UNIQUE GENE EXPRESSION PROFILE AMONG THE CHEMICALS IN THE TOXICOGENOMICS PROJECT IN JAPAN.

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National Institute of Health Sciences and 17 pharmaceutical companies have started a five-year project, the Toxicogenomics Project in Japan (TGJP) in 2002. The objective of TGJP is to construct a large-scale database of over 150 drugs on rats and develop a system which will forecast the toxicity of new chemical in the early stage of drug development. In order to characterize the effects of chemicals by gene expression, we employed clustering analyses using multi-dose (3 dose levels), multi time-point data (3, 6, 9, or 24 hr after single dose and 24 hr after repeated dose for 3, 7, 14, or 28 days) by Affymetrix GeneChip. K-means or hierarchical clustering with gene expression in the liver revealed that thioacetamide (TAA) and methapyrilene (MP) were strikingly similar among 25 chemicals during repeated administration. In clinical biochemistry both chemicals caused a similar pattern, e.g., decrease in triglyceride, increase in serum enzymes. Similarity in gene expression was also noticed with several gene subsets categorized by physiological functions. However, the similarity was much less within 24 hrs after single administration. A gene set modulated in common for TAA and MP at 24hr was extracted by K-means clustering. Hierarchical clustering using this gene set showed high similarity between these two chemicals. Of these genes, up-regulated ones were categorized into protein metabolism, biosynthesis, stress response, cell cycle, whereas down-regulated ones were into fatty acid metabolism/transport. Modulated genes common between single and repeated dosing were categorized into response to stress/stimulus, whereas those exclusively modulated in repeated dosing group included genes related to cell cycle/proliferation, protein synthesis or transcription. In conclusion, we could extract a gene set that characterize chronic phenotype from the data of single treatment. This would be a model case of toxicity prediction by gene expression data.

## 1903

### GENE EXPRESSION IN PORPHYRIA INDUCED BY 2, 3, 7, 8-TETRACHLORO-DIBENZO-P-DIOXIN IN SUSCEPTIBLE MICE.

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The mechanism by which TCDD induces hepatic porphyria, a disruption of heme synthesis, is unknown but as with other toxic actions it is mediated through the AH receptor (AHR) and must be associated with a cascade of gene expression changes. C57BL/6J mice (high affinity AHR) are the most susceptible and DBA/2 (low affinity AHR) are a highly resistant strain. Previous studies have implicated iron metabolism and an oxidative mechanism with susceptibility linked to polymorphism in other genes in addition to the *Ahr* gene. Male C57BL/6J and DBA/2 mice were dosed (orally) with TCDD (75  $\mu$ g/kg in corn oil) and hepatic gene expression examined after 3 days, 2 weeks and 5 weeks by which time hepatic porphyria developed in the former strain but not in the latter. Hepatic gene expression changes were analysed primarily with cDNA microarrays constructed from approximately 3000 IMAGE clones of candidate genes. At this dose of TCDD maximum induction of most AH battery genes including those for CYP1A1/CYP1A2 occurred by 3 days in both strains similarly declining slowly over 5 weeks. In contrast, expression of the *Cyp1b1* gene fell much quicker in DBA/2 mice than C57BL/6J. Expressions of some genes of heme metabolism (*Alad* and *Hmbs*) were down-regulated only in C57BL/6J not DBA/2 mice over the experimental period whereas *Alas1* and *Hmox1*, associated with heme regulation, were up-regulated. There was no compensatory up-regulation of the gene for uroporphyrinogen decarboxylase. The iron homeostasis genes cytosolic aconitase (*Aco1*) and transferrin receptor 2 (*Trf2*) were down regulated whereas haemastatin (*Heph*) was up-regulated, again in the susceptible strain. Hepcidin (*Hamp*) a hormonal peptide regulator of iron absorption was unaffected. Some genes associated with oxidative stress showed changes in expression concomitant with porphyria development (*Fab*, *Car3*, *Sod3*, *Cat*) in C57BL/6J but not DBA/2 mice. The results are consistent with an association of TCDD induced-porphyria with disturbances of iron homeostasis and an oxidation mechanism.

## 1904

### STRESS- AND INFLAMMATORY-RELATED MNRA INDUCTION IN HEPATOCYTES AND NON-PARENCHYMAL CELLS RESPONSE TO OXIDATIVE STIMULI.

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Oxidative stress plays a major role in various hepatic injuries. However, the gene expression in hepatocytes and non-parenchymal cells in response to oxidative stimuli has not been well known. In this study, stress- and inflammatory-related gene expressions were analyzed in hepatocytes and non-parenchymal for two liver injury models, ischemia/reperfusion and acetaminophen (APAP)-administration. Male SD rats were subjected to ischemia for 45 min, followed by 1 or 3hr of reperfusion, and sacrificed under anesthesia. As for APAP-administration, the animals were treated at 1000 mg/kg (po) and sacrificed under anesthesia at 24hr after treatment. After the liver was removed, hepatocytes and non-parenchymal cells were separated out by collagenase perfusion method. The levels of gene expression in the whole liver, hepatocytes and non-parenchymal cells were determined by RT-PCR. In ischemia/reperfusion model, the expressions of HSP70 and IL-6 were increased in whole liver at 1hr-reperfusion. At 3hr- reperfusion, HO-1, HSP70, iNOS and IL-6, were the genes which expression was enhanced. When the liver was separated the two components, HO-1 expression was increased in hepatocytes, the expressions of iNOS and IL-6 were seen in non-parenchymal cells. Furthermore, HSP70 expression was increased to a great extent in hepatocytes and non-parenchymal cells. The increases in expressions of iNOS and inflammatory cytokines were detected from 1hr-reperfusion in non-parenchymal cells, while these changes were seen only at 3hr-reperfusion in whole liver. The gene expressions in hepatocytes and non-parenchymal cells after APAP treatment were similar to those of ischemia/reperfusion model. Thus there were obvious differences in the induction of oxidative stress-related genes between hepatocytes and non-parenchymal cells. These results suggest that the intracellular response in hepatocytes and non-parenchymal cells to oxidative stimuli might be controlled with different mechanisms.

## 1905

### DEVELOPMENT OF PROTEIN MICROARRAY TECHNOLOGY TO FACILITATE IDENTIFICATION OF PROTEINS ISOLATED BY GEL FILTRATION AND ION-EXCHANGE CHROMATOGRAPHIES.

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Protein microarray technology was developed to facilitate protein purification. This technology can be utilized to produce a reference protein mixture to be used for an antibody microarray analysis with a reference design. Hepatic microsomes and cytosol were obtained by differential centrifugation from male Sprague-Dawley rats treated with phenobarbital (100 mg/kg/day for 3 days). Cytosolic proteins were fractionated by gel filtration chromatography. Seventy two fractions collected from gel filtration chromatography were spotted (spotting volume, 1 nl) in quadruplicate (4 blocks) on a nitrocellulose-based chip using split pin technology. GST-alpha IgG was hybridized with the cytosolic protein fractions spotted on the chip in a hybridization buffer containing blocking proteins and a detergent. Secondary hybridization of the chip was carried out with a Cy5-conjugated anti-rabbit IgG. Scanning of the slide revealed dots with a strong red signal in the middle section of all 4 blocks. The dots corresponded to fractions No. 25 through No. 36. No background signal was detected. Western blot analysis with GST-alpha IgG verified the result obtained by protein microarray analysis. Microsomal proteins were fractionated using anionic exchange chromatography. Every third fraction out of 240 fractions were spotted on a nitrocellulose-based chip and hybridized with cytochrome P450 2C23 antibody followed by a secondary hybridization with a Cy5-conjugated anti-rabbit IgG. Dots with a strong red signal corresponded to fractions No. 183 through No. 195. The results demonstrated that protein array technology facilitated identification of proteins isolated by gel filtration and ion-exchange chromatographies. Supported by NIEHS SBIR Phase I contract ES35506.

## 1906

### HEPATIC GENE EXPRESSION PROFILES OF RATS EXPOSED TO PERFLUOROOCTANESULFONATE (PFOS) IN UTERO.

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Perfluorooctanesulfonate (PFOS) is a surfactant that occurs primarily as a degradation product of some materials used for oil and water resistance. PFOS is persistent and widely dispersed in the environment. Toxicity testing indicates that PFOS interferes with intermediary metabolism by inducing the proliferation of peroxisomal

bodies via transactivation of PPAR- $\alpha$ , both in adult and neonatal rodent species. The purpose of this investigation was to assess whether the observed modulation of lipid and fatty acid metabolism in liver of exposed neonates is reflected at the transcriptional level. Gravid Sprague-Dawley rats were exposed by gavage to 3 mg/kg PFOS/K $^{+}$  beginning on gestational day (GD) 2. On GD21, liver was harvested from fetuses for RNA extraction and cRNA was generated for hybridization to Affymetrix 230A chips. Raw intensities were adjusted for background and normalized by the robust multichip analysis method. Statistical analysis revealed treatment-related differences in the degree of expression of 153 genes between the control and PFOS exposed animals (91 upregulated and 62 downregulated, p<0.01). The most prominent changes were the increased expression of both lipid synthesis and degradation pathways, as well as mitochondrial and peroxisomal  $\beta$ -oxidation genes, with no change in expression of the mitochondrial electron transport chain genes. On average, there was a two-fold induction of genes related to metabolism. The results of this investigation are in good concordance with previously observed changes in metabolic enzyme activity and intermediate profiles, suggesting that the metabolic effects associated with *in utero* PFOS exposure in rodents are manifested at the transcriptional level and are likely related to PPAR $\alpha$  activation. Supported by the 3M Company. This abstract does not reflect EPA policy.

**1907 LIVER SLICE MODEL SYSTEM FOR BILIARY CELL EFFECTS: CHANGES IN CLINICAL CHEMISTRY PARAMETERS.**

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Hepatobiliary toxicants induce histological changes in rat liver slice cultures similar to their known effects *in vivo* (Poster No. \_\_\_\_). Demonstrating a relationship between diagnostic biomarkers and histological findings *in vitro* would strengthen confidence in the use of roller slice culture systems for investigations and assays of biliary toxicant effects. In this aspect of the work, concurrently with histological tissue examination, we measured the effects of various hepatobiliary toxicants on clinical chemistry parameters in precision-cut slices from male F-344 and Sprague-Dawley rat livers. Geldanamycin (GEL), 17-allylaminogeldanamycin (17AAG),  $\alpha$ -naphthylisothiocyanate (ANIT), and 4, 4'-methylene dianiline (MDA), compounds known to induce biliary duct necrosis and/or cell hyperplasia in these strains, were examined. GEL and 17AAG induced concentration-dependent alterations in biomarker levels, with the most prominent being (in order of percentage change relative to corresponding control slice levels): ALP $\downarrow$ , LDH $\downarrow$ , GGT $\downarrow$ , ALT $\downarrow$ , Bili $\uparrow$ , AST $\downarrow$  in the effect range. The loss of slice enzyme content was associated with increased levels in the medium. The biomarker changes occurred at physiologically relevant exposures, and GEL was appreciably more toxic than 17AAG over the test concentration range. In contrast, ANIT and MDA appeared to induce increased slice contents of the enzyme biomarkers relative to control slice levels (most notably in ALP and/or LDH and GGT) up to concentrations at which necrosis was evident histologically. The biomarker profiles were similar to those seen in rats *in vivo*. At these latter exposures the increases in slice enzyme parameters were markedly reversed, indicative of a loss in cell viability. The biomarker patterns associated with BEC necrosis and hyperplasia were distinctly different and consistent with the type of injury histologically present in the slice tissues. These results provide further support for the reliability of the model system for studies and assays of biliary cell effects. Supported by NIH grant R21/R33 CA093262.

**1908 LIVER SLICE MODEL SYSTEM FOR BILIARY CELL EFFECTS: HISTOLOGICAL CHANGES.**

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Precision-cut slices which maintain intact biliary duct structures are an attractive option for studying biliary cell effects but are limited in value for this application because slice viability in culture deteriorates too rapidly. Advances in preparation and roller culture methods have resulted in better quality tissues and integrity over 10 days of incubation (Behrsing et al., *Biochem. Biophys. Res. Commun.* 312: 209-213, 2003) and more recently up to 21 days. In the studies reported here, we examined histological changes in liver slices from male F-344 and Sprague-Dawley rats incubated with and without hepatobiliary toxicants known to induce biliary epithelial cell (BEC) necrosis and hyperplasia *in vivo*. Control slice preparations in roller organ cultures supplemented with CMH1a medium under a 70-75% O $_2$ -containing atmosphere retained 60-70% hepatocellular and 80-90% BEC viability over the experimental period as assessed by semi-quantitative histological examination. Geldanamycin (GEL) and 17-allylaminogeldanamycin (17AAG) at 0.40-5.0  $\mu$ M caused pronounced injury to BEC; hepatocytes were also affected though generally to a lesser extent. GEL-induced injury was more severe than that due to 17AAG in the same concentration range. In contrast,  $\alpha$ naphthylisothiocyanate (ANIT) and 4, 4'-methylene dianiline (MDA) in the 1-200  $\mu$ M range induced

BEC proliferation (assessed by H&E staining and BrdU immunohistochemistry) at most concentrations and BEC necrosis at the highest concentrations. These compound-induced effects were either absent or less severe in control tissues throughout the incubations, and the severity of each identified effect was concentration-dependent. These observations encourage more extensive examination of this precision-cut liver slice system as a general model for biliary cell effects in addition to hepatotoxicity. Supported by NIH grant R21/R33 CA93262.

**1909 IMPAIRED G1 TO S-PHASE SIGNALING EXPLAINS INHIBITED TISSUE REPAIR IN THIOACETAMIDE-TREATED TYPE 1 DIABETIC RATS.**

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A nonlethal dose of thioacetamide (TA, 300 mg/kg) causes 90 % mortality in diabetic (DB) rats primarily due to impaired tissue repair (-50% inhibition of S-phase). Objective of the present study was to investigate the molecular events that orchestrate G1 to S-phase progression of the cell cycle to explain inhibited tissue repair in TA-treated DB rats. Immunoblotting revealed that EGFR-MAPKs pathway was upregulated in the TA-treated nondiabetic (NDB) group. DB-associated down regulation of EGFR-MAPKs pathway was further downregulated after TA treatment. EGFR-MAPKs stimulate transcription factor NF-kB, activating transcription of CD1. Gel shift assay revealed adequate NF-kB/DNA binding in the TA-treated NDB liver, resulting in higher CD1 gene expression. In contrast, in the DB group receiving the same dose of TA, down regulation of EGFR/MAPK pathway inhibited NF-kB/DNA binding, resulting in lower CD1 gene expression as measured by real time RT-PCR. Furthermore, microarray analysis revealed that gene expression of extracellular matrix (ECM) proteins like integrin, laminin, collagen IV, which are known to stimulate CD1 expression were also upregulated in the TA-treated NDB rats. In contrast, gene expression of ECM proteins was inhibited in the TA-treated DB group, leading to inhibited CD1 expression. In association with cdk4 and cdk6, CD1 phosphorylates retinoblastoma (Rb) and G1 to S-phase progression. Immunoblotting revealed that higher CD1 expression, coupled with higher cdk4 and cdk6 in the NDB group, led to phosphorylation of Rb, explaining prompt and efficient tissue repair. Whereas inhibited expression of CD1, cdk5 and Rb explains inhibited hepatic tissue repair in the TA-treated DB rats. These findings suggest that impaired CD1-centred signaling underlies inhibited tissue repair and higher sensitivity of DB rats to TA hepatotoxicity. These findings suggest that impaired CD1-centred signaling may be utilized as a risk assessment tool for screening novel antidiabetic drugs for potential hepatotoxicity in DB patients.

**1910 MOLECULAR MECHANISMS OF INHIBITED COMPENSATORY LIVER REPAIR UPON HEPATOTOXIC CHALLENGE IN TYPE 2 DIABETIC RATS.**

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Earlier we showed that higher sensitivity to CCl<sub>4</sub> hepatotoxicity and mortality in type 2 diabetes is due to inhibited liver tissue repair. The objectives of present studies were to investigate the molecular mechanisms of higher sensitivity of type 2 diabetic (DB) rats to CCl<sub>4</sub> hepatotoxicity and to further characterize the sensitivity of type 2 DB rats to hepatotoxicants other than CCl<sub>4</sub>. MAPK pathway was investigated (0 to 24 h) after CCl<sub>4</sub> treatment. Expression and activation of ERK 1/2 were down regulated while p38 activation was increased as early as 3 h in the DB rats after CCl<sub>4</sub> administration. Furthermore, NF $\kappa$ B-DNA binding was high in the DB rats at 0 h compared to non-DB rats but was severely inhibited following CCl<sub>4</sub> administration. Cyclin D1 and CDK 4/6 expression were low in the DB rats compared to the non-DB rats following CCl<sub>4</sub> administration, resulting in decreased phosphorylation of retinoblastoma protein. Thus, down regulation of mitogenic signaling in the DB rats leads to problems at the G<sub>0</sub>/G<sub>1</sub> to S phase advance, impeding cell cycle progression needed for stimulation of tissue repair thereby explaining the progression of liver injury after CCl<sub>4</sub> administration. Time course (0 to 96 h) study of thioacetamide (TA, 300 mg/kg, ip) toxicity revealed higher liver injury in DB rats. Interestingly, covalent binding of <sup>14</sup>C-TA to liver tissue of DB rats did not differ from the non-DB rats, indicating identical bioactivation-based liver injury. Compensatory cell division measured via <sup>3</sup>H-T pulse labeling and PCNA immunohistochemistry was inhibited in DB rats leading to progression of injury, liver failure (hyperbilirubinemia), and death (100% mortality) between 36 to 48 h after TA administration. Investigation of whether MAPK pathway plays a role in potentiation of TA-induced liver injury in DB rats is underway. In conclusion, type 2 diabetes increases sensitivity to structurally and mechanistically dissimilar hepatotoxins (CCl<sub>4</sub> and TA) due to impaired compensatory tissue repair.

**1911**

PPAR $\beta$ -MEDIATED HEPATOTOXICITY IN A DIETARY MODEL OF NONALCOHOLIC STEATOHEPATITIS (NASH).

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NASH, an advanced stage of liver injury associated with obesity, type 2 diabetes, and hyperlipidemia, is characterized by hepatic steatosis associated with lipid peroxidation, inflammation, and fibrogenesis and can progress to cirrhosis and liver cancer. The mechanisms that mediate transition from steatosis to NASH remain unclear. Recent studies linked peroxisome proliferator-activated receptor $\beta$  (PPAR $\beta$ ) to attenuation of liver toxicity induced by environmental chemicals. The present study investigated the function of PPAR $\beta$  in a mouse model of dietary-induced NASH. Wild-type and PPAR $\beta$ -null mice were fed a methionine and choline deficient (MCD) diet and a marginal MCD diet for 5 weeks, and markers of hepatotoxicity were evaluated. Wild-type mice treated with the marginal or the MCD diets exhibited hepatocellular injury, indicated by elevated serum ALT levels (~10-fold and ~40-fold) as compared to controls. PPAR $\beta$ -null mice fed the marginal MCD diet exhibited a similar extent of liver injury as the wild-type mice but were not as severely affected by the MCD diet as were the wild-type mice. Both the marginal and the MCD diets induced lipid peroxidation in wild-type mice (~3.75-fold and ~5-fold) and to a lesser extent in PPAR $\beta$ -null mice (~1.5-fold and ~3.25-fold). As a measure of hepatic redox status, the ratio of reduced to oxidized glutathione was determined. In wild-type mice, glutathione ratios were decreased as compared to controls with both the marginal and the MCD diets (by ~36% and ~74%). In PPAR $\beta$ -null mice, glutathione ratios were decreased to the same extent by both diets (by ~54%). While these data indicate that PPAR $\beta$  may mediate increased lipid peroxidation and oxidative stress, histological evidence from mice fed the marginal MCD diet showed the presence of hyperplastic lesions in PPAR $\beta$ -null mice but not in wild-type mice, suggesting that PPAR $\beta$  attenuates hepatic injury. Additional studies are underway to delineate the mechanism contributing to this disparity.

**1912**

INDUCTION OF CYTOTOXICITY IN PRIMARY RAT HEPATOCYTES AND HUMAN HEPG2 CELLS BY SELECTED PPAR $\gamma$  AGONISTS.

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Peroxisome proliferator activated receptor gamma (PPAR $\gamma$ ) agonists are used to treat insulin resistant diabetes and type 2 diabetes. These agents have a proven efficacy for reducing plasma glucose levels in type 2 diabetic patients. Several members of the thiazolidinedione (TZDs) class are PPAR $\gamma$  agonists. Three TZDs have been approved by FDA. However, troglitazone was removed from the market due to idiosyncratic drug toxicity. In order to gain insight into the mechanism of hepatic toxicity of some members of this class of agents, we investigated the relative toxicities of five PPAR $\gamma$  agonists, troglitazone, ciglitazone, rosiglitazone, pioglitazone, and JTT-501 to primary rat hepatocytes and HepG2 cells. Toxicity was assessed by use of the MTT assay. Exposure of primary rat hepatocytes and HepG2 cells to five PPAR $\gamma$  agonists for 2, 6 and 16 hours at various concentrations (0, 1, 5, 10, 20, 40 and 100 $\mu$ M) demonstrated that troglitazone, ciglitazone, and JTT-501 exhibited a dose and time dependent cytotoxicity. The TUNEL assay was used to demonstrate that the observed cell death was apoptotic in nature. The observed toxicity was independent of PPAR $\gamma$  activation based on RNAi experiments and on comparison of the rank order of the published relative binding affinity and the IC<sub>50</sub> of the observed hepatotoxicity.

**1913**

INDUCTION OF EARLY FIBROGENESIS IN PRECISION-CUT HUMAN AND RAT LIVER SLICES AFTER CARBON TETRACHLORIDE TOXICITY.

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**Introduction:** Hepatic stellate cell (HSC) activation is a key event in the development of fibrosis. Current *in vitro* models for liver fibrosis contribute significantly to the understanding of HSC biology and fibrogenesis but lack a physiologic milieu that can incorporate effects of cell sociology and extracellular signals. We have developed a liver slice system in which HSC activation following injury by standard hepatotoxins can be studied in a more physiologic context, both in human and rat liver tissue. **Methods:** Precision-cut rat (RPCLS) or human liver slices (HPCLS) were incubated for 3 or 16 hours with carbon tetrachloride (CCl<sub>4</sub>). Expression of

markers for HSC and for early fibrogenesis was studied using real-time PCR. Viability was assessed by ATP-content or LDH leakage. **Results:** Liver slices remained viable during control incubation. Incubation with CCl<sub>4</sub> led to a decrease of viability, which was observed at lower doses in RPCLS. Both in RPCLS and HPCLS mRNA expression of the early fibrogenesis/HSC-activation markers heat-shock protein 47 and  $\alpha$ B-crystallin was induced 3 hours after addition of CCl<sub>4</sub> and further increased after 16 hours of incubation (induction: rat 2.1 $\pm$ 0.1 and 16.4 $\pm$ 113.3; human 10.1 $\pm$ 2.8 and 28.6 $\pm$ 15.0, all  $P$ <0.05). In HPCLS higher doses of CCl<sub>4</sub> were needed to induce HSC activation. Gene expression of synaptophysin (HPCLS) and desmin (RPCLS), markers for quiescent as well as activated HSC, was only modestly increased after CCl<sub>4</sub> treatment (1.4 $\pm$ 0.1,  $P$ <0.05, and 1.6 $\pm$ 0.5) indicating a relatively constant number of HSC in the liver slices. **Conclusion:** We developed a method to induce and track early HSC activation in precision-cut liver slices that can be applied to both human and rat liver tissue. This model enables to study activation of HSC within their physiological milieu and provides the unique opportunity to investigate species-specific mechanisms underlying early fibrogenesis.

**1914**

HUMAN LIVER AND KIDNEY SLICE VIABILITY IS EXTENDED AND PATHWAYS OF FIBROSIS ARE CHARACTERIZED.

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The extension of human liver slices (7 days) and kidney slices (11 days) expands the use of this *in vitro* model for studying pathways of injury and repair, including fibrosis. Organ slice quality is sustained by preparing slices in a preservation solution, from organs perfused and maintained in a preservation solution, and with slice intracellular K<sup>+</sup> levels >70  $\mu$ moles/mg slice wet weight. In culture the organ slices are supported by a cellulose filter atop the titanium mesh roller-insert, which contributes to a stabilization of slice wet weight throughout the culture time. Medium is replaced daily to mimic multiple dosing. The slices are metabolically active, maintaining slice ATP and glutathione levels, and organelle integrity is preserved. By 72 hr of culture the organ slices begin to exhibit pathways of repair and a fibrogenic response. An increased deposition of collagen IV is evident at 72 hr and remains elevated for the duration of the cultures. Global gene expression profiling using U133A Affymetrix gene chip arrays revealed an increased expression of extracellular matrix genes including collagens, laminins, contractile proteins, and markers of proliferation in both liver and kidney slices. Some metabolic genes and lipoprotein genes were repressed in the liver slices only. The data supports the activation of tissue stellate cells in the induction of fibrogenic pathways. The recent improvements in organ acquisition and slice preparation and culturing methods demonstrates that slice viability, integrity and morphology is extended for several days in culture. The 3-dimensional cellular complexity increases the utility of this *in vitro* model system to study pathways of injury and repair including fibrosis, and can be extended to understanding mechanisms of fibrotic liver and kidney diseases.

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THE EFFECT OF THE PPAR $\alpha$  AGONIST WY-14, 643 ON MITOCHONDRIAL MEMBRANE POTENTIAL IN HUMAN AND RODENT LIVER CELL LINES.

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PPAR $\alpha$  agonists have been found to induce peroxisome proliferation and hepatomegaly in rodents. Due to the coordinated function of peroxisomes and mitochondria in fatty acid metabolism and the occurrence of skeletal muscle toxicity in rodents treated with PPAR $\alpha$  agonists, it is possible that some of these compounds may also have mitochondrial effects in some species. The ability of the PPAR $\alpha$  agonist WY-14, 643 to affect the mitochondrial membrane potential in human (Fa2N-4) and rat (Clone 9) liver-derived cell lines was monitored using the fluorescent probe JC-1. These changes could be monitored by measuring either an increase in JC-1 monomer or a decrease in aggregated JC-1 molecules. Measuring levels of JC-1 monomer was far more sensitive than measuring levels of the aggregate. The PPAR $\alpha$  agonist elicited a dose dependent increase in mitochondrial membrane permeability in the rat cell line after a 20 minute exposure. This effect was more pronounced in the rat cell line than in the human cell line. Changes in cell viability from this compound were also measured in both rat and human cells at 1 and 24 hrs using either the ATP production luminescence assay or the MTT viability assay. Changes in cell viability using the more sensitive ATP assay were also more pronounced in the rat cells. Thus, an association between changes in mitochondrial membrane permeability and induction of cell death was best monitored within a 1 hr exposure period in rat liver cells. In addition, it can be concluded that the PPAR $\alpha$  agonist WY-14, 643 causes a decrease in the mitochondrial membrane potential in the Clone 9 cell line, but not in the Fa2N-4 cell line, as measured by an increase in JC-1 monomer. Research funded by Pfizer Global Research and Development.

IN SITU QUANTITATIVE EVALUATION OF PEROXISOMAL PROLIFERATION IN THE NON-HUMAN PRIMATE LIVER VIA LASER SCANNING CYTOMETRY (LSC).

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Laser scanning cytometry (LSC) and biochemical markers were employed to evaluate the effect of a peroxisome proliferator-activated receptor (PPAR) mixed beta/alpha agonist under development for the treatment of dyslipidemia. Two- to 3.5-year old male cynomolgus monkeys received test compound at 0, 0.1, 0.3, 1, 3, or 10 mg/kg by oral gavage for 28 consecutive days. At necropsy, there were no drug-related gross pathologic findings. Immunohistochemical staining for the peroxisomal membrane protein (PMP70) within the hepatocytes was qualitatively increased in drug-treated monkeys compared to controls. Quantitative evaluation of the immunohistochemical labeling of peroxisomes via LSC confirmed an increase in peroxisomal labeling in all dose groups, which plateaued at drug levels  $\geq$  0.3 mg/kg. Similarly, liver biochemical analyses revealed increased peroxisomal beta-oxidation and acyl CoA oxidase activities in all dose groups, which also plateaued at  $\geq$  0.3 mg/kg. These changes correlated with increases in absolute and relative liver weights. The liver weight changes corroborated the histopathologic findings (hepatocellular hypertrophy) at  $\geq$  0.3 mg/kg. The hepatocellular hypertrophy was characterized by an increase in cell size due to increased quantities of granular eosinophilic cytoplasm. Interestingly, the plasma and liver concentrations of the test compound increased with increasing dosage. However, the above data suggests that maximal peroxisomal activity was achieved at 0.3 mg/kg indicating a saturation effect. In conclusion, quantitative evaluation of hepatocellular peroxisome levels was achieved *in situ* via LSC, which correlated well with biochemical activities, organ weight changes, and histopathology.

COMPARISON OF ALDEHYDE DEHYDROGENASE (ALDH) ACTIVITY IN THE LIVER AND LUNG.

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Aldehydes are highly reactive molecules present in the environment and can be produced during biotransformation of xenobiotics. Aldehyde dehydrogenases (especially classes 1, 2 and 3) are important in their detoxification catalyzing oxidation to carboxylic acids. The lung is a major target for aldehydes toxicity. However, ALDH activity in the lung has been poorly studied compared to the liver. In this study, ALDH activity was measured in 4 subcellular fractions from liver and lung to compare the enzymatic capacity of aldehyde removal. Adult (60 days) female Wistar-Han-IGS rats were used to prepare S1 (postnuclear fraction), mitochondrial, cytosol, and microsomal fractions. Propionaldehyde or hexanal (50 uM) were used as substrates for mitochondrial ALDH2 or microsomal ALDH3, respectively. Propionaldehyde (10mM) was used to measure total ALDH activity in each subcellular fractions. ALDH activity in S1 fractions was considered to be an indicator of the total ALDH in the tissue. In the liver, the mitochondrial ALDH2 showed the majority of ALDH activity (i.e., 52% of the total S1 activity). Activity in the cytosol and microsomes was 37% and 29% of the total S1 activity, respectively. In the lung, however, the major ALDH activity was found in the cytosol showing 56% of total S1 activity. Total mitochondrial ALDH activity contributed 41 % of total lung S1 activity. Compared with the liver, lung had 15 % of total S1 ALDH activity. Mitochondrial ALDH2 and microsomal ALDH3 activities were 5 and 10 fold higher in the liver than in the lung, respectively. These data show that the liver and lung had differences in distribution of ALDH among subcellular fractions. Greater cytosolic contribution to the total ALDH activity in the lung indicates that the major isoform(s) in the lung may be different from the liver. These finding indicates that aldehyde metabolizing capacity may respond differently to environmental and/or physiological factors in the lung compared with liver. (Supported by EPA-NRC #CR82879001. This abstract may not represent official EPA policy.)

ETHANOL HAS BIPHASIC EFFECTS ON EXPRESSION OF STEROL REGULATORY ELEMENT BINDING PROTEIN-1 (SREBP-1) IN RAT FGC-4 HEPATOMA CELLS WITH INHIBITION AT HIGH DOSES RESULTING IN INDUCTION OF ADH CLASS I.

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SREBPs are transcription factors which regulate fatty acid and cholesterol metabolism. Ethanol has long been known to perturb lipid homeostasis. However, effects of ethanol on SREBP expression have been an area of dispute with some investiga-

tors reporting a significant increase and some a decrease. In the current study we examined the dose responsive effects of ethanol on expression of SREBP-1 in the rat FGC-4 hepatoma cell line with which we previously demonstrated ethanol induction of ADH class I gene transcription. Ethanol had a biphasic effect on expression of the mature form of SREBP-1 protein in cellular homogenates. At low doses of ethanol (< 10 mM) SREBP-1 expression was increased ( $p \leq 0.05$ ) with a 2-fold increase evident at 5 mM. In contrast, at higher doses of ethanol a dose-dependent decrease in SREBP-1 expression was observed and this correlated with increased expression of ADH class I ( $p \leq 0.05$ ). The liver X-receptor agonist T0901317, a known activator of SREBP-1 transcription, was shown to produce a dose-dependent decrease in ADH expression which was completely abolished by ethanol. These data confirm in an *in vitro* model that SREBP-1 is a negative regulator of ADH transcription and that ethanol at high doses induces ADH via suppression of SREBP-1 protein expression. (Supported in part by R01 AA088645 TMB).

ETHANOL INDUCES RAT HEPATIC ALCOHOL DEHYDROGENASE (ADH) CLASS I BY INTERFERING WITH POST-TRANSLATIONAL REGULATION OF STEROL REGULATORY ELEMENT BINDING PROTEIN-1 (SREBP-1).

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Continuous intragastric infusion of ethanol as part of total enteral nutrition results in pulses of blood and urine ethanol with a 6-7 d. cycle. The cycle is driven by cyclical changes in transcription of the liver ADH Class I gene. Electrophoretic mobility shift analysis of the ADH I promoter revealed decreased binding of nuclear proteins from ethanol-exposed rats to two canonical sterol response element sites at -63 to -53 and -52 to -40 base pairs relative to the transcription start site on the ADH promoter ( $p \leq 0.05$ ). Specificity of SREBP-1 for the response element was confirmed by chromatin immunoprecipitation (ChIP). Transcriptional inhibition of ADH by SREBP-1 was confirmed by showing the stimulatory effects of SREBP-1 antibodies in *in vitro* transcription assays. SREBP-2 antibodies had no effect on *in vitro* transcription of ADH. Although ethanol treatment elevated the SREBP target genes fatty acid synthase and cholesterol 7-alpha hydroxylase ( $p \leq 0.05$ ) in liver, this appears to be due to increased expression of SREBP-2 ( $p \leq 0.05$ ). Mature SREBP-1 protein levels in nuclear extracts were suppressed by ethanol ( $p \leq 0.05$ ) but SREBP-1 mRNA levels were unaffected suggesting an ethanol inhibition of post-translational processing of this transcription factor. (Supported in part by R01 AA088645 TMB).

MICE WITH HEPATOCYTE-SPECIFIC RXR $\alpha$  DEFICIENCY HAVE ALTERED ALCOHOL METABOLISM.

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There is substantial overlap between vitamin A and alcohol metabolism and their mediated signaling pathways. First, the same enzymes metabolize vitamin A and ethanol. Second, alcohol toxicity and vitamin A deficiency cause the same congenital abnormalities. Third, alcohol ingestion depletes vitamin A stored in the liver. Retinoic acid (RA) is the active metabolite of vitamin A and retinoid X receptor alpha (RXR $\alpha$ ) mediates the action of RA. To investigate the interaction between alcohol and RA, alcohol metabolism and alcohol-induced liver disease were studied in hepatocyte-specific RXR $\alpha$ -deficient mice. Hepatocyte RXR $\alpha$  deficiency resulted in 50% more liver alcohol dehydrogenase (ADH) activity. Consistent with this finding, RXR $\alpha$ -deficient mice exhibited a higher rate of ethanol clearance. However, none of the seven genes that comprise the five-family mouse ADHs showed elevated mRNA. Additionally, antibodies against yeast ADH and human ADH1 showed no difference in ADH level between mutant and wild type (WT) mice. Thus, it appears that the increase in ADH activity might be a result of post-translational ADH enzyme regulation or an increase in activity of the microsomal ethanol oxidizing system. Hepatocyte RXR $\alpha$  deficiency also resulted in a 20% reduction in acetaldehyde dehydrogenase (ALDH) activity. The expression of *aldb1A1* and *aldb3A2*, but not *aldb2*, mRNA was lower in hepatocyte RXR $\alpha$ -deficient mice. In agreement with enzyme activity and mRNA level, western blot analysis demonstrated that ALDH1A1 protein was lower in the mutant mice. These findings indicate that RXR $\alpha$  might regulate expression of the *aldb1A1* and *aldb3A2* genes. Morphological analysis showed that hepatocyte RXR $\alpha$ -deficient mice were more susceptible to ethanol-induced liver damage than the WT mice, presumably because of higher levels of acetaldehyde in the liver resulting from the increase in ADH, and decrease in ALDH, activity. Taken together, our data demonstrate the importance of the vitamin A/RA signaling pathway in regulating alcohol metabolism. (supported by NIH AA014147 and CA053596)

TEMPORAL AND ZONAL EXPRESSION PATTERNS OF LIVER MRP4 AND NTCP FOLLOWING HEPATOTOXICANT EXPOSURE.

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Hepatic membrane transporters are important in the disposition of xenobiotics and endogenous compounds. Following acute chemical injury to the liver, hepatocytes are generally more resistant to toxicant re-exposure. Alterations in the expression of transporters may contribute to this resistance by preventing the accumulation of oxidative products or potentially toxic chemicals. Previous data showed the concomitant reduction of uptake transporters and induction of efflux transporters mRNA expression during chemical liver injury. Notably, administration of carbon tetrachloride ( $CCl_4$ ) resulted in 70% reduction in mRNA expression of the uptake transporter  $Na^+$  taurocholate co-transporting polypeptide (Ntcp) and 37-fold increased mRNA levels of the efflux transporter multidrug resistance protein 4 (Mrp4). This study further characterizes the expression of Mrp4 and Ntcp protein in mouse liver following  $CCl_4$  treatment. Male C57BL/6J mice received a hepatotoxic dose of  $CCl_4$  (25  $\mu$ l/kg). Liver samples were collected at 6, 24 and 48 hrs for western blot and immunofluorescence analysis. Hepatic Ntcp protein expression was reduced 50 and 30% at 24 and 48 hrs, respectively. Conversely, induction of Mrp4 protein was prominently detected in  $CCl_4$ -treated mice at 24 and 48 hrs in comparison to almost unnoticeable expression in control mice. Double immunofluorescence staining of liver sections demonstrated the simultaneous down-regulation of Ntcp and up-regulation of Mrp4 in centrilobular hepatocytes. These localized changes in immunofluorescence coincided with areas of necrosis in  $CCl_4$  treated mice. These data suggest that centrilobular hepatocytes attempt to limit intracellular accumulation of Mrp4 and Ntcp substrates (i.e., bile acids, oxidative by-products and xenobiotics) in response to damage. The selective zonal expression of transporters may reduce the overall chemical burden of an injured liver during repair and regeneration and contribute to the resistance of hepatocytes to subsequent toxicant exposure.

METHYLENEDIANILINE INJURY TO LIVERS OF TR<sup>-</sup> [LIVER CANALICULAR MRP2 TRANSPORTER-DEFICIENT] RATS IS NOT LINKED TO OXIDATIVE STRESS.

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**Background.** Methyleneedianiline (DAPM) is an industrial chemical used in the production of polyurethanes and epoxy resins. DAPM initially injures biliary epithelial cells (BEC) in rats with normal canalicular transport mechanisms. In contrast, DAPM injury is shifted from BEC to hepatocytes in mutant transport-deficient (TR<sup>-</sup>) rats, which lack expression of the canalicular multispecific organic anion transporter (cMOAT or MRP2) and have an impaired capacity for biliary excretion of organic anions, GSH and GSH conjugates. Because DAPM hepatic injury in TR<sup>-</sup> rats is both rapid and severe, our objective was to assess the effects of DAPM exposure on parameters indicative of oxidative stress. **Methods.** Female TR<sup>-</sup> rats (19-22 wks old) were given 50 mg DAPM/kg po in 35% ethanol and sacrificed at 16 hr. Liver and blood were removed for analysis; liver mitochondria were isolated by differential centrifugation. **Results.** Serum Serum ALT levels of DAPM rats were elevated ~5-fold without an increase in serum bilirubin and bile salt levels. **Histology.** Liver weights in treated females were only mildly increased ( $p > 0.08$ ). Livers of DAPM rats showed mild inflammatory infiltrate around central veins and scattered, small foci of apparently oncotic cells in centrilobular to midzonal regions. Midzonal hepatocytes were frequently vacuolated, reminiscent of mild fatty change. BEC injury was not observed. **Oxidative Stress.** Reduced (GSH) and oxidized (GSSG) glutathione levels in livers were comparable between control and treated rats. Similarly, mitochondrial GSH and GSSG levels were not altered by DAPM treatment. Immunohistochemical staining for 4-HNE was not discernably increased in liver sections of DAPM-treated rats. **Conclusion.** The observed lack of detectable changes in indices of oxidative stress, namely GSH, GSSG, and 4-HNE, after DAPM treatment suggests that oxidative stress is not an important mechanism for the hepatocellular injury induced by this toxin in TR<sup>-</sup> rats. (Supported by NIH grants ES06348; DK56494)

PLASMA ESTERASE ACTIVITY PROTECTS AGAINST COCAINE HEPATOTOXICITY IN FEMALE MICE.

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Oral cocaine administration results in hepatic necrosis, increased plasma transaminase concentration, and decreased anti-oxidative capability which is potentiated by lipopolysaccharide (LPS) in male CF-1 mice. Females administered the same treat-

ment regimen display none of the hepatotoxic effects seen in their male counterparts. This study was conducted to further dissect the mechanisms responsible for this gender difference in cocaine hepatotoxicity (CH) and lipopolysaccharide potentiation of CH. Male and female CF-1 mice were orally administered 20 mg/kg cocaine hydrochloride once daily for seven days. Four hours after the last cocaine administration the mice were administered  $12 \times 10^6$  EU LPS intraperitoneally. The activity of plasma esterase (butyrylcholinesterase), the enzyme responsible for the major pathway of cocaine metabolism to non-hepatotoxic metabolites, was measured. Aminotransferase release and histological analysis were used to determine hepatotoxicity. The concentration of the hepatotoxic precursor norcocaine was measured in the plasma and liver. Regardless of treatment, males were shown to have only 30% of the plasma esterase activity displayed by females. In addition, administration of testosterone to ovariectomized females resulted in a 70% reduction in plasma esterase activity when compared with surgically unaltered females. Moreover, hepatic norcocaine was not detected in the plasma or liver of surgically unaltered female animals while it was present in males and testosterone supplemented ovariectomized females. These results indicate that plasma esterase activity is heavily influenced by sex hormones, predominantly testosterone, in CF-1 mice. Suppression of plasma esterase by testosterone correlates with decreased norcocaine production and is therefore responsible, in part, for the increased CH seen following oral administration with and without LPS exposure in male CF-1 mice.

IDENTIFICATION OF NOVEL SUBSTRATES AND INHIBITORS OF SOLUBLE EPOXIDE HYDROLASE N-TERMINAL PHOSPHATASE ACTIVITY BASED ON ITS SUBCELLULAR LOCALIZATION.

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Recently, soluble epoxide hydrolase (sEH) has been found to possess an N-terminal phosphatase activity in addition to its C-terminal epoxide hydrolase activity. Endogenous hydrolase substrates include linoleic acid and arachidonic acid epoxides, some of which have been suggested to be involved in regulating blood pressure and inflammation. However, there are limited data regarding possible endogenous substrates for the N-terminal phosphatase. In this study, we applied immunofluorescence and confocal microscopy techniques using markers for different subcellular compartments to evaluate sEH co-localization in an array of human tissues. We then evaluated possible substrates based on our sEH subcellular localization findings. Results showed that sEH is both cytosolic and peroxisomal in human hepatocytes and renal proximal tubules. Isoprenoid phosphate metabolites, which are synthesized in peroxisomes as precursors for cholesterol biosynthesis and protein isoprenylation were found to be substrates of the N-terminal phosphatase, with farnesyl monophosphate having a  $K_m$  of 24.16 ( $\pm 7.85$ )  $\mu$ M and  $V_{max}$  of 684.2 ( $\pm 92.7$ ) nmole/min/mg. Based on the isoprenoid phosphate structure we were able to identify several N-terminal phosphatase inhibitors with IC<sub>50</sub> values in the low micromolar range. To our knowledge, this is the first report showing 1) the subcellular localization of sEH in a wide array of human tissues 2) farnesyl, geranyl and geranylgeranyl mono- and pyrophosphates as possible endogenous substrates of the sEH N-terminal phosphatase and 3) sEH phosphatase inhibitors. Our data suggests a possible role of sEH in the cholesterol biosynthesis pathway and/or protein isoprenylation. These results warrant further investigation of the biological significance of sEH in these pathways.

EFFECTS OF BETAINE SUPPLEMENTATION ON HEPATIC METABOLISM OF SULFUR-CONTAINING AMINO ACIDS IN MICE.

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We previously reported that acute betaine treatment induced significant changes in the hepatic glutathione and cysteine levels in mice and rats. The present study was aimed to determine the effects of dietary betaine supplementation on the metabolism of sulfur-containing amino acids. Alterations in the enzyme activities and metabolites involved in the transsulfuration reactions were determined in male mice supplemented with betaine (1%) in drinking water for two weeks. Betaine intake increased hepatic methionine, S-adenosylmethionine, and S-adenosylhomocysteine levels significantly, whereas homocysteine, cystathione, and taurine were decreased. Hepatic methionine adenosyltransferase activity was elevated to 3-fold of control. Cysteine dioxygenase activity was decreased suggesting an inhibition of cysteine catabolism to taurine. Glycine levels in liver and plasma were decreased significantly, but hepatic serine was elevated. Despite the significant changes in the transsulfuration reactions, neither hepatic cysteine nor glutathione level was altered by betaine intake. The results indicate that betaine supplementation enhances recycling of homocysteine for the generation of methionine and S-adenosylmethionine

while reducing its utilization for the synthesis of cystathione and cysteine in liver. However, the hepatic level of cysteine or glutathione is not changed due to the depression of cysteine catabolism to taurine in betaine-fed mice. The pharmacological/toxicological significance of this finding is discussed.

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APPLICATION OF QSARS TO EVALUATE MOLECULAR MECHANISMS OF HALOBENZENE INDUCED CYTOTOXICITY IN RAT HEPATOCYTES.

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Halobenzenes are ubiquitous environmental contaminants, which are hepatotoxic in rodents and humans. Their toxicological effects however, are believed to be mediated by their metabolites. Accordingly, an understanding of the molecular mechanism of halobenzene induced cytotoxicity is desirable. We applied quantitative structure-activity relationships (QSAR) and accelerated cytotoxicity mechanism screening (ACMS) to evaluate the cytotoxicity of halobenzene congeners in rat hepatocytes. Rat hepatocytes were freshly isolated by 2-step collagenase perfusion method, and maintained at 37°, in a 95%O<sub>2</sub>/5%CO<sub>2</sub> atmosphere. Cytotoxicity was determined by trypan blue exclusion tests. The LD<sub>50</sub> of 16 halobenzene congeners in rat hepatocytes *in vitro* at 2 h determined by ACMS correlated to the hepatotoxicity, measured as plasma ALT levels, reported in rats *in vivo*. ACMS revealed that halobenzene cytotoxicity was markedly increased by sulfotransferase, UDP-glucuronosyl transferase and NADPH:Quinone Oxidoreductase inhibitors suggesting that toxicity was due to P450 oxidation to phenols and quinones and not epoxides. We derived a QSAR and found that the cytotoxicity of halobenzene LD<sub>50</sub> values correlated with dipole moment,  $\mu$ (symmetry), and log  $P$ (hydrophobicity): log LD<sub>50</sub>(2h)=−0.641(±0.124) $\mu$ −0.685(±0.205)log  $P$ +5.713. The most cytotoxic halobenzenes were those that had asymmetrically distributed halogen substituents and were more hydrophobic. This suggests that unsymmetrical halobenzenes were well activated by microsomal metabolizing enzymes to phenols and quinones whereas symmetrical halobenzenes were poorly activated by metabolizing enzymes. In conclusion, the derived QSAR implies that halobenzene cytotoxicity depends on P450 activation to reactive metabolites.

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ACTIVATION OF PROMUTAGENS WITH HUMAN LIVER S9 AND HUMAN HEPATOCYTES USING CHINESE HAMSTER OVARY MICRONUCLEUS ASSAY.

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*In vitro* genetic toxicity test systems require the addition of external metabolic activation sources to detect promutagens or proclastogens. The use of induced rat liver S9 is a well established metabolic activation system for detection of potential genotoxic activity of xenobiotics. However, species often respond differently to a variety of chemical carcinogens. Species-specific differences in drug metabolism are believed to be a key determinant of differences in drug toxicity. For the genetic safety assessment of human pharmaceuticals, the use of human liver S9 and/or human liver may be more appropriate than to use induced rat liver S9. This would have greater relevance to human exposure as it would result in the metabolism of drug closer to human *in vivo* condition. We evaluated the proficiency of human hepatocytes and human liver S9 to activate three different class of promutagens cyclophosphamide (CP), dimethylnitrosamine (DMN) and dimethylbenzanthracene (DMBA) using Chinese hamster ovary cells. We used cytokinesis-blocked *in vitro* micronucleus assay. Multiple endpoints e.g. apoptosis, necrosis, multinucleated cell, induction of micronuclei, and cell proliferation were evaluated in this assay. Our results indicate that all three promutagens formed genotoxic metabolites in presence of human hepatocytes and human liver S9. Results also indicated CP as a strong inducer of apoptosis and necrosis (2-3 folds) in comparison to DMN and DMBA. Results were comparable to that of Rat S9. Results of the study suggest that human hepatocytes and human liver S9 can activate efficiently various classes of promutagens and may be applied in routine *in vitro* genotoxicity assays.

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RUBRATOXIN B INDUCED TISSUE INHIBITOR OF METALLOPROTEINASES (TIMP)-1 SECRETION IN HEPG2 CELLS.

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Rubratoxin B is a potent hepatotoxic mycotoxin produced by various *Penicillium* fungi. Last annual meeting, we reported that stress-activated MAP kinases (SAPKs) regulate rubratoxin B-caused cytotoxicity and cytokine secretion in human he-

patoma cell line HepG2. In this study, the effect of rubratoxin B on the secretion of tissue inhibitor of metalloproteinases (TIMP)-1 was investigated using HepG2 cells. TIMP-1 has several physiological activities such as promotion of cell proliferation and anti-apoptosis activity. Furthermore, TIMP-1 is considered to be in charge of liver fibrosis and cancer metastasis. Rubratoxin B apparently induced TIMP-1 secretion in HepG2 cells. According to RT-PCR, it is likely that rubratoxin B-induced TIMP-1 secretion is chiefly regulated transcriptionally. We previously reported that active (phosphorylated) forms of SAPKs were detected in rubratoxin B-treated HepG2 cells, indicating the possibility that SAPKs (JNKs and p38s) are involved in rubratoxin B-induced TIMP-1 secretion. JNK inhibitor SP600125 did not affect rubratoxin B-induced TIMP-1 secretion. Conversely, p38 inhibitor SB203580 augmented rubratoxin B-induced TIMP-1 secretion. The results of RT-PCR showed that SB203580 increased the amount of TIMP-1 mRNA. However, compared with the increase in TIMP-1 secretion, the increase in mRNA was not significant, suggesting that p38 impairs rubratoxin B-induced TIMP-1 secretion both transcriptionally and post-transcriptionally. Our results lead us to the hypothesis that rubratoxin B causes liver fibrosis via the increase in TIMP-1 secretion. In fact, the treatment of rubratoxin B increased TIMP-1 secretion in mice sera. This result supports our hypothesis.

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FUMONISIN B<sub>1</sub> AND TNF $\alpha$  INDUCED INCREASES IN SPHINGANINE AND SPHINGOSINE IN HEPG2 HEPATOCYTES ARE ATTENUATED BY SP600125, A JNK INHIBITOR.

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Sphinganine and sphingosine, the most common sphingolipid bases in mammalian cells, are important mediators in fumonisin B<sub>1</sub> (FB1) toxicity and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) mediated transplant rejection and cardiovascular dysfunction. *In vivo*, we have shown that FB1 induces hepatotoxicity, cardiotoxicity and alteration of sphingolipids in pigs and horses. *In vitro*, we have demonstrated a sustained increase in sphinganine and a transient increase in sphingosine in HepG2 hepatocytes after 24 hr exposure to FB1 and TNF $\alpha$ , respectively. To determine the signaling pathway responsible for the alteration in sphingolipid metabolism, we investigated the effects of c-Jun N-terminal kinase (JNK) inhibitor, SP600125 (10  $\mu$ M), and transcription factor NF- $\kappa$ B inhibitors, Helenalin (1  $\mu$ M) and Bay 11-7082 (10  $\mu$ M), on the amounts of sphinganine and sphingosine in HepG2 cells following 24 hr treatment with FB1 (10 M) and TNF $\alpha$  (10 ng/mL). Pretreatment (30 min) of HepG2 cells with the JNK inhibitor, SP600125, decreased intrinsic sphingosine, and attenuated FB1 and TNF $\alpha$  induced increases in sphinganine and sphingosine, respectively. In contrast, pretreatment with NF- $\kappa$ B inhibitors Helenalin and Bay 11-7082 did not affect the increases in sphinganine and sphingosine. The results suggest an interaction between the JNK signaling pathways and the sphingolipid metabolic pathways in HepG2 cells, and a potential novel application of JNK inhibitors for the treatment of FB1 and TNF $\alpha$  mediated diseases. Supported by the Society of Toxicologic Pathology/Abbott Laboratories Clinical Research Award and International Life Sciences Institute.

1930

AUGMENTATION OF LIPOPOLYSACCHARIDE-INDUCED GENE EXPRESSION AND LIVER INJURY BY RANITIDINE BUT NOT FAMOTIDINE.

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Rats cotreated with nonhepatotoxic doses of lipopolysaccharide (LPS) and ranitidine (RAN) develop liver injury, whereas LPS/famotidine (FAM)-cotreated rats do not. This result matches the relative propensity of these two drugs to precipitate idiosyncratic liver injury in humans. We tested the hypotheses that 1) early changes in hepatic gene expression could distinguish LPS/FAM-cotreated rats from rats cotreated with LPS/RAN and 2) cotreatment with RAN but not FAM alters the expression of some LPS-regulated genes. Rats were treated with a nontoxic dose of LPS (44 x 10<sup>6</sup> EU/kg, iv) or its vehicle, then two hours later with RAN (30 mg/kg, iv), FAM (6 mg/kg; a pharmacologically equi-efficacious dose, or 29 mg/kg; an equimolar dose, iv), or vehicle. They were killed 2 or 6 h after drug treatment for evaluation of hepatotoxicity and liver gene expression (2 h only). Hierarchical clustering of gene expression data from Affymetrix Rat Genome 230 2.0 arrays identified the LPS-treated rats as a unique group. RAN-treated groups were distinguishable from FAM-treated groups in the animals co-treated with LPS and also in the

animals co-treated with Veh. Relative to the Veh/Veh group, a similar set of transcripts was altered in the groups treated with LPS alone and with LPS/FAM. For the vast majority of these transcripts (6307 of 6321), the magnitude of change was similar in these two groups. Within this group of probe sets, LPS/RAN cotreatment either enhanced or prevented the LPS-induced change in expression of several genes encoding inflammatory mediators, transcriptional regulators, and others. The results suggest that LPS/RAN cotreatment, but not LPS/FAM cotreatment, alters the expression of some genes regulated by LPS. Protein products of several of these genes (e.g., COX-2 and MIP-2) have actions that could play a role in LPS/RAN-induced liver injury (Supported by NIH grant DK061315).

### 1931

#### 1H-NMR BASED METABONOMICS STUDY OF GALACTOSAMINE, METHYLENE DIANILINE, AND CLOFIBRATE IN RATS.

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Efficacy of 1H-NMR based metabolomics was studied in rat liver toxicity models. Galactosamine (GAL), methylene dianiline (4, 4'-diaminodiphenylmethane; DAPM), or clofibrate (CLO) was intraperitoneally (GAL) or orally (DAPM and CLO) administered to male Crj:CD (SD) IGS rats (8 weeks old) on Day 1. Control animals received the vehicle in the same manner. The following examinations were conducted: blood chemistry on Days 2, 3, and 5, 24-hour urine samples for 1H-NMR from Day 1 (pretreatment) to Day 5, and histopathology. After administration, apparent increases in liver enzymes were shown in animals given GAL or DAPM, and histopathology revealed typical changes following hepatocyte deaths and bile duct damage in animals given GAL and DAPM, respectively. Principle component analysis of 1H-NMR spectra showed differences between treatment and control groups, and in addition, the CLO treated group was discriminated from the GAL and DAPM treated groups. Changes in the 1H-NMR spectra over time showed characteristic peaks that suggested possible biomarkers for the respective toxicities.

### 1932

#### EVALUATION OF A NOVEL ANTI-TUMOR DRUG USING *IN VITRO* TOXICITY SCREENING IN RAT HEPATOMA (H4IIE) CELLS, NORMAL RAT KIDNEY (NRK) CELLS, AND RAT PRIMARY HEPATOCYTES.

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Cytoreg® is a novel drug candidate shown to have cytotoxic activity against cancer cells. The compound is acidic in nature and has the potential of being an effective first line treatment for various cancer indications. This study was designed to identify potential cytotoxicity not related to the pharmacology and to determine whether the drug can distinguish tumor from non-tumor cells. Rat primary hepatocytes, normal rat kidney (NRK) cells and rat hepatoma (H4IIE) cells were used as the test systems. *In vitro* toxicity assays were used to measure membrane leakage, cell number, mitochondrial function, oxidative stress, and apoptosis. Dosing was based on a series of sponsor-requested dilutions, including dilutions of 2000, 1000, 500, 100, 50, 10, and 1. The highest dilution represented the lowest exposure concentration. The effect of pH in the absence of drug was also determined. Cells were cultured in 96-well plates and exposed for 6 and 24 hr. In H4IIE cells, the compound was lethal to more than 90% of the cells at the 500 dilution at 24 hr. These effects occurred prior to any cytotoxic effect related to low pH. Dilutions of 100 and lower showed cytotoxicity that could not be distinguished from effects due to low pH. Rat primary hepatocytes were used to investigate cytotoxic effects on non-tumor cells. Exposure dilutions of 2000, 1000, and 500 did not produce any acute cytotoxicity in these cells, as was observed in H4IIE cells at the 500 dilution, but did produce effects on the mitochondria. NRK cells were evaluated to investigate specificity and selectivity in another non-tumor cell line. No cytotoxic effects were observed up to and including the 500 dilution after 6 and 24 hr. Significant effects were observed on the mitochondria at dilutions of 100 and lower, consistent with effects due to low pH. These data indicate that the tumor cell line under conditions of near physiologic pH is considerably more sensitive to the cytotoxic effects of Cytoreg® than are non-tumor cells based on cell number and membrane integrity.

### 1933

#### UPREGULATION OF STEM CELL-DERIVED TYROSINE KINASE (STK) EXPRESSION IN LIVER MACROPHAGES AND ENDOTHELIAL CELLS DURING ACUTE ENDOTOXEMIA IS INDEPENDENT OF TUMOR NECROSIS FACTOR RECEPTOR-1.

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Acute endotoxemia is associated with tissue injury caused by excessive release of proinflammatory and cytotoxic mediators from activated liver macrophages and endothelial cells. Macrophage stimulating protein (MSP) is an immunoregulatory

growth factor produced mainly by hepatocytes that is thought to play a critical role in inflammation and tissue repair. MSP exerts its action by binding to a membrane receptor kinase, stem cell-derived tyrosine kinase (STK), on responsive cell types. In the present studies, we analyzed STK expression in liver macrophages and endothelial cells during acute endotoxemia. The role of TNF- $\alpha$  in STK expression was also evaluated. Macrophages and endothelial cells were isolated from the livers of wild type (WT) and TNF- $\alpha$  receptor (TNFR1, p55) knockout mice treated i.p. with 3 mg/kg endotoxin or control. We found that freshly isolated macrophages and endothelial cells from control WT and TNFR1-/- mice constitutively expressed STK mRNA as determined by real time RT-PCR. Treatment of mice with endotoxin caused a time-dependent increase in STK mRNA expression in both hepatic macrophages and endothelial cells, which was maximal at 3 hr. No major differences were noted between the mouse strains. In endothelial cells isolated from WT mice 24 hr after endotoxin administration, TNF- $\alpha$  (5 ng) or IL-1 $\beta$  (5 ng) treatment (24 hr) upregulated STK expression. This was not observed in cells from control animals. These data suggest that MSP may play a role in nonparenchymal cell activation and tissue injury during acute endotoxemia. However, this activity is independent of TNFR1. Upregulation of STK in endothelial cells by TNF- $\alpha$  may occur via activation of alternative cell surface receptors. Supports: NIH GM34310, ES04738 and ES05022.

### 1934

#### EVALUATION OF SYSTEMIC TOXICITY IN MIXTURES OF TRICHLOROETHYLENE (TCE), HEPTACHLOR (HEPT), AND DI(2-ETHYLHEXYL)PHTHALATE (DEHP) ASSESSED IN A 5X5X5 DESIGN.

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While a large number of experiments have evaluated the interactive toxicity of environmental chemicals with the same mechanism or similar modes of action, fewer have examined the interactions of chemicals with diverse modes of action. This study examined the interactive toxicity of three diverse chemicals: TCE, DEHP and HEPT. Female F-344 rats were dosed daily by gavage for 10 consecutive days. A 5x5x5 full-factorial design with 125 treatment groups (n=10/group) was used in which rats received either one of five dose levels of TCE alone (0, 40, 200, 800, 1200 mg/kg/day), DEHP alone (0, 50, 100, 150, 200 mg/kg/day) or HEPT alone (0, 1, 3, 10, 14 mg/kg/day) or else one of the possible binary and tertiary combinations. Eleven endpoints (body weight loss, brain weight, relative thymus weight, relative adrenals weight, relative spleen weight, relative kidney weight, relative liver weight, serum bilirubin, serum alanine aminotransferase, liver pathology and kidney pathology) were analyzed by response-surface methodology. The overall test for interaction was significant for seven endpoints. Ten of the 33 binary interaction terms were statistically significant; nine of these ten were less than response additive and one (the interaction of TCE and DEHP on liver pathology) was greater than response additive. Three-way interactions were detected for three endpoints (relative liver weight, relative kidney weight and bilirubin); their effect was a dose-dependent decrease in the magnitude of the antagonistic two-way interactions. In summary, nonadditive binary interactions were frequently detected; most were less than additive. Three-way interactions were less frequent and acted to lessen the severity of the antagonistic binary interactions. Response-surface experiments are labor- and time-intensive, in conduct, analysis and interpretation, pointing out the need for more efficient mixtures designs and analyses. (This abstract may not reflect EPA policy)

### 1935

#### FIRST-PASS EFFECTS ON THE BIOAVAILABILITY OF HISTAMINE DIHYDROCHLORIDE. A MULTIPLE-CANNULAE DOG MODEL FOR INVESTIGATION OF HEPATIC ELIMINATION.

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Histamine dihydrochloride, (Ceplene™, 2-(1H-imidazol-4-yl) ethanamine) is a synthetic analog of histamine, which is being tested subcutaneously in cancer patients. An oral formulation (HD-O) is being developed to target liver diseases. Animal models are an essential aspect of the testing of alternative formulations of histamine due to its extensive first-pass effect. A dog model was developed for investigation of the hepatic elimination of histamine. The study involved intra-duodenal (ID) infusion in conscious animals of HD-O with or without aminoguanidine sulfate (AGS), a potent diaminoxidase inhibitor. Following a 10-minute infusion with saline or AGS (50 mg/kg), HD-O (0.5 to 15 mg/kg) was infused for 10 minutes. Histamine concentrations were measured in portal (PV), caval (CV)

and jugular vein (JV) up to 4 hours. The hepatic extraction ratios (ER) were calculated as the difference between PV and CV histamine levels relative to PV values. Maximum oral bioavailability (OBA) was calculated as 1-ER. The intrinsic hepatic clearance ( $Cl_{H_2}$ ) was calculated as  $LPF \times ER$  where LPF is the estimated liver plasma flow. Mean arterial pressure (MAP) was measured indirectly. The mean ER values for histamine were 0.94 and 0.79 with or without AGS, respectively indicating that HD-O exhibited an extensive first-pass effect. Mean OBA values ranged from 5.9 to 21.3%. Mean  $Cl_{H_2}$  ranged from 0.69 to 1.03 L/hr/kg. This data indicate that the disposition of HD-O could be influenced by changes in liver blood flow. Based on pharmacokinetic/pharmacodynamic (PK/PD) analyses, a significant reduction in MAP from baseline (as low as 40 mmHg) was observed which correlated with plasma histamine levels in both JV ( $p=0.004$ ) and CV ( $p=0.024$ ). The present data demonstrate the utility of this model for the investigation of hepatic first-pass effects on histamine as well as PK/PD correlations.

### 1936

#### REACTIVITY AND MODELING STUDIES PREDICT THAT FLUORINATED AMINOPHOSPHONATES (FAP) INHIBIT CHOLINESTERASES (CHE) VIA P—C BOND CLEAVAGE.

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Previous kinetic studies showed that FAP of general formula  $(RO)_2P(O)C(CF_3)_2NHS(O)_2C_6H_5$  irreversibly inhibit CHE, despite their lack of a typical leaving group. QSAR, x-ray, and chemical reactivity studies of FAP phosphinate analogs suggested that FAP inhibition of CHE proceeds by P—C bond cleavage. The present research examined the molecular properties of FAP using chemical reactivity studies and molecular modeling, with a view toward understanding their mechanism of inhibition of CHE. <sup>19</sup>F and <sup>31</sup>P NMR showed that heating an equimolar mixture of the diethyl (diEt)-FAP and water in dimethylformamide yields mainly diEt phosphate,  $(EtO)_2P(O)OH$ , and the amide,  $(CF_3)_2CHNHS(O)_2C_6H_5$ , demonstrating the high lability of the P—C bond in FAP. To determine why this bond is unusually labile, a conformational search was performed for the most probable 3D structures with subsequent quantum-chemical calculations of FAP and the isosteric molecule  $(EtO)_2P(O)C(CH_3)_2NHS(O)_2C_6H_5$ , diEt-AP, which does not have  $CF_3$  groups. Energy barriers (kcal/mol) for breaking bonds in diEt-FAP assessed through virtual stretching of covalent bonds from 1.5 to 3.5 Å, were 33.1 (P—C), 76.0 and 101.8 (P—O), and 80.7 (N—S). Thus, the P—C bond was the most labile. In contrast, the energy barrier for breaking the corresponding P—C bond in the diEt-AP molecule (58.2 kcal/mol) was far greater. Moreover, modeling results showed that the P—C bond in diEt-FAP had significant lengthening (1.946 Å) compared with the corresponding P—C bond in diEt-AP (1.679 Å), in agreement with x-ray studies. The results support the hypothesis that CHE inhibition by FAP proceeds by cleavage of the P—C bond, which is stabilized by the presence of two  $CF_3$  groups. (Supported by CRDF RB2-2035 & RB2-2488 and ARO DAAD19-02-1-0388).

### 1937

#### X-RAY CRYSTAL STRUCTURES OF FLUORINATED AMINOPHOSPHONATE (FAP) COMPOUNDS SHOW AN ELONGATED P—C BOND AND DIMERIZATION.

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FAP compounds,  $(RO)_2P(O)C(CF_3)_2NHS(O)_2C_6H_5$ , R = alkyl, inhibit serine esterases. It is thought that inhibition involves phosphorylation of the active site serine, although this mechanism would involve an unusual reaction requiring scission of the P—C bond. Moreover, FAP compounds exhibit a time-dependent increase in anti-esterase potency after being dissolved in organic solvents, while their NMR spectra do not change. To gain insight into the unusual properties of FAP compounds, the x-ray crystal structures of the ethyl, *n*-propyl, isopropyl, and *n*-butyl analogs were determined. X-ray intensities were measured at 113 K and refined to R values of 0.028-0.035. The structural studies revealed that the compounds have an elongated P—C bond (1.897 Å) compared to the normal P—C ( $\sigma^3$ ) bond (1.844 Å). In addition, all of the compounds form dimers due to intermolecular H-bonding (P=O—H—N). Dimerization is favored by the geometry of the  $CF_3$  groups. Using the ethyl analog as an example, the  $CF_3$ —C— $CF_3$  angle is 109.3°. This angle allows for close intermolecular proximity of the N—H and P=O moieties without incurring steric hindrance from the  $CF_3$  groups. Whereas the intermolecular H-bond distance is 2.0 Å, the intramolecular H-bond distance is 2.3 Å.

Furthermore, the intramolecular H-bond angle is 119.0°, while the intermolecular H-bond angle is 157.7°. The intermolecular H bond angle is more open and stronger than the relatively closed and weaker intramolecular H bond. The observed dimerization may explain why FAP compounds require prolonged solvation for maximum inhibitory potency. Moreover, the elongated (and therefore weaker) P—C bond supports the hypothesis that this bond is cleaved during inhibition of serine hydrolases. (Supported by CRDF RB2-2035 & RB2-2488 and ARO DAAD19-02-1-0388).

### 1938

#### MASS SPECTROMETRY REVEALS THAT SERINE ESTERASES ARE PHOSPHORYLATED AND AGED BY FLUORINATED AMINOPHOSPHONATE (FAP) COMPOUNDS.

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Serine esterases are inhibited by dialkyl FAP compounds of general formula,  $(RO)_2P(O)C(CF_3)_2NHS(O)_2C_6H_5$ , where R = alkyl. It has been hypothesized that the active site serine attacks the phosphoryl moiety resulting in formation of a dialkyl phosphate adduct that can age by net loss of an R group. However, this mechanism would require an unusual inhibition reaction involving scission of a P—C bond. The present work tested this hypothesis by identifying FAP adducts on serine esterases before and after aging using peptide mass mapping with surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS). Esterases were horse serum butyrylcholinesterase (BChE) and human neuropathy target esterase catalytic domain (NEST). Dialkyl FAP compounds included R = ethyl, *n*-propyl, isopropyl, and *n*-butyl. For the diethyl analog, mass shifts (mean  $\pm$  SE, average mass  $m/z$  for inhibited and aged, respectively) were 135.6  $\pm$  0.6 and 106.9  $\pm$  0.5 Daltons (Da) for BChE ( $n \geq 8$ ), and 135.5  $\pm$  0.3 and 107.4  $\pm$  0.4 Da for NEST ( $n = 2$ ). These values were statistically identical to the theoretical mass shifts for the intact and aged diethyl phosphoryl adduct of 136.1 and 107.0 Da, respectively, presuming aged species are deprotonated. Similarly, mass shifts for intact and aged adducts of the other FAP analogs on BChE and NEST were within 0.0–0.8 Da of their respective theoretical values. The results support the hypothesis that both BChE and NEST are inhibited by scission of the P—C bond, in agreement with predictions from computational modeling and x-ray studies. Furthermore, aging occurs in each case yielding a monoalkyl phosphoryl adduct. Determination of the protonation state of the aged species detected by MS will require further research. (Funded by CRDF RB2-2035, 2488; ARO DAAD19-02-1-0388).

### 1939

#### COMPARISON BETWEEN ELLMAN AND RADIOMETRIC METHODS FOR ASSESSING CHOLINESTERASE (CHE) INHIBITION IN RATS TREATED WITH *N*-METHYL CARBAMATE INSECTICIDES.

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Carbamylated ChE is unstable and readily reactivates. This reactivation, promoted by increasing temperature and dilution, could have an impact on *ex vivo* ChE assays by decreasing apparent ChE inhibition. To assess the best method for measuring ChE inhibition in brain and RBCs from animals treated with one of 5 different carbamate insecticides, ChE was analyzed using both the Ellman method (encourages reactivation through tissue dilution and higher temperature) and a radiometric method (limits reactivation with lower tissue dilution and lower temperature). All tissues were prepared in exactly the same manner, and only diluted immediately before assay. Each tissue was assessed using both assays on the same day. Adult male rats were dosed orally with carbaryl, methomyl, methiocarb, oxamyl, or propoxur. At the time of peak effect, following a single dose of these compounds, the Ellman method underestimated the level of ChE inhibition; the degree of reactivation depended on the carbamate and ranged from 10-40% for the brain and 10-45% for the RBCs. Comparison of the dose-response curves showed an even greater difference between the methods: at the extreme, the Ellman method showed no brain ChE inhibition in any propoxur dose group, whereas radiometric data showed a significant, predictable dose-response relationship. In addition, the variability in the data was increased using the Ellman method. The combined coefficients of vari-

ation (CV; (StDev/mean) x 100) for all dosage groups for all compounds were 11% (brain) and 25% (RBC) for the radiometric method, but for the Ellman method, CVs were increased: 21% (brain) and 38% (RBC). These data indicate that the radiometric method is far superior to the Ellman method for assessing ChE inhibition in animals treated with *N*-methyl carbamate insecticides. This is an abstract of a proposed presentation and does not reflect Agency policy.

1940

TIME COURSE AND DOSE RESPONSE ASSESSMENT OF CHOLINESTERASE (CHE) INHIBITION IN ADULT RATS TREATED ACUTELY WITH CARBARYL, METHOMYL, METHIOCARB, OXAMYL OR PROPOXUR.

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To compare the toxicity of 5 *N*-methyl carbamates, the time course and dose response profiles for ChE inhibition were established for each. For the time course comparison, adult male Long Evans rats (n=5 dose group) were dosed orally with either carbaryl (CB; 30 mg/kg in corn oil), methomyl (MM; 3 mg/kg in water); methiocarb (MC; 25 mg/kg in corn oil); oxamyl (OM; 1 mg/kg in water) or propoxur (PP; 20 mg/kg in corn oil). Brain and blood were taken at 0.5, 1.0, 2.0, 4.0 and 24 hrs after dosing for analysis of ChE activity using a radiometric method which limits the amount of reactivation of the ChE activity. This level of dosing produced at least 40% brain ChE inhibition with all compounds. The time-course study revealed that the time of peak effect (brain and RBC ChE inhibition) was very similar for all 5 carbamates: 0.5-1.0 hr after dosing. Two compounds, methomyl and oxamyl, however, showed significant recovery by 1.0 hr after dosing. By 24 hours after dosing, brain and RBC activity in all animals had returned to normal levels. For the dose-response study, each compound was administered at 5 different levels (plus vehicle control) and the tissues (brain and RBC) taken at 40 minutes after dosing for assessment using the radiometric assay. The lowest dose that significantly inhibited ChE activity in RBC was 15 mg/kg (CB); 1.25 mg/kg (MM); 25 mg/kg (MC); 0.5 mg/kg (OM) and 10 mg/kg (PP), and for brain was 7.5 mg/kg (CB); 0.6 mg/kg (MM); 2 mg/kg (MC); 0.5 mg/kg (OM) and 3 mg/kg (PP). Although linear regressions comparing ChE inhibition in brain and RBCs showed generally close correspondence, brain ChE was usually the more sensitive measure, especially at the lower dosages. The exception was OM where RBC ChE inhibition appeared to be more sensitive than brain ChE inhibition. *This is an abstract of a proposed presentation and does not reflect Agency policy.*

1941

COMPARISON OF ACUTE NEUROBEHAVIORAL EFFECTS OF *N*-METHYL CARBAMATE INSECTICIDES.

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The acute neurobehavioral and cholinesterase (ChE)-inhibiting effects of *N*-methyl carbamate insecticides have not been systematically compared. We evaluated five carbamates - carbaryl (CB), propoxur (PP), oxamyl (OM), methomyl (MM), and methiocarb (MC). Adult male Long-Evans rats (n=10/dose) were dosed by oral gavage with either CB (3-50 mg/kg), PP (0.3-20 mg/kg), OM (0.07-1.5 mg/kg), MM (0.1-2.5 mg/kg) or MC (0.5-25 mg/kg). Ten minutes after dosing, rats were scored for obvious signs of cholinergic toxicity ("tox score"). Horizontal (HA) and vertical (VA) activity were tabulated during subsequent 20-minute sessions in a figure-eight chamber. Brain and blood tissues were taken immediately thereafter to determine ChE activity. Exposure to each of the carbamates resulted in decreased motor activity. Based on no-effect levels (NOELs), activity was equally (PP, MC) or less (CB, OM, MM) sensitive than ChE inhibition. Within-subject regressions indicated a better correlation between motor activity and brain ChE inhibition than with blood ChE. Activity measures decreased faster than did the brain ChE activity (i.e., slope<1), with the exception of CB (slope=1). The VA dose-response curves were significantly different from HA, with greater decreases in VA at the higher doses. Nonetheless, the correlation between HA and VA was good, indicating a high concordance of effect on both measures. The ranking of toxicity as measured by the tox score ranged from no effect (CB, MC, OM) to a dose-related increase in the incidence and severity (PP, MM). Thus, some carbamates showed pronounced effects on motor activity at doses that produced little or no obvious toxicity as evaluated by this crude score. This illustrates that observable signs of cholinergic toxicity are not predictive of the magnitude of behavioral neurotoxicity. Furthermore, activity, especially VA, is a very sensitive outcome of ChE inhibition produced by carbamates. This is an abstract of a proposed presentation and does not reflect Agency policy.

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THE ORGANOPHOSPHOROUS INSECTICIDE FENTHION DOES NOT AFFECT PHAGOCYTOSIS OF ROD OUTER SEGMENTS BY RETINAL PIGMENT EPITHELIUM CELLS IN CULTURE.

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Exposure to the organophosphorous insecticide fenthion has been associated with retinal degeneration in occupational studies. It has also been associated with pigmentary changes of the retina. Because retinal degeneration and pigmentary changes may be due to dysfunction of the retinal pigment epithelium (RPE) at the back of the eye, we tested RPE cell function and viability with exposure to fenthion. Human-derived RPE cells, ARPE-19, were grown to confluence in 12 well plates. Cells were exposed for 24 hours to  $10^{-7}$  -  $10^{-5}$  M fenthion, a range of concentrations that has been shown to affect cell viability and function in other neurally-derived cell lines. Cell viability was assayed with a calcein/propidium iodide live/dead (L/D) assay. The ability of the RPE cells to phagocytize rod outer segments (ROS), one of the important homeostatic functions of the RPE, was tested by co-incubating the cells with ROS labeled with fluorescein isothiocyanate, then evaluating them with fluorescent phase microscopy. Phagocytosis was also evaluated under conditions that enhance (incubation with insulin) or inhibit this function (incubation in cold or in serum-free medium). ARPE-19 cells showed no decrease in viability after treatment with  $10^{-7}$  -  $10^{-5}$  M fenthion. Positive control experiments with hydrogen peroxide demonstrated the sensitivity of the L/D assay. This concentration range of fenthion also had no notable effect on phagocytosis of ROS. Phagocytosis could be enhanced by incubation with insulin or inhibited by cold or incubation in serum-free medium. Exposure to the OP fenthion did not produce dramatic effects on RPE cell viability or their ability to phagocytize ROS. Further work will establish the sensitivity of these cells to the oxon metabolite of fenthion. ARPE-19 cells appear to be less sensitive than neuroblastoma and PC-12 cells to the effects of fenthion. This abstract does not reflect USEPA policy.

1943

CONCURRENT EXPOSURE TO REPEATED STRESS AND CHLORPYRIFOS ALTERED NMDA AND TOTAL MUSCARINIC RECEPTOR RESPONSES IN HIPPOCAMPUS, CEREBRAL CORTEX AND HYPOTHALAMUS.

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Sustained stress causes reversible impairment to the hippocampus, possibly related to glutamatergic effects. Effects of concurrent repeated stress and chlorpyrifos (CPF) on NMDA and total muscarinic receptors in the brain were studied in Long-Evans rats. Groups of rats (n=8) were handled 5 days/week (control); restrained 1 hour/day for 5 days/week; swum 30 minutes for 1 day/week; or restrained 4 days/week and swum for 1 day/week, for 28 days. On day 24, each group was injected either with corn oil or CPF 160 mg/kg sc 4 hours after restraint. On day 28, blood samples were collected for plasma corticosterone. Brains were dissected into hippocampus, cerebral cortex, and hypothalamus to determine maximum binding density (Bmax) and equilibrium dissociation rate constant (Kd) of NMDA and total muscarinic receptors. Swim and restraint with swim elevated plasma corticosterone more than handling and restraint alone. Restrained rats (1.839 +/- 0.140 nM) had higher Kd of NMDA receptors in the hippocampus than control (1.386 +/- 0.150 nM) and restrained with swim rats (1.333 +/- 0.140 nM); however, Bmax was similar. CPF decreased Bmax and Kd of total muscarinic receptors in the cerebral cortex of swum rats (237.64 +/- 17.36 fmol/mg protein, 0.216 +/- 0.023 nM) and CPF also decreased Bmax of total muscarinic receptors in the cerebral cortex of restrained rats (229.08 +/- 17.36 fmol/mg protein). There were no effects of stress, CPF, and interactions of stress and CPF on NMDA receptors in the cerebral cortex and on total muscarinic receptors in the hippocampus although CPF down-regulated total muscarinic receptors of swum and restrained rats. Therefore, restraint stress increased binding affinity of NMDA receptors in the hippocampus and decreased Bmax of total muscarinic receptors in the cerebral cortex. Restraint with swim decreased both Bmax and Kd of total muscarinic receptors in the cerebral cortex.

1944

NEUROTROPHIN CONCENTRATION AFTER *IN VIVO* EXPOSURE TO NEUROPATHIC AND NON-NEUROPATHIC ORGANOPHOSPHATES.

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Organophosphorous (OP) esters are used as insecticides, petroleum additives, and modifiers of plastics (Ecobichon, 2004), and have also been employed as weapons of terrorism. Exposure to OP compounds that inhibit neurotoxic esterase (NTE)

induces a delayed neuropathy (OPIDN) characterized by Wallerian-like degeneration of long axons in certain animals, including humans. A previous study in our laboratory (Pope et al., 1995) discovered neurite outgrowth comparable to that of cells exposed to nerve growth factor (NGF) occurred 24 hours after incubation of SH-SY5Y neuroblastoma cells with spinal cord extracts from chickens with active OPIDN. Further studies showed that NGF, brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) are expressed in the lumbar spinal cord of chickens after neuropathic OP exposure (Pomeroy et al., SOT 2004, Abstract 1102). We hypothesized that fiber degeneration induced by neuropathic OP administration causes upregulation of neurotrophins, particularly NGF, BDNF, and NT-3, in susceptible regions of the nervous system prior to clinical signs. We exposed juvenile chickens to a neuropathy-inducing OP (PSP, 2.5mg/kg), an OP compound that does not induce neuropathy (paraoxon, 0.10 mg/kg), and vehicle (DMSO, 0.5ml/kg) intramuscularly. By day 8, all PSP-treated birds demonstrated clinical signs of OPIDN. Chickens were sacrificed by pentobarbital overdose at 4, 8, 24, and 48 hours, and 5 and 10 day post-treatment. We confirmed NTE inhibition in birds treated with PSP and performed enzyme-linked immunosorbant assays to determine the concentrations of NGF, BDNF, and NT-3 in lumbar spinal cord. Data indicate that levels of BDNF and NT-3 did not increase following neuropathy-inducing OP exposure; NGF concentration increased 24 hours post-dosing in all treatment groups. These data suggest that NGF, BDNF, or NT-3 are not individually responsible for *in vitro* neurite outgrowth.

#### 1945 CHLORPYRIFOS INDUCES APOPTOSIS IN OLIGODENDROCYTE PROGENITOR (CG-4) CELLS.

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Virtually all children in the United States have detectable levels of the organophosphate pesticide, Chlorpyrifos (CPF), in their bodies. Although, CPF has been shown to be relatively safe in adult animals, newly discovered evidence suggests that juveniles (animals and humans) may be more sensitive to CPF toxicity than adults. In young animals, CPF inhibits neural cell replication, interferes with cell differentiation, evokes oxidative stress, and alters synaptic neurotransmission. The adverse effects of CPF on neurons have been relatively well documented; however, few studies have focused on the developmental effects of chronic exposure to CPF on glial cells. Because glial cells are integral to neuronal survival and function, we studied the effects of CPF and its metabolites on oligodendrocyte viability. The viability assays utilizing Alamar Blue and Crystal Violet yielded results in which Chlorpyrifos (CPF) and its metabolite Chlorpyrifos-oxon (CPO) caused dose-dependent cytotoxicity within seventy-two (72) hours of exposure. The IC50 values for CPF and CPO were approximately 62.5  $\mu$ M and 31  $\mu$ M respectively. Nuclear morphological staining with Hoechst 33342 revealed that exposure to both CPF and CPO produced nuclear condensation as well as fragmentation, which are indicative of apoptosis. Wortmannin and PD98059 failed to reverse CPF- and CPO-induced cytotoxicity suggesting that CPF- and CPO-induced apoptosis occurs independently of PI-3 kinase and p42/44 MAP kinase activation. Taken collectively, the experimental results suggest that oligodendrocytes are sensitive to CPF and its, metabolite CPO. Furthermore, preliminary results suggest that CPF-induced neurotoxicity may be mediated, in part, by the disruption of oligodendrocyte survival and function.

#### 1946 ROLE OF A-ESTERASES IN THE AGE-RELATED DIFFERENCES IN ORGANOPHOSPHATE DETOXICATION.

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The role of the calcium dependent A-esterases in the detoxication pathways of organophosphate (OP) insecticides was investigated in rats with respect to age. The OP compounds chosen for the study were the active metabolites (oxons) of commercial insecticides or model compounds. The OP compounds were either dimethyl or diethyl phosphates and had a variety of "leaving groups". The detoxication of 12 OP compounds by liver homogenates of adult male rats was determined by comparing the decrease in OP concentration following a 15 min incubation in homogenate containing 1.0mM  $\text{Ca}^{++}$  to that containing 1.0mM EDTA. In both preparations, carboxylesterase activity was eliminated using 3 $\mu$ M 4-nitrophenyl diphenylphosphinate. The OP's were assayed at concentrations near their IC80 for bovine brain AChE, the enzyme used to quantify the residual OP concentration. Only five OP's tested were significantly detoxified by liver A-esterase. Four OP's were selected for subsequent studies with adult serum and 12-day old rat liver and serum. Because of limited tissue available, only two OP's were tested with 1-day old rat liver. As expected based on paraoxonase activities using 5mM paraoxon as a sub-

strate, detoxication of OP's tested was greater in liver than in serum, and was highest in adult tissues. Results agree with findings of greater sensitivity of young animals to the acute toxicity of some OP insecticides. (Supported by NIH ES R01 ES 11287).

#### 1947

#### EFFECT OF ORGANOPHOSPHATE INSECTICIDE EXPOSURE ON DOPAMINE METABOLISM IN STRIATAL SLICES.

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Chlorpyrifos (CP) and parathion (PT) are organophosphate (OP) insecticides which, after metabolic activation to chlorpyrifos-oxon (CPO) and paraoxon (PO), respectively, act as cholinesterase (ChE) inhibitors. While most of the neurotoxic effects associated with exposure to OPs are a direct consequence of ChE inhibition, non-cholinergic effects have been proposed. Limited evidence indicated that CP has a direct, ChE inhibition-independent, effect on brain development. Epidemiological studies have associated exposure to certain pesticides with increased incidence of Parkinson's Disease (PD), but OPs, despite their widespread use, have not been implicated in PD. The present study had two objectives: (1) to determine whether selected OPs, CP and PT, have an effect on dopamine (DA) metabolism, and (2) to assess whether the eventual effects on DA metabolism are associated with ChE inhibition. Rat striatal tissues were exposed *ex vivo* to various concentrations of CP, PT, CPO, and PO for 4 h. At the end of exposure, concentrations of DA and its metabolites DOPAC and HVA in the media and tissue were determined. In addition, tissue ChE activity was analyzed. Overall, CP and CPO were more potent than the PT and PO in decreasing tissue DA and DOPAC levels, but otherwise behaved similarly, *i.e.*, the parent compound was just as potent as the oxon. Only the highest (500  $\mu$ M) dose of PT reduced tissue DA and DOPAC levels, whereas PO, even at 500  $\mu$ M, was ineffective. On the other hand, as expected, CPO and PO were far more potent in inhibiting cholinesterase than the CP and PT. Taken together, results from this study suggest that (1) CP and its oxon are more effective in modulating DA metabolism than PT and its oxon, (2) the DA modulating effects appear to be independent of cholinesterase inhibition, and (3) rather large amounts of OPs, especially PT, are required for DA metabolism to be affected. Supported in part by: NIH T35 RR07071.

#### 1948

#### THIOFLAVIN-T, AN ACETYLCHOLINESTERASE PERIPHERAL SITE LIGAND, DECREASES NG108-15 CELL PROLIFERATION.

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Acetylcholinesterase (AChE) is widely studied for its role in the hydrolysis of acetylcholine. This enzymatic activity is inhibited by a number of organophosphate insecticides. Allowable human exposure to insecticide levels are set by their ability to inhibit AChE enzymatic activity and cause varying degrees of cholinergic symptoms. However, recent evidence has accumulated indicating AChE may also have a non-cholinergic function. Concern has been raised that this non-cholinergic function of AChE may be altered from exposure to environmental pesticides and lead to effects on neuronal development. Furthermore, studies have implicated the peripheral anionic binding site of AChE in its noncholinergic, neurotrophic action. Thioflavin-T binds selectively to the AChE peripheral site. In order to investigate whether this binding alters the non-cholinergic function of AChE in the nervous system, we used a cultured hybrid mouse neuroblastoma-rat glioma cell line, NG108-15, which expresses AChE in its undifferentiated and differentiated state. These cells were grown on tissue culture plates on a substratum containing varying amounts of AChE and the degree of growth assessed by counting adherent viable cells. The change in cell number over time was quantitated by calculating the Area Under the Curve (AUC) and the maximum concentration of cells (Cmax) achieved. Previously, an increase in cell adhesion was found when these cells were grown on AChE coated plates and this increase was blunted by the presence of Thioflavin-T. However, there was no change in the AUC or the Cmax of adherent viable cell numbers when cells were grown on a substratum containing AChE compared to controls. In addition, Thioflavin-T decreased both the AUC and the Cmax of NG108-15 cell growth at concentrations as low as 500 nM. This decrease in cell growth in the presence of Thioflavin-T was found both in control plates and tissue culture plates coated with AChE. The decrease in AUC (35%) and Cmax (36%) was lower than the AUC (76%) and Cmax (36%) for cells grown on the substratum containing AChE.

TOXICITY OF CHLORPYRIFOS AND CHLORPYRIFOS OXON IN A TRANSGENIC MOUSE MODEL OF THE HUMAN PARAOXONASE (PON1) Q192R POLYMORPHISM.

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The Q192R polymorphism of paraoxonase (PON1) has been shown to affect the hydrolysis of organophosphorous compounds *in vitro*. PON1Q192 and PON1R192 exhibit equivalent catalytic efficiencies of hydrolysis for diaxonon, the oxon form of the pesticide diazinon. However, PON1R192 has a higher catalytic efficiency of hydrolysis than does PON1Q192 for chlorpyrifos oxon (CPO), the oxon form of chlorpyrifos (CPS). Here, we examined the relevance of the Q192R polymorphism for exposure to CPS and CPO *in vivo*, through the use of a novel transgenic mouse model. Using BAC clones comprised of the entire human PON1 gene and its 5 prime and 3 prime regulatory regions, transgenic mice were generated that expressed either human PON1Q192 or PON1R192 at equivalent levels in the absence of endogenous mouse PON1. Dose response and time course experiments were performed on mice exposed dermally to CPS or CPO. After exposure, brain and diaphragm cholinesterase (ChE) activities were measured and morbidity was assessed. Mice expressing PON1Q192 were significantly more sensitive to CPO, and to a lesser extent CPS, than were mice expressing PON1R192. Compared to PON1Q192 mice, PON1R192 mice required nearly twice the dosage of CPO for 50% inhibition of brain ChE. The time course of inhibition following exposure to 1.2 mg/kg CPO revealed maximum inhibition of brain ChE at about 6 hours, with PON1Q192 and PON1R192 mice exhibiting 65% and 40% ChE inhibition, respectively. These results indicate that individuals carrying the PON1Q192 allele are more sensitive to the adverse effects of CPO or CPS exposure, especially if combined with lower plasma levels of PON1, which can vary as much as 13-fold among individuals. Supported by ES09883, ES11387, ES09601/EPA-R826886, ES04696, ES07033, T32 AG00057.

EFFECTS OF DIAZINON AND CYPERMETHRIN ON DIFFERENTIATING NEURONAL AND GLIAL CELL LINES.

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The main objective of the work was to study key morphological and proteomic changes associated with exposure of differentiating neuronal (N2a) and glial (C6) cells to sub-lethal concentrations of the sheep dip pesticides diazinon and cypermethrin. This was addressed by a combination of molecular and morphological approaches to the analysis of differentiating cells and cell extracts prepared following exposure of cells to sheep dip pesticides. Mouse N2a neuroblastoma and rat C6 glioma cells were induced to differentiate by serum withdrawal and the addition of cAMP or sodium butyrate, respectively, in the presence and absence of various concentrations of each pesticide. Exposure of cells was under co-differentiation (4 h or 24 h after the toxin was added at the point of induction of cell differentiation) or post-differentiation (cells were pre-differentiated for 24 h prior to exposure for 4 h) conditions. Concentration up to 10  $\mu$ M of both compound had no significant effect on the viability of either differentiating cell line, as determined by MTT reduction assays. Concentrations of 1  $\mu$ M and 10  $\mu$ M diazinon but not cypermethrin caused a significant decrease in neurite outgrowth in N2a cells but only after 24 h exposure under co-differentiation conditions. By contrast, no major changes were observed in the morphology of differentiating C6 cells. The results indicate that exposure to diazinon but not cypermethrin causes inhibition of axon outgrowth in differentiating N2a cells. In current work we are studying the molecular basis of these effects by indirect immunofluorescence and western blotting analysis, using antibodies that recognize specific cytoskeletal and axon-growth associated proteins.

GLUCOSE CONSUMPTION ENHANCES PARATHION TOXICITY: FUNCTIONAL AND NEUROCHEMICAL CORRELATES.

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Sugar feeding increases the toxicity of the organophosphorus (OP) insecticide, parathion. OP insecticides elicit toxicity by acetylcholinesterase (AChE) inhibition, leading to accumulation of acetylcholine (ACh) in the nervous system. We studied the effects of sugar feeding and parathion exposure on brain ACh levels using *in*

*vivo* microdialysis. Guide cannulae were surgically implanted in the striatum four days prior to beginning glucose feeding (15% in drinking water for 5 days), and OP was given after three days. Experimental groups (n= 5-6 per group) included: 1) drinking water only and vehicle (peanut oil, 1 ml/kg, sc), 2) glucose and vehicle, 3) drinking water and parathion (18 mg/kg), and 4) glucose and parathion. Body weight and cholinergic signs of toxicity (SLUD signs and involuntary movements) were monitored throughout. Two days after OP, dialysis probes were inserted (under isoflurane) and perfused with artificial cerebrospinal fluid (1.5  $\mu$ l/min). After 42 min, six dialysis samples (40 min each) were collected. Rats were then decapitated and the frontal cortex, striatum, hippocampus, and diaphragm were collected for AChE measurement. Cholinergic signs following parathion treatment were significantly greater in rats drinking glucose compared to those drinking water. Parathion caused extensive AChE inhibition (>85%) in all tissues, but no statistical differences were noted between water and sugar-fed groups. In contrast, extracellular ACh levels following parathion were significantly higher in glucose-fed vs. water only rats (2, 906  $\pm$  711 vs. 965  $\pm$  338 fmole/fraction). There was a trend (p=0.09) towards a correlation between striatal AChE and acetylcholine levels in treated rats. The results indicate that regulation of synaptic acetylcholine turnover is modified by sugar feeding. While difficult to demonstrate statistically, a minimal further decrease in synaptic AChE activity in glucose-fed rats could be biologically important, leading to further accumulation of synaptic ACh and enhanced toxicity. (Supported by OSU Board of Regents and OSU College of Veterinary Medicine).

IMMEDIATE EARLY ALTERATION AND DIFFERENTIAL PERSISTENCE OF PKA (PROTEIN KINASE A) / P-CREB (PHOSPHORYLATED CREB) PATHWAY IN THE CENTRAL NERVOUS SYSTEM OF HENS DEVELOPING DELAYED NEUROTOXICITY (OPIDN) BY A SINGLE DOSE OF DIISOPROPYLPHOSPHOROFLUORIDATE (DFP) TREATMENT.

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Diisopropylphosphorofluoridate (DFP) produces OPIDN in the chicken that results in mild ataxia in 7-14 days and severe or paralysis as the disease progresses with a single dose. Hens were treated with DFP (1.7 mg/kg, sc) after prophylactic treatment with atropine (1 mg/kg, sc) in normal saline and eserine (1 mg/kg, sc). Control groups were treated with vehicle (propylene glycol, 0.1 ml/kg, sc). The hens were sacrificed at 2, 4, and 8 hours as well as 1, to 20 days, and the tissues cerebrum and spinal cord were quickly dissected and frozen. Subcellular fractionation, SDS-PAGE and immunoblotting of the nuclear and supernatant fractions were done according to standard protocols. Protein levels of PKA, CREB and p-CREB were assessed. Histological sections of the brain and spinal cord prepared from a similar treatment approach, were subjected to immunostaining for p-CREB. There was an increase in PKA level in spinal cord nuclear fraction after 4 hrs (130%) and 8 hrs (133%), while cerebrum nuclear fraction showed decrease (77%) at 4 hrs and remained at the same level at 8 hrs. No change was seen in either spinal cord or cerebrum soluble fraction at any time points. There was an increase CREB level in the supernatant (133%) after 5 days, while nuclear and supernatant fraction of the cerebrum did not show any alterations at any time point. P-CREB was induced in the spinal cord nuclear fraction, after 1 day (150%) and 5 days (173%) of treatment, in contrast to the decreased levels P-CREB (72%) at 10 days in cerebrum nuclear fraction. Supernatant fraction of spinal cord and cerebrum did not show any changes. Immunohistochemistry data confirmed the differential alteration of P-CREB. Thus, the differential protein expression of PKA, CREB, P-CREB, reflect the susceptibility of the tissues studied, thereby confirming a role in the pathogenesis of OPIDN.

ACTIONS OF PYRETHROID INSECTICIDES ON THE SPONTANEOUS RELEASE OF GLUTAMATE FROM CULTURED HIPPOCAMPAL NEURONS.

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Pyrethroid insecticides increase the excitability of the central and peripheral nervous systems. Modulation of voltage-gated sodium channels is likely to play a primary role in this effect, but recent studies have suggested that pyrethroid effects on other ion channels may contribute to increased excitability of neurons. Pyrethroid modulation of neuronal excitability, regardless of the target site, ultimately manifest as changes in neurotransmitter release. However, very few studies have utilized neurophysiological approaches to examine directly pyrethroid effects on neurotransmitter release in either the central or peripheral nervous systems of mammals. The present studies examined the effects of two pyrethroids on glutamate release using whole-cell patch clamp recordings from pyramidal neurons in mixed hippocampal

cultures. Under control conditions, spontaneous action potential-induced glutamate release was observed as regular bursts of excitatory post-synaptic currents with average interevent intervals of 143 msec (range = 50 - 300 msec, n = 33 neurons). In the presence of both the Type I pyrethroid permethrin and the Type II pyrethroid deltamethrin, the average interevent interval within each burst increased in a concentration dependent manner between 10 nM and 10  $\mu$ M with approximate EC<sub>50</sub>s of 710 and 37 nM for permethrin (n = 3-5 neurons/concentration) and deltamethrin (n = 3-6 neurons/concentration), respectively. At 10  $\mu$ M, interevent intervals were  $\geq$  90% by deltamethrin (n = 3) and permethrin, (n = 3), respectively. This increase in time between events within individual bursts of glutamate release indicates that exposure to these pyrethroids alters the basal pattern of glutamate release from hippocampal neurons in culture. The role of various ion channels in this effect remains to be investigated. (This abstract does not necessarily reflect EPA policy).

#### 1954 EFFECT OF PYRETHROIDS ON GLUTAMATE RELEASE IN THE HIPPOCAMPUS OF FREELY MOVING RATS.

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We have been studying effects of pyrethroids on the release of various neurotransmitters in brain regions of freely moving rats. In the present study, we investigated the effect of pyrethroids on the hippocampal glutaminergic transmission. Pyrethroids, allethrin (type I), cyhalothrin (type II) and deltamethrin (type II) were found to affect the release of glutamate differently in the hippocampus of freely moving rats as measured by *in vivo* microdialysis. The basal release of glutamate of untreated rats was 0.312 pmol/20  $\mu$ l/10 min. Control vehicle injections had no effect on the efflux of glutamate in any of the experiments. Allethrin had interesting dual effects on glutamate release, at the doses of 10 and 20 mg/kg i.p. increasing the efflux to about 175 and 150% of baseline with a peak time of 60 min and decreasing it to about 50% of baseline at 60 mg/kg i.p. up to 3 hrs after administration. Cyhalothrin 10, 20 and 60 mg/kg i.p. inhibited the release of glutamate dose-dependently to about 60, 50 and 30% of baseline, respectively, with a peak time of 40-60 min after administration. Deltamethrin 10 and 20 mg/kg i.p. increased the efflux of glutamate to about 190% of baseline with a peak time of 40 min after administration and 60 mg/kg i.p. increased it to about 275% of baseline and remained at a steady level during the rest of the 3 hr experiment. Above results suggest that these pyrethroids are able to alter the release of glutamate in opposite direction than GABA release, which will be presented by a co-author at this section, in the hippocampus of rat brain.

#### 1955 EFFECTS OF EXPOSURE TO THE PESTICIDE ATRAZINE ON STRIATAL NEUROCHEMISTRY IN MALE C57BL/6 MICE ARE AGE-DEPENDENT.

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Young and/or aged animals appear to be more sensitive than adults to certain neurotoxicants. We recently demonstrated that the herbicide atrazine (ATR) decreased striatal dopamine (DA) levels in striatal slices. The present study had the objective of investigating the ability of ATR to modulate DA metabolism *in vivo* in the context of age. One, 3, and 10-month-old male C57BL/6 mice were exposed to ATR by daily gavage for 14 days at doses of 5, 25, 125, 250 mg/kg x BW<sup>-1</sup>. Groups of mice were sacrificed and brains collected at 1, 7 and 49 d post termination of ATR exposure. Sub-groups of mice were challenged with 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP; 4 X 10 mg/kg). DA and its metabolites, 3, 4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), were analyzed in the striatum by HPLC. At 1, but not at 7 d, after the last dose, ATR (125 and 250 mg/kg) decreased striatal DA, DOPAC and HVA levels in the 1, but not in the 3- and 10-month-old mice. As expected, MPTP decreased striatal levels of DA and its metabolites in all mice with the degree of decrease being the greatest in the 10-month-old mice. The decrease of DOPAC and HVA, but not of DA, was greater in the young mice that were exposed to ATR prior to the MPTP challenge; only HVA levels were decreased further in the 3-month-old mice, and no interaction between MPTP and ATR was observed in the 10-month-old mice. Thus, it appears that, pertaining to striatal neurochemistry, young mice are more sensitive to ATR than both adult and older animals and that previous ATR exposure enhances the dopaminergic toxicity of MPTP mainly by augmenting its effects on DA metabolism. Supported by: P20 RR017661 (NIH).

#### 1956

#### EFFECTS OF 14-DAY EXPOSURE TO THE HERBICIDE ATRAZINE ON DOPAMINE NEURONS IN THE SUBSTANTIA NIGRA AND VENTRAL TEGMENTAL AREA OF JUVENILE MALE C57BL/6 MICE.

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Recently, using *ex vivo* striatal slices, we demonstrated that the herbicide atrazine (ATR; a commonly used pesticide in the US) decreased striatal dopamine (DA) levels. Moreover, ATR altered striatal neurochemistry in experiments with juvenile male C57BL/6 mice *in vivo*. Daily gavage with ATR, at doses of 5, 25, 125, 250 mg/kg-BW<sup>-1</sup> for 14 days decreased striatal DA levels, as well as the levels of its metabolites DOPAC and HVA on day 15 at the two highest doses. This effect was evident on day 22, but not on day 64 of the experiment and only on the DA metabolites, but not on DA itself. An MPTP challenge on day 15 to sub-groups of mice resulted in significant decreases of striatal DA, DOPAC, and HVA on day 22; in the case of DOPAC and HVA the decreases were potentiated by ATR. To extend these findings, using the same dosing paradigm, we analyzed the number of the dopaminergic (tyrosine hydroxylase [TH]-positive) neurons in the substantia nigra (SN) and ventral tegmental area (VTA), as well as the number of all neurons (cresyl violet [CV]-positive) in these areas for specific (SN/VTA) and non-specific (CV) neuronal loss by unbiased stereology. Up to date, brains collected at days 15 and 22 were processed immunohistochemically for neuronal counting, whereas day 64 samples are currently being analyzed. Our preliminary findings suggest that ATR decreases the number of TH-positive neurons in both SN and VTA with the effect in the SN being more prominent. In addition, in the juvenile mice we used here, there does not appear to be a synergy between MPTP and ATR. Because the number of CV-positive neurons was not affected by ATR, it appears that dopaminergic neurons, more so in the SN than in the VTA, are uniquely sensitive to the effects of ATR. Supported by: P20 RR017661 (NIH).

#### 1957

#### DECREASE OF THE STRIATAL DOPAMINE RELEASE CAUSED BY ACUTE ATRAZINE EXPOSURE IS BLOCKED BY HALOPERIDOL ADMINISTRATION.

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The herbicide atrazine (ATR) is widely used to control weeds and grass in a wide variety of crops. Given its abundant use, ATR has been found as a contaminant in ground and surface water and results in human exposure. Previous rodent studies have reported that chronic atrazine exposures cause hormonal, behavioral and neurochemical disruptions. Chronic ATR decreases levels of striatal dopamine and hypothalamic serotonin. Acute ATR decreases basal striatal DA release as measured by microdialysis, decreases also present following a high potassium solution (60 mM) challenge to the system. To examine potential mechanisms in this study by which ATR decreases DA release, rats implanted with microdialysis probes in the dorsal striatum were exposed to either 1% methyl cellulose (vehicle) or 100 mg/kg ATR i.p. and then challenged with a low autoreceptor active dose of 0.01 mg/kg haloperidol or saline s.c. Haloperidol alone increased striatal DA release. However, co-administration of ATR and haloperidol reversed the decrease in DA release produced by ATR alone, producing DA levels similar to control. No significant changes in the DA metabolites DOPAC or HVA, were observed. Two possible mechanisms for the decreases in DA release caused by ATR exposure may be the inhibition of tyrosine hydroxylase or a decrease in the firing rate of dopaminergic neurons. Both of these effects are known to be blocked by D2 specific antagonists acting on presynaptic autoreceptors, which increase dopamine synthesis and subsequently release. This data also demonstrates that although the effects of ATR on the DA system may be subtle, a further challenge to this system can uncover adverse effects. Given the widespread use of ATR and its deleterious effects on the central nervous system, understanding its mechanism of action holds important implications in particular for risk assessment. ES05903, 10791 and 05017.

#### 1958

#### GENDER DIFFERENCES IN A PESTICIDE MODEL OF PARKINSON'S DISEASE.

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Epidemiological and clinical studies have demonstrated that the incidence of Parkinson's disease (PD) is greater in men than women, suggesting that female gender may be protective for the development of PD. It has been suggested that estrogen plays a role in modulating PD with it being neuroprotective, symptomatic or both. We have developed a combined pesticide exposure model of PD where a po-

tentiated decrease in dopamine function is observed in mice treated with paraquat (PQ) + maneb (MB) compared to either alone in male mice. To examine the possible gender-related differences in this model, male and female mice were exposed to saline, 10 mg/kg PQ, 30 mg/kg MB or PQ+MB twice a week for 6 weeks. Both male and female mice exposed to PQ+MB demonstrated a significant decrease in locomotor activity immediately after exposure. These male mice also failed to recover 24hrs after exposure from the 6th injection onwards, whereas females exposed to PQ+MB showed recovery at this time point. Similarly, 7 days after the last pesticide exposures, only males treated with PQ+MB still exhibited significant decreases in locomotor activity. Additionally, male mice treated with PQ+MB demonstrated greater striatal dopamine and metabolite loss compared to their female counterparts. When lipid peroxidation levels in the midbrain were evaluated, both PQ alone and PQ+MB increased lipid peroxides in both genders. However, this increase was greater in males compared to females for both treatment groups. The concentration of PQ in the striatum was similar between males and females following an acute exposure to PQ. However, only the males showed an elevation in PQ levels in the striatum following acute PQ+MB compared to PQ alone. This suggests that there are gender-related differences in toxicokinetics of PQ, partially explaining the difference in susceptibility. Studies to further understand the differential gender vulnerability are crucial in determining the mechanism of neuroprotection. Moreover, this could lead to new and better therapeutic interventions for PD. Supported by ES10791

**1959**

#### SIGNALING PATHWAYS OF PARAQUAT-INDUCED APOPTOSIS: A MODEL FOR PARKINSON'S DISEASE.

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Parkinson's disease (PD) is the second most common age-related neurodegenerative disorder. It is characterized by a loss of dopaminergic neurons in the substantia nigra which leads to decreased dopamine production in the brain. Such a loss can result in bradykinesia, tremor at rest, loss of postural reflex, and a shuffling gait. Currently, the causes and mechanisms underlying PD are still unknown. Epidemiological studies have suggested a link between pesticide use and PD. Paraquat (PQ) is an herbicide of particular interest because of its structural similarities with MPTP, a known neurotoxicant that produces Parkinson-like symptoms. Furthermore, PQ has been shown to cause Parkinson-like symptoms in animal studies *in vivo*. In this study, we use PC12 cells, a rat dopaminergic cell line, and PQ as a model to study mechanisms of cell death induced by environmental toxins in PD. Two mitogen-activated protein (MAP) kinase signaling pathways of interest are the p38 and the stress-activated protein kinase/ Jun-N-terminal kinase (SAPK/JNK) pathways. These pathways have been shown to be activated in response to stress and toxicants to cause apoptosis. Such information could prove important for better understanding of the mechanisms of PD and elucidating new drug targets. PC12 cells were dosed with 50-200uM PQ for varying time points and analyzed for cell death. Apoptosis was scored by evaluating nuclear morphology via Hoechst staining and visualization through DAPI filter. In addition, TUNEL and MTS assays were performed to further confirm apoptosis and cell viability. Activation of the p38 and JNK pathways was determined by western blot analysis and attenuation by DN constructs. Immunocytochemistry for JNK was performed to determine nuclear translocation of JNK with PQ exposure. Ventral mesencephalic cultures from embryonic day 14 rat will be used to determine if results from PC12 cell experiments apply to real neurons.

**1960**

#### THE ORGANOCHLORINE PESTICIDE METHOXYCHLOR ALTERS BRAIN MITOCHONDRIAL RESPIRATION, H<sub>2</sub>O<sub>2</sub> PRODUCTION AND CALCIUM/CAMP RESPONSE ELEMENT BINDING PROTEIN LEVELS.

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Methoxychlor, an environmental contaminant widely used as an insecticide is implicated in decreasing antioxidant enzyme activity and increasing hydrogen peroxide production resulting in oxidative stress. Phosphorylation of the Ca<sup>2+</sup>/cAMP response element binding protein (CREB) has been demonstrated in many studies to increase in response to oxidative stress. In the current study, we tested the hypothesis that methoxychlor inhibits mitochondrial respiration, increases mitochondrial hydrogen peroxide production and alters the phosphorylation state of mitochondrial CREB. When isolated rat or mouse brain mitochondria were exposed to increasing concentrations of methoxychlor (1-10 µg/mL), state 3 (ADP-stimulated) O<sub>2</sub> consumption was inhibited and respiration-dependent H<sub>2</sub>O<sub>2</sub> production was stimulated in a dose-dependent manner. Analysis by ELISA demonstrated a dose-

dependent increase in phosphorylated CREB in the mitochondrial lysates in the absence or presence of respiratory substrates. These results suggest that *in vitro*, acute methoxychlor application causes mitochondrial metabolic and oxidative stress. Methoxychlor also increases mitochondrial pCREB but in a manner that does not require mitochondrial H<sub>2</sub>O<sub>2</sub> generation. (Supported by NIH grants ES07263 (R.S.) and NS34152 (G.F.), and USAMRMC grant DAMD 17-99-1-9483 (G.F.))

**1961**

#### DIELDRIN EXPOSURE CAUSES OXIDATIVE DAMAGE IN DOPAMINE NEURONS.

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Numerous epidemiological studies suggest that pesticide exposure is a significant risk factor for Parkinson's disease (PD). In particular, elevated levels of the organochlorine pesticide dieldrin have been found in the brains of post-mortem PD patients. However, the mechanism(s) by which dieldrin damages the dopaminergic system are not well established. *In vitro* studies have determined that dieldrin is capable of generating free radicals and causing oxidative damage. In this study, we used immortalized rat mesencephalic cells (N27) and mice repeatedly exposed to dieldrin to determine whether dieldrin promotes oxidative damage and dopaminergic dysfunction. Exposure of N27 cells to 60 or 100 µM dieldrin for 6 hr resulted in a 36% and 62% increase in protein carbonyl formation without causing cell death. In mice treated with 1 or 3 mg/kg of dieldrin every 3 days for 1 month, protein carbonyl levels in the striatum were increased by 56 and 90%. Similar to the cell culture experiments, there appeared to be no overt dopaminergic neuron loss in the striatum, as tyrosine hydroxylase levels were unchanged. However, dieldrin exposure significantly increased striatal dopamine transporter levels (DAT) by 25 and 60%. We have previously demonstrated that the organochlorine pesticide heptachlor similarly increases DAT levels in mice, while acting as an inhibitor of the vesicular monoamine transporter (VMAT2) *in vitro*. As DAT is responsible for transport of dopamine into the cell and VMAT2 sequesters cytosolic dopamine, alteration of these transporters by dieldrin may increase cytosolic dopamine resulting in oxidative damage. These results suggest that dieldrin may specifically damage the dopamine system through up-regulation of DAT and enhanced oxidative damage which may provide mechanistic insight to the epidemiological link between organochlorine exposure and PD. (Supported by NIH U54ES012068 (GWM) and F32ES013457 (JRR)).

**1962**

#### MECHANISM OF SELECTIVE TOXICITY OF IVERMECTIN IN INSECTS AND MAMMALS.

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Ivermectin is a broad-spectrum anthelmintic drug that has been widely used in domestic animals and in humans. Ivermectin is thought to act on glutamate-activated chloride channels (GluCls) and GABA-activated receptors. However, the mechanism of selective toxicity in invertebrates over mammals is not fully understood. The present study was conducted to further elucidate the mechanism of agonistic and modulatory actions of ivermectin on ligand-activated chloride channels in cockroach and rat neurons using the whole-cell patch clamp technique. Ivermectin induced chloride currents with slow activation, slow deactivation and slow desensitization in cockroach neurons with an EC<sub>50</sub> of ~50 nM, and in rat neurons with an EC<sub>50</sub> of ≥ 300 nM. In cockroach neurons, the GABA receptors and the slow desensitizing GluCls, but not the fast desensitizing GluCls, were desensitized by ivermectin, and the ivermectin-induced currents were only partially blocked by picrotoxinin and fipronil at the concentrations that would completely block GABA receptors and GluCls. After exposure to ivermectin, GABA receptors and GluCls were became less sensitive to picrotoxinin or fipronil block. In rat neurons, ivermectin also activated and modulated the GABA<sub>A</sub> receptors, but unlike cockroach neurons, picrotoxinin completely blocked ivermectin-induced currents. In conclusion, ivermectin has agonistic and modulatory actions on ligand-activated chloride channels in cockroach neurons, and the higher sensitivity of cockroach neurons than rat neurons is partially responsible for the selective toxicity of ivermectin. Supported by NIH grant NS14143.

**1963**

#### SULFONE METABOLITE OF FIPRONIL BLOCKS GABA- AND GLUTAMATE-ACTIVATED CHLORIDE CHANNELS IN MAMMALIAN AND INSECT NEURONS.

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Fipronil sulfone is a major metabolite of fipronil in mammals and insects. To better understand the insect and mammalian toxicology of fipronil, we examined the effects of fipronil sulfone on GABA- and glutamate-activated chloride channels

(GluCls) in cockroach thoracic ganglion neurons and in rat dorsal root ganglion neurons, using the whole-cell patch clamp technique. Fipronil sulfone blocks of both fast desensitizing and slow desensitizing GluCls in the cockroach required receptor activation. Furthermore, channel activation was required for unblock of the fast desensitizing GluCls, but not for the slow desensitizing GluCls. The  $IC_{50}$  of fipronil sulfone against activated slow desensitizing GluCls was 8.8 nM. In cockroach and rat neurons, fipronil sulfone blocked GABA receptors in both activated and resting states. The  $IC_{50}$  for blocking the activated GABA receptors were 15.4 nM and 46.6 nM, respectively, for cockroach and rat. Kinetic analysis of fipronil sulfone block of the activated chloride channels revealed that its blocking and unblocking rate constants for cockroach GABA receptors were similar to those for fipronil, explaining their similarity in blocking potency. The blocking rate constant of fipronil sulfone for rat  $GABA_A$  receptors was 7-fold larger than that of fipronil explaining the sulfone's higher blocking potency. In conclusion, fipronil sulfone is a potent inhibitor of cockroach GABA receptors and GluCls, and a weaker inhibitor of rat  $GABA_A$  receptors. Its potent blocking action on insect GluCls appears to contribute to selective toxicity against insects over mammals. Since fipronil sulfone is rapidly formed *in vivo*, the toxicological effects are likely due to the sulfone. Supported by NIH grant NS14143.

## 1964

### IMMORTALIZED MICROGLIAL CELLS AS A MODEL SYSTEM FOR OXIDATIVE STRESS: PESTICIDE-INDUCED GENOMIC CHANGES.

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In risk assessment there is a need to accelerate toxicological evaluation of vast numbers of chemicals. New programs focus on identifying common modes of action and on model systems for rapid screening. In this study we address both these issues. Oxidative stress is a good candidate as a mode of action for neurotoxicity testing. A role for reactive oxygen species has been well documented in both disease (e.g. Parkinson's disease, Alzheimer's disease, etc.) and toxicity (e.g. Paraquat) of the nervous system. Microglial cells are the major mediator of the brain's immune system. When activated in response to injury, they release an oxidative burst that is initially protective, but may become toxic in excess. We are investigating the use of immortalized murine microglial cells as a model test system for identifying chemicals that elicit the oxidative burst. Microglia were treated with the herbicide, Paraquat (1.0  $\mu$ M), or the insecticide, Rotenone (0.125  $\mu$ M), as prototypic compounds that generate reactive oxygen species (ROS). Hydrogen peroxide (0.002 mM) and lipopolysaccharide (25 ng/ml) are chemicals known to elicit oxidative stress in cell culture and were used as positive controls. Four hours after exposure, RNA was extracted for analysis on Affymetrix mouse genome 430 2.0 chips. Preliminary analysis showed similar numbers of up and down regulated genes across treatment groups with approximately equal distribution across ontology areas. The greatest proportions of genes identified were related to signal transduction (~41%), enzymes (~20%) or cell communication (~18%). Cluster analysis showed good correlation between samples from a given treatment group. However, only 5 genes were up regulated 2 or more fold by all 4 treatments and only 9 were down regulated. These data support oxidative stress as a mode of action where multiple pathways can contribute to a single outcome. (This abstract does not necessarily reflect USEPA policy).

## 1965

### NEUROTOXIC EFFECTS OF MANCOZEB AND MANEB *IN VIVO* AND *IN VITRO*.

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Mancozeb (MZ), an ethylene-bis-dithiocarbamate fungicide, is widely utilized on golf courses, residential lawns, and agricultural lands in the US. The adverse effects of MZ on humans and other living organisms have not been widely studied. Other dithiocarbamate fungicides, including maneb (MB), have been implicated in selective dopaminergic neurotoxicity in cell culture, rodents, and humans (Ferraz et al., 1988; Morato et al., 1989; Soleo et al., 1996; Thiruchelvam et al., 2000; Zhang et al., 2003). Consistent with this, we found a dose-dependent decrease in dopamine (DA) and an increase in DA metabolites and GABA levels in mice (C57BL/6J) exposed to 30-120 mg/kg MZ for 1-72 hrs. It has been postulated that these dithiocarbamates exert toxicity via mitochondrial inhibition (Zhang et al., 2003; Zhou et al., 2004). Primary mesencephalic cells isolated from Sprague-Dawley E15 rat embryos were exposed *in vitro* to MZ or MB to better understand the mechanism of toxicity of these compounds. To assess toxicity, a functional assay of selective transporter activity was performed in cells exposed to 0-60  $\mu$ M of MZ or MB for 24 hrs.

Exposures resulted in a dose-dependent decrease in the uptake of both DA and GABA, with MB being more potent than MZ. Despite findings that MZ (60  $\mu$ M) and MB (60  $\mu$ M) inhibited oxygen consumption by 42% and 78% respectively in whole mitochondria preparations isolated from adult rats, ATP levels as analyzed by a bioluminescent ATP assay and lactic acid production were not altered by fungicide exposure in cells. In conclusion, our findings suggest that while MZ and MB have toxic effects in DA neurons *in vitro* and *in vivo* and can inhibit mitochondrial respiration in isolated mitochondrial preparations, *in situ* their toxic effects are exerted via mechanisms other than mitochondrial inhibition. Alternative mechanisms of action for these fungicides will be evaluated in future studies. This research is supported by the NIEHS training grant and the Cook College NJAES grant.

## 1966

### EFFECT OF PYRETHROIDS ON GABA RELEASE IN THE HIPPOCAMPUS OF FREELY MOVING RATS.

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Previously, we reported that pyrethroids, allethrin (type I), cyhalothrin (type II) and deltamethrin (type II) modulate the ACh release in the hippocampus of freely moving rats. In the present study, we studied changes in outflow of extracellular level of GABA in the hippocampus of conscious rats after the exposure of three pyrethroids as measured by *in vivo* microdialysis. GABA was detected by high performance liquid chromatography with electrochemical detector. A microdialysis probe was implanted into the left hippocampus of male SD rats. Rats were treated i.p. with pyrethroids or the same volume of vehicle. Control vehicle injections had no effect on the efflux of GABA in any of the experiments. The extracellular level of GABA was decreased by 10 and 20 mg/kg allethrin to about 50% of baseline and increased to about 250% of baseline by 60 mg/kg with a peak time of 90 min after injection. Cyhalothrin 10, 20 and 60 mg/kg stimulated GABA release dose-dependently to about 130, 210 and 300% of baseline, respectively with a peak time of 40-90 min after administration. Deltamethrin 10, 20 and 60 mg/kg inhibited GABA release dose-dependently to about 75, 55 and 40% of baseline, respectively with a peak time 60-90 min after injection. Local infusion of tetrodotoxin (1 micro M) with Ringers solution through the dialysis probe completely prevented the effect of allethrin, cyhalothrin and deltamethrin, but not the effect of deltamethrin at 60 mg/kg on GABA release. Our results suggest that there may be a relationship between cholinergic and GABAergic neuronal network in hippocampus which plays a role in the neurotoxicity of pyrethroids.

## 1967

### EFFECTS OF PHENYL SALYGENIN PHOSPHATE (PSP) AND PHENYLMETHANE SULFONYL FLUORIDE (PMSF) ON RAT DORSAL ROOT GANGLIA (DRG) CULTURES.

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PSP is a direct acting organophosphate that causes axonal degeneration (organophosphate induced delayed polyneuropathy, OPIDP). The mechanism involves inhibition of neuropathy target esterase (NTE) and subsequent aging of the phosphorylase enzyme. PMSF is a direct inhibitor of esterases that does not cause OPIDP. PMSF protects from OPIDP when given before the neuropathic OP because it causes NTE inhibition that is not followed by aging. However, PMSF exacerbates OPIDP when given after the neuropathic OP and this effect is related to the interaction with another target. Cultures of rat DRG were tested as a possible model for protection and exacerbation of OPIDP. Desheathed DRGs from 13-16 day old rats were dissected and plated on poly-L-lysine precoated dishes. Neuronal processes were allowed to grow for 72 hours before incubation with inhibitors. PSP caused massive degeneration of neuronal processes at 100  $\mu$ M for 3 hours and no effect at 10  $\mu$ M for 24 hours or more. Intermediate effects were seen at 50-70  $\mu$ M. PMSF caused no effect (1 mM), partial (1.5 mM) or massive (2 mM) degeneration of the processes. At 2 mM maximum effect was obtained with incubations of 24 hours or more but no effect for incubations of less than 5 hours. No significant cell death was observed with either compound. The morphological characteristics of axonal degeneration were different: PSP caused a beading-like damage whereas PMSF caused a rapid dying-back-like damage. When PMSF (1 mM) was added 24 hours before PSP (50  $\mu$ M) incubation minimal effects were observed. When PMSF (1.5 mM) was added 6 or 24 hours after incubation with 10  $\mu$ M PSP there was a dying-back-like massive destruction of processes. Different concentrations and times of incubation as well as other compounds with different characteristics need to be tested.

## 1968 CROSS-SPECIES TOXICOLOGY IN THE AGE OF GENOMICS.

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While animal models have been the cornerstone of experimental toxicology and safety assessment for the last two centuries, the question often arises whether the results seen in one species are relevant to what may be expected in another, particularly when the extrapolation is being made to man. Genomic information could change this problem dramatically. At one level, comparisons of coding regions and upstream regulatory regions across genomes can provide clues as to similarities and differences between species vis-à-vis the molecular components of a cell and their regulation. One example where this approach has provided valuable insights is the nuclear receptor gene family. These genomic comparisons can also be used to inform the use of non-mammalian models of toxicity such as *Caenorhabditis elegans*. Complementary to such genomic comparisons, mRNA profiling with microarrays allows a global view of toxicant-induced transcriptome alterations in various cell types, tissues and species, and allows an experimental view of similarities and differences in signaling and response pathways. Thus transcriptome alterations can be compared *in vivo* between rat and canine models, and *in vitro* between rat, canine, and human hepatocytes. The promise is that such tools will allow for any given toxic response a truly molecular assessment of the relevance of various animal models to one another and to man.

## 1969 INSIGHTS INTO EVOLUTION OF XENOBIOTIC METABOLISM IN MAMMALS FROM COMPARATIVE GENOMICS OF THE NUCLEAR RECEPTOR GENE FAMILY.

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The partial and complete sequencing of 3 mammalian genomes has enabled detailed comparative studies of the nuclear receptor gene family and genes with which they interact. Overall, the nuclear receptors have changed very slowly on the time-scale of mammalian evolution. Pregnen X receptor (PXR;NR1I2) and constitutive androstane receptor (CAR;NR1I3) manifest a rate of change 5-10 times the average rate for the family. Structural analysis of ligand binding domain of PXR from mouse, rat and human reveals a cluster of variable amino acids in the ligand binding pocket and another cluster along alpha helix 9. The cytochrome P450 genes regulated by PXR and CAR have also evolved rapidly, suggesting the possibility of co-evolution of two major gene families that cooperate in xenobiotic metabolism in mammals.

## 1970 COMPUTATIONAL AND EMPIRICAL INVESTIGATION OF ESTROGEN AND DIOXIN ELICITED EFFECTS: A COMPARATIVE ANALYSIS.

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Emerging omic technologies, novel computational approaches, and the availability of human, mouse and rat genome sequences has provided unprecedented opportunities to investigate mechanisms of toxicity at the molecular level that support risk assessment. The incorporation of comparative methods provides a more powerful approach to identify conserved responses that play important roles in toxicity and facilitate the elucidation of the underlying biochemical networks and mechanisms of action. In this presentation, comparative empirical and computational approaches will be presented that describe (i) the use of homology models and estrogen receptor - ligand binding preferences for multiple species (i.e. human, mouse, chicken, *Xenopus laevis*, green anole and rainbow trout) to identify critical residues within the binding pocket that are important for endocrine disruptor interactions, (ii) differences in estrogen elicited genome-wide gene expression in human and rodent models using customized orthologous cDNA microarrays, and (iii) the computational identification of 5763, 3539, and 1190 putative DREs in 17, 882 human, 11, 697 mouse, and 3, 896 rat genes, respectively. Results from these studies not only further our molecular knowledge of estrogen receptor and Ah receptor-mediated toxicity, but also begin to address the validity of risk assumption extrapolations between surrogate test species to human and ecological relevant species based on the current understanding of the molecular mechanisms of action. This work is supported by the United States Environmental Protection Agency (826301) and the National Institutes of Health (ES11271 and ES12245).

## 1971 GENOMIC CHARACTERIZATION OF THE ACUTE PHASE RESPONSE IN MICE, DOGS, AND RATS.

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Genome-scale analysis using microarrays is a powerful means to characterize responses in model systems. Genomic tools to monitor responses in species most often utilized in drug development are now available (and of sufficient quality) where interspecies physiological responses to insult can be mapped. To characterize these responses and search for biomarkers of target organ toxicity that could be applied in preclinical drug development, microarray data from LPS treated rat, mouse, and dog were compared. LPS was administered to mice (4.0 mg/kg), rats (1.0 mg/kg), and dogs (0.2 mg/kg) using doses designed to produce 'equivalent' inflammatory responses, and liver gene expression was assessed using commercial or internally developed microarrays. The kinetics of hepatotoxicity associated with LPS administration differed markedly between species. Gene expression of the acute phase reactants serum amyloid A and alpha-2-macroglobulin correlated with protein levels measured in serum. The expression levels of other transcripts, such as cytochrome c, correlated with hepatic damage as measured by serum ALT. Comparing gene expression between the liver and kidney of LPS-treated dogs revealed differential expression of vascular markers such as E-selectin. These findings begin to describe mechanistic details of differential organ toxicity observed in response to LPS. Although cross-species annotation with murine, rat, and canine microarrays posed a significant challenge, analysis of these data have proven to be important in interpreting species-specific responses to inflammatory stimuli.

## 1972 CROSS-SPECIES COMPARISONS OF TRANSCRIPTIONAL RESPONSES IN PRIMARY HEPATOCYTES.

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Primary hepatocytes offer a unique system for examining the molecular and transcriptional responses to toxicant treatment. Furthermore, the availability of primary hepatocytes from humans as well as species commonly used in preclinical testing allows direct comparisons of dysregulated genes and pathways across species under similar conditions of exposure. Such comparisons can provide insight into the relevance to humans of a particular toxic response in a non-clinical species. For example, Wy-14643 belongs to a class of compounds that elicit peroxisome proliferation (PP) as well as hepatocarcinogenesis in rodents. The weight of evidence is that the PP response to these compounds is rodent-specific. To examine the species-specific responses on a genome-wide basis, cultures of primary rat hepatocytes or primary human hepatocytes were treated with 100 microM or 200 microM WY-14643, respectively, for 24 hr, and mRNA levels were analyzed on Affymetrix GeneChips®. Cross-species analysis was accomplished by matching genes on the human Hu-133A GeneChip® with those on the rat RG\_U34A GeneChip® by protein homologies: by this analysis about 3000 non-redundant genes are present in common on both microarrays. Using standard statistical cut-offs, 166 non-redundant genes were regulated by WY-14643 treatment of rat hepatocytes, in contrast to 20 for treatment of human hepatocytes. And while 135 Wy-14643-regulated rat genes were also present on the human array, only 2 of these were also regulated in human hepatocytes by Wy-14643 treatment. Such studies offer evidence for species-species responses and provide a mechanistic approach to using animal data for assessment of human risk.

## 1973 TOXICO- AND PHARMACOGENETIC ANALYSIS IN A NOVEL MODEL OF PARKINSON'S DISEASE: DOPAMINE NEURON DEGENERATION IN *C. ELEGANS*.

R. Nass, C. Nichols, M. Fullard, M. Garrett and M. Marvanova. *Anesthesiology and Pharmacology, and Center for Molecular Neuroscience, Vanderbilt University Medical Center, Nashville, TN*. Sponsor: B. Mattes.

Most animal models for idiopathic Parkinson's disease (PD) rely on inducing nigrostriatal damage in mammals with the neurotoxins 6-hydroxydopamine (6-OHDA) or MPP<sup>+</sup>, or overexpressing the presynaptic protein  $\alpha$ -synuclein. The dopamine transporter DAT, which is the target for many psychoactive drugs, provides the cellular gateway for the accumulation of the neurotoxins that evoke neuronal death and Parkinson-like syndrome and has been shown to interact with  $\alpha$ -synuclein. We have recently established a novel model system using *C. elegans* to evaluate *in vivo* dopamine (DA) neuron degeneration (Nass et al., PNAS, 2002; Nass and Blakely, Annu. Rev. Pharmacology Toxicol., 2003). We have shown that a brief exposure to 6-OHDA causes selective loss of the DA neurons, pharmacological agents that interfere with DAT or genetic ablation of the transporter blocks the degeneration, and that the degeneration likely occurs via a novel cell death pathway.

We have also shown that overexpressing human wild-type (WT) or mutant A53T  $\alpha$ -synuclein in *C. elegans* also confers DA neuron degeneration. We have now instituted genetic and chemical screens for regulators of toxin- and  $\alpha$ -synuclein-mediated cell death, and we have isolated several mutants and compounds that suppress the neurodegeneration. We have also identified several heavy metals that exasperates the neurodegeneration. Finally we have instituted genome-wide microarray analyses of gene expression in WT, DAT knockout, and human A53T  $\alpha$ -synuclein-expressing animals following exposure to control or 6-OHDA. Our studies suggest that genes that modulate DA production, DA regulation, cell signaling, and oxidative stress play a role in the neurotoxicity. These results will also be presented in the context of utilizing this system via high throughput screening assays to explore the molecular basis of toxin-induced neurodegeneration in mammals.

 **1974** ONTOGENY OF HUMAN HEPATIC PHASE I AND PHASE II ENZYMES: IMPLICATIONS FOR DIFFERENTIAL TOXICANT SUSCEPTIBILITY.

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Over the past several years, there has been considerable interest in the dynamic changes that occur in toxicokinetic factors during early life stages and how these changes may impact differential toxicant susceptibility. Advances in molecular and analytical techniques have allowed a better characterization of these changes, as well as some of the underlying control mechanisms. The objectives of this symposium are to: 1) present examples of the information gained about major members of the oxidative Phase I enzymes and how this information has led to the identification of common developmental themes; 2) present information on the developmental expression of two conjugative phase II enzyme classes, N-acetyl transferases and sulfotransferases, and how, combined with the information regarding phase I enzymes, can inform regarding possible differential susceptibility during ontogeny; and 3) demonstrate how such information regarding developmental expression can be integrated into physiological-based toxicokinetic models for predicting temporal-specific changes in toxicant disposition useful for early life stage risk assessment. These advances offer the promise and challenge of predicting changing dose-response relationships during early life and the possible prevention of developmental toxicity.

 **1975** HUMAN HEPATIC PHASE I ENZYME DEVELOPMENTAL EXPRESSION.

R. N. Hines. *Pediatrics, Med. Col. Wisconsin, Milwaukee, WI*.

The phase I enzymes are important for prototoxicant bioactivation and toxicant bioinactivation. Earlier studies suggested significant phase I enzyme expression changes during human hepatic development. Such temporal-specific expression would impact toxicant susceptibility. To gain a better understanding of phase I enzyme ontogeny, a tissue bank was established containing 240 human liver samples representing ages from 8 wks gestation to 18 yrs. Information available on most postnatal samples and many fetal samples included age, ethnicity, gender, body weight, liver weight, postmortem interval, and cause of death. Tissue samples from individuals succumbing to diseases involving liver pathology were excluded. Using recombinant protein standards and regression analysis, enzyme specific contents were measured by western blot. Specific activity determinations were performed where probe substrates were available. As an example, an examination of the two major hepatic forms of the CYP2C gene family revealed low CYP2C9 expression in some individuals from 8-24 wks gestational age (GA) with increasing expression levels during the remainder of gestation. In contrast, CYP2C19 was detectable at 8 wks GA, but values were similar throughout gestation. CYP2C9 expression increased in a birth-dependent process such that 50% of individuals <4 mos postnatal age (PNA) expressed near-adult values. However, the first 4 mos PNA represented a period of high interindividual variability. Unlike CYP2C9, 2C19 values increased linearly over the first 5 mos PNA to adult values in most individuals, suggesting birth was necessary but not sufficient for increased expression. From 5 mos to 10 yrs, CYP2C19 expression was highly variable. The ontogeny of CYP2C9 and 2C19 were dissimilar among both fetal and 0-4 mos postnatal samples. These 2 examples highlight common themes observed across many phase I enzyme systems, including: windows of hypervariability due to interindividual differences in expression onset or timing of increased expression, and temporal asynchrony among members of the same gene family suggesting different developmental regulatory mechanisms (PHS CA53106 & GM68797).

 **1976** SULFOTRANSFERASE EXPRESSION: IMPLICATIONS FOR PRENATAL TOXICITY.

M. Runge-Morris. *Inst. Envir. Health Sciences, Wayne State University, Detroit, MI*.

In the developing human, hepatic sulfotransferase (SULT) expression represents a primary early defense system against xenobiotic insult. The sulfonation of toxic endogenous intermediates and harmful exogenous species by members of the SULT

gene family is a critical step in xenobiotic detoxification. In addition, since sulfonated hormones are generally receptor inactive, the differential expression of SULT enzymes affects the delicate balance of hormone homeostasis in developing organs. Knockout mouse models demonstrate that the inactivation of estrogen sulfotransferase (SULT1E) produces a condition of estrogen excess and leads to significant reproductive abnormalities. Human aryl sulfotransferase (SULT1A1) sulfonates thyroid hormones and therefore is essential to the progression of normal embryonic growth. Because of the impact of sulfonation on reproductive outcome, the immunoreactive expression of SULT1E, SULT1A1 and hydroxysteroid sulfotransferase (SULT2A1) was examined in pre- and post-natal liver samples using quantitative ECL immunoblot analysis. Human hepatic SULT enzymes were determined to be differentially expressed during development. SULT1A1, a major conjugating enzyme in adult human liver, was variably expressed in both pre and postnatal liver samples. SULT1E was most robustly expressed during early periods of hepatic gestation. By contrast, the levels of human hepatic SULT2A1 increased following birth, and unlike in rodent species, did not demonstrate a gender-dependent pattern of expression. The results of this study shed light on the determinants of prenatal toxicity and hormone homeostasis in the developing human.

 **1977** DEVELOPMENTAL EXPRESSION OF HUMAN AND MURINE ARYLAMINE N-ACETYLTRANSFERASES(NAT): IMPLICATIONS FOR AROMATIC AMINE GENOTOXICITY.

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Children and adults often respond differently to xenobiotics. This may be due, at least in part, to alterations in the expression of biotransformation enzymes. The isoforms of NAT, NAT1 and NAT2, catalyze the acetylation of therapeutic drugs and environmental carcinogens. Pre- and postnatal human liver samples were evaluated using antibodies generated against human NAT sequences. NAT isoforms were found at an estimated gestational age of 73 days to age 18 years. NAT1 was detected in the first trimester, increasing with gestational age. There was no significant change in the early neonatal period followed by higher levels in the first year of life. NAT2 was also expressed in the first trimester showing an increase with age. There were no differences in samples from premature or full-term births indicating that expression of NATs are not dependent on changes triggered by birth. The age-related differences in NATs could affect susceptibility to chemical toxicity by altering the formation of reactive and non-toxic products. This was tested in a mouse model with 4-aminobiphenyl (4ABP). Activation of 4ABP to genotoxic products can occur via NATs. In C57Bl/6J mice, NAT1 and NAT2 mRNAs and functional enzymes were detected pre- and postnatally showing a pattern of hepatic expression similar to what was observed in human liver. Neonatal exposure to 4ABP resulted in levels of 4ABP-DNA adducts significantly lower than in adult liver. The magnitude of changes in expression of genes associated with responses to genotoxicity was less in young animals than adults. The data illustrate that lower expression of NAT1 and NAT2 occurring in neonates limits acetylation, a key reaction in the activation of 4ABP, and contributes to decreased susceptibility to 4ABP genotoxicity compared to adults. Based on the similarities in expression of NATs in mice and humans, children would be predicted to have a higher risk of toxicity for chemicals that are inactivated by NATs compared to adults but lower risk for xenobiotics activated by these enzymes. (ES09812, ES10047)

 **1978** DEVELOPMENT OF PHYSIOLOGICALLY-BASED TOXICOKINETIC MODELS FOR EARLY LIFE STAGES: IMPLICATIONS FOR DIFFERENTIAL TOXICANT SUSCEPTIBILITY.

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Immaturities in metabolic and renal function have been associated with slower clearance of numerous therapeutic drugs in the first months of life. These immaturities and other developmental factors are likely to affect the internal dose of environmental toxicants. This presentation describes PBTK inputs we use for children's modeling and presents case studies where this has been applied to therapeutic drugs (caffeine and theophylline) and an environmental toxicant (acrylamide). Caffeine and theophylline are oxidized primarily by CYP1A2 to demethylated products. In spite of their close structural similarity, caffeine clearance is 3 times slower in neonates than theophylline. This inter-drug differential does not exist in adults, who clear both drugs considerably faster than neonates. PBTK modeling was able to simulate caffeine and theophylline metabolic and renal clearance in both neonates and adults. The considerably faster metabolic clearance for theophylline is explained by a novel methylation pathway for theophylline that is not available for caffeine and is not detectable in adults. This points out the potential importance of

novel metabolic pathways in neonates which can become predominant due to immaturities in the major pathway (CYP1A2) in adults. Acrylamide is a neurotoxicant and multi-site carcinogen to which there is widespread human exposure. Both the parent compound and a CYP2E1 metabolic product (glycidamide) are active toxicants, with glycidamide the best candidate for genetic activity/cancer. Both compounds are detoxified via glutathione conjugation and epoxide hydrolase can also detoxify glycidamide. PBTK modeling of the dosimetry of acrylamide and glycidamide has been calibrated to hemoglobin adduct levels found in exposed rats and adult humans. This modeling has been adapted to early life stages to determine how child/adult differences in key distributional factors and metabolic steps may affect internal dose and carcinogenic risk.

## 1979 SYSTEMS BIOLOGY: APPROACHES AND APPLICATIONS TO TOXICOLOGY.

W. Slikker<sup>1</sup> and T. B. Knudsen<sup>2</sup>. <sup>1</sup>*Division of Neurotoxicology, NCTR/FDA, Jefferson, AR* and <sup>2</sup>*Department of Molecular, Cellular and Craniofacial Biology Birth Defects Center, University of Louisville, School of Dentistry, Louisville, KY.*

Genomics and proteomics provide information on the cellular reaction to drug and chemical exposures but this is only part of what is needed to understand complex developing systems, where susceptibilities to exposure may advance through transitional states of varying susceptibilities. The NIH Director's Road Map has focused on the need to provide new tools to investigators, to speed the process of discovery, to encourage interdisciplinary research, and to promote translational research. Systems biology will contribute to this mission. Systems biology is the application of systems theory to solving biological problems and is a means to analyze complex behavior in a composite system that may be decomposed into subsystems to facilitate understanding and modeling. In toxicology, it provides a means for identifying pathways that are critical to disease and to discovering on- and off- target effects of compounds. Devising computational models and integrating these models with empirical data provide important insight into complex systems-level behaviors, ultimately striving to deliver the mechanism connecting small molecules (drug or chemical) with a clinical endpoint (phenotype or disease) with regards to metabolic and regulatory networks. The integrative topic of systems biology and application examples focused on several organ systems and stages of development will interests toxicologists with backgrounds in mechanisms, reproduction, development, neuroscience, modeling, and safety and risk assessment.

## 1980 EXTRACTING MEANING FROM EXPRESSION DATA.

J. Quackenbush. *Institute for Genomic Research, Rockville, MD.* Sponsor: T. Knudsen.

The popular literature is rife with proclamations regarding the coming genomics revolution, but the application of genome scale techniques, including genome sequencing, transcript profiling, proteomics, and metabolomics, have largely been to cataloging response rather than developing a mechanistic interpretation of the patterns involved. While many balk at the challenges posed by "too much data," in fact the problem may be not enough data. The value of large datasets is that they can reveal features of the underlying biology provided they are filtered appropriately, and one approach that has proven successful is to integrate large datasets with other large datasets. In this way, genetic mapping and microarray data can be used to identify genes that are differentially expressed and genetically linked to a particular phenotype. Or changes in gene expression can be linked to changes in protein representation or metabolic flux. Similarly, prior knowledge about gene functional classes, or pathway membership, or interaction can be used as additional constraints on the data, as can constraints posed by evolutionary conservation of genes and pathways. The challenge moving forward is not one of collecting and analyzing data, but of integrating data to produce a comprehensive understanding of the biological systems under study.

## 1981 MODELING GENE NETWORKS IN THE NEURONAL ADAPTATION TO ALCOHOL.

J. S. Schwaber. *Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, PA.* Sponsor: W. Slikker.

The mammalian neuronal response to environmental inputs typically involves coordinated transcriptional regulatory responses <2-fold for large numbers of genes. Microarray technology can provide global datasets for systems approaches but for smaller changes is limited by precision and accuracy. We engineered several modifications to enhance quantitative reliability of microarray data and also developed bioinformatics tools and analysis approaches to improve our ability to derive networks, pathways and relationships for modeling and simulation of cellular dynam-

ics. This integrated approach was applied to chronic alcohol exposure in a region of the rat brain associated with addiction and withdrawal (nucleus tractus solitarius). Initially 542 genes were found differentially expressed ( $p < 0.05$ ); these were further analyzed to produce a list of associated transcription factors (TF) and attendant transcriptional regulatory network. We performed meta-analysis for sensitive dependence of the results on initial conditions to determine robustness of the networks. The surviving interaction results are input to associative network building tools using (1) natural language processing (NLP) of literature and databases to associate genes with known protein relationships and (2) a searchable database of hand-curated gene and pathway associations to build an initial model network containing differentially expressed genes, relevant transcription factors, cellular signaling processes and functional interactions in the context of relevant cellular pathways, e.g. p38 MAPK, NF- $\kappa$ B, ERK-MAPK. Results include hypothesized integrated functional systems influenced by alcohol adaptation and withdrawal, critical predictions of gene-TF-pathway interactions for experimental verification, and qPCR, ChIP, and pathway inhibitor results.

## 1982 SYSTEMS BIOLOGY AND DOSE RESPONSE ASSESSMENT.

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Systems biology involves the quantitative evaluation, through laboratory experiments and computer modeling, of the organization of components in biological systems that give rise to biological function. Toxicity is a perturbation in those biological processes that lead to adverse responses. These perturbations are often related to chemically-induced activation or silencing of specific signaling pathways through non-linear switches embedded in transduction cascades. The goal in toxicology is to define those doses where perturbations are expected to be large enough to pose a risk in an exposed population. Systems biology methodologies now provide the ability to understand the molecular basis of toxicant dose-response relationships and produce detailed biological models of signal transduction systems that predict dose-response relationships. This talk begins with several examples of receptor auto-regulation and kinase cascades to demonstrate the manner in which signaling networks can produce specific, non-linear dose-response relationships. The second part of the talk will discuss the set of genomic and computational tools available for delineating and modeling signaling networks that are targets for perturbations in cells and tissues. Methods for deconvoluting signaling pathways include a variety of traditional and emerging genomic tools including microarray analysis and high coverage reverse genetic screens using full-length gene sets and inhibitory RNA libraries. Bioinformatic tools are used to store results in relational databases and assemble the cellular components static network diagrams. The static signaling networks are parameterized and converted into computational models to simulate the temporal and dose response behavior of the system. This talk emphasizes the integration of high coverage genomic methods with bioinformatic tools and computational modeling to implement a systems biology approach in toxicology and dose-response assessment. We will also discuss the approach in reference to current work with NF- $\kappa$ B signaling in kidney epithelial cells and steroidogenesis in mouse Leydig cells.

## 1983 AN $\pm$ OMICS $\pm$ APPROACH TO ELUCIDATE MECHANISMS OF DISRUPTED NEPHROGENESIS AND FUNCTIONAL INTERACTIONS BETWEEN AHR AND WILMS $\pm$ TUMOR SUPPRESSOR GENE.

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Supported by NIH grant ES04917 and NRSA — Loss of aryl hydrocarbon receptor (AHR) function by gene deletion, as well as unregulated activation of AHR signaling following ligand binding, are associated with deficits in murine nephrogenesis. Disrupted nephrogenesis *in vitro* is evidenced by changes in the ratio of Wilms $\pm$ tumor suppressor gene (Wt1) splice variants following AHR activation. To evaluate these interactions,  $\pm$ omics $\pm$  approaches were used to study the role of AHR during mesenchymal-to-epithelial transition (MET) and identify gene networks controlled by AHR and WT1 in embryonic kidney cells. Using organ cultures of E11.5 metanephric blastema from C57BL/6 AHR $+/+$  and AHR $-/-$  mice treated for 4 consecutive days with 3 micromolar benzo(a)pyrene (BaP) identified glial derived neurotrophic factor, frizzled receptor, insulin-like growth factor, insulin-like growth factor binding protein (ILGFBP), syndecan, laminin, Sry, oncostatin M, pinin, GATA-3, Sox-18, Nrf-2, cyclin G and fibulin as targets of developmental interference. Co-determination of transcriptional states and multivariate nonlinear coefficient of determination revealed strong interactions between AHR, opn and ILGFBP among others. Glycolysis, the tricarboxylic acid cycle, and amino acid metabolism were implicated as metabolic targets of developmental interference by

using NMR and GC-MS. AHR knockdown by small interfering RNA was associated with significant accumulation of +exon5 Wt1 splice variant and a shift in WT1 expression from a predominant 52 kDa isoform toward a smaller 40 kDa isoform. A subset of signature genes essential for nephrogenesis, including genes regulated by Wt1, was found to be regulated by AHR. These data suggest that *omics* approaches can help elucidate molecular mechanisms of developmental interference by environmental agents.

#### 1984 COMPUTATIONAL SYSTEMS ANALYSIS OF DEVELOPMENTAL TOXICITY.

T. B. Knudsen and A. V. Singh. *Department of Molecular, Cellular and Craniofacial Biology, University of Louisville, Louisville, KY.*

The availability of genomic sequences from a growing number of human and model organisms has fueled an explosion of data, information and knowledge regarding biological systems and disease processes. Database Network infrastructure is needed that can enable high volume data processing and management as applied to dynamical biological attributes, such as the embryo's reaction to drug and chemical exposures. Birth Defects Systems Manager (BDSM) is being developed as a resource to advance this goal for developmental toxicology. The BDSM prototype is a mid-level E-server for storage/retrieval of applications and data as well as tools for research discovery efforts, computational, and theoretical analysis of developmental processes and toxicities. In the long-term BDMS aims to capture systems-level properties of the developing mouse embryo, encapsulate these properties as network objects, and map possible outcomes following a point of molecular dysfunction. Holistic genomic data, for example, can lead to computational gene network prediction models for the chemical interactions between adaptive (homeostatic) and isothermal (developmental) processes but the key challenge is to build comprehensive network identification libraries that cross platforms, studies, and even species. From here, statistical methods can begin to assign probabilities of dysfunction based on actual experimental evidence regarding phenotypes and appropriate experiments to test for interesting relationships that are descriptive of potential outcomes. Funded by NIH grants AA13205 and ES09120

#### 1985 CELLULAR/MOLECULAR MECHANISMS INVOLVED IN ENVIRONMENTAL CHEMICALS- INDUCED DOPAMINERGIC NEUROTOXICITY AND THE CONSEQUENCES ON NEURODEGENERATIVE DISEASES.

A. G. Kanthasamy. *Iowa State University, Ames, IA.*

Environmental neurotoxic chemical exposures are increasingly recognized as dominant risk factors in the etiology of many neurodegenerative disorders including Parkinson's disease, Alzheimer's disease Amyotrophic Lateral Sclerosis and Huntington's disease. Exposure to environmental neurotoxic agents (metals, pesticides, PCBs and others) is often superimposed with the pathological hallmarks of neurodegenerative disorders. In recent years, cell death mechanisms have been investigated extensively. Despite this growing amount of information regarding the cell death process, little effort has been made to integrate this body of knowledge with the area of neurotoxicology and establish its relevance to neurodegenerative diseases. This workshop is designed to fill this gap by entertaining discussion of key cell death signaling molecules and other cellular targets during neurotoxic chemical exposures that may impact the disease process of neurodegenerative disorders. Specifically, novel findings obtained from both *in vitro* and *in vivo* models of neurotoxicity using state-of-the-art approaches including toxicogenomics, transgenics, knock-outs, and RNA interference (RNAi) will be presented at this workshop. The workshop presentations are expected to uncover unifying concepts and set the groundwork for translation of key analogies between neurotoxicology research and neurodegenerative disorders. Also, questions provided by the speakers will serve as catalysts for discussion with the audience. Overall, this workshop is likely to accelerate understanding of the role of environmental neurotoxic agents in the etiopathogenesis of neurodegenerative disorders as well as to foster the development of novel therapeutic strategies.

#### 1986 OXIDATIVE STRESS-SENSITIVE KINASE IN NEUROTOXINS-INDUCED SELECTIVE DOPAMINERGIC CELL DEATH: RELEVANCE TO PARKINSON'S DISEASE.

A. G. Kanthasamy. *Biomedical Sciences, Iowa State University, Ames, IA.*

Environmental exposure to pesticides and metals has been linked to the etiopathogenesis of Parkinson's disease. The cellular mechanisms underlying the selective degeneration of nigral dopaminergic neurons following environmental neurotoxin ex-

posure remain elusive. Recent studies from our laboratory demonstrate that dopaminergic neurotoxins such as MPP+, manganese and diethdrin induce apoptosis in dopaminergic cells via caspase-3-dependent proteolytic activation of the proapoptotic kinase PKC $\delta$ . Also, we examined the expression pattern of PKC $\delta$  and its functional role in the degenerative process associated with chemical-induced Parkinson's disease. Western blot analysis revealed a high expression level of PKC $\delta$  in rat mesencephalic dopaminergic neuronal cells (N27 cells) as well as in the substantia nigra of the mouse brain. Confocal imaging revealed co-localization of PKC $\delta$  in TH-positive neurons in primary rat mesencephalic neuronal cultures, and distribution of PKC $\delta$  in the cell body as well as in the dendrites. Double labeling of TH and PKC $\delta$  further confirmed their co-localization in the mouse substantia nigra. To further examine the functional consequence of PKC $\delta$  activation in environmental chemicals-induced dopaminergic degeneration, we employed the RNA interference (RNAi) gene silencing procedure. Dopaminergic neurotoxins proteolytically activated PKC $\delta$  in a caspase-3 dependent manner in rat mesencephalic dopaminergic neuronal cells. The proteolytically cleaved catalytic fragment of PKC $\delta$  (PKC $\delta$ -CF) further translocated to the nucleus and subsequently initiated DNA fragmentation. Furthermore, specific knock down of PKC $\delta$  by siRNA rescued dopaminergic cells from neurotoxins-induced apoptosis, demonstrating the proapoptotic role of PKC $\delta$  in dopaminergic degeneration. Collectively, these results suggest that PKC $\delta$  is highly expressed in dopaminergic neurons and the activation of this oxidative stress sensitive kinase might contribute to the selective degenerative processes in Parkinson's disease [NIH grants ES10586 and NS 45133].

#### 1987 ROLE OF PROINFLAMMATORY CYTOKINES IN CHEMICALLY-INDUCED DOPAMINERGIC NEURODEGENERATION.

J. O'Callaghan. *Neurotoxicology Laboratory, CDC/NIOSH, Morgantown, WV.*

The pathogenic mechanisms underlying Parkinson's disease (PD) remain enigmatic. In an effort to identify early molecular events associated with PD, we profiled genomic and proteomic changes in the MPTP mouse model of PD. Specifically, we focused on the role of TNF- $\alpha$ , IL-1 $\alpha$ , IL-6 and MCP-1, since enhanced expression of these proinflammatory cytokines and chemokines have been found in association with glial cells of patients with PD. MPTP caused a time-dependent increase in the mRNA expression of these cytokines in the striatum, but not in the hippocampus, and their expression preceded striatal dopaminergic degeneration (loss of dopamine and tyrosine hydroxylase), activation of JAK/STAT3 pathway and astrogliosis (upregulation of GFAP). Deficiency of the IL-6 gene did not alter striatal nerve terminal loss, but attenuated astrogliosis. However, in transgenic mice lacking TNF $\alpha$  receptors (TNFR-DKO), loss of striatal dopaminergic markers, phosphorylation of STAT3, upregulation of GFAP and astrocyte hypertrophy were nearly abolished. Interestingly, the lack of TNF $\alpha$  receptors exacerbated hippocampal neuronal damage (increased Fluoro Jade-B staining and loss of MAP-2 immunoreactivity) after MPTP. These findings implicate a region-specific role for TNF- $\alpha$  in the brain: a promoter of neurodegeneration in striatum and a protector against neurodegeneration in hippocampus. From a Parkinson's disease perspective, these findings are suggestive of a primary role for TNF- $\alpha$  in the pathogenesis of this disorder. As deficiency of TNF receptors or IL-6 gene attenuated phosphorylation of JAK-STAT3 and upregulation of GFAP, the findings implicate these signaling pathways in upregulation of GFAP. Since the activation of these pathways are early events in MPTP neurotoxicity, they may serve as potential therapeutic targets for modulation of neuronal loss and/or glial response following dopaminergic neurodegeneration.

#### 1988 BIOLOGICAL BASES FOR PCB INDUCED ALTERATION IN DOPAMINE-MEDIATED NEUROLOGICAL FUNCTION.

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Polychlorinated biphenyls (PCBs) are associated with developmental deficits in children and behavioral and neurochemical changes in laboratory animals. These latter changes include reductions in dopamine (DA) concentrations and the number of tyrosine hydroxylase positive neurons in the substantia nigra of adult rodents and non-human primates (NHPs). This loss of function involves inhibition of monoamine transporters, including the vesicular monoamine transporter, metabolism of free cytosolic DA, induction of oxidative stress and cell death. Indeed, in a midbrain DA cell line (N27), PCBs induce oxidative stress and apoptotic cell death, thus supporting the hypothesis that PCBs alter DA-mediated neuronal function and behavior. In order to test this hypothesis we are studying the neurological,

behavioral and structural consequences of prior long-term high level exposure to PCBs in a cohort of former workers. We will determine if prior PCB exposure alters DA mediated behaviors, including motor function, memory and the density of basal ganglia DA terminals using  $\beta$ -CIT SPECT imaging. This epidemiological study will allow us to begin to determine whether laboratory-derived data demonstrating that PCBs lead to DA neuronal dysfunction and cell death can be extrapolated to humans, thus providing additional evidence for the role of environmental neurotoxicants in the etiology of neurodegenerative disorders, including Parkinson's disease. (Supported by grants from the US Army Medical Research and Materiel Command, NIEHS and EPA to RFS, and NIH to AGK).

 **1989** ROLE OF NITRIC OXIDE IN METHAMPHETAMINE-INDUCED DOPAMINERGIC NEUROTOXICITY IN MICE.

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Methamphetamine (METH) induced dopaminergic neurotoxicity is thought to be associated with the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Recently, we have reported that copper/zinc(Cu/Zn)-superoxide dismutase transgenic mice are resistant to METH- induced neurotoxicity. In the present study, we examined the role of the neuronal nitric oxide synthase (nNOS), susceptibility of nNOS knockout (KO) in mice after neurotoxic doses of METH. Male Swiss Webster mice were treated with or without 7-nitroindazole (7-NI) along with METH (5 mg/kg, ip, q 3h X 3) and were sacrificed 72 h after the last METH injection. Dopamine (DA) and dopamine transporter (DAT) binding sites were determined in striatum from saline and METH-treated animals. 7-NI completely protected against the depletion of DA, and DAT in striatum. In follow up experiments nNOS KO mice along with appropriate control (C57BL/6N, SV129 and B6JSV129) mice were treated with METH (5 mg/kg, ip, q 3hX3) and were sacrificed 72 h after dosing. This schedule of METH administrations resulted in only 10-20% decrease in tissue content of DA and no apparent change in the number of DAT binding sites in nNOS KO mice. However, this regime of METH resulted in a significant decrease in the content of DA as well as DAT binding sites in the wild type animals. Therefore, these studies strongly suggest the role of nitric oxide, nNOS, peroxynitrite and DA system in METH-induced neurotoxicity.

 **1990** SAFETY ASSESSMENT OF BIOLOGICAL THERAPEUTIC PRODUCTS — DEFINING THE SCIENTIFIC AND REGULATORY ISSUES.

A. Weir<sup>1</sup> and B. J. Mounho<sup>2</sup>. <sup>1</sup>*FDA/CDER, Rockville, MD* and <sup>2</sup>*Amgen Inc., Thousand Oaks, CA.*

Biological therapeutic products (BTPs) are proteins derived from living organisms or produced via biotechnology means that have provided the medical community with novel, highly targeted therapies for the diagnosis and treatment diseases in humans. An integral part of the safety evaluation of these products is toxicology studies. BTP—induced toxicities are typically limited to their pharmacological mechanism of action; therefore, toxicology studies need to be conducted in an animal model that expresses the receptor or epitope that is recognized by the product. Frequently, a non-human primate (NHP) is the relevant model. In recent years, the quality of NHPs and the availability of methods for assessing toxicity in these animals have increased. In spite of these advances, many challenges remain in the safety assessment of BTPs. For example, only a very limited toxicological assessment can be conducted if the only relevant model is a chimpanzee. In such cases, toxicologists use innovative approaches, including the development of surrogate molecules, to conduct toxicology studies. Therefore, identification of novel methods is an ongoing effort in the BTP arena. Regardless of the animal model used, the potential for animals to mount an immune response to BTPs (immunogenicity) exists. Because immunogenicity can confound interpretation of toxicology studies, it is another challenge facing toxicologists that can result in the need for innovative approaches to safety assessment. Additionally, because immunogenicity can occur in humans receiving BTPs, the development of animal models to predict this effect in humans is an area of ongoing research. The need for innovative, flexible approaches when assessing the safety of BTPs is reflected in USFDA and international regulatory documents. The topics covered in this workshop will provide toxicologists with the most current information on the unique scientific properties of BTPs and with state-of-the-art approaches to safety assessment of BTPs.

 **1991** DIFFERENCES BETWEEN SMALL MOLECULES AND BIOLOGICAL THERAPEUTIC DRUG PRODUCTS.

B. Mounho. *Toxicology, Amgen, Inc., Thousand Oaks, CA.*

The majority of pharmaceutical drugs are chemically-synthesized, small molecular weight products. Over the years, however, biological therapeutic products (BTPs) have been approved for various clinical indications. BTPs are large molecular

weight molecules typically derived from genetic manipulation of living cells, such as bacteria, yeast, or mammalian cells; examples of BTPs include cytokines, growth factors, fusion proteins, and monoclonal antibodies. BTPs have several distinctive properties, which can create unique challenges in conducting nonclinical toxicology studies that typically are not issues for small molecule products; conversely, there are challenges unique to small molecule products (e.g. metabolism) that do not apply to BTPs. Due to the target specificity of BTPs, it is necessary to conduct toxicology studies in a pharmacologically relevant animal model. Relevant animal models for BTPs are often limited to nonhuman primates where limited safety data can be obtained and thus may require the use of alternative models such as transgenic mice or surrogate molecules. BTPs intended for humans are frequently immunogenic in animals, and can result in the formation of anti-drug antibodies; such antibodies can impact the outcome of toxicology studies by altering the pharmacokinetics or neutralizing the pharmacologic activity of the BTP. Because of their unique properties and challenges, the types of nonclinical toxicology studies conducted for BTPs are often science driven and can vary for each agent; regulatory expectations for nonclinical safety studies required for small molecule products, however, are more clearly defined due to the extensive experience and established database with small molecules. Furthermore, because there are fundamental differences between BTPs and small molecule products, the toxicology studies routinely conducted for small molecules (e.g. genotoxicity testing), may not be relevant for BTPs. Likewise, nonclinical studies conducted for particular BTPs (e.g. tissue-cross reactivity for monoclonal antibodies) may be inappropriate for small molecule products.

 **1992** IMMUNOGENICITY- IMPACT ON TOXICOLOGY STUDIES AND BEYOND.

D. Wierda and H. Smith. *Immunotoxicology, Eli Lilly Research Laboratories, Greenfield, IN.*

The design and interpretation of preclinical safety studies with human biotherapeutics are complicated by the formation of antibodies directed against the therapeutic molecule. A first design consideration is the selection of a toxicology testing species in which the test molecule is pharmacologically active. Most molecules are primate specific, but in cases where it is active in multiple species, the question arises as to whether a non-primate species should be used if immunogenicity is expected. Another consideration is the relative half-life of a molecule, especially with monoclonal antibodies, since a wash out period should be included to allow for clearance and to avoid free drug interference in the immunoassay. Longer lead times are also required for immunoassay development and to allow for the generation of positive antibody controls. Each of these factors significantly lengthens the timeline for conducting preclinical safety studies. In some cases, consideration must be given to the use of surrogate antibodies, or immunodeficient rodents, to avoid antibody neutralization of pharmacology. In safety studies, the principal impact of immunogenicity is on whether or not the antibodies neutralize pharmacology or alter pharmacokinetics as these could invalidate the toxicology study. The next consideration is whether any adverse effects are associated with an unexpected immune response such as immune complex deposition, allergy or, in the case of recombinant proteins, neutralization of endogenous proteins. It has been customary to dismiss the latter findings as an artifact due to differences in species homology, but recent experiences with protein replacement therapies such as erythropoietin demonstrate that such findings in animal studies may be predictive of potential clinical sequelae. These clinical findings indicate that further efforts are warranted for developing less immunogenic molecules and better models for predicting immunogenicity.

 **1993** THE NONHUMAN PRIMATE AS AN ANIMAL MODEL FOR THE SAFETY EVALUATION OF BIOLOGICAL THERAPEUTIC PRODUCTS.

J. Kapeghian. *Discovery and Development Services, Charles River Laboratories, Sierra Division, Sparks, NV.*

The nonhuman primate has become the default "species of choice" for the nonclinical development of biological therapeutics primarily for two reasons: 1) the therapeutic target is often expressed at levels comparable to humans and "monitorable" within the confines of IND-enabling toxicology studies, and 2) the phylogenetic similarities of nonhuman primates to humans reduces the potential for immunogenicity. Although many species of nonhuman primate are used in safety evaluation studies, the nonclinical safety of biologics have been typically tested in young adult rhesus and cynomolgus monkeys. The chimpanzee is important in selected types of disease and research programs; however, it is rarely used in toxicology studies due strict protection as a highly-threatened species. Essentially all nonhuman primates used for nonclinical research are now purpose-bred, and are imported from offshore colonies; there are also, however, domestic breeding sources within the US for laboratory use of these species. Due to recent supply shortages and increased cost of

rhessus monkeys, the cynomolgus monkey, a closely-related macaque, became the primary nonhuman primate species used in nonclinical studies within the US. Both the rhesus and cynomolgus are Old World species and have been well-characterized from a "historical data perspective" to allow relatively low numbers of animals to be used in toxicology test designs, and often, these animals provide the only relevant nonclinical safety data prior to dosing humans with biological therapeutics. For this reason, pharmacokinetic/pharmacodynamic, immunologic, and clinical pathology endpoints are routinely modeled in macaques for comparable monitoring in human trials, often with correlative predictive value. The similarities in genetic, physiologic, and pharmacologic responses between humans and macaques used in nonclinical safety evaluation of biologic therapeutics add to the rising importance of the rationale for the use of nonhuman primates in this role.

**1994 ALTERNATIVE METHODS FOR THE SAFETY EVALUATION OF BIOLOGICAL THERAPEUTIC PRODUCTS — SURROGATE ANTIBODIES AND BEYOND.**

*J. Clarke, BiogenIdec, Cambridge, MA.*

The safety evaluation of biological therapeutic products (BTPs) can be challenging because it can necessitate the use of alternative methods due to the pronounced species specificity and immunogenic potential of these products. BTPs being developed for chronic indications such as psoriasis, peripheral neuropathy or rheumatoid arthritis present toxicologists with the challenge of assessing the potential for reproductive toxicity and carcinogenicity. Novel approaches to this challenge include conducting toxicology studies using surrogate molecules and/or in knock-out or knock-in models. Surrogate molecules are specific for the epitope or receptor expressed in the desired species of laboratory animal. This approach, while it has gained acceptance and been used for several BTPs, remains a challenging and customized technique, highly influenced by the pharmacology and physical properties of the BTP. Examples will be discussed including Raptiva, a monoclonal antibody to LFA-1 for the treatment of psoriasis, and Remicade, a monoclonal antibody for the treatment of Crohn's Disease. Assessing the carcinogenic potential of BTPs remains challenging for toxicologists, but recent, highly novel approaches are being investigated, including tumor xenograft models and specialized mouse strains. Finally, as in other areas of toxicology, transcriptional profiling of cells and tissues using DNA arrays is an unfolding area in terms of its application to BTPs.

**1995 SAFETY ASSESSMENT OF BIOLOGICAL PRODUCTS — A REGULATORY PERSPECTIVE.**

*H. Ghantous, FDA/CDER/ODEVI/DTBIMP, Rockville, MD.*

The development of biological therapeutic products (BTPs) for the diagnosis and treatment of human diseases is a cooperative effort between biopharmaceutical companies and regulatory agencies. In the US, USFDA's Center for Drug Evaluation and Research (CDER) is responsible for regulating monoclonal antibodies for human use, cytokines, enzymes, growth factors, thrombolytics, and extracted proteins in addition to small molecular-sized drugs. The types of pharmacology and toxicology studies considered scientifically appropriate to support the safe development and, ultimately, the approval of BTPs are defined in a series of guidance documents. These documents include those generated within the International Conference on Harmonisation (ICH) process and within CDER. Due to the relatively unique properties of BTPs (e.g. large molecular size, target specificity, and immunogenicity), their safety assessment differs from that of small molecular-sized drugs. The scientific need for this different approach is reflected in the guidance documents. For example, the ICH S6 document, Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals, is specific for BTPs. In addition, other guidance documents define the relevance of specific types of studies (e.g. safety pharmacology) to BTPs. This presentation will address how the available guidance documents define a scientifically sound path for the development of BTPs.

**1996 NOVEL REPORTER GENE ASSAY FOR DEVELOPMENTAL TOXICITY TESTING.**

*S. Schwengberg<sup>1</sup>, A. Ehlich<sup>1</sup>, H. Marquardt<sup>2</sup>, J. Hescheler<sup>3</sup> and H. Bohlen<sup>1</sup>.  
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Animal-based developmental toxicity testing is often a bottleneck in compound development in the chemical and pharmaceutical industry. *In vivo* tests need large amounts of compounds, are time- and cost-consuming, and lack public acceptance. During the last years, *in vitro* alternatives have been examined to overcome the limitations of the current test strategies, among which the embryonic stem cell test is

the only animal-free test based on mammalian cells (Spielmann H et al., *In Vitro Toxicol* 1997, 10:119). However, this test is not suitable for screening large compound libraries. We have developed two different reporter gene assays based on the differentiation of mouse embryonic stem cells towards cardiac tissue (R.E.Toxicology FL: GFP expression, R.E.Toxicology SL: expression of a soluble enzyme). In a double-blind study, we have tested 70 compounds either recommended by ECVAM (Brown NA, ATLA 2002, 30:177) or tested by the National Toxicology Program (<http://ntp-server.niehs.nih.gov/htdocs/pub-TT0.html>). 93% of all compounds were identified correctly by R.E.Toxicology (strong embryotoxic: 100%, weak embryotoxic: 85%, non embryotoxic: 94%). Moreover, the effect of Thalidomide can be clearly demonstrated by R.E.Toxicology, although its teratogenicity cannot be detected in rodents *in vivo*. Compounds that are not classified correctly belong mainly to chemical classes that need metabolic activation (e.g. glycolethers and some phthalates). Therefore, the addition of a metabolic system (e.g. S9 mix, precirculation with hepatocytes) should be considered to further enhance the significance of the assay. In conclusion, R.E.Toxicology allows the monitoring of embryotoxic and teratogenic effects of chemicals, pharmaceuticals, and environmental agents in an animal-free, time- and cost-effective assay. Moreover, R.E.Toxicology needs only minimal amounts of compounds, which allows shifting developmental toxicity testing to early stages of compound development.

**1997 VALIDATION STATUS OF THE HENS EGG TEST-CHORIOALLANTOIC MEMBRANE (HET-CAM) TEST METHOD.**

*N. Choksi<sup>1,2</sup>, D. Allen<sup>1,2</sup>, C. Inhof<sup>1,2</sup>, J. Truax<sup>1,2</sup>, R. Tice<sup>1,2</sup> and W. Stokes<sup>1</sup>.  
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Concerns about animal welfare and interest in higher throughput testing have led researchers to develop alternative *in vitro* test methods for the current rabbit eye test. NICEATM evaluated four *in vitro* ocular test methods for their ability to identify substances that cause irreversible or severe irritation or corrosion. One of these test methods, HET-CAM, is a model that is proposed to mimic the mucosal tissues of the eye. The ability of HET-CAM to correctly identify ocular corrosives and severe irritants using available HET-CAM data that evaluated the time to appearance of endpoints and corresponding *in vivo* eye irritation data was evaluated according to current hazard classification schemes for the USEPA (n=54), the European Union (n=54), and the UN Globally Harmonized System (n=52). Depending on the classification scheme used, HET-CAM had a false positive rate of 20-27%, and a false negative rate of 0-7%. Lack of published intra- and interlaboratory data for this analysis method precluded an evaluation of test method reliability. A proposed standardized test method protocol and a proposed recommended list of reference substances have been developed for future validation and testing studies to further assess the accuracy, reliability, and the applicability domain of HET-CAM for the detection of ocular corrosives and severe irritants. HET-CAM may be useful in a tiered-testing strategy where positive results can be used to classify and label a substance, while substances with negative results would undergo additional testing to identify false negative ocular corrosives/severe irritants and to identify those chemicals with reversible ocular effects. This approach would reduce the number of animals used for eye irritation testing and reduce the number of animals experiencing pain and distress by identifying substances that are severe irritants/ corrosives. ILS staff supported by NIEHS contract N01-ES 35504.

**1998 GENE EXPRESSION SIGNATURES FOR CADMIUM, MERCURY, AND ACRYLAMIDE EXPOSURE IN CAENORHABDITIS ELEGANS.**

*D. Jackson<sup>1</sup>, M. Szilagyi<sup>2</sup>, E. Gehman<sup>2</sup> and E. Clegg<sup>1</sup>. <sup>1</sup>US Army Center for Environmental Health Research, Fort Detrick, MD and <sup>2</sup>Geo-Centers, Inc., Fort Detrick, MD.*

To ascertain whether distinct biological responses to different chemical toxicants could be discerned at the level of gene expression in the nematode *C. elegans*, we exposed synchronized cultures of mid-L4 larvae to two heavy metals, cadmium chloride, and mercuric chloride, and to the chemically dissimilar compound, acrylamide. Exposures were for 4 and 8 h. We used doses sufficient to reproducibly arrest development of approximately 10%, 50% or 90% of the larvae in the L4 stage 24 h after exposure. Unexposed worms molt and enter the adult stage about 17 h after the mid L4 stage. Exposures were performed in triplicate in axenic medium to eliminate potential confounding effects due to using *E. coli* as food source for the worms. Microarray analyses were performed with RNA isolated from whole control and exposed worms using Affymetrix whole genome *C. elegans* GeneChips. As judged by ANOVA, each chemical induced a distinct pattern of

gene expression, even within metabolic pathways, such as glutathione synthesis, which are affected by all the chemicals. While there were some developmental differences between worms exposed for 4 or 8 h, toxicant induced alterations in gene expression were generally similar at both exposure durations. Disclaimer: The views, opinions, and/or findings contained in this report are those of the authors and should not be construed as official Department of the Army position, policy, or decision, unless so designated by other official documentation.

**1999**

**CYTOKINE SECRETION PROFILES OF MOUSE DENDRITIC CELLS: IMPACT OF CELL TRAUMA.**

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Cutaneous immune responses such as chemical contact allergy are regulated by cytokines produced by skin cells including Langerhans cells (LC) and keratinocytes. LC-like DC can be generated in culture from precursor cells, facilitating investigations of alternative approaches for skin sensitization hazard identification. DC were generated from bone marrow cells of BALB/c strain mice and cytokine secretion profiles measured following 24h culture with the water soluble allergen dinitrobenzene sulfonic acid (DNBS) and the non-sensitizing analogue, benzene sulfonic acid (BSA). Cells were exposed to concentrations of DNBS (0.05 and 0.5mM) or BSA (5 and 50mM) that were without effect on cell viability or that provoked significant cytotoxicity (40 to 100% cell death), respectively. Control cells were cultured with medium alone or with 1 µg/ml bacterial lipopolysaccharide (LPS), a potent stimulator of DC that is without effect on cell viability. In the majority of experiments (n=8), DC expressed constitutively interleukin (IL)-1β, IL-1α, IL-6, IL-12 p40 and IL-12 p70. Compared with medium alone, culture with high dose DNBS up-regulated IL-6 and IL-12 p40 secretion (5- and 20-fold). In contrast, culture with high dose BSA provoked increases in IL-1α (2-fold) and IL-1β (3-fold) expression. Culture of DC with 1µg/ml LPS resulted in marked increases in cytokine expression, with for example a 260-fold increase in IL-6 secretion and a 46-fold increase in IL-12 production compared with medium controls. In addition, increased levels of tumor necrosis factor-α, IL-10 and IL-17, cytokines that were not expressed constitutively, were provoked by treatment with LPS. These data show that the modest cytokine production induced by chemical is dependent, in this instance, upon cytotoxicity. Under conditions of equivalent cell injury, different cytokine profiles were induced by sensitizers and non-sensitizers, suggesting that it may be possible to identify chemical allergens on the basis of differential cytokine secretion.

**2000**

**MATRIX METALLOPROTEINASES AS BIOMARKERS FOR DIOXIN EXPOSURE IN DEVELOPING JAPANESE MEDAKA (ORYZIAS LATIPES).**

V. L. Prince, V. LaPrete, C. M. Villano and L. A. White. *Biochemistry and Microbiology, Rutgers University, New Brunswick, NJ.*

Dioxin like compounds (DLC), such as 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), are global environmental contaminants that are particularly toxic to early stages of vertebrate development. In the developing fish, TCDD disrupts cardiovascular development and function that is manifested in decreased blood flow and edema in the caudal region, in addition to edema in the pericardium and/or yolk sac. Our hypothesis is that TCDD and related DLCs disrupt vascular formation and function through activation of the matrix metalloproteinases (MMPs). MMPs are a family of endopeptidases that degrade the proteins of the extracellular matrix. Expression and activity of the MMPs are required for tissue remodeling, and are expressed during vascular development and wound regeneration. We propose that MMP expression is a pre-lesion biomarker for dioxin exposure, and that activation of MMP expression and activity is a critical step in resulting lesions. Reports demonstrate that developmental TCDD exposure of medaka (*Oryzias latipes*) results in hemorrhaging and edema. Our data further define the sensitive period of TCDD exposure to between 30 and 80 hpf. Medaka embryos exposed to TCDD from 1-2 hpf (hours post fertilization) or from 30 hpf resulted in hemorrhaging by 120 hpf and 83-87% pre-hatch mortality. Medaka embryos exposed from 80 hpf, showed similar lesions, however, fewer embryos were affected (59%). Using quantitative RT-PCR, we show that MMP-2 and MMP-9 are expressed at critical times for vascular development, with MMP-2 having two peaks at 30 hpf and 120 hpf. Following TCDD exposure, CYP1A1 expression was increased over vehicle control, demonstrating activation of the AhR pathway. MMP-2 expression was increased in response to TCDD (2-5 fold) at 120 hpf. TCDD-induced expression of MMP-9 was not observed at this time, but was observed at 216 hpf. This suggests that TCDD activation of the AhR pathway alters MMP expression and activity, resulting in abnormal vascular development and edema.

**2001**

**EPIOCULAR™ HUMAN CELL CONSTRUCT: TISSUE VIABILITY AND HISTOLOGICAL CHANGES FOLLOWING EXPOSURE TO SURFACTANTS.**

M. E. Blazka<sup>1</sup>, M. Diaco<sup>2</sup>, J. W. Harbell<sup>2</sup>, H. Raabe<sup>2</sup>, A. Sizemore<sup>2</sup>, N. Wilt<sup>2</sup> and D. M. Bagley<sup>1</sup>. <sup>1</sup>Colgate-Palmolive Co, Piscataway, NJ and <sup>2</sup>Institute for In Vitro Sciences, Inc., Gaithersburg, MD.

The ability of the EpiOcular™ construct to predict the eye irritation potential of surfactants and surfactant-based formulations has been the subject of a formal validation program. EpiOcular™ correlates a test article's potential for ocular irritation with the time it takes to reduce tissue viability by 50% (ET<sub>50</sub>) as measured by the tissue's ability to reduce MTT. An algorithm is used to convert the ET<sub>50</sub> value to a 'predicted Draize' score which can then be compared to *in vivo* data. This study investigated whether the histological changes following exposure are in agreement with the MTT results. Eight surfactants were selected from the validation study; 4 surfactants whose *in vitro* ocular irritation potential agreed with the *in vivo* data (cetyl alcohol; 3% sodium lauryl sulfate; 50% didecyldimonium chloride; sodium sulfolaurate mixture) and 4 whose *in vitro* data differed from the *in vivo* data (10% cetylpyridinium bromide; 3.2% benzethonium chloride; C10-12 alcohol ethoxylate; quaternium-18). Exposure times used in this study bracketed ET<sub>50</sub> values established in the validation study. For all surfactants, the results showed a good relationship between the degree of histological damage with changes in tissue viability. An increase in the depth and severity of tissue damage was associated with a decrease in tissue viability. Histological changes ranged from subtle cellular changes such as vacuolization and punctate chromatin condensation to overt tissue loss and cell necrosis. Loss of or damage to the surface squamous epithelium was associated with <20% decrease in viability, while the degree of damage to the central squamous epithelium was directly related to a 20-80% decrease in viability. In conclusion, the nature and severity of the histological changes were in agreement with the MTT results. Understanding the progression and types of cellular changes associated with tissue damage may be able to help distinguish the degrees of ocular irritation.

**2002**

**LONG TERM REPRODUCIBILITY OF EPIOCULAR™, A THREE-DIMENSIONAL TISSUE CULTURE MODEL OF THE HUMAN CORNEAL EPITHELIUM.**

M. Klausner, J. E. Sheasgreen, J. Kubilus and P. J. Hayden. *MatTek Corporation, Ashland, MA.*

The EpiOcular tissue model (OCL-200) is an organotypic model of the human corneal epithelium (HCE) cultured from normal human keratinocytes using serum free medium. Paraffin embedded, H & E stained histology cross-sections show the structure of EpiOcular closely parallels that of the HCE; large nucleated basal cells lie beneath 5-6 stratified cell layers which progressively flatten out, ending with a highly squamous, non-keratinized layer at the tissue's apical surface. Since commercial introduction in 1995, EpiOcular has been increasingly used by many personal care and household product companies to determine the ocular irritancy of their products without using animals. Currently, validation of the EpiOcular model as a replacement for the Draize rabbit eye test is underway in the US. In addition, a validation study sponsored by ECVAM is scheduled to begin in 2005. For commercial and regulatory purposes, it is very important to know that the model is reproducible both within a given lot and between lots, especially over extended periods. Quality control of weekly batches of EpiOcular is performed using the MTT assay, which historically has been the *in vitro* endpoint of choice for European and US regulators. The exposure time needed to reduce the viability to 50% (ET-50) for 0.3% Triton X-100 is determined. Yearly average ET-50 values have ranged from 20.6 minutes (2000) to 25.0 minutes (1998). The coefficients of variation (CV) for the negative control tissue (exposed to ultrapure H<sub>2</sub>O) have averaged under 6% for every year since 1997. In addition, the yearly average CV for all tissues has never exceeded 6.5%. These results over the past 8 years of commercial production show EpiOcular to be a highly reproducible, stable toxicological model that is ideally suited for industrial and regulatory ocular irritancy studies.

**2003**

**HIGH-THROUGHPUT *IN VITRO* MODELS OF HUMAN EPIDERMIS AND OCULAR EPITHELIUM FOR PRECLINICAL SAFETY AND EFFICACY TESTING OF CONSUMER PRODUCTS AND PHARMACEUTICALS.**

G. R. Jackson, J. Kubilus, M. Klausner, J. E. Sheasgreen and P. J. Hayden. *MatTek Corp., Ashland, MA.*

Prior to introduction of new consumer products and cosmetics, or human testing of new therapeutics, animal experiments are traditionally utilized to screen for safety and efficacy. However, large numbers of lead candidates generated by modern high throughput technologies renders animal testing expensive and impractical. A growing need exists for high throughput *in vitro* models which can provide rapid, reliable safety and efficacy screening. The current poster describes development of

*in vitro* models of human epidermal and ocular cultures in 96-well high-throughput screening (HTS) formats compatible with robotic manipulation. The HTS models are derived from normal human cells cultured at the air/liquid interface in 96-well microporous membrane plates to produce three-dimensional organotypic cultures. Culture histology of both models was evaluated by H&E staining of formalin fixed paraffin sections. The epidermal model displays a stratified and cornified differentiated structure, similar to native epidermis. The 96-well HTS ocular cultures are stratified squamous epithelium typical of *in vivo* ocular epithelium. Well to well (intraplate) and plate to plate (interlot) variability, as determined by the MTT viability assay, was generally found to be less than 10%. The HTS ocular model was also evaluated for compatibility with the EpiOcular Prediction Equation developed for predicting *in vivo* Draize ocular irritation scores with MatTek Corporation's standard EpiOcular product(OCL-200). When tested with various concentrations of chemicals including sodium dodecyl sulfate, Triton X-100, benzalkonium chloride, sodium hydroxide and others, the HTS ocular model gave predicted Draize scores that correlated well with standard EpiOcular predicted Draize scores and historical *in vivo* Draize scores. HTS epidermal and ocular models such as these may thus help to lessen bottlenecks currently encountered in safety and efficacy testing of consumer products and topical therapeutics. This work was supported by NIEHS grant 5R44 ES010237-03.

## 2004

### EVALUATION OF A TISSUE ENGINEERED HUMAN SKIN EPIDERM 3-D CULTURE AS A MODEL TO STUDY IRRITATION AFTER JET FUEL EXPOSURE.

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The purpose of the present study was to evaluate human skin epiderm 3-D culture, EPI-200 (MatTek Corp, Ashland, MA) as a model to study the skin irritation of various jet fuels. Upon receipt, EPI-200 tissues were placed in six-well plates containing maintenance medium (EPI-100-NMM) and incubated for 24-72h. The cultures were subjected to different doses (5, 10, 50  $\mu$ l) of JP-8 and JP-8+100. After each exposure, the tissue viability was measured by MTT assay and the morphology was studied by hematoxylin and eosin (H&E) staining of histological sections. The expression of IL-1  $\alpha$  in the tissue culture media was determined by enzyme immunoassay (EIA). The EPI-200 exposures showed an interesting dose dependent relationship between IL-1 $\alpha$  release and cell viability. At the lowest dose (5 $\mu$ l) of JP-8 and JP-8+100, there was no cell kill at 24 h, but at 48 and 72 h both the chemicals reduced the tissue viability to 61-65 %, with no significant difference between the two time points. JP-8 and JP-8+100 exposures at 24h significantly increased the expression of IL-1 $\alpha$  to 13 and 51 folds respectively over the control. This finding was very interesting considering the fact that there was no cell kill detected after 24 h. At 48 and 72h the IL-1 $\alpha$  level was lower than at 24h which was due to reduction in the cell viability. After 24h, 10 $\mu$ l of JP-8 and JP-8+100 exposures demonstrated around 45-50% cell viability while 50  $\mu$ l of the jet fuels reduced the cell viability to zero. Control tissues were 100% viable at all time points. H&E staining of the tissues after 24h indicated that the jet fuels disrupted the barrier function of stratum corneum. The effect was more severe after 48h, while after 72h the tissue started losing their integrity. The findings with EPI-200 correlate well with our earlier findings from skin irritation studies conducted on CD Hairless rats with JP-8 and JP-8+100. Overall, our experiments demonstrate that EPI-200 can be used as a valuable model to evaluate the skin irritation potential of jet fuels and hydrocarbon chemicals.

## 2005

### VALIDATION STATUS OF THE BOVINE CORNEAL OPACITY AND PERMEABILITY (BCOP) TEST METHOD.

C. Inhof<sup>1,2</sup>, N. Choksi<sup>1,2</sup>, D. Allen<sup>1,2</sup>, J. Truax<sup>1,2</sup>, R. Tice<sup>1,2</sup> and W. Stokes<sup>1</sup>.  
<sup>1</sup>NICEATM, NIEHS, Research Triangle Park, NC and <sup>2</sup>ILS, Inc., Research Triangle Park, NC.

Concerns about animal welfare and an interest in higher throughput testing have led researchers to develop alternative *in vitro* test methods for the current rabbit eye test. NICEATM evaluated four *in vitro* ocular test methods for their ability to identify substances that cause irreversible or severe irritation or corrosion. One of these test methods, BCOP, is an organotypic model that provides short-term maintenance of normal physiological and biochemical function of the cornea in an isolated system. The ability of BCOP to correctly identify ocular corrosives and severe irritants using available BCOP and corresponding *in vivo* eye irritation data was evaluated according to current hazard classification schemes for the USEPA (n=101), the European Union (n=141), and the UN Globally Harmonized System (n=104). Depending on the classification scheme used, BCOP had a false positive rate of 20-26%, and a false negative rate of 22-26%. In terms of reliability, the assay was determined to have acceptable intra- and inter-laboratory reproducibility. A proposed standardized test method protocol and a proposed recommended list of reference substances have been developed for future validation and testing studies to further assess the accuracy, reliability, and the applicability domain of BCOP for the detection of ocular corrosives/severe irritants. Investigators should consider using BCOP prior to eye irritation testing in animals. BCOP may be useful in a tiered testing strategy where positive results can be used to classify and label a sub-

stance, while substances with negative results would undergo additional testing to identify false negative ocular corrosives/severe irritants and to identify those chemicals with reversible ocular effects. This approach would reduce the number of animals used for eye irritation testing and reduce the number of animals experiencing pain and distress. ILS staff supported by NIEHS contract N01-ES 35504.

## 2006

### ASSESSMENT OF THE SKINETHIC RECONSTITUTED HUMAN EPIDERMAL MODEL FOR THE PREDICTION OF THE DERMAL IRRITATION POTENTIAL OF PHARMACEUTICAL PROCESS MATERIALS.

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<sup>1</sup>GlaxoSmithKline, Ware, United Kingdom, <sup>2</sup>SafePharm Laboratories, Derby, United Kingdom, <sup>3</sup>SkinEthic Laboratories, Nice, France and <sup>4</sup>University of Ghent, Ghent, Belgium.

Occupational dermatitis is an ongoing issue and identification of the irritant potential of materials is an important component in chemical control. Traditionally, testing for irritation has relied upon *in vivo* models. Recent developments, including ethical and scientific concerns regarding the rabbit model and availability of new technologies, have driven the development of promising systems for the prediction of dermal irritation. This report describes a study to assess the performance of a reconstituted human epidermal (RHE) model produced by SkinEthic Laboratories, Nice, France when tested with 23 diverse pharmaceutical process materials at two separate laboratories. The collection included chemicals producing a range of reactions from non-irritant to severe irritation *in vivo*. Untreated cultures were used as negative controls and 0.1% Triton as a positive control. Substances were applied topically, as supplied to triplicate RHE cultures for periods of 4 or 24-hours. At the end of treatment, cultures were assessed for viability (MTT), histological change and IL-1 alpha and IL-8 release. Results for MTT, histopathology and interleukin-1 alpha were shown to be reliable and reproducible, both within and between laboratories. Results for interleukin-8 showed greater variation. Concordance with *in vivo* results at the two laboratories was excellent, being 78% and 83% respectively, as were values for sensitivity and specificity. Absence of cytotoxicity at 24 hours was shown to be a reliable predictor of non-irritant chemicals. Time-related cytotoxicity (at 24-hours only) may be a useful model for mild irritants. RHE cultures are shown to have promise as an alternative for the identification of the dermal irritation hazard of pharmaceutical process materials.

## 2007

### VALIDATION STATUS OF THE ISOLATED CHICKEN EYE (ICE) TEST METHOD.

D. Allen<sup>1,2</sup>, N. Choksi<sup>1,2</sup>, C. Inhof<sup>1,2</sup>, J. Truax<sup>1,2</sup>, R. Tice<sup>1,2</sup> and W. Stokes<sup>1</sup>.  
<sup>1</sup>NICEATM, NIEHS, Research Triangle Park, NC and <sup>2</sup>ILS, Inc., Research Triangle Park, NC.

Concerns about animal welfare and interest in higher throughput testing have led researchers to develop alternative *in vitro* test methods for the current rabbit eye test. NICEATM evaluated four *in vitro* ocular test methods for their ability to identify substances that cause irreversible or severe irritation or corrosion. One of these test methods, ICE, is an organotypic model that provides short-term maintenance of normal physiological and biochemical function of the eye in an isolated system. The ability of ICE to correctly identify ocular corrosives and severe irritants using available ICE and corresponding *in vivo* eye irritation data was evaluated according to current hazard classification schemes for the USEPA (n=91), the European Union (n=121), and the UN Globally Harmonized System (n=93). Depending on the classification scheme used, ICE had a false positive rate of 8-10% and a false negative rate of 30-40%. In terms of reliability, the assay has acceptable interlaboratory reproducibility; intralaboratory reproducibility could not be assessed. A proposed standardized test method protocol and a proposed recommended list of reference substances have been developed for future validation and testing studies to further assess the accuracy, reliability, and the applicability domain of ICE for the detection of ocular corrosives/severe irritants. Investigators should consider using ICE prior to eye irritation testing in animals. When used in a tiered testing strategy, positive results could be used to classify and label a substance, while substances with negative results would undergo additional eye irritation testing to identify false negative ocular corrosives/severe irritants and to identify those chemicals with reversible ocular effects. This approach would reduce the number of animals used for eye irritation testing and reduce the number of animals experiencing pain and distress. ILS staff supported by NIEHS contract N01-ES 35504.

## 2008

### VALIDATION STATUS OF THE ISOLATED RABBIT EYE (IRE) TEST METHOD.

J. Truax<sup>1,2</sup>, N. Choksi<sup>1,2</sup>, C. Inhof<sup>1,2</sup>, D. Allen<sup>1,2</sup>, R. Tice<sup>1,2</sup> and W. Stokes<sup>1</sup>.  
<sup>1</sup>NICEATM, NIEHS, Research Triangle Park, NC and <sup>2</sup>ILS, Inc., Research Triangle Park, NC.

Concerns about animal welfare and interest in higher throughput testing have led to the development of alternatives to the current rabbit eye test. NICEATM evaluated four *in vitro* ocular test methods for their ability to identify irreversible or se-

were irritants/corrosives as alternatives to the rabbit eye test. One of these test methods, IRE, is an organotypic model that maintains normal physiological and biochemical function of the isolated eye. The ability of IRE to correctly identify ocular corrosives/severe irritants using available IRE and corresponding *in vivo* irritation data was evaluated according to current hazard classification schemes defined by the USEPA (n=77), the European Union (n=149), and the UN Globally Harmonized System (n=81); accuracy (48-86%), and false positive (22-59%)/false negative (0-57%) rates varied widely but consistently across regulatory systems. When fluorescein retention and/or epithelial integrity assessment were included (EU system only; n=65), accuracy was 77-86%; false positive and false negative rates were 23-34% and 0%, respectively. Lack of published intra- and interlaboratory data for this assay version precluded an evaluation of reliability. A proposed standardized test method protocol and a proposed recommended list of reference substances have been developed for future validation/testing studies to further assess the accuracy, reliability, and the applicability domain of IRE for the detection of ocular corrosives/severe irritants. IRE may be useful in a tiered testing strategy where positive results can be used to classify and label a substance, while substances with negative results would undergo additional testing to identify false negative ocular corrosives/severe irritants and to identify those chemicals with reversible ocular effects. This approach would reduce the number of animals used for eye irritation testing and reduce the number of animals experiencing pain and distress. ILS staff supported by NIEHS contract N01-ES 35504.

## 2009

### ASSESSMENT OF THE SKINETHIC RECONSTITUTED HUMAN CORNEAL EPITHELIUM MODEL FOR PREDICTION OF THE OCULAR IRRITATION POTENTIAL OF PHARMACEUTICAL PROCESS CHEMICALS.

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New process chemicals are continually required to develop novel drugs and to improve synthesis of existing medicines. Determination of the eye irritancy potential of process chemicals is essential because of the serious effects that could occur after accidental workplace exposure to severe irritants. Despite the development of several promising *in vitro* methods for assessment of eye irritancy, none has yet been fully validated and the use of animals is still necessary. Following an initial trial in 2003, the skinethic reconstituted human corneal model (RHC) was assessed for its predictive ability with regard to the irritancy of 21 process chemicals. Testing was conducted blind at two laboratories, SafePharm, UK and SkinEthic, France. Based on *in vivo* or *ex vivo* data 7/21 chemicals had previously been classified as irritant (EU R36 or R41). Chemicals were applied topically to triplicate rhc cultures for periods of 5, 10 and 60 minutes. Triplicate tissues were similarly treated with a buffered salt solution (negative control) or SDS 0.5% or 1% (positive control). At the end of treatment cultures were assessed for viability (MTT), histological changes and cytokine release (IL-1 $\alpha$ ). Results for MTT were shown to be the best indicator of irritancy. Histological changes were nearly always associated with decreased viability. IL-1 $\alpha$  release was low and was not considered useful for classification purposes. Some variability was noted between results obtained in the two laboratories. However, using data from safePharm, a concordance of >80% and sensitivity of 100% was obtained. Furthermore, 2/3 R36 irritants were correctly identified and a third was overpredicted as R41. The concordance was improved by evaluation of the results in conjunction with other safety data available for the chemicals. The RHC model shows promise for identification of irritant pharmaceutical process chemicals.

## 2010

### MELANOCYTE CONTAINING HUMAN ORGANOTYPIC EPIDERMIS AS A MODEL TO EVALUATE TOXICITY OF MELANIN BINDING SUBSTANCES.

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The present study was performed in order to investigate how the presence of melanocytes alters toxicity of the melanin binding substance chloroquine in reconstituted organotypic human epidermis *in vitro*. To this aim, effects in two slightly different human epidermis constructs were compared; i) the well described epidermis produced by SkinEthic Laboratories and ii) the epidermis model containing melanocytes in addition to keratinocytes. Both tissues were generated at SkinEthic using the same stock of keratinocytes and kept in culture like described earlier (De Brugerolle de Fraissinette A et al. 1999). Both tissues were treated in parallel in five replicates with 0.019, 0.19 and 1.9mM of chloroquine or the non-toxic control substance trizma (= Tris buffer) dissolved in the culture medium. Sodium lauryl sul-

fate (SLS, 1.7mM) served as severely cytotoxic control. After 24h treatment the release of lactate dehydrogenase (LDH) and of the pro-inflammatory cytokines IL-1 $\alpha$  and IL-8 were determined. SLS induced severe cytotoxicity independently from the presence or absence of melanocytes as indicated by strong LDH release, whereas trizma treatment in all tested concentrations did neither induce cytokine nor LDH release. The melanin binding substance chloroquine induced release of LDH and IL-1 $\alpha$  in all tested concentrations in the absence of melanocytes. When melanocytes were present chloroquine was less toxic, causing LDH and IL-1 $\alpha$  release only at the highest concentration tested. In conclusion, melanocytes reduced toxic effects of the melanin binding substance chloroquine in human reconstituted epidermis. The melanocyte containing epidermis models may be a useful model to investigate toxicity of melanin binding substances.

## 2011

### VALIDATION OF A FLOW CYTOMETRY-BASED PHOTO-LOCAL LYMPH NODE ASSAY (PHOTO-LLNA) FOR THE IDENTIFICATION AND CHARACTERIZATION OF PHOTO-ALLERGENS.

M. K. Reeder, T. L. Ripper, D. R. Cerven and G. L. DeGeorge. *MB Research Laboratories, Spinnerstown, PA*.

The increasing concerns of both industry and regulatory agencies to identify and protect consumers from the photosensitizing properties of substances (chemical plus UVR exposure-induced allergic contact dermatitis) has driven the need for the development of photo-allergy (photosensitization) screening assays. Rapid, cost-effective tests that can identify and distinguish photo-allergic from photo-irritating or sensitizing substances, are totally lacking. Currently, the Photo-Buehler Guinea Pig Sensitization Assay, a subjective test that uses a large amount of animals, is commonly used to assess photosensitization potential of chemicals. This Photo-LLNA test is based on our recently validated "Enhanced Local Lymph Node Assay using Flow Cytometry" (eLLNA). Although the LLNA has been validated by both EU and USA regulatory agencies, and is now the "default, preferred test method" for the OECD and EPA, the use of radioactivity has limited its widespread application. The eLLNA, as well as the Photo-LLNA, substitutes the use of BrdU, a thymidine analog, in place of 3H-thymidine for measuring proliferation, eliminating the need for hazardous radioactivity. Other advantages of the Photo-LLNA include: 1) reduction of the number of test animals used, 2) decreased time and expense of testing, and 3) reduced pain and distress to test animals. The Photo-LLNA increases the discriminating power of the basic LLNA and GP photo-allergy assays by including immunophenotyping of lymphocyte subpopulations (CD3+ and B220+) and measuring "activation marker expression" such as CD69 and I-Ak. Using these additional endpoints, the Photo-LLNA identifies and discriminates photosensitizers from non-photosensitizers with over 92% accuracy.

## 2012

### COMPARISON OF ALTERNATIVE TOXICITY TEST METHODS FOR OCULAR IRRITATION USING THE AVIAN CHORIOALLANTOIC MEMBRANE: HET-CAM VS. CAMVA.

A. C. Gilotti, D. R. Cerven, S. Craig and G. L. DeGeorge. *MB Research Laboratories, Spinnerstown, PA*.

Industry and some regulatory agencies are seeking alternatives to the Draize Eye Irritation Test in rabbit to address the three R's of decreased animal testing. Decades of research and validation have produced two non-animal-based ocular toxicity tests, the CAM Vascular Assay (CAMVA) and the Hen's Egg Test-CAM (HET-CAM). The HET-CAM assay has been nominated by the EPA for evaluation as an alternative to the Draize test for ocular irritancy in rabbits. In the HET-CAM assay, test substances are applied topically to the CAM (at 10, 2.5 and 1% w/v). Ocular irritancy is predicted by calculating an irritation score (IS) based on the length of time until the exposure results in hemorrhage, lysis or coagulation. This reaction time is determined by visual inspection of the CAM through the test chemical. Turbid test chemicals prevent the reaction time from being determined, and thus they cannot be evaluated in the HET-CAM assay. This presents a serious problem for determining the ocular irritancy potential of mixtures and cosmetic formulations. We routinely use the CAM Vascular Assay (CAMVA) to evaluate turbid or insoluble formulations of cosmetics and other products. In the CAMVA, the CAMs are exposed to various concentrations of a test substance for 30 minutes and vascular effects are monitored to determine a RC50 for each test substance. We have used a 5-minute exposure time in the HET-CAM assay to determine the RC50 of various test chemicals. HET-CAM derived RC50 values were comparable to those determined in the CAMVA. In addition, the IS scores of translucent test substances were compared to the HET-CAM derived RC50 values to develop a model for predicting ocular irritancy based on a concentration-dependent modification to the HET-CAM protocol.

**2013****EXPOSURE OF MUCOCILIARY HUMAN LUNG EPITHELIAL CULTURES TO WHOLE CIGARETTE SMOKE.**

A. Richter, J. Phillips, N. Newland and E. D. Massey. *R&D, British American Tobacco, Southampton, United Kingdom.* Sponsor: J. Seagrave.

We have designed an exposure system for investigating the biological effects of whole cigarette smoke on lung epithelial cultures. In this study, primary human bronchial epithelial cells were differentiated to generate 3-dimensional mucociliary cultures resembling the normal tracheobronchial epithelium. The cultures were exposed to serial dilutions of mainstream cigarette smoke at an air-liquid interface for 1 hour. In addition, control cultures were exposed to filtered air. The cells were then incubated for a further 20 hours in serum-free medium. The trans-epithelial electrical resistance (TER) of the cultures was then determined and cell viability measured by the active uptake of neutral red dye. The levels of interleukin-8 (IL-8), interleukin-6 (IL-6), interleukin-1beta (IL-1beta) and matrix-metalloprotease-1 (MMP-1) in the conditioned medium were determined by enzyme-linked immunoassay. High concentrations of smoke reduced cell viability in a dose-dependent manner. Cytotoxicity was paralleled by a reduction in TER. Cigarette smoke stimulated the secretion of IL-8, IL-6 and MMP-1 from the epithelial cultures. By comparison, IL-1beta was not detectable in the conditioned medium of control or treated cultures. This study demonstrates a new experimental system for whole smoke exposure of cells at an air-liquid interface that may be useful for studying the molecular basis of smoking-related lung disease. Moreover, it may be adaptable for studying the effects of other aerosols and the use of such an *in vitro* system could potentially reduce use of animals for inhalation toxicology.

**2014****USE OF PLATEABLE CRYOPRESERVED RAT HEPATOCYTES AS A MODEL TO ASSESS HEPATOTOXICITY AND DRUG-DRUG INTERACTIONS.**

T. A. Moeller, S. Lloyd, P. M. Silber and N. S. Jensen. *In Vitro Technologies, Baltimore, MD.*

Freshly isolated rat hepatocytes are widely used to study drug metabolism, toxicity, and drug-drug interactions. However, the preparation of fresh rat hepatocytes is labor- and time-intensive and can be subject to interday variability with respect to both the quantity and quality of the isolated hepatocytes. We have recently identified cryopreserved rat hepatocytes (PCRH) that form monolayers on collagen-coated plates, and which can be used to assess long-term (>24 hour) toxicity and drug-drug interactions. To evaluate the PCRH model, hepatocytes from SD rats were thawed, and allowed to attach to collagen-coated plates overnight in a 37°C, 5% CO<sub>2</sub> incubator. Plates were then washed to remove nonadherent cells. After washing, all hepatocyte monolayers were >70% confluent. Hepatotoxicity was determined by treating hepatocyte monolayers with tamoxifen (0-100 µM), followed by measurement of MTT reduction and intracellular ATP levels at 24- and 48-hour time points following dosing. Tamoxifen demonstrated hepatotoxicity that was both dose and time dependent with an LD<sub>50</sub> of 23 µM and 15 µM at 24 and 48 hours, respectively when measured by MTT reduction. The LD<sub>50</sub> as measured by ATP levels was 30 µM and 18 µM at 24 and 48 hours, respectively. These results demonstrated the increased sensitivity of these hepatocytes to hepatotoxins with increasing exposure time. PCRH were also evaluated for their ability to support induction of cytochrome P450 1A activity. Hepatocyte monolayers were dosed with β-naphthoflavone (10 µM) on days 3 and 4 post plating. The metabolism of ethoxresorufin was measured on day 5. An 8-fold increase in ethoxresorufin O-deethylase (EROD) activity was observed following treatment with β-naphthoflavone as compared to the untreated vehicle controls. Viability as measured by MTT reduction remained high throughout the 5-day culture. In summary, PCRH combine the benefits of fresh and cryopreserved rat hepatocytes, and allow the long-term study of drug toxicity, metabolism, and drug-drug interactions in a reproducible model.

**2015****THE SLUG MUCOSAL IRRITATION TEST: A USEFUL TOOL FOR EVALUATING THE LOCAL TOLERANCE OF PHARMACEUTICAL FORMULATIONS.**

E. Adriaens, M. Dhondt and J. Remon. *Lab. Pharmaceutical Technology, Ghent University, Gent, Belgium.* Sponsor: C. Seaman.

Several studies have shown that the Slug Mucosal Irritation (SMI) test is a useful tool for evaluating the local tolerance of pharmaceutical formulations. The mucosal irritation potency can be estimated by the mucus production (MP) and the release of proteins and enzymes from the body wall of the slug *Arion lusitanicus* after treatment with the substances. During a 5-day procedure the slugs are daily placed on the undiluted substance for 30 min. After each contact period the MP, protein and enzyme release (LDH and ALP) are measured. Based on the 4 endpoints a classifi-

cation prediction model was developed that distinguishes into irritation and membrane damage. The MP is used to classify the formulations into 4 irritation classes (NI, mild, moderate and severe) whereas the release of proteins and enzymes is a measure of membrane damage (No, slight, moderate and severe). In this study the mucosal irritation potency of 13 nasal, 4 buccal and 16 vaginal or rectal formulations covering different irritation classes was evaluated with the SMI test. The classifications obtained with the SMI test were compared with published *in vitro* and *in vivo* (animal and clinical) data on the nasal, buccal, vaginal and rectal irritation potency of the formulations. There was an excellent agreement between the SMI and the *in vivo* data, only one vaginal formulation was overestimated. The SMI test can be used early in the R&D phase of new pharmaceuticals and this can result in the reduction of the use of laboratory animals.

**2016****ESTABLISHING THE TOXICOLOGY OF SELENIUM IN THE MODEL ORGANISM, CAENORHABDITIS ELEGANS.**

M. A. Nascarella and S. M. Presley. *Department of Environmental Toxicology, Texas Tech University, Lubbock, TX.*

Selenium is an essential component in a number of proteins, and is a required element for normal immune system function. Selenium levels that exceed both nutritional requirements and endogenous detoxification mechanisms are highly toxic; animals chronically exposed to these levels have physiological insults ranging from hair loss and nail brittleness to severe developmental and neurological abnormalities. The nematode *Caenorhabditis elegans* is a widely used model organism whose response to selenium has not been characterized. The aim of this study was to establish the baseline response of *C. elegans* (wild-type N2 strain) to selenium, with a future goal of evaluating the role of selenium in nematode immune system function. We have exposed both adults and embryos to concentrations of sodium selenite ranging from 0 to 1000 ppm (w/v selenium). Adult worms can tolerate acute (24 hr) exposure in selenium concentrations as high as 1000 ppm, with an approximate LC<sub>50</sub> of 14 ppm. *C. elegans* embryos that are exposed to 10 ppm of selenium are able to develop into apparently healthy adults, but are 85% smaller than control animals. Embryos exposed to concentrations exceeding 10 ppm fail to develop into viable ovigerous adult worms. These animals have shortened, often malformed bodies with disproportionately corpulent heads. At concentrations above 214 ppm, embryonic development is arrested and eggs fail to hatch. Our results show that selenium exposure above 10 ppm may cause both developmental arrest and congenital malformations to *C. elegans*. These findings are similar to reports in other animals (including humans) that are exposed to high concentrations of selenium.

**2017****PROTEOMIC INDICATORS OF PESTICIDE TOXICITY IN A CAENORHABDITIS ELEGANS MODEL.**

J. A. Lewis<sup>1</sup>, W. E. Dennis<sup>1</sup>, M. Szilagyi<sup>1</sup> and D. A. Jackson<sup>2</sup>. <sup>1</sup>*Geo-Centers, Inc., Ft. Detrick, MD* and <sup>2</sup>*US Army Center for Environmental Health Research, Ft. Detrick, MD.*

A comparative proteomic analysis using *C. elegans* was performed to determine if there are signature protein changes that are indicative of exposure to each of three pesticides. The toxicants are all classical acetylcholinesterase (AChE) inhibitors but are structurally distinct, and only two of them, aldicarb and dichlorvos, inhibit AChE reversibly; phenamiphos does not. Synchronized cultures of mid-L4 larvae grown in axenic medium were exposed for eight hours to three doses of each chemical. Axenic medium was used to eliminate the confounding effect of having *E. coli* in the culture as a food source. Exposure time was limited to 8 hrs since the unexposed worms molt and mature into adults about 17 hours after the mid-L4 stage. Exposure concentrations were set at a level that prevented a percentage of larvae (10%, 50% or 90%) from developing into adults within 24 hours. Protein extracted from whole worms was analyzed using 2-D gel electrophoresis and mass spectrometry to quantify differences between exposures and identify the proteins. Phenotypically, there was little distinction between the effects of the three chemicals; each produced a stumpy morphology, protruding vulva, abnormal cuticle formation and motility abnormalities. At the molecular level, a subset of the proteins shows a similar perturbation for all three chemicals, but substantial differences are also present that can distinguish among the three pesticides. Disclaimer: The views, opinions, and/or findings contained in this report are those of the authors and should not be construed as official Department of the Army position, policy, or decision, unless so designated by other official documentation.

**2018****ZEBRAFISH: A GOOD MODEL FOR DETECTING OTOTOXICITY.**

C. Ton and C. Parng. *Phylonix Pharmaceuticals, Inc., Cambridge, MA.* Sponsor: D. Monteith.

Drug-induced ototoxicity can lead to hair cell death, causing hearing loss and vestibular disorders. Currently, there is no *in vivo* quantitative method to determine drug-induced hair cell damage. Using zebrafish as a model organism, we aim to de-

velop a rapid, quantitative *in vivo* assay to assess drug effects on inner ear hair cell and screen protective agents for ototoxicity. The mechanosensory hair cells of zebrafish neuromasts are very similar in structure and function to the hair cells of the inner ear in mammals. These hair cells are comprised of bundles of hairlike projections that bend in response to sound waves. Cells convert this mechanical force into electrical impulses that are carried by the auditory nerves to the brain. Since zebrafish larvae are optically clear, hair cells can be visually assessed after DASPEI staining providing a rapid assessment of pharmacological effects. To examine hair cell damage, apoptosis and proliferation, we used DASPEI, acridine orange and BrdU staining to examine hair cell morphology after drug treatment. We showed that both aminoglycosides and cisplatin induced apoptosis and caused neuromast hair cell loss supporting the utility of zebrafish in inner hair cell assessment. In addition, we tested different classes of antioxidants for their ability to protect zebrafish against gentamicin and cisplatin-induced ototoxicity. These antioxidants, which have shown efficacy in mammals, include glutathione and its precursors and free radical scavengers. Zebrafish are treated with gentamicin or cisplatin alone or in combination with these compounds. DASPEI staining is used to stain hair cells and morphometric analysis is used to semi-quantify the fluorescent intensity. The results show that co-treatments of antioxidants and cisplatin significantly increase hair cells survival in a dose-dependent manner, whereas only methioninebenzoic acid and glutathione protect hair cell loss-induced by gentamicin. The results suggest that the toxicity of gentamicin and cisplatin are mediated by different pathways and demonstrate the utility of zebrafish in assessing potential ototoxicity and screening hair cell protective agents.

## 2019

### THE ROLE OF RECOMBINANT ZEBRAFISH CYP1A IN THE METABOLIC ACTIVATION OF BENZO(A)PYRENE (BaP) AND IN THE GENERATION OF REACTIVE OXYGEN SPECIES.

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The zebrafish is a sensitive non-mammalian model for polycyclic aromatic hydrocarbon (PAH)-induced chemical carcinogenesis. The susceptibility of zebrafish to PAH-induced carcinogenesis might be related to the ability of the zebrafish P450s to activate these carcinogens. As a part of our overall effort to identify the various P450 enzymes that are involved in the activation and detoxification of PAHs in zebrafish, we have examined the ability of recombinant zebrafish CYP1A (zCYP1A) expressed in yeast (Chung et al., *Aquat. Toxicol.*, in press) to metabolize BaP *in vitro*. Like rainbow trout CYP1A1, cDNA-expressed zCYP1A was found to oxidize BaP to phenols, quinones and diols (BaP-7, 8-diol and BaP-9, 10-diol). BaP-7, 8-diol is the precursor of the ultimate carcinogen, BaP-7, 8-diol-9, 10-epoxide (BaPDE). The ability of zCYP1A to activate BaP was confirmed by the formation of DNA adducts when calf thymus DNA was added to the incubation mixture. BaP-DNA binding was enhanced by the addition of human epoxide hydrolase to the incubation mixture. HPLC analysis of the [<sup>33</sup>P]-post-labeled DNA adducts showed the formation of two major adducts by zCYP1A, one of which was identified as anti-BaPDE. zCYP1A also mediated the binding of BaP to protein, providing further evidence that this enzyme is capable of oxidizing BaP to reactive metabolites that bind to macromolecules. In addition, the metabolism of BaP by zCYP1A in the presence of NADPH resulted in the generation of reactive oxygen species as indicated by the formation of hydrogen peroxide in the incubation medium. Taken together, these results suggest that zCYP1A may play a role in BaP-induced carcinogenesis in the zebrafish model by catalyzing the sequential formation of the ultimate carcinogenic metabolites (diol epoxides) of BaP and by increasing the generation of active oxygen species which are known to contribute to chemical carcinogenesis. (Supported by NIH grants Nos. ES 00210 and ES 11587).

## 2020

### DEVELOPMENT OF SPECIFIC ANTIPEPTIDE ANTIBODIES AGAINST ZEBRAFISH XENOBIOTIC METABOLISING FORMS OF CYTOCHROME P450.

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Nine zebrafish putative xenobiotic metabolizing cytochrome P450s (CYPs) have been cloned in our laboratories. They are CYP1A, 1B1, 2AD3, 2K6, 2K7, 2K8, 2V1, 3A65 and 3C1. Since we are interested in studying the translational expression of those CYPs, the close structural similarities among the CYPs persuaded us to use antipeptide antibodies for our research. Here we report the preliminary development and testing of specific antipeptide antibodies against each CYP. Peptide sequences were 16-20 amino acids, selected with consideration of hydrophobicities

from alignments of the 9 CYPs. Each short peptide region selected was unique to that specific gene. High titer antibodies, as judged by ELISA, were produced in rabbits immunized with these peptides coupled to keyhole limpet hemocyanin. Antipeptide antibodies were affinity purified from antisera using CNBR-activated peptide-Sepharose 4B. Immunoblotting studies using yeast or baculovirus/insect cells expressed recombinant CYPs are being used to examine the specificities and cross-reactivity of the antipeptide antibodies. We have produced these antipeptide antibodies with the intention of using them in the investigation of their translational expression of the corresponding CYPs in immunohistochemical studies. (Supported by NIH grants Nos. ES 00210 and ES 11587).

## 2021

### ORAL ABSORPTION OF WATER SOLUBLE AND LIPOPHILIC CONTAMINANTS IN THE JAPANESE MEDAKA.

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Small aquarium fish such as the Japanese Medaka (*Oryzias latipes*), are increasingly used as an alternative to mammalian models for chronic toxicity testing. The most common exposure route used in Medaka and other chronic dosing studies with fish is continuous water exposure, which is physiologically analogous to bronchial (inhalation) exposure in mammals. This limits the utility of fish models by preventing direct comparisons with rodent studies, which typically rely on oral dosing. Also, chronic exposure to certain toxicants such as those with low water solubility can be difficult and costly to maintain. Orally dosing fish can be challenging, particularly with water soluble contaminants which can leach into the aquarium water prior to ingestion. We developed a method of bioencapsulation using newly hatched Artemia to study the toxicokinetics of two classes of contaminants in the Medaka: a series of five chlorinated and brominated halogenated acetic acids (HAAs), which are drinking water disinfection by-products and a polybrominated diphenyl ether (BDE), which are a class of highly lipophilic compounds used as flame retardants. The HAAs and BDE were bioencapsulated by freshly hatched Artemia nauplii after incubation in concentrated solutions of the study chemicals for 4-18 hr. Aliquots of the nauplii were quantitatively removed for chemical analysis and fed to individual fish. We observed that an adult Medaka would consume 500 nauplii in less than 5 min, simulating a bolus dose. At select times after feeding (up to 96 hrs for HAAs and 21 days for BDE), fish are euthanized, the GI tract removed and the HAA or BDE content separately determined by GC-ECD. The absorption of HAAs was qualitatively similar to previous studies in rodents: rapid absorption with peak body levels occurring within 1 hr of dosing then rapidly declining with elimination half-life of 3-6 hrs depending on HAA. BDE was more slowly absorbed with an apparent lag-time of 2 hr before absorption began with peak levels occurring by 12 hr. BDE was very slowly eliminated with a elimination half-life > 14 days.

## 2022

### INTERACTION TRAPPING WITH A NON-TCDD BINDING ARYL HYDROCARBON RECEPTOR FROM *MYA ARENARIA*.

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The aryl hydrocarbon receptor (AHR) is typically associated with TCDD-induced toxicity. However, there is increasing evidence suggesting AHR has a role independent of dioxin binding and conversely, that TCDD can exert toxic effects through non-AHR mediated pathways. AHR is the only member of the PAS superfamily of transcriptional regulators to be ligand activated. However, previous work demonstrated an apparent significant divergence in function between the vertebrate and invertebrate AHRs: *M. arenaria*, *D. melanogaster* and *C. elegans* AHR homologues lack the ability to bind prototypical ligands ([<sup>3</sup>H]TCDD and [<sup>3</sup>H]β-naphthoflavone). Therefore, one might speculate that the invertebrate AHR homologues may not have the same requirement as vertebrate AHRs for ligand activation. Further investigation into the expression of *Mya arenaria* AHR revealed a positive correlation between AHR protein levels and the stage of ovogenesis. To better understand AHR function in the female clam reproductive tissue, a truncated AHR (amino acids 1-400) was used as the 'bait' protein to screen a yeast two-hybrid cDNA expression library prepared from the gonad of a female with mature gametes. Yeast colonies containing potential interactors were selected up to 6 days post-plating. Positive yeast yielded plasmids bearing library cDNA for 117 potential AHR interactors. Sequence homology has revealed an array of putative AHR-interacting proteins, several of which have been previously identified by others as essential for early zebrafish, yeast, worm and fly development (e.g. ribonucleoside reductase small subunit, ribosomal proteins L7 and L13, nascent polypeptide-associated complex αsubunit and zinc finger 183). We hypothesize that interaction with one or more of these critical developmental genes may indicate an endogenous function for the AHR. Work in our laboratory continues to verify the initially positive clam AHR interactors.

**2023****GENOMICS RESPONSES AND TOXICOLOGICAL ENDPOINTS AMONG FISH SPECIES EXPOSED TO ESTROGEN AND POTENTIAL EXTRAPOLATION OF FISH TOXICITY ASSAYS TO MAMMALIAN SPECIES.**

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The National Research Council (1998) has recommended the use of fish toxicity testing to evaluate the viability of augmenting drinking water supplies with reclaimed water. A consideration includes the ability to capture a range of effects, including responses to estrogen. Recognizing the clear differences in physiology and pharmacodynamics, the use of considering a simple screening assay using fish may provide insights into potential human impacts. The increasing use of genomics data can provide additional tools in the interpretation of potential mechanisms of action of selected chemicals that may be present in complex mixtures. Comparison of the gene expression profiles by microarray analysis in fathead minnows, bass and sheepshead minnows exposed to 17- $\beta$  estradiol revealed several similarities, including robust up-regulation of vitellogenin, the egg precursor protein. The production of vitellogenin as a biomarker of estrogenic exposure has been shown among numerous other fish species. Some notable differences were also observed, one of which included robust up-regulation of choriogenin genes in the liver tissue of both largemouth bass and sheepshead minnow, but not in fathead minnows. It is speculated that the choriogens, which form the covering of the egg, are expressed in the gonadal tissue and not the liver of fathead minnows. The specificity and sensitivity of these molecular responses were also compared to dose response studies and endpoints reported for these and other fish species. These traditional toxicity studies have illustrated differences in endpoints and sensitivity among various fish species. While the clear physiological differences in the responses in fish as compared to humans, the NRC suggests information on underlying control mechanisms may be identified. This supports the necessity to incorporate microarray data with traditional endpoints identified in the numerous dose-response studies that may be available.

**2024****THE USE OF A ROTAROD IN CNS SAFETY PHARMACOLOGY STUDIES – A COMPARISON OF TRAINING AND TESTING METHODS.**

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The Rota-Rod is a useful technique to assess motor-coordination in rodents. Given that Rota-Rod performance is considered to be an acquired behavior dependent on skill (Cartmel et al 1991), the intent of this study was to evaluate the use of an accelerating versus fixed speed rotarod for use as a motor performance assessment tool as well as to design a suitable training regime for rats used on these studies. Morphine (5 mg/kg IV) and diazepam (1.5 mg/kg IV) were selected for use as reference compounds in this study due to their known ability to decrease motor performance (Cartmel et al 1991, Capacio et al 1992). Male Sprague-Dawley rats, approximately 7 weeks of age were selected as the test system. Prior to performance of the study, animals were placed through one of the following training regimes: 1) no training, 2) one day of acclimation to a fixed speed of 12 rpm (which occurred the day prior to dosing) and 3) pre-selection and additional training. Various rejection criteria were employed for animals undergoing the different selection and training methodologies, however in all cases repetitive falls from the rod lead to exclusion of that animal from the study. For the final motor performance study, rats were assessed 15, 30 and 45 minutes after dosing. Animals were tested using either a fixed speed of 12 rpm over 2 minutes or with the rod accelerating from 0 to 12 rpm over the 2 minute period. In both cases, the duration of time spent on the rod was recorded. For both morphine and diazepam treated animals, a marked decrease in motor performance was noted at 15 and 30 minutes post dose when compared to the control (saline) group, however performance on the fixed-speed rod was better than for the accelerating system. Animals that received more training were seen to have performed the Rota-Rod task with a greater success rate for both systems. The results were consistent with the literature and the accelerating Rota-Rod, preceded by selection and training is considered the best approach for motor performance assessments of this type.

**2025****EVALUATION OF AN INTEGRATED TELEMETRY SYSTEM (ITS) FOR MEASUREMENT OF CARDIOVASCULAR AND RESPIRATORY PARAMETERS IN CYNOMOLGUS MONKEYS.**

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The telemetric measurement of cardiovascular and respiratory parameters in conscious, freely moving animals is an essential component of safety pharmacology studies. It is well known that heart rates as well as blood pressure values are differ-

ent when measured in freely moving rather compared to restrained and/or anaesthetized animals. Hence, data obtained by telemetry are considered to reflect the physiological condition. The aim of this study was to evaluate an ITS system for the measurement of aortic blood pressure, ECG waveforms, temperature and intra-thoracic-pressure (ITP). Two mature, male cynomolgus monkeys (>6.0 kg) were dosed on two successive days with sotalol, ketamine, theophylline and chlorpromazine, respectively with saline as the corresponding vehicle. Biopotential signals were measured two hours before dosing and for a 6 hour period thereafter. Ketamine at 20 mg/kg caused a marked decrease in body temperature and in maximum ITP. QT-interval was markedly prolonged, heart rates and mean-arterial-blood-pressure were decreased at 10 mg/kg sotalol. Respiratory rate was decreased whereas the minimum ITP was slightly elevated following 25 mg/kg chlorpromazine administration. Theophylline at 20 mg/kg elicit no effect. On repeated occasions, pair- and single-housing of the two animals were compared: Heart rates were reproducibly lower during pair-housing compared to single housing, whereas blood pressure remained unaltered. In conclusion, our findings suggest that the ITS system is a valid tool for the selective detection of drug induced changes in cardiovascular and respiratory parameters in conscious, freely moving cynomolgus monkeys.

**2026****VALIDATION OF SAFETY PHARMACOLOGY ASSESSMENT OF CARDIOVASCULAR FUNCTION IN CONSCIOUS DOGS WITH TWO POSITIVE CONTROLS (SOTALOL AND IBUTILIDE).**

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Drug-induced effects on the QT-interval with the possibility of inducing fatal arrhythmias have become a new challenge for the drug development and regulatory approval. Antiarrhythmic class III drugs have been associated with development of a polymorphic ventricular tachycardia (PVT) known as torsades de pointes. The purpose of this study was to validate the method of safety pharmacology assessment of cardiovascular function using two class III agents, ibutilide and d, l-sotalol, in a conscious telemetric canine model. The duration of QT-interval has been used as the major determinant of the risk of drug-induced PVT. A telemetry transmitter (D70-PCI) from Data Sciences International (DSI) was implanted subcutaneously into the flank of each dog following the standard surgical procedures. The animals blood pressure, ECG, heart rate, body temperature and locomotor activity were continuously monitored for at least 24 hours prior to and following a single bolus intravenous dosing of saline (vehicle control). The d, l-sotalol at 10 mg/kg or ibutilide at 0.15 mg/kg were injected intravenously three days later. ECG waveforms and above cardiovascular parameters were recorded simultaneously for at least 24 hours following each drug administration. The heart rate-adjusted QT intervals (Fridericia correction QTc) were determined using the rate-correction formula in DSI PhysiostatTM ECG analysis program. Both sotalol and ibutilide significantly increased QT-interval with similar pattern in the beagle dogs. These data demonstrated that the assay is capable of detecting relevant changes in cardiovascular function in conscious beagle dogs. This validated telemetry canine model and either sotalol or Ibutilide as a positive control can be used to study the potential undesirable pharmacodynamic effects of the test compounds on QT-interval for assessing clinical benefits and risks in accordance to the ICH safety pharmacology S7B guideline.

**2027****INTEGRATED ASSESSMENT OF SMALL MOLECULE INTERACTION WITH THE HERG CHANNEL: WHAT DOES THE HERG BINDING ASSAY MISS?**

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Block of the hERG channel, a voltage gated potassium channel responsible for the current ( $I_{Kv}$ ) which produces the rapid phase of ventricular repolarization, is the basis of QT interval prolongation and predisposes individuals to Torsades de Pointes (TdP). The hERG binding assay, a competitive radioligand displacement assay using membranes from HEK293 cells stably transfected with hERG channel cDNA, offers a high-throughput screen for small molecule interaction with the hERG channel. A study was undertaken to compare the results of this assay with other *in vitro* methods of assessing hERG interactions. Data from the hERG binding assay is well correlated with electrophysiology data ( $r^2 = 0.83$ ). Safety ratios calculated for marketed compounds run in these two assays allowed rank ordering of compounds in a similar fashion. Because QT interval prolongation may also be precipitated by a reduction in native  $I_{Kv}$  current resulting from interference with trafficking of the mature hERG protein to the cell membrane, the HERG-lite assay (Chantest, Inc.) monitors channel block along with hERG channel surface expression. Of 26 compounds evaluated, the HERG-lite assay and the hERG binding assay correctly predicted for the clinical outcomes of 19 and 16 compounds, respectively. Arsenic trioxide, which was not shown to interact with the hERG chan-

nel by patch-clamp or binding methods, was shown to interfere with hERG trafficking in the hERG-lite assay. hERG-lite was more adept at predicting the pro-arrhythmic potential of compounds for which there are rare reports of TdP, such as nifedipine and kentaserin, which were missed by the hERG binding assay. Ebastine and diltiazem, for which there are no reports of TdP, appeared as false positives in the hERG-lite assay but classified correctly by the hERG binding and patch-clamp assays. A paradigm including all three of these assays may offer the best hope for the selection of a drug candidate free of hERG interaction and potential for *in vivo* QT prolongation.

## 2028

### MEASUREMENT OF CARDIOVASCULAR EFFECTS OF PHARMACEUTICALS IN MINIPIGS USING TELEMETRY.

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The use of the Gottingen minipig as a non-rodent species in pre-clinical safety testing is increasing. Because of this it is logical that this species should also be used in safety pharmacology. We have therefore performed a cardiovascular study in minipigs using a telemetric system (Data Science International (DSI)). The objective of this study was to establish our ability to measure blood pressure, heart rate, ECG and temperature in minipigs, and to measure effects on these parameters following administration of three compounds, Propranolol, Isoprenalin and Sotalol. These compounds have a known effect upon blood pressure, heart rate, and ECG. Dose levels intended to cause measurable effects were chosen. Propranolol is a highly potent beta-receptor blocker with no intrinsic sympathomimetic activity. Its known effect is to decrease the force and the rate of heart contractions by blocking beta-adrenergic receptors of the autonomic nervous system. Isoprenalin is a synthetic sympathomimetic amine. It is a beta-adrenergic agonist that can increase the heart rate, increase cardiac contractility and decrease diastolic pressure. Sotalol is a class III antiarrhythmic drug which, in addition to increasing action potential duration (APD) by blocking K<sup>+</sup> channels, also competitively antagonizes beta-adrenergic receptors. This antiadrenergic activity can depress slow channel tissue by decreasing cAMP-dependent calcium entry. Sotalol is known to decrease the atrioventricular node automaticity by prolonging atrioventricular node APD and decreasing contractility leading to decreasing heartbeat and prolonging the QT interval. Because of the current focus on potential of drugs to prolong QT interval, this was an important aspect of the study. The study was performed as a cross over study with a minimum of 7 days wash out period between each phase. Measurements were made for up to 4 hours after dosing. The data presented demonstrate that the minipig is a suitable model for use in standard cardiovascular safety pharmacology studies.

## 2029

### VALIDATION OF A RADIOTELEMETRIC SYSTEM FOR THE MEASUREMENT OF CARDIOVASCULAR PARAMETERS AND TEMPERATURE IN THE CONSCIOUS CYNOMOLGUS MONKEY.

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The current ICH S7A guidelines state that 'in conducting *in vivo* studies it is preferable to use unanesthetized animals' and that 'data from unrestrained animals that may be chronically instrumented for telemetry or other suitable instrumentation methods for conscious animals...are preferred.' Conscious restrained animals of all species have artificially elevated heart rates and sympathetic drive that can easily confound interpretation of cardiovascular data. Similarly, unconscious animals typically exhibit depressed cardiovascular parameters that may permit the oversight of meaningful, drug-induced changes. However, before any particular animal model is used for the generation of GLP data, the test system must be fully-validated. To this end we would like to report on the validation of a telemetry system for the monitoring of cardiovascular parameters and temperature in conscious nonhuman primates, macaca fascicularis. The primary objectives of this study were to biologically validate the ITS telemetry system and evaluate its effectiveness in detecting toxicologically-induced changes in the cardiovascular system through the use of specific selected pharmacologic agents. Four male cynomolgus monkeys, each approximately 6 years old weighing 5-8 kg, were surgically implanted with telemetric transducers (Integrated Telemetry Systems). Pharmacologic standards were then selected to test the ability of the system to clearly define quantifiable changes in blood pressure, heart rate and electrocardiographic parameters as well as temperature. The specific pharmacologic agents used were: ketamine, an anesthetic agent; d, l-sotalol, a nonselective beta-antagonist used as an antiarrhythmic agent; and isoproterenol, a beta-1, beta-2 adrenergic agonist. The data presented demonstrate that the ITS telemetry system provides a sensitive, specific, and precise means for the measurement of quantifiable drug induced changes in cardiovascular parameters including the assessment of QT Interval Prolongation and body temperature in conscious cynomolgus monkeys.

## 2030

### A REVIEW OF THE RELATIONSHIP BETWEEN HEART RATE AND QT INTERVAL IN THE BEAGLE DOG.

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Electrocardiography is now one of the standard investigations performed in pre-clinical toxicology studies. One of the key parameters is the QT interval, as certain types of drugs have the capacity to delay cardiac repolarization, an effect seen in the electrocardiogram traces as a prolongation of the QT interval. This is an undesired effect when caused by non-cardiac drugs, and has lead to drugs such as Astemizole®, Sertidole® and Grepafloxacin® being removed from the market. Even when this effect is part of the therapeutic mechanism of an anti-arrhythmic drug, excessive QT interval prolongation is also undesirable as this can provoke new arrhythmias (torsades de pointes) and sudden death. The detection of drug induced QT interval changes is sometimes masked by concurrent heart rate changes and therefore it is normalized by means of various formulae (such as Bazetts, Frederica, Van der Waters and Linear regression analysis) into a heart rate independent corrected value known as the QTc interval. The purpose of this publication is to present electrocardiography data obtained from naive beagle dogs (*Canis familiaris*) from two North American suppliers and from control animals during the course of toxicology studies at CTBR (N=652) between February 2001 and February 2004. A plot of QT versus heart rate was prepared for the whole data set. Linear regression analysis was performed to describe the HR-QT relationship. The relationship between QT-RR was tested and a correlation coefficient of -0.70 was obtained for the whole data set. The analysis was also applied to the data sets for the different suppliers and values of -0.71 and -0.73 were obtained, indicating no difference between the two suppliers. Access to such a comprehensive background data base may help put individual study results into context where individual variation in control animals may mask drug related effects.

## 2031

### DEVELOPMENT OF THE METHODOLOGY FOR RESPIRATORY RATE AND VOLUME ASSESSMENT USING LINEAR PNEUMATACHS AND BUXCO BIOSYSTEM XA SYSTEM IN THE CONSCIOUS DOG AND CYNOMOLGUS MONKEY.

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The purpose of this study was to establish the methodology to perform respiratory rate, tidal volume and minute volume assessments which are required by ICH S7A guideline in conscious dogs and monkeys or may be a required parameter in inhalation toxicology studies. The dogs and monkeys, which had been previously trained and/or acclimated, were manually held with a dental rubber diaphragm sealed face mask over their nose and mouth while standing on a cart (dog) or restrained in a purpose built non-human primate restraint chair (monkey). Air exhaled into the mask was directly through a calibrated in-line linear pneumatach (Hans Rudolph Model Number 3500A-calibrated flow range 0-35 L/min). Room air was allowed to freely enter and leave the facemask through the two-way valves and exited through the pneumatach into the room air. Pressure data from the pneumatach was passed to a Buxco Max II preamplifier, which was used to convert the signal to the range from 0 to -5V DC. The output from the preamplifier was connected to a computer loaded with Buxco BioSystem XA integrating software. Respiratory rate and tidal volume were evaluated by the software package and the respiratory minute volume was derived for each animal. These values were determined for a continuous period of at least 60 seconds; however, if the period of data capture was interrupted by vocalization or struggling, the target parameters were determined from data for a number of shorter intervals. Values obtained in this study in dogs and monkeys were within acceptable ranges for these species. In conclusion, we have demonstrated the suitability of using linear pneumatachs and Buxco BioSystem XA system in dogs and cynomolgus monkeys to assess respiratory pumping apparatus function for toxicology and safety pharmacology assessments in our Testing Facility environment.

## 2032

### USE OF GAMMA SCINTIGRAPHY FOR THE STUDY OF GASTRIC EMPTYING IN SAFETY PHARMACOLOGY.

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Gastric emptying is a useful parameter to assess gastrointestinal motility for physiological or pharmacological purposes. In rats, phenol red recovery is the gold standard for the determination of gastric emptying. However, this method requires the sacrifice of the animal and yields data at only one time point. In contrast, scintigraphy, the gold standard method in humans, allows sequential and serial measurements of gastric emptying in the same subject. Here, gastric emptying measured

with a dedicated gamma camera in unanesthetized rats was compared with phenol red recovery. Normal values for the gastric emptying of semi-liquid (methylcellulose), solid (egg white) and fatty meals were established. The effects of L-NAME, an inhibitor of gastric emptying, and cisapride, a prokinetic drug, were quantified using both methods. Raw data included absorbance by spectrophotometry for phenol red and the activity of the regions of interest for scintigraphy. Both were converted into % of gastric retention. The calculation of the half emptying time ( $T_{1/2}$ ) by non linear regression was used to evaluate gastric emptying. The gastric emptying of methylcellulose measured by scintigraphy ( $T_{1/2}=8.4$  min;  $n=5$ ) did not differ from that measured by phenol red recovery in non-treated animals ( $T_{1/2}=9$  min;  $n=20$ ). The inhibitory effect of L-NAME on methylcellulose emptying was demonstrated by scintigraphy ( $T_{1/2}=18.1$  min;  $n=5$ ). The effect of cisapride on methylcellulose could only be demonstrated with the phenol red method ( $T_{1/2}=5.5$  min;  $n=20$ ). Scintigraphy also confirmed that solid ( $T_{1/2}=49.8$  min;  $n=4$ ) and fatty meals ( $T_{1/2} > 300$  min) are emptied more slowly than semi-liquid meals ( $T_{1/2}=8.4$  min;  $n=5$ ). Dedicated rodent gastric-emptying scintigraphy is a reliable tool to investigate gastrointestinal function in safety pharmacology and significantly reduces the number of animals needed for statistical analysis.

## 2033

### EFFECTS OF TCDD ON THE LEVELS OF BIOGENIC AMINES IN DIFFERENT BRAIN REGIONS OF RATS AFTER SUBCHRONIC EXPOSURE.

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The effects of subchronic exposure to TCDD on the levels of biogenic amines and their metabolites in different brain regions have been studied in rats. Groups of female Sprague-Dawley rats were administered p.o. either TCDD at doses of 22 and 46 ng/kg/day (treated groups) or the vehicle used to dissolve TCDD (control group), for 90 days. The rats were sacrificed at the end of the exposure period and their brains were dissected into different regions including, hippocampus, cerebral cortex, cerebellum, and brain stem. The concentrations of different biogenic amines, including, nor epinephrine (NE), dopamine (DA), 3, 4-dihydroxy phenyl acetic acid (DOPAC), 5-hydroxy indole 3-acetic acid (5-HIAA), 5-hydroxy tryptamine (5-HT) and 4-hydroxy, 3-methoxy-phenyl acetic acid (HVA) were determined in those brain regions, using a High Performance Liquid Chromatography (HPLC) system with an electrochemical detector. Results of analyses indicate significant increases in the levels of DA, DOPAC and HVA in the hippocampus and cerebral cortex but not in cerebellum or brain stem in response to the two doses of TCDD, as compared with the corresponding controls. However, the level of NE was significantly increased in the hippocampus and cerebral cortex of rats treated with 46 ng TCDD /kg/day and also in the cerebellum of rats treated with either doses of TCDD, when compared with the corresponding controls. These results are well-correlated with the previously observed increases in oxidative stress in the brain regions of rats in response to TCDD and may suggest the contribution of those biogenic amines to the observed increases in the biomarkers of oxidative stress in those regions. (Supported by NIH/NIEHS grant ES11048).

## 2034

### IGF-1 MEDIATES THE EFFECTS OF TCDD AND HxCDD ON BODY WEIGHT AND THE INHIBITION OF PEPCK.

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TCDD affects rat intermediary metabolism with effects on glycemia, gluconeogenesis and circulating thyroid hormone levels. These effects combined with a reduction in feed intake, culminate in a decrease in body weight in 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated rats. In this study, we investigated the effects of steady state levels of TCDD (loading doses of 0.0125, 0.05, 0.2, 0.8, and 3.2  $\mu$ g/kg) or iso-equivalent doses of 1, 2, 3, 4, 7, 8-hexachlorodibenzo-*p*-dioxin (HxCDD) (loading doses of 0.3125, 1.25, 5, 20, and 80  $\mu$ g/kg) on body weight and biochemical effects after 2, 4, 8, 16, 32, 64, and 128 days of administration. At the 0.05 and 1.25  $\mu$ g/kg dose of TCDD and HxCDD, respectively, there was a slight increase in body weight by day 64, while at the 3.2 and 80  $\mu$ g/kg dose of TCDD and HxCDD, respectively, by day 32 the body weight gain of the rats was significantly decreased and remained decreased throughout the remainder of the study. TCDD and HxCDD dose-dependently inhibited PEPCK activity at time-points after day 16 through day 128. PEPCK RNA and protein were also decreased at doses and the time point which inhibited PEPCK activity. Serum IGF-I levels of the 3.2 and 80  $\mu$ g/kg TCDD- and HxCDD- treated rats after day 16 decreased by about 60% of the levels seen in controls by day 16, remaining low for the duration

of the study. There was no effect with any dose of TCDD or HxCDD on circulating insulin or glucose. The 3.2  $\mu$ g/kg dose of TCDD significantly decreased circulating total thyroxine (TT4) as compared to rats which received 0.05  $\mu$ g/kg TCDD or corn oil alone. Hepatic phosphorylated AMP kinase- $\alpha$  protein was increased in a dose-dependent manner with TCDD or HxCDD administration although at low doses it appeared to be decreased. In conclusion, at a dose of TCDD or HxCDD where there is an inhibitory effect on body weight gain in female rats, there is also an inhibitory effect on gluconeogenesis, IGF-I and TT4 levels.

## 2035

### COMPARATIVE 14-WEEK TOXICITY STUDIES OF 2, 3, 7, 8-TETRACHLORODIBENZO-*P*-DIOXIN (TCDD) IN FEMALE HARLAN SPRAGUE-DAWLEY (HSD) AND FISCHER 344 (F344) RATS.

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The purpose of the study was to determine if the TCDD response in female HSD rats is predictive of the response in female F344 rats. In a previous NTP study, female HSD rats (10 rats/group) were administered TCDD in corn oil:acetone (99:1) by gavage at dosages of 0, 3, 10, 22, 46 or 100 ng TCDD/kg body weight for 14 weeks. Mean body weights of 100 ng/kg females were 5% lower than vehicle controls. Thyroxine ( $T_4$ ) levels in groups receiving  $\geq 22$  ng/kg were significantly lower than vehicle controls. In the 46 and 100 ng/kg groups, triiodothyronine ( $T_3$ ) and thyroid stimulating hormone (TSH) levels were significantly higher than vehicle controls. Hepatic EROD, A-4-H and pulmonary EROD activities were significantly higher in all dosed groups; maximal inductions were 70, 6 and 20-fold relative to vehicle controls, respectively. In the F344 rat study, TCDD was administered in corn oil:acetone (99:1) by gavage (10 rats/group) at dosages of 0, 1, 10, 50, 100, and 200 ng/kg for 14 weeks. Mean body weights were 7-10% less than vehicle controls in groups receiving  $\geq 50$  ng/kg. The F344 strain was less sensitive to thyroid hormone alterations. No changes were observed in  $T_3$  or TSH; slight decreases in  $T_4$  levels were observed in the 200 ng/kg group. Hepatic EROD, A-4-H and pulmonary EROD activities were significantly higher in all dosed groups; maximal inductions were 42, 6 and 54-fold relative to vehicle controls, respectively. Hepatocellular hypertrophy was correlated with increased liver weights in both strains. Thymus weights were decreased and thymic atrophy was increased in both strains. Slight differences in histopathology were observed between strains. Hepatic granulomatous and chronic active inflammation and splenic hemosiderin accumulation were only observed in F344 rats. In conclusion, TCDD administration to F344 rats produced similar toxicity as observed in the HSD rat.

## 2036

### EFFECTS OF 2, 3, 7, 8-TETRACHLORODIBENZO-*P*-DIOXIN (TCDD) ON PROLACTIN (PRL) GENE EXPRESSION *IN VIVO* AND *IN VITRO*.

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Exposure to TCDD decreases plasma levels of PRL, a hormone important for reproduction functions in males and females. These effects are likely due, at least in part, to TCDD disruption of pituitary function, but the mechanisms are not clear. The effects of dioxin are mediated by the arylhydrocarbon receptor (AhR), a basic helix-loop-helix PAS transcription found in a variety of tissues, including the pituitary gland. In many of these tissues, TCDD interferes with actions of estradiol (E2), a hormone that directly regulates PRL synthesis. In previous work using cDNA microarrays and Northern blotting, we found that E2-induced increases in PRL gene expression were blocked by TCDD exposure in ovariectomized rats. However, it was not clear whether this was through a direct action of TCDD on the pituitary gland or indirect actions mediated by the hypothalamus. Therefore, in these studies, we performed dual-label *in situ* hybridization and found PRL mRNA and AhR mRNA colocalized in pituitary cells. We then used transient transfection analysis in pituitary-derived GH3 cells to examine the effects of TCDD and E2 on PRL promoter activity. We cloned a region of the PRL promoter that included both an estrogen response element (ERE) and a putative dioxin response element (DRE) into pGL3-basic luciferase reporter vector and transfected it into GH3 cells. Using steroid-free medium conditions for 16 h, transfected GH3 cells were treated with various doses of TCDD and/or E2. Cell lysates were collected after 24 or 48 hours and analyzed using Dual-Luciferase Reporter (DLR) Assay kits. We found that TCDD alone had no consistent effect on promoter activity. However, at 1, 10, and 50 nM TCDD interfered with E2 stimulation of the promoter with maximal effects seen at 48 h. Interestingly, at 500 nM TCDD had no effect on promoter activity at either 24 or 48 h. These findings indicate that TCDD may disrupt PRL secretion by interfering with E2 regulation of PRL gene expression.

INVOLVEMENT OF PROSTAGLANDIN PATHWAY IN CIRCULATION FAILURE INDUCED BY 2, 3, 7, 8-TETRACHLORODIBENZO-P-DIOXIN IN DEVELOPING ZEBRAFISH.

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Using zebrafish embryos as a model system for developmental toxicology, we have reported that 2, 3, 7, 8-tetrachlorodibenz-p-dioxin (TCDD) increases the occurrence of apoptosis in dorsal midbrain with very good correlation with a decrease in local blood flow. We also found that mesencephalic circulation failure is associated with an increase in permeability in mesencephalic vein, by which mechanism was unclear. Recently, it has been reported that TCDD promotes prostaglandin (PG) synthesis of the mouse liver cells. In this study, we will show the effects of several prostaglandin synthesis inhibitors on developmental toxicity in zebrafish exposed to TCDD. As the results, TCDD-induced mesencephalic circulation failure was markedly inhibited by NS398, a selective inhibitor for cyclooxygenase 2 (COX2) and by indomethacin, a general inhibitor for COX1 and COX2, but not by nordihydroguaiaretic acid (NDGA), a selective inhibitor for 5-lipoxygenase (5-LOX). Morpholino antisense oligos against COX2 (COX2-MO) also recovered mesencephalic circulation failure by TCDD. On the other hand, pericardial edema caused by TCDD was significantly inhibited by all three inhibitors for COXs and 5-LOX. These results raise the possible involvement of PG synthesis pathway in developmental toxicology by TCDD.

DOSE- AND TIME-DEPENDENT HEPATIC GENE EXPRESSION PROFILING IN FEMALE RATS EXPOSED TO PCB126.

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DNA microarray analysis was used to identify unique hepatic gene expression patterns in female Harlan Sprague-Dawley rats associated with subchronic (0 or 1000 ng/kg/day for 13 weeks) and chronic (0, 30, 300, or 1000 ng/kg/day for 52 weeks) exposures to 3, 3', 4, 4', 5-pentachlorobiphenyl (PCB126), the most potent dioxin-like PCB. Pathological reports obtained from the National Toxicology Program (NTP) show that both test groups displayed liver inflammation and exhibited a dose dependent increase in hepatocyte hypertrophy. Animals from the 52 week exposure group also displayed a dose dependent increase in the incidence of preneoplastic markers, including multinucleated hepatocytes, cellular pigmentation, and bile duct hyperplasia. Following 2 years of exposure, PCB126 produced cholangiocarcinoma, hepatocellular adenoma, toxic hepatopathy, multinucleated hepatocytes, diffuse fatty change, and liver pigmentation. Global gene expression profiles of the 8, 799 gene probe sets contained on Affymetrix RGU34A GeneChips exhibited a dose-dependent progression when the 52 wk group was analyzed by principal components analysis (PCA). The dose-dependent pattern of global gene expression may be the result of AhR activation and/or the hepatic injury resulting from chronic PCB126 exposure. Many genes were co-expressed during the 13 and 52 wk PCB126 exposures, including classical AhR regulated genes and genes recently characterized by our group as being dioxin-responsive, such as the up-regulation of carcinoembryonic-cell adhesion molecule 4 (C-CAM 4) and the down-regulation of CYP 3A9 (Vezina et al., 2004). Real time RT-PCR confirmed the altered expression of these genes as well as the up- or down-regulation of several other novel dioxin-responsive genes. Dose- and time-dependent gene expression data may provide new biomarkers of exposure and/or effect and help elucidate biological mechanisms for toxicity. (Supported in part by NIEHS ES09440, SOT, and University at Buffalo).

EFFECTS OF AMMONIUM PERCHLORATE ON LIVER ENZYMES AND THE THYROID AXIS OF RATS PRETREATED WITH PCB126.

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Ammonium perchlorate and 3, 3', 4, 4', 5-pentachlorobiphenyl (PCB126) are environmental contaminants that are known to disturb thyroid hormone (TH) homeostasis by well defined modes of action that lead to hypothyroidism in the rat. PCB126 increases phase II conjugation of T4 by inducing hepatic enzymes and

perchlorate blocks thyroidal uptake of iodide. To investigate the low dose interaction between perchlorate and PCB126 adult male Sprague-Dawley rats were administered a single oral bolus dose of 0, 7.5, or 75 ug PCB126/kg-bw dissolved in corn oil on Day 0. On Day 9, ammonium perchlorate was administered in drinking water to obtain target doses of 0, 0.01, 0.1, or 1 mg perchlorate/kg-bw per day for 14 days. Rats were euthanized on Day 22. Total P450 levels were significantly higher than controls in rats dosed with 75 ug PCB126/kg and EROD activity, a marker for CYP1A1 activity, was significantly induced by 7.5 and 75 ug PCB126/kg dose groups. Rats exposed to both PCB126 and perchlorate exhibited EROD activity corresponding to that of the PCB126 doses. T4-UDPGT was increased only in the 75 ug PCB126/kg dose group; no significant interaction between PCB126 and perchlorate was observed. A dose related increase in TSH was observed for both compounds. Under these experimental conditions, TSH serum levels of rats that were co-administered PCB126 and perchlorate were less than the sum of the % increase when these chemicals were administered individually. Analyses of serum thyroid hormones and organ 5'-deiodinase activity are ongoing. Biologically based pharmacokinetic models for the thyroid axis in the rat are under development in our laboratory to characterize dose response relationships for endocrine disrupting chemical mixtures such as PCB126 and perchlorate. ATSDR #U61/ATU472105-01

2, 3, 7, 8-TETRACHLORODIBENZO-P-DIOXIN MODULATES A TRANSLOCATION OF PKC- $\beta$ II VIA RECEPTOR FOR ACTIVATED C KINASE (RACK-1) IN DEVELOPING NEURONAL CELLS IN CULTURE.

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2, 3, 7, 8-Tetrachlorodibenz-p-dioxin (TCDD) induces motor dysfunction and cognitive impairment as neurodevelopmental outcomes in humans and animals. PKC signaling pathways have been implicated as important factors in learning and memory processes. PKC requires a binding to receptor for activated C kinases (RACKs) for its activation. Although RACK is important for regulating PKC function and TCDD affects PKC activities, roles of RACK in TCDD-mediated signaling pathways remain unknown. To understand the mechanism of TCDD-induced signaling pathway in developing neurons and identify possible intracellular target molecules, the present study attempted to analyze effects of TCDD on PKC signaling pathways in cerebellar granule cells in culture. Cells exposed to TCDD were fractionated and subsequently immunoblotted against RACK-1 or PKC- $\beta$ II antibody. TCDD induced a significant increase (% of control: 129 $\pm$ 12 at 1nM, 131 $\pm$ 8 at 10nM and 127 $\pm$ 11 at 100nM) of RACK-1 levels. 10nM TCDD induced a significant increase of RACK-1 (% of control: 152 $\pm$ 17 at 5min, 192 $\pm$ 15 at 15 min, 204 $\pm$ 22 at 30min and 168 $\pm$ 28 at 1hr) in a time-dependent manner. The induction was AhR-dependent. TCDD induced a significant decrease of PKC- $\beta$ II in cytosol (% of control: 51 $\pm$ 4 at 1nM and 32 $\pm$ 6 at 10nM) and a significant increase in membrane (% of control: 121 $\pm$ 8 at 1nM and 145 $\pm$ 16 at 10 nM) fractions in a dose-dependent manner. The translocation from cytosol to membrane fractions was AhR-dependent. When TCDD-induced increase of RACK-1 levels was blocked with antisense oligonucleotides against RACK-1, translocation of PKC- $\beta$ II was also blocked. The study demonstrated that TCDD activates PKC- $\beta$ II thru the modulation of RACK-1. It is believed that this study is a first report identifying an adaptor protein, RACK-1, as a possible target molecule for TCDD exposure and contributes to better understanding of TCDD-induced signaling pathways in developing neurons.

TCDD ELICITS TYPICAL DEFENSIVE CELL STRESS RESPONSES FROM MCF10A CELLS: A STUDY ON THE MECHANISM OF ITS CELL RESCUING ACTION FROM UV-INDUCED APOPTOSIS.

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It has been previously proposed from this laboratory that one of the action mechanisms of TCDD is to elicit typical cell stress responses. To assess the effectiveness of TCDD as a stressor, we have compared it to other typical stressors with respect to its potency to rescue cells from apoptosis in a human mammary epithelial cell line, MCF10A. We found in this cell line that TCDD can antagonize apoptosis that was induced by a variety of cell damaging treatments, such as UV- and gamma-irradiation, growth factor starvation and trypsinization, or treatments with H<sub>2</sub>O<sub>2</sub>, TGF $\beta$ , and staurosporine. Such anti-apoptotic action of TCDD resembles that of exogenously added EGF or TGF $\alpha$ . Furthermore, other typical inducers of cell stress responses, such as LPS, Fe<sup>3+</sup>, nitric oxide and hypoxia could also antagonize UV induced apoptosis just as in the case of TCDD. The anti-apoptosis action of TCDD was blocked by 1 $\mu$ M of a-naphthoflavone (500  $\mu$ M) or 1,10-phenanthroline (3 $\mu$ M), indicating the essential role of the Ah receptor. Furthermore, the action of

TCDD to induce stereotypical cell stress responses in this cell line has been verified by its action of increase the mRNA expression of cox-2, TNF $\alpha$ , and NF $\kappa$ B in addition to c-Src and ERK as assessed by qRT-PCR. We then tested a variety of diagnostic agents to reverse the effect of TCDD. Antagonists of TCDD which were found to be effective in this way were (a) inhibitors of c-Src kinase, such as PP-2 and CGP77675, (b) those known to block the action of TGF $\alpha$ , such as anti-TGF $\alpha$  antibody and a1-antitrypsin, (c) PD98059, a specific inhibitor of ERK activation, but not SB202190 (an inhibitor of p38 MAPK activation) or SP600125 (a JNK inhibitor). These results indicate that TCDD acts as an anti-apoptotic agent by mimicking the action of EGF through activation of the c-Src/ERK signaling pathway. They also support the idea that there are types of cell stressors including TCDD that elicit typical cell stress response reactions in order to protect cells from apoptosis.

## 2042

### SELECTIVE MODIFICATION OF *BHLH/PAS* GENE EXPRESSION BY TCDD IN RAT HYPOTHALAMUS.

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2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD) induces a wide variety of toxic and biochemical effects via an AH receptor (AHR)-mediated signalling pathway. A lethal dose of TCDD leads to a drastically and permanently reduced feed intake and wasting by an unknown mechanism. Both wasting syndrome and acute lethality show a striking sensitivity difference between TCDD-sensitive Long-Evans (Turku/AB) (L-E) and TCDD-resistant Han/Wistar (Kuopio) (H/W) rat strains. We utilized this large difference to study hypothalamic effects of TCDD on expression of bHLH/PAS proteins potentially involved in wasting syndrome. Quantitative RT-PCR-method was used to measure mRNA levels of genes taking part in the signalling cascade of TCDD (AHR, AHRR, ARNT, ARNT2), feed intake regulation (*SIM1*) and circadian rhythmicity (*PER2*). In addition, two well-established target genes of TCDD, *CYP1A1* and *CYP1A2* were also examined. The measurements were done from total hypothalamic blocks, since hypothalamus is known to be one of the major centres of food intake and body weight regulation. At both 6 and 96 h after a single dose of 50  $\mu$ g/kg TCDD (lethal to L-E rats), significant elevations were found in mRNA levels of AHRR, CYP1A1 and CYP1A2, but not those of AHR, ARNT or ARNT2. Likewise, TCDD did not alter the expression of *SIM1*, implicated in the suppressive impact of TCDD on food intake, nor that of *PER2*. Differences between H/W and L-E rats appeared in constitutive levels of AHR and ARNT and in TCDD-induced levels of CYP1A2, AHRR, AHR and ARNT, which all were about two- to fourfold lower in H/W rats. The expression of all principal genes of the AHR-signalling pathway in rat hypothalamus suggests that it might be a critical target for TCDD.

## 2043

### COMPARISON OF THE TCDD-INDUCED CYP1A1 GENE EXPRESSION PROFILE IN LYMPHOCYTES FROM MICE, RATS AND HUMANS.

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2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin (TCDD) exerts its toxicity by binding and activating a transcription factor, the aryl hydrocarbon receptor (AhR). C57BL/6 mice express an AhR that has high affinity for TCDD, and they strongly express target genes, including the most sensitive target, CYP1A1, and develop severe toxic effects when exposed to TCDD. By contrast, DBA/2 mice have a low-affinity form of AhR, weakly express target genes, and are hardly affected by TCDD. Although humans express an AhR whose affinity for TCDD is comparable to that of the AhR of DBA/2 mice and should be refractory to TCDD toxicity, their sensitivity to TCDD has not been precisely investigated. In this study we compared the TCDD-induced CYP1A1 gene expression profiles in lymphocytes from humans, C57BL/6 mice, DBA/2 mice, and SD rats to get a clue for clarifying human sensitivity to TCDD. Lymphocytes were prepared from the blood of individual animals, and after culturing them in the presence of TCDD, total RNAs were prepared. The mRNAs for CYP1A1 and housekeeping genes were measured by RT-PCR or real-time PCR using primers designed for regions that are 100% homologous in the individual genes of all the species. The PCR efficiency for CYP1A1 in the four species yielded similar values, ranging around 1.7 - 1.8. A time-course study showed that TCDD-induced CYP1A1 expression peaked at 2 h in DBA/2 mice and SD rats and at 6 h in C57BL/6 mice and humans. Quantification of the amount of CYP1A1 mRNA at the individual peak times yielded the following order of amount: humans > C57BL/6 mice > DBA/2 mice > SD rats. These results suggest the existence of a major factor(s) that affects the AhR-dependent gene expression in the lymphocytes, in addition to the affinity of the AhR for TCDD. They also suggest that the TCDD sensitivity of human lymphocytes may be similar level to that of the lymphocytes of C57BL/6 mice.

## 2044

### ANALYSIS OF TEQ-EQUIVALENT AROCLOR AND TCDD TREATED RATS REVEALS DIFFERENT GENE DOSE-RESPONSE PROFILES AMONG DIOXIN RESPONSIVE GENES.

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DNA microarray technology offers a great potential to address questions regarding the characteristic profiles of the multi-gene response to AhR ligands fundamental to validation of the TEQ (dioxin toxic equivalency) approach. TEQ equivalent dosages were administered to SD rats. Rats (n=4) were administered corn oil or 0.3 or 3.0  $\mu$ g/kg TCDD or 0.6, 6.0, or 60 mg/kg Aroclor 1254 mg/kg po for 3 consecutive days. The Aroclor 1254 lot has a TEQ (WHO98) of 46 ppm. Hepatic RNA was analyzed using Affymetrix RG U34 arrays. A dose-response model was fitted to each gene probe set. Treatment (corn oil, TCDD, Aroclor), TEQ dosage, and gender effects were treated as fixed effects and probe number as a random effect. Plausible interactions such as gender x treatment, TEQ x treatment, gender x TEQ, and gender x TEQ x treatment, were incorporated into the model. As expected, the primary early response genes included the CYP1 family genes. When normalized to TEQ, preliminary results show that well characterized AhR responsive genes, CYP1A1, CYP1A2, and CYP1B1, each follow very different response profiles. Further, the observed Aroclor TEQs for CYP1A1, CYP1A2, and CYP1B1 were 8.4, 46, 0.05 ppm, respectively. The CYP1A1 and CYP1B1 dose-response curves showed only treatment dependency; CYP1A2 showed only gender dependency. These results, which are based on measurements of the *in vivo* response of several dioxin responsive genes, demonstrate that the current TEFs used to calculate the Aroclor 1254 TEQ resulted in a 5.5 fold overestimate of the primary dioxin responsive gene, CYP1A1. Further, each gene had a unique response profile that may be a combination of factors including gender, specific ligand, and/or ligand concentration. Findings from these early response indicators do not support the requirement of the TEQ approach of parallel dose-responses across endpoints.

## 2045

### DIOXIN-RESPONSIVE DOWN-REGULATION OF SERPINA7, CYP3A9/3A13, AND CES3 REQUIRES A FUNCTIONAL ARYL HYDROCARBON RECEPTOR.

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Previously, thyroxin binding globulin (Serpina7), cytochrome P450 3A9 (CYP3A9) and carboxylesterase 3 (CES3) mRNA were demonstrated to be down-regulated in the liver following subchronic (13 week) exposure of female Sprague-Dawley rats to the aryl hydrocarbon receptor (AhR) ligands 2, 3, 7, 8-TCDD, 2, 3, 4, 7, 8-PeCDF and PCB126 (Vezina et al., 2004). No down-regulation of these genes was observed following exposure to the non-AhR ligand PCB153, suggesting that Serpina7, CYP3A9 and CES3 are novel dioxin-responsive genes. To investigate whether this response was a primary event associated with exposure to AhR ligands, the hepatic expression of these genes was investigated in female Sprague-Dawley rats at 24 and 72 hrs following a single exposure to TCDD (5 $\mu$ g/kg, po). Real time RT-PCR confirmed that Serpina7, CYP3A9, and CES3 were down-regulated -3-4, 5-10 and 3-7 fold, respectively. To establish whether the down-regulation of these genes was species- and AhR-dependent, female AhR wild type (AhR +/+) and AhR knockout (AhR -/-) mice were exposed to a single dose of TCDD (5 $\mu$ g/kg, po). At 72 hrs following exposure, hepatic Serpina7, CYP3A13 (the mouse homologue of CYP3A9), and CES3 were down-regulated -18, 2, and 7-fold in AhR +/+ mice, respectively. In AhR -/- mice Serpina7 and CYP3A9 were up-regulated -2-fold and CES3 was unchanged. These results indicate that the dioxin-responsive down-regulation of Serpina7, CYP3A9, and CES3 mRNA is an early, primary response to TCDD exposure that is dependent on a functional AhR. The functional and toxicological significance of these novel AhR-dependent down-regulated genes is currently under investigation. (Supported in part by NIEHS ES09440, SOT, and University at Buffalo).

## 2046

### COPLANAR POLYCHLORINATED BIPHENYLS ACTIVATE ARYL HYDROCARBON RECEPTOR IN DEVELOPING TISSUES OF TWO TCDD-RESPONSIVE LACZ MOUSE LINES.

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*In utero* exposure to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) can have an immediate impact on developmental processes that may then lead to long-term deficits in function. To define the tissues affected by TCDD exposure during devel-

opment, our laboratory developed a *lacZ*-reporter gene mouse which models activation of the Aryl Hydrocarbon Receptor (AhR). Exposure of the fetus via maternal oral gavage of 30 µg/kg TCDD on gestational day (GD) 14 results in strong activation of the *lacZ* transgene in numerous tissues including palate, paws, ear and genital tubercle. In order to further establish the utility of our mouse model, fetuses were exposed to selected polychlorinated biphenyls (PCBs) to test for a response to alternative dioxin-like toxicants. The coplanar PCB congeners 3, 4, 5, 3', 4'-pentachlorobiphenyl (C126) and 3, 4, 3', 4'-tetrachlorobiphenyl both induced staining in fetal tissues identical to that observed following TCDD exposure, although C126 was much more effective. Exposure of fetuses to the PCB mixture Aroclor 1254 and the non-coplanar congener 2, 3, 6, 2', 5'-pentachlorobiphenyl did not result in activation of the *lacZ* transgene. In addition to the testing of alternative ligands, another line of reporter mice was generated to determine the potential influence of the site of insertion of the *lacZ* transgene on the reported observations. Both TCDD and the coplanar PCBs induced a similar pattern of staining in the new line as compared to that observed in the original *lacZ* reporter mouse line, suggesting that the site of insertion of the transgene does not mediate the observed response. The ability of AhR ligands, in addition to TCDD, to activate the AhR-mediated *lacZ* transgene, in combination with the insertion-site independence of the response, strengthens the data previously derived from this model and validates the use of this system for investigations examining AhR-mediated events during development. Funded by NIH Grants ES09430, ES07026 and ES01247.

## 2047

### CCN4 AND CCN5 ARE DOWN REGULATED IN RESPONSE TO THE ADIPOGENIC STIMULATION WHILE 2, 3, 7, 8 - TETRACHLORODIBENZO-P-DIOXIN (TCDD) ELEVATES CCN5 MESSAGE IN C3H10T1/2 MOUSE EMBRYO FIBROBLASTS.

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Adipogenesis in mouse embryo fibroblasts is inhibited at an early stage by 2, 3, 7, 8 - tetrachlorodibenzo-p-dioxin (TCDD). We and others have shown that administration of TCDD inhibits both early and late markers of fully mature adipocytes. The critical window in which TCDD must be present for successful attenuation of the central mediator of adipogenesis, the transcription factor PPAR $\gamma$ , is 6 – 16h after stimulation by an adipogenic hormonal cocktail (IDM); furthermore, we have demonstrated that this TCDD mechanism of suppression is MEK/ERK dependent. To determine what gene expression changes TCDD and MEK/ERK signaling are causing, we have used Affymetrix microarrays. Under adipogenic conditions, the arrays identified 45 genes in which the additional presence of both TCDD and EGF substantially and cooperatively inhibited the IDM stimulation. Changes in expression of some of these 45 genes may be necessary for differentiation and, when blocked by TCDD and EGF, lead to inhibition on adipogenesis; if a gene(s) from this set of 45 is required for the TCDD and EGF mechanism of inhibition, changes in message should occur within the 6 – 16 hour window of TCDD sensitivity. This window also corresponds to a period of IDM-induced cell contraction, which is cooperatively blocked by TCDD and EGF. Quantitative Real Time PCR (qRT-PCR) has been used to establish CCN4 and CCN5 as genes which are both IDM sensitive and are highly responsive in the opposite direction in the additional presence of TCDD and EGF. CCN4 is a typical member of the matrix-associated CCN family, which binds to integrins and affect cell adhesion; CCN5 is unique from other CCN family members because it lacks the integrin binding domain. Notably, both CCN4 and CCN5 are regulated by Wnt signaling, which suppresses adipogenesis. This research addresses whether CCN4 and CCN5 are possible mediators of TCDD inhibition of adipogenesis or are merely responding to these signaling changes.

## 2048

### DEVELOPMENT OF A NEW DIOXIN-ANALOGUE: 2, 3, 7, 8-TETRACHLOROPHENOTHIAZINE.

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Inspired by interest in comparative studies, a new dioxin-analogue was designed and subsequently synthesized. The chemical characterization of the hitherto unknown compound 2, 3, 7, 8-tetrachlorophenothiazine (TCPT) revealed the desired structural resemblance with the archetype 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD). TCPT was synthesized in low yield by an intra-molecular Ullmann-coupling of the corresponding diarylamine. The successful synthesis was accomplished in a three-step process. In addition to spectroscopic and spectrometric methods, the

identity and conformation of TCPT were definitively confirmed by the resolution of the crystal structure. As expected for a phenothiazine, the molecule was found to have a butterfly shape, folded along the heteroatom axis. Thus, its deviation from planarity (by 18.5 °) distinguishes it slightly from the strictly planar dioxins.

## 2049

### COMPARISON OF GC/MS AND CALUX® BY XDS FOR DETECTION OF DIOXIN LIKE COMPOUNDS IN ENVIRONMENTAL SAMPLES.

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The CALUX® by XDS bioassay measures the level of activation of the aryl hydrocarbon receptor (AhR)-dependent gene expression of chemical extracts providing an estimate of the relative potency and toxic potential expressed as Toxic Equivalents (TEQs) for dioxin like compounds. The CALUX® by XDS bioassay detects dioxin like compounds, using mouse hepatoma 6.1 cells stably transfected with an AhR luciferase reporter gene plasmid. A validation study was conducted with ash, soil and exhaust gas samples using both gas chromatography/mass spectrometry (GC/MS) and the CALUX® by XDS bioassay. Samples were sent to the Hiyoshi Corporation of Japan and Xenobiotic Detection Systems, Inc. (XDS) in a double blind study later decoded by a third party statistician. A double blind SITE study was conducted with the EPA and the Battelle Corporation in which soil samples were sent to several labs (including XDS) and a GC/MS lab. Samples from Lockheed Martin, Sumika Corp., and Umea University / Örebro University were sent to XDS for other validation studies. This report shows the cumulative data from all five direct sample comparisons. The results from the SITE study showed a 0.94 R<sup>2</sup> value for dioxins/furans (PCDD/PCDF), and a 0.81 R<sup>2</sup> for PCBs. The average Soil / soil extract correlation was a 0.91 R<sup>2</sup> value for PCDDs/PCDFs. The Ash / ash extracts tested had an average 0.93 R<sup>2</sup> correlation value for PCDDs/PCDFs. Exhaust extract demonstrated an R<sup>2</sup> correlation for PCDDs/PCDFs at 0.94. The solution samples tested demonstrated a 0.91 R<sup>2</sup> correlation for PCDDs/PCDFs. The overall correlation for PCDDs/PCDFs for all samples tested between GC/MS and the CALUX® by XDS bioassay was a 0.95 R<sup>2</sup> correlation. This study has been submitted to the EPA for SW846 validation and clearly demonstrates the CALUX® by XDS assay can be used in the detection of dioxin like compounds and is predictive of GC/MS results.

## 2050

### INDUCTION OF EROD-ACTIVITY *IN VITRO* BY 2, 3, 7, 8-TETRACHLOROPHENOTHIAZINE.

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2, 3, 7, 8-Tetrachlorophenothiazine (TCPT), a newly developed dioxin-analogue, was the subject of an investigation for the induction of *in vitro* EROD-activity. Its effects were compared to those of its structural analogue 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), the most potent dioxin congener. The time-dependency of induction was also measured after 24, 48 and 72 h of induction. After 24 h, a 370-times lower potency was calculated for TCPT as compared to TCDD. The efficacy of TCPT to induce EROD-activity was 77.6 % of that of TCDD as indicated by the respective maxima. TCPT appeared to be rapidly metabolized and was not found to be cytotoxic at the applied concentrations. The findings of this study indicate that TCPT is a partial agonist of the aryl hydrocarbon receptor (AhR) despite of its non-planar conformation.

## 2051

### LYMPHOCYTES CHOLINERGIC MUSCARINIC RECEPTORS: *IN VIVO* AND *IN VITRO* EFFECTS OF THE COMBINED EXPOSURE TO POLYCHLORINATED BIPHENYLS AND METHYLMERCURY.

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Cerebral cholinergic muscarinic receptors (MR) have been suggested as a possible common target for the widespread seafood contaminants methylmercury (MeHg) and polychlorinated biphenyls (PCBs). *In vivo*, MeHg has been shown to alter MR binding both in the rat brain and lymphocytes, supporting the use of MR in blood cells as a surrogate marker of CNS changes. The effects of MeHg and PCBs alone or in combination were evaluated on rat lymphocyte MR binding (using [<sup>3</sup>H]QNB as specific muscarinic ligand) *in vivo* and *in vitro*. For comparison, *in*

*vitro* studies were also performed on human lymphocytes. In rat splenic lymphocytes obtained from adult rats orally exposed *in vivo* to 1 mg MeHg/kg/day for 21 days and sacrificed 14 days later, MR density (Bmax) was increased by 240%. PCB153, 20 mg/kg/day, fed for 7 days caused a slight decrease in MR density, and completely masked MeHg effect when given together with it. In *in vitro* studies on human and rat lymphocytes, MeHg caused 20–40% inhibition of MR binding at 1  $\mu$ M. The concentration-dependent effects (from 10 nM to 100  $\mu$ M) of various PCBs (PCB28, PCB77, PCB105, PCB118, PCB153, 4-OH-PCB107) were also evaluated on lymphocyte MR binding in the presence or absence of 1 microM MeHg. In rat splenic lymphocytes, no significant differences in IC50 values were detected among the different PCBs, tested either alone or together with MeHg. All IC50s were about 30  $\mu$ M. Even in human blood lymphocytes, PCBs IC50s were in the high micromolar range (from 20 to 50  $\mu$ M) and unaffected by the presence of MeHg. These *in vivo* results provide evidence of an interaction between PCB153 and MeHg on rat lymphocyte MR binding. However, this interaction does not seem to occur directly at the receptor level because of the negligible direct effect of PCB153 on this endpoint according to our *in vitro* data. The sensitivity of lymphocyte MRs to PCBs was neither species-related (rat and human) nor PCB congener-dependent. (EU Grants: QLK4-CT-2001-00186; FOOD-CT-2003-506543).

## 2052

### THE ROLE OF SUPEROXIDE ANION IN 2', 4, 4'-TETRACHLOROBIPHENYL-INDUCED UPREGULATION OF COX-2 IN HL-60 CELLS.

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Polychlorinated biphenyls (PCBs) are persistent environmental contaminants that affect a number of cellular processes in a variety of cells, including neutrophils. Previous studies in a promyelocytic leukemia cell line (HL-60) differentiated with DMSO to a neutrophil-like phenotype have shown that ortho-substituted, non-coplanar PCBs such as 2, 2', 4, 4'-tetrachlorobiphenyl (2244-TCB) cause increased arachidonic acid release and superoxide anion production accompanied by upregulation of cyclooxygenase-2 (COX-2). We tested the hypothesis that PCB-mediated production of superoxide anion is required for upregulation of the COX-2 gene in HL-60 cells. 2244-TCB (30  $\mu$ M) significantly increased superoxide anion levels in both quiescent cells and in cells treated with the immunostimulatory compound, phorbol myristate acetate (PMA). In addition, 2244-TCB treatment increased both [<sup>3</sup>H]-arachidonic acid (<sup>3</sup>H-AA) release and COX-2 mRNA expression. Apocynin, an inhibitor of the superoxide anion-producing enzyme NADPH oxidase, and 4-hydroxy TEMPO (4-HT), an intracellular free radical scavenger, both significantly reduced superoxide anion levels in 2244-TCB-treated cells. Release of <sup>3</sup>H-AA was significantly reduced by apocynin, but not by 4-HT, in vehicle, 2244-TCB-treated or PMA-stimulated cells. COX-2 mRNA was decreased by apocynin in vehicle-treated cells as well as in 2244-TCB-treated cells both in the absence and presence of PMA. 4-HT treatment had no effect on COX-2 mRNA levels under any condition. The results suggest that superoxide anion is not required for 2244-TCB-mediated 3H-AA release or COX-2 expression and that apocynin decreases COX-2 expression through a mechanism independent of inhibition of superoxide anion production. (Supported by ESO4911 and training grant T32 ES007255).

## 2053

### RYR1 MALIGNANT HYPERTHERMIA POINT MUTATION R615C ENHANCES SUSCEPTIBILITY TO NON-COPLANAR 2, 2', 3, 5'-PENTACHLOROBIPHENYL (PCB 95).

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Malignant Hyperthermia (MH) is an inherited pharmacogenetic disease of skeletal muscle in human and animals possessing specific point mutations within the ryanodine receptor (RyR1), which are calcium channels found on the sarcoplasmic reticulum (SR). Since patients with MH point mutations do not always have a fulminant MH episode when exposed to triggering agents, and there are different degrees of severities in patients possessing the same point mutation, we examined the effect of an environmental contaminant, 2, 2', 3, 5'-pentachlorobiphenyl (PCB 95), on <sub>MH</sub>RyR1 compared to that of <sub>wt</sub>RyR1. In this study, we demonstrated that there is no significant difference in ryanodine protein expression level of <sub>wt</sub>RyR1 and <sub>MH</sub>RyR1 in isolated porcine SR membranes (p-value <0.84649 by ANOVA one-way comparison). Using [<sup>3</sup>H]ryanodine receptor binding to access ryanodine receptor function in response to endogenous agonists/antagonists of <sub>wt</sub>RyR1 and <sub>MH</sub>RyR1, we found that <sub>MH</sub>RyR1 is more sensitive to channel activation by  $Ca^{2+}$ , and less sensitive to channel inhibition by  $Ca^{2+}$  and  $Mg^{2+}$  compared to <sub>wt</sub>RyR1. In conditions that mimic resting intracellular  $Ca^{2+}$ , PCB 95 has significantly lower threshold and higher efficacy toward <sub>MH</sub>RyR1 compared to <sub>wt</sub>RyR1. Furthermore, PCB 95 disrupts feedback inhibition of the channel by calcium and magnesium to a greater extent in <sub>MH</sub>RyR1 than <sub>wt</sub>RyR1. In the presence of PCB 95 (2  $\mu$ M), the

shifts in IC50s for  $Ca^{2+}$  and  $Mg^{2+}$  were 3.6- and 2-fold greater in <sub>MH</sub>RyR1 compared to <sub>wt</sub>RyR1, respectively. In summary, our data suggest PCB 95 has a more profound effect on <sub>MH</sub>RyR1 channels than it does to <sub>wt</sub>RyR1. These studies are the first to identify a specific gene-environment interaction that influences PCB neurotoxicity.

## 2054

### ANALYSIS OF THE TUMOR PROMOTING POTENCY OF PCB 28 AND PCB 101 IN RAT LIVER.

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Polychlorinated biphenyls (PCBs) are potent persistent environmental pollutants exhibiting neurotoxic, developmental and tumor promoting effects in experimental animal models. Complex technical PCB mixtures have been shown to cause liver cancer in rodents while very few data are available on the carcinogenic potency of individual PCB congeners. Among the ortho-substituted, 'non-dioxinlike' PCBs a few congeners including PCB 28 and 101 are present in environmental and food samples. In this study, the effect of both compounds on the promotion of enzyme-altered hepatic foci was investigated in female Wistar rats after initiation with diethylnitrosamine. After eight and sixteen weeks of PCB treatment at dose levels of 50 and 150  $\mu$ mol/kg body weight per week, both congeners were recovered in the liver according to the dose levels applied, with PCB 28, at the same dose level, showing six- to sixteen-fold higher hepatic levels than PCB 101. Both congeners slightly induced 7-ethoxyresorufin O-deethylase (EROD) and 7-pentoxyresorufin O-depetylase (PROD) activity. The focal volume of adenosine triphosphatase-(ATPase) negative foci, but not the volume of the placental form of glutathione S-transferase- (GSTP) positive foci, was enhanced significantly by treatment with both congeners after sixteen weeks. No significant increase in the number of ATPase-negative or GSTP-positive foci was observed. Our results indicate a weak promoting potency of PCB 28 and 101 in the liver of female rats correlated, in the case of PCB 28, with a significant increase in relative liver weight.

## 2055

### TRANSCRIPTIONAL PROFILING OF RAT HEPATIC RESPONSES TO DIETARY EXPOSURE TO AROCLOR 1254.

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Dietary intake is a primary route for biomagnification of environmentally persistent polychlorinated biphenyls (PCBs) within humans and animals. Aroclor 1254, a mixture of PCBs, is associated with a variety of toxic responses in cell culture and in whole animals including alterations in blood and lipid biochemistry, tumor promotion, immune suppression, and impaired neurological function. Pretreatment of cells and live animals with Aroclor 1254, a broad spectrum MFO inducer, has also been used to enhance metabolic activation of mutagens. Measurement of the transcriptional responses to sub-acute and sub-chronic dietary exposure to Aroclor 1254 has not been reported in the literature to date, and is particularly important given the persistence and pervasiveness of PCBs, and the range of toxicities associated with exposure. Changes in expression of hepatic genes in male Fisher 344 rats were assessed by Affymetrix microarray analysis to identify important physiological pathways affected by PCB intake. Seven week old animals were fed control diet or diet containing 10 ppm or 50 ppm Aroclor 1254 for 7 days and 84 days. Three RGU34A arrays were prepared from liver mRNA for each treatment group and time point. Animal health at the time of sacrifice was also assessed by measuring serum levels of cholesterol, triglycerides, bilirubin, ALT, AST, ALP, and GGT. Most of the significant effects of Aroclor treatment on animal health were seen in the high dose groups; these included enlarged livers and elevated cholesterol levels, and decreases in serum ALT and AST. Analysis of microarray data showed upregulation of several cytochrome P450s, oxidative stress response, and detoxification genes in treated animals compared to controls, at one week and after twelve weeks of feeding. Significant changes were also observed in transcription of genes involved in long chain fatty acid transport and metabolism, cell cycle, and transcription.

## 2056

### COMPARISON OF AROCLOR AND TCDD RESPONSIVE GENES BETWEEN *IN VIVO* AND *IN VITRO* EXPOSURES USING DNA MICROARRAYS.

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DNA microarrays were used to characterize gene expression in hepatic cells exposed either *in vivo* or *in vitro* to two chemicals known to act through the aromatic hydrocarbon receptor, AhR. Sprague-Dawley (SD) rats were administered corn oil,

TCDD, or Aroclor 1254 *po* for 3 consecutive days. Primary cultures of SD rat hepatocytes were treated with TCDD or Aroclor 1254. RNA samples were analyzed on Affymetrix RG U34 arrays. Only genes whose expression changed significantly ( $p \leq 0.001$ ) by  $>2$  fold were considered. Comparisons were made between chemical treatments, exposure method, single genes, gene networks, and network functions. Of 156 genes responding to TCDD, 16 were common to both *in vivo* and *in vitro* exposures, but 69 were unique to *in vivo* and 71 unique to *in vitro*. Of 225 genes responding to Aroclor, 16 were common to both exposures, but 163 were unique to *in vivo* and 46 unique to *in vitro*. Thirteen of the 16 genes common to both exposures of either TCDD or Aroclor were the same, showing remarkable consistency across chemical and exposure regime. Using Ingenuity Pathways Analysis ([www.ingenuity.com](http://www.ingenuity.com)), each gene was evaluated for its association with other genes, i.e., gene networks. The 13 common genes were strongly associated with the AhR and involved transcription, expression, differentiation, and binding. There were also networks unique to each chemical that were also different *in vivo* or *in vitro*. These generally involved cellular functions such as synthesis, translation, viability, and oxidative stress. This preliminary work suggests that there is useful correlation between the primary pathway genes between *in vivo* and *in vitro* exposures to each of these chemicals. However, this work also indicates that TCDD uniquely influences genes, gene networks, and functions that are different from those affected by Aroclor.

## 2057

### ACCUMULATION OF PBDE-47 IN PRIMARY CULTURES OF RAT NEOCORTICAL CELLS.

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Studies have examined the actions of PBDEs using *in vitro* cell culture models. However, there is little data reporting the final concentration of PBDEs in cells after *in vitro* exposure. The present study examined the concentration- and time-dependent accumulation of 2, 2', 4, 4'-tetrabromodiphenyl ether (PBDE-47) in primary cultures of rat neocortex. Neuronal cultures were prepared from the neocortex of newborn rats and grown for 7 days *in vitro*. Cells were then exposed to freshly prepared serum-free culture media containing <sup>14</sup>C-PBDE-47. Radiolabel associated with the cells or remaining in the media was determined by liquid scintillation spectrometry. Exposure to 0.01 - 3.0  $\mu$ M PBDE-47 for 60 min resulted in a concentration-dependent accumulation in cells. At each concentration approximately 15% of the applied PBDE-47 was associated with the cells, resulting in a 100-fold magnification of the applied concentration (e.g., exposure to 1  $\mu$ M resulted in 100  $\mu$ M concentration in the cells); 55% of the PBDE remained in the media and 30% was associated with the plastic culture dish. Exposure to 1  $\mu$ M PBDE-47 resulted in a linear increase in PBDE-47 in cells with time for the first 60 min, which began to saturate at 120 min. Addition of serum proteins to the media decreased accumulation; at 10% serum in the media only 3% of the applied PBDE-47 was associated with the cells and 96% remained in the media after 60 min. The volume of exposure also influenced accumulation. Doubling the volume of serum-free exposure media (from 2 ml to 4 ml) but leaving the concentration constant (1  $\mu$ M) resulted in an 1.5-fold increase in PBDE concentration in the cells. These data show that a number of factors, including duration of exposure, volume of exposure, and concentration of serum proteins in the media, can influence the accumulation of PBDE in cells *in vitro*. For this highly lipophilic compound, use of media concentration underestimates tissue concentration by up to 2 orders of magnitude. (This is an abstract of a proposed presentation and does not necessarily reflect EPA policy).

## 2058

### MIXTURE EFFECTS OF PCBs AND PBDES WITH OTHER CONTAMINANTS/DRUGS.

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Polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) are ubiquitous in humans. The troubling question remains whether the toxicity of these contaminants at apparently innocuous concentrations may be enhanced by co-exposure to other chemicals. To gain insight into this question, we exposed cells in culture to three commercial PBDE-mixtures, a PCB mixture, or two PCB congeners alone or in combination with compounds that induce peroxisome proliferation, redox-cycling or reduction of intracellular glutathione (GSH). Three different cell lines were chosen which represent two different tissues and two species, i.e. human colon carcinoma cells (CaCo-2), and hepatoma cells HepG2 (human) and H-4-II-E (rat). Cytotoxicity was determined by Neutral Red uptake and intracellular GSH content by enzyme recycling. No toxicity was observed in HepG2 and CaCo-2 with PCBs or PBDEs at the highest soluble concentration (5 - 50  $\mu$ M). However, H-4-II-E cells were more sensitive, with cytotoxicity of up to 40% (penta-PBDE mixture) and 50% (Aroclor 1254). None of the compounds had an

effect on intracellular GSH levels in H-4-II-E, but all PCBs tested slightly decreased GSH in CaCo-2 cells. The penta- and octa-PBDE mixtures increased GSH in HepG2 cells. Co-incubation with non-cytotoxic concentrations of Dicamba (100  $\mu$ M), Diquat (1  $\mu$ M), or Ciprofibrate (50  $\mu$ M) did not change toxicity and GSH content in any of the cell lines. BSO (0.5 mM) alone completely reduced GSH in all three lines, but the only effect of co-incubation was a slight increase of cytotoxicity with PCB-77 in H-4-II-E cells. Co-incubation with a non-cytotoxic concentration of PFDA (100  $\mu$ M) increased cytotoxicity of all PCB and PBDE exposures in both rat and human hepatoma cells without significantly altering total GSH. This shows that co-exposure of PCBs or PBDEs, with non-toxic concentrations of the peroxisome proliferator PFDA, may cause toxic effects in hepatoma cells, an effect that is not mediated through GSH depletion. (Supported by CHEEC, DAMD17-02-1-0241, R-82902101 from EPA, ES 07380 from NIEHS)

## 2059

### EFFECT OF PBDES ON TCDD-INDUCED CYP1A1 ACTIVITY (EROD) IN PRIMARY HEPATOCYTES OF CYNOMOLGUS MONKEYS.

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Polybrominated diphenylethers (PBDE) are used as additive flame-retardants in plastics, textiles and circuits boards. Some PBDE congeners have been found world-wide in wildlife and human tissues during the last decades. The structural similarity of certain PBDE congeners to other polyhalogenated aromatic hydrocarbons such as PCBs has raised concerns that these compounds might be agonists for the aryl hydrocarbon receptor (AhR). To study the possible dioxin-like effects of environmentally relevant PBDEs (BDE47, 77, 99, 100, 153, 154, 183, 209), the Ah receptor-mediated induction of CYP1A1 was studied in primary cynomolgus monkey (*Macaca fascicularis*) hepatocytes. The monkeys served as donors for the production of the poliomyelitis vaccine. After the liver was perfused *in situ* with PBS, it was removed and transported to our laboratory where the hepatocytes were isolated using a two-step collagenase perfusion technique. Ethoxresorufin-O-deethylation (EROD) activity was used as a marker for CYP1A1 activity. The cells were seeded in 12-well plates and exposed for 48h to PBDEs (0.01-10  $\mu$ M), the positive control TCDD (0.001-2.5 nM) and negative control (DMSO). There was a concentration-dependent increase in EROD activity measured after 48h exposure to the positive control TCDD. The PBDEs (BDE47, 77, 99, 100, 153, 154, 183) did not show any induction of EROD activity. This indicates that these PBDEs were not agonists of the Ah receptor. The lack of CYP1A1 induction by these PBDEs further supports the exclusion of these compounds in the TEF concept for dioxin-like compounds. However, when the hepatocytes were exposed to various concentrations of BDE77 in combination with TCDD for 48h, there was a concentration dependent inhibitory effect on the TCDD-induced EROD activity. Co-exposure to TCDD (1 nM) and BDE77 (10  $\mu$ M) resulted in a 88% decrease of the TCDD-induced EROD activity. Other PBDEs showed similar effects, although quantitative differences were observed.

## 2060

### 2, 2'-DIBROMODIPHENYL ETHER BINDS AND INDUCES CALCIUM RELEASE THROUGH THE RYANODINE RECEPTOR IN AN FKBP12-DEPENDENT MANNER.

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Polybrominated diphenyl ethers (PBDEs) are widely used flame retardants that have been identified as persistent environmental pollutants since the late 1990s. The bioaccumulation and potential neurotoxicity warrant further investigation into their biological activity and mechanism of action. It has previously been reported that the non-coplanar polychlorinated biphenyls (PCBs) specifically bind the ryanodine receptor calcium release channel (RyR). Because of structural and toxicological similarities between the PBDEs and the non-coplanar PCBs, we investigated whether the PBDE congener 2, 2'-dibromodiphenyl ether (BDE-4) also binds to the skeletal isoform of the RyR (RyR1). BDE-4 dose-dependently enhances [<sup>3</sup>H]ryanodine binding to RyR1 (EC<sub>50</sub> = 5.60  $\mu$ M), whereas diphenyl ether and 4, 4'-dibromodiphenyl ether (BDE-15) showed no activation, suggesting ortho substitution is required for activity at RyR1. BDE-4 dose-dependently decreases the EC<sub>50</sub> of RyR1 activation by Ca<sup>2+</sup> (vehicle EC<sub>50</sub> = 1  $\mu$ M Ca<sup>2+</sup> vs 8  $\mu$ M BDE-4 EC<sub>50</sub> = 191 nM Ca<sup>2+</sup>). In addition, inhibition of RyR1 activity by mM Ca<sup>2+</sup> and Mg<sup>2+</sup> was attenuated in the presence of BDE-4 (Ca<sup>2+</sup> inhibition: vehicle IC<sub>50</sub> = 101  $\mu$ M vs 8  $\mu$ M BDE-4 IC<sub>50</sub> = 125  $\mu$ M Ca<sup>2+</sup>; Mg<sup>2+</sup> inhibition: vehicle IC<sub>50</sub> = 190  $\mu$ M vs 8  $\mu$ M BDE-4 IC<sub>50</sub> = 90  $\mu$ M Mg<sup>2+</sup>). Microsomal Ca<sup>2+</sup> flux measurements suggest a similar increase in RyR1 activity with BDE-4. These effects were abolished in the presence of the RyR blocker ruthenium red but was unaffected by inhibition of the SR/ER ATPase by 375 nM thapsigargin, suggesting the effect is specific to alterations of

RyR1 activity. Rapamycin 0 to 50  $\mu$ M selectively prevented activation of RyR1 channels by BDE-4 in both [ $^3$ H]ryanodine binding and microsomal  $\text{Ca}^{2+}$  flux measurements. These results suggest RyR1 is a molecular target for the ortho-substituted BDE-4, and that this activity is FKBP12 dependent.

2061

THE BROMINATED FLAME RETARDANT HBCDD INDUCES CYP2B AND CYP3A BUT NOT CYP1A IN RAT LIVER.

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Among the major brominated flame retardants (BFRs) hexabromocyclododecane (HBCDD) is found regularly in environmental samples and in mother's milk. Some BFRs have been suspected to act as 'endocrine disrupters' and/or to affect the development of the unborn. Induction of drug metabolism may play a role in such effects by changing the body's homeostasis of hormones such as steroids, thyroxine and others. In this study adult Wistar rats were treated with HBCDD (0.3, 1, 3, 10, 30, 100 mg/kg body weight per day) over 28 days. The effects of treatment on the hepatic levels of cytochrome P450 (CYP) 1A1/2, 2B1/2, and 3A1/3 were analyzed by Real Time PCR, Western blotting, an determination of microsomal 7-ethoxyresorufin O-deethylase (EROD), 7-pentoxyresorufin O-depentylase (PROD), and luciferinbenzylether-debenzylase (LBD) activity, respectively. Doses of up to 100 mg/kg per day did not lead to any effects on CYP1A1/2 expression. There was a significant trend of increase with dosage for CYP2B mRNA in male but not in female animals. Levels of CYP3A mRNA were highly increased in females, more than 20 times at the highest dose. Male samples gave evidence for an inducing trend. LBD activity similarly unveiled high induction for females (up to 9 times), contrarily there was no indication for an increase in male animals. These findings suggest that oral treatment with HBCDD, has a significant inducing effect on the hepatic drug-metabolizing enzymes CYP2B and CYP3A. This pattern of CYP induction may cause sex specific variations in hormone metabolism, thus leading to endocrine effects.

2062

NEONATAL EXPOSURE TO POLYBROMINATED DIPHENYL ETHERS, PBDE 183, PBDE 203, AND PBDE 206, CAUSES NEUROTOXIC EFFECTS IN ADULT MICE.

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Polybrominated diphenyl ethers (PBDEs) are used as flame-retardants, and have been shown to increase in the environment and in human mother's milk. Previous studies have shown that lower brominated PBDEs, such as, tetra-, penta-, and hexa-brominated diphenyl ethers, can cause developmental neurotoxic effects. This is also seen for the full-brominated PBDE, deca-brominated diphenyl ether, although the developmental neurotoxic effects are suggested to be caused by a metabolite (possible de-brominated one), of PBDE 209. The present study have shown that 2, 2', 3, 4, 4', 5', 6'-heptaBDE (PBDE 183), 2, 2', 3, 4, 4', 5, 5', 6-octaBDE (PBDE 203) and 2, 2', 3, 3', 4, 4', 5, 5', 6-nonaBDE (PBDE 206), can induce developmental neurotoxic effects, such as aberrations in spontaneous behavior. Neonatal NMR mice were orally exposed on day 3 or day 10 to 15.2 mg PBDE 183/kg body weight, 16.8 mg PBDE 203/kg body weight, or 18.5 mg PBDE 206/kg body weight. These doses are all the same on molar basis (21  $\mu$ mol/kg body weight). Defects in spontaneous behavior (locomotion, rearing or total activity) was observed in 2-month-old mice, neonatally exposed to PBDE 203 and PBDE 206, on postnatal day 10, and to PBDE 183 and PBDE 203, on postnatal day 3. Furthermore, mice exposed to PBDE 203 and PBDE 206 showed also reduced habituation capability. The developmental neurotoxic effects were most pronounced in mice exposed to the octa-brominated diphenyl ether, PBDE 203. These developmental effects are in accordance with earlier reported developmental neurotoxic effects for other PBDEs, and certain PCBs.

2063

EFFECT OF PERFLUOROOCTANE SULFONATE (PFOS) AND PERFLUOROOCTANOATE (PFOA) ON L-TYPE CALCIUM CURRENT IN GUINEA PIG VENTRICULAR MYOCYTES.

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Perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) are chemicals ubiquitously found in the environment, wildlife and humans. Their toxicity is still unknown. There are several reports that PFOS and PFOA may change physiological properties of cell membranes. These effects on membrane properties, however,

have not been investigated from viewpoint of cellular functions. We herein show the effect of PFOS and PFOA on L-type calcium current in isolated guinea pig ventricular myocytes using the whole cell patch-clamp recording technique. PFOS (1, 3, 10, 30, 100  $\mu$ M) increased the voltage-activated peak amplitude of I(Ca, L) in a concentration-dependent manner. PFOS decreased the maximal activation voltage of I(Ca, L) ( $\text{EC}_{50} = 6.7 \mu\text{M}$ ). PFOS shifted the steady-state inactivation curve of I(Ca, L) to the left ( $\text{EC}_{50} = 12.6 \mu\text{M}$ ). PFOA also had the same effects of PFOS but its dose-response curves shifted to the higher doses by about 10-fold larger than those of PFOS. These effects were also observed in sodium dodecyl sulfate, which is known to change surface charge, suggesting the importance of amphiphilic nature for the effects on Ca-channel. In this study, perfluoroalkyl acids were considered to alter the L-type calcium channel kinetics in ventricular myocytes through the change of surface charge. This surface charge alteration may modify physiological properties of other channels, leading perturbation of signal transduction through various ion channels by PFOS and PFOA. Further studies are needed to bridge over a gap between *in vitro* finding and *in vivo* toxicity.

2064

RENAL CLEARANCES OF PERFLUOROOCTANE SULFONATE AND PERFLUOROOCTANOATE IN HUMANS, AND SPECIES-SPECIFIC EXCRETION OF THESE CHEMICALS.

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Perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) are chemicals ubiquitously found in the environment, wildlife and humans. However, the bioaccumulation mechanism of perfluorooctanoates in humans are unknown. Half-lives of these chemicals in human were longer than in other species. In rat study, renal clearances of PFOA in female rat were higher than those in male rat, and were regulated by sex hormones. First, we measured the concentrations of PFOA and PFOS in subjects who had lived in Kyoto City for more than 10 yr. The serum concentrations of PFOA and PFOS increased with age in females ( $r=0.460$  and  $r=0.524$ ,  $p<0.05$ , respectively), but not in males. The levels in females caught up in males at ages  $\geq 60$  yr. Second, we determined the renal clearances of PFOA and PFOS in young (20-40 yr of age,  $N=5$  for each sex) and old ( $\geq 60$  yr of age,  $N=5$  for each sex) subjects of both sexes. All the young females had menstruation cycles while the old females did not. The renal clearances were  $10^{-5}$ -fold smaller than the glomerular filtration rate, suggesting almost absence of renal excretion in humans. The sex differences in renal clearance that have been observed in rats and Japanese macaques, were not found in humans. Renal clearance of PFOA in human was about 1000 times lower than in rat and macaque. The lower serum levels of PFOA and PFOS in females than in males might be associated with elimination through menstruation. The results of the present study suggest species-specific renal excretion of these chemicals.

2065

MAMMALIAN METABOLISM AND DISTRIBUTION OF PERFLUOROOCTYL ETHANOL AND ITS OXIDATION METABOLITES.

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Perfluorinated compounds (PFCs) have been shown to be globally distributed, bioaccumulative, persistent and potentially toxic. It has been hypothesized that many precursor fluorinated compounds, including the telomer alcohols, degrade or metabolize to the common metabolite PFOA. Currently, little information is available on the mammalian metabolism of the telomer alcohols. Both  $\alpha$  and  $\beta$  oxidation pathways are proposed, resulting in the formation of perfluorononanoic acid (PFNA) and perfluorooctanoic acid (PFOA), respectively. An animal model is needed to fully elucidate the mechanisms of metabolism and translocation as it relates to humans. The objective of this study was to investigate the distribution and metabolism of the telomer alcohol in mice. Male CD-1 mice received a gavage dose of 20 mg 8-2 telomer alcohol/kg BW in a propylene glycol/water (1:1) vehicle. Animals were serially sacrificed at 1, 3, 6, 12 and 24 hours post-treatment and organs excised for PFC analysis. Telomer alcohol and metabolite concentrations were analyzed with EI-GC/MS. Based on preliminary studies, the telomer alcohol temporally decreased becoming non-detectable by 12 hours in both liver and serum. In liver, the telomer alcohol was seen at peak concentrations at 1 hour and in serum was quantifiable only at 1 and 3 hours. In serum the most abundant metabolite was PFOA with a concentration of 186-212 ng/mL at 6 hours post-treatment and decreased by 22% at 12 and 24 hours. In liver, PFOA is also the major metabolite reaching peak concentrations (38-44 ng/g) at 12 hours post-treatment. The highest concentration of PFNA coincides with peak PFOA concentrations and both metabolites decreased at 24 hours. In conclusion, the appearance of PFNA in the liver suggests that  $\alpha$  oxidation occurs although it does not appear to be the pre-

dominant metabolism pathway. However, these results suggest the telomer alcohol is metabolized by both  $\alpha$  and  $\beta$  oxidation pathways. Analysis of the brain, kidney, spleen, gonads and fat is ongoing.

## 2066

### MEASUREMENT OF THYROID HORMONES IN RAT SERA CONTAINING PERFLUOROOCTANESULFONATE (PFOS).

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Perfluorooctanesulfonate (PFOS), a persistent and bioaccumulative acid, is widely distributed in humans and wildlife. Prior studies with PFOS (rats and monkeys) have observed decreased total and free thyroid hormones (TH) in serum without a rise in thyrotropin (TSH). Measurement of serum free TH by analog methods can result in negative bias if serum binding capacity (SBC) and serum protein-bound TH (PBTH) are reduced. We tested the hypothesis (*in vivo* and *in vitro*) that PFOS reduces SBC and PBTH, resulting in a negative bias in analog assays for free TH. *In vivo*, adult female rats (10/group) were given 3 daily oral doses of 5 mg/kg PFOS or vehicle. Sera and liver were harvested and flash frozen 24 hours after the last dose. Both chemiluminescent (CL) and RIA methods were used to measure serum total thyroxine (TT4), free thyroxine (FT4), and TSH. FT4 was also measured by equilibrium dialysis-RIA (ED-RIA), a reference method not subject to negative bias. Hepatic mRNA transcripts for malic enzyme (ME),  $\alpha$ -glycero-3-phosphate-dehydrogenase ( $\alpha$ G3PD) (both responsive to TH), and UDP-GST-1A were quantified by RT-PCR. *In vitro*, rat sera were mixed with PFOS (0-100  $\mu$ g/mL) and assayed for TT4 and FT4 (CL and ED-RIA). *In vitro*, PFOS decreased TT4 and FT4 by CL and RIA. TSH, by CL and RIA, and FT4, by ED-RIA, were comparable to controls. ME and UDP-GST-1A transcripts were elevated 20% ( $p<0.05$ ) and 38% ( $p<0.001$ ), respectively, by PFOS.  $\alpha$ G3PD transcripts were unchanged. *In vitro*, TT4 was unchanged, and FT4 was positively correlated with PFOS concentration by both CL and ED-RIA up to 32% and 89% over control, respectively, at 100  $\mu$ g/mL PFOS. Thus, PFOS in serum resulted in a negative bias in analog FT4 measurements and did not reduce either FT4 by ED-RIA or the liver response to TH. These observations suggest that prior reports of reduced free TH were artifacts of the analog methods. [This does not reflect EPA policy.]

## 2067

### PROLONGATION OF K<sup>+</sup>-INDUCED BACKWARD SWIMMING OF *PARAMECIUM CAUDATUM* BY PFOS AND PFOA.

Y. Yamazaki, K. Harada, A. Morikawa, T. Yoshinaga and A. Koizumi. *Health Environmental Sciences, Kyoto University Graduate School of Medicine, Kyoto, Japan.* *Paramecium caudatum* is a protozoan and has been used to investigate the effects of chemicals on physiological processes. When cells are transferred to a K<sup>+</sup>-rich solution, increase in Ca<sup>2+</sup> conductance by stimulation of Ca<sup>2+</sup>-channel, results in initiation of backward swimming. Cells begin whirling in consequence, when the increase in Ca<sup>2+</sup> conductance is attenuated. Thus if a given chemical has some effects on the processes of Ca<sup>2+</sup>-channel kinetics, a series of swimming behavior will be modified. In the present study, we investigated the effects of perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) on K<sup>+</sup>-induced swimming behavior. After cultivating for four days, cells were transferred in K<sup>+</sup>-rich solution [20mM KCl, 1mM CaCl<sub>2</sub>, and 1mM Tris-HCl buffer (pH 7.2)], PFOS solutions (1, 10 and 100 ppm in K<sup>+</sup>-rich solution) and PFOA solution (1, 10 and 100 ppm in K<sup>+</sup>-rich solution) and were observed with a stereoscopy. In comparison with a period in K<sup>+</sup>-rich solution (M $\pm$ SE, sec) [25.5 $\pm$ 1.6 (n=10)], backward swimming was significantly prolonged by PFOS: 60.7 $\pm$ 8.8 at 1ppm (n=10), 162.4 $\pm$ 20.3 at 10 ppm (n=10). PFOA prolonged significantly only at 100 ppm [65.85 $\pm$ 3.0 (n=10)]. At 100 ppm PFOS was lethal and all cells died within 5 min. We further investigated the effect of a typical anionic amphiphile, sodium dodecyl sulfate: it also prolonged backward swimming at 10 ppm [317.2 $\pm$ 31.0 (n=5)]. The present study clearly demonstrated that PFOS has effects on Ca<sup>2+</sup> conductance at pharmacological dose levels likely through an action on the cellular membrane. Since the Ca<sup>2+</sup> signaling plays important roles in homeostasis, PFOS may perturb cellular functions through Ca<sup>2+</sup> signaling.

## 2068

### A SIGNIFICANTLY LARGER BIOCONCENTRATION FACTOR OF PFOS THAN THAT OF PFOA IN WILD TURTLES: THE AI RIVER ECOLOGICAL STUDY IN JAPAN.

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Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) are ubiquitously found contaminants. We recently found that surface waters in Kinki district, are more profoundly contaminated with PFOS and PFOA than other districts in

Japan. The Ai River (located in Setsu city, Kinki district), in particular, was found to be most heavily contaminated with PFOA when compared with other reported values in the world so far. In this study, we investigated with bioconcentration factors of PFOS and PFOA in the turtles in the Ai River. Because the turtle is located in the highest rank on the food-chain in the river, this species is considered suitable for predicting biomagnifications of these chemicals through food-chain. *Trachemys scripta elegans* turtles (N=46) and *Chinemys reevesii* turtles (N=51) were captured with baited traps in the Ai River, its branch and other uncontaminated sites from September to October 2003 and from April to June 2004. Surface water samples were collected simultaneously from each site when we caught turtles. Blood samples were collected by heart puncture through the plastron. PFOS and PFOA concentrations were measured by LC/MS. Concentrations of PFOS in serum ranged from 2.4 to 486 ( $\mu$ g/L) and those in the water ranged from 2.9 to 37 ( $\mu$ g/L). The geometric mean (GM) (geometric standard deviation: GSD) of the PFOS bioconcentration factors (BCFs: Serum concentration divided by corresponding surface water concentration) was 10964 (2.5). These data indicate that PFOS is bioconcentrated at 10,000 times in the body of turtle. In contrast, the water level of PFOA ranged from 16.7 to 87100 ( $\mu$ g/L) and the serum level ranged from 0.2 to 870 ( $\mu$ g/L). The GM (GSD) of BCFs of PFOA was 3.7(7.9). Furthermore BCFs of PFOA decreased as contaminations of PFOA increased. Although the chemical structures of PFOS and PFOA are almost the same, PFOS is preferentially bioconcentrated in biota while PFOA is not.

## 2069

### ANALYTICAL METHOD VALIDATION FOR QUANTITATION OF 1, 2, 3, 4, 6, 7-HEXACHLORONAPHTHALENE FROM RAT ADIPOSE TISSUE.

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The compound 1, 2, 3, 4, 6, 7-hexachloronaphthalene (PCN 66) has been used as a dielectric fluid in capacitors, transformers, and cables. Because of its extensive use, PCN 66 has been selected for toxicological evaluation by the NIEHS, Environmental Toxicology Program (ETP). Method development/validation was performed for PCN 66 in rat adipose tissue to support an ETP reproductive toxicology/toxicokinetics study. The method used gas chromatography with micro-electron capture detection (GC/ $\mu$ ECD). A nine-point spiked matrix curve was prepared at a PCN 66 concentration range of  $\sim$  20 to  $\sim$  4000 ng/g in matrix or  $\sim$  2.2 to  $\sim$  450 ng/mL in hexane. Each spiked tissue was extracted with 1.5 mL of hexane, then a 500- $\mu$ L aliquot of the hexane layer and 100  $\mu$ L of isodrin dissolved in hexane (internal standard solution) were transferred to a vial for analysis. The spiked matrix and corresponding solvent curves were found to be linear, with correlation coefficients  $\geq$  0.999; accurate, with recoveries ranging from 96.2 to 101.9 percent of the expected spiked concentration and 77.6 to 89.5 percent relative to the corresponding solvent standards; precise, with percent RSD for n=6 preparations of 6.0 percent at the low concentration. For PCN 66 in rat adipose tissue, the estimated LOD for the method was 0.24 ng/g, the estimated LOQ was 0.80 ng/g, and the ELOQ was 19.335 ng/g. An analysis period stability study was conducted at three concentrations,  $\sim$  219 ng/mL,  $\sim$  36 ng/mL, and  $\sim$  4 ng/mL PCN 66 in rat adipose tissue. A sample from each concentration and a blank were stored under ambient, refrigerated ( $\sim$  5 °C) and freezer ( $\sim$  -20 °C with 3 freeze/thaw cycles) conditions. The percent recoveries were: Ambient, 90.3 to 94.5 percent; Refrigerated, 91.1 to 96.5 percent; Freezer, 88.8 to 93.0 percent (high concentration), 90.3 to 97.0 percent (middle concentration), and 121.4 to 124.2 percent (low concentration). PCN was not detected in any of the blanks.

## 2070

### 14-WEEK COMPARATIVE STUDY OF 1, 2, 3, 4, 6, 7-HEXACHLORONAPHTHALENE (PCN 66) AND 1, 2, 3, 5, 6, 7-HEXACHLORONAPHTHALENE (PCN 67) IN FEMALE HARLAN SPRAGUE-DAWLEY (HSD) AND FISCHER 344 (F344) RATS.

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The purpose of the study was to compare the toxicity of PCN 66 and PCN 67 in female HSD and F344 rats in order to determine whether the responses are strain dependent. Formulations of PCN 66 or PCN 67 in corn oil:acetone (99:1) were administered by gavage (10 rats/group) at dosages of 0, 1000, 10, 000, 50, 000, 100, 000 and 200, 000 ng/kg for 14 weeks. Two F344 rats receiving 200, 000 ng/kg PCN 66 died before scheduled sacrifice. Mean terminal body weights were 36% lower than vehicle controls in F344 rats receiving 200, 000 ng/kg PCN 66. Body weights for the other PCN 66 groups and all PCN 67 groups were generally

within 5-10% of vehicle controls. Thyroxine ( $T_4$ ) levels were decreased in HSD rats receiving  $\geq 1000$  ng/kg PCN 66 and F344 rats receiving  $\geq 50,000$  ng/kg PCN 66. Triiodothyronine ( $T_3$ ) was decreased 44% from vehicle controls in F344 rats receiving 200,000 ng/kg PCN 66. Thyroid stimulating hormone (TSH) was increased in both strains receiving  $\geq 100,000$  ng/kg PCN 66. Administration of PCN 67 to HSD rats resulted in decreased  $T_4$  and increased TSH. Only  $T_4$  was decreased in F344 rats receiving 200,000 ng/kg PCN 67. Liver weights were increased and thymus weights were decreased in both strains receiving PCN 66 or PCN 67. Hepatocellular hypertrophy, thymic atrophy and lesions in the lymph nodes were observed in both strains receiving PCN 66 or PCN 67. Forestomach epithelial hyperkeratosis was observed in both strains receiving 200,000 ng/kg PCN 66. F344 rats in this dose group also had lesions in the blood vessels, heart and ovary. Increased hepatic EROD, A-4-H and pulmonary EROD activities were observed for both strains receiving PCN 66 or PCN 67. In conclusion, PCN 66 had greater toxicity in both strains relative to equivalent dosages of PCN 67. PCN administration generally resulted in similar target organs but the HSD rat often developed lesions at lower dosages compared to the F344 rat.

## 2071 SOURCES AND DISTRIBUTION OF POLYCYCLIC AROMATIC HYDROCARBONS IN NEW ORLEANS AND DETROIT SOILS.

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Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous organic pollutants that have been found at elevated concentrations in urban environments such as air particulates, soils, and sediments. Incomplete combustion of petroleum products and coal has been identified as the primary source of high concentrations of PAHs in cities. The purpose of the study was 1) to determine and compare the concentration of PAHs in soils taken from two major US cities: New Orleans and Detroit, and 2) to examine the main sources of PAHs in urban soils by diagnostic PAH ratios. A total of 103 Detroit soil samples were taken from 6 different census tracts with 16-19 samples each per census tract. Similarly, some 107 New Orleans soil samples were analyzed for comparison, also from 6 census tracts. The mean total PAH concentration of Detroit soils was measured to be 7843 g/kg, compared to 5100 g/kg for New Orleans soils. Several diagnostic PAH concentration ratios were calculated for source determination, such as phenanthrene/[phenanthrene+anthracene] ratio (0.81 for Detroit and 0.87 for New Orleans), fluoranthene/(fluoranthene+pyrene) ratio (0.56 for Detroit and 0.55 for New Orleans), and indeno (1, 2, 3-cd) pyrene/[indeno (1, 2, 3-cd) pyrene+benzo (g, h, i) perylene] ratio (0.68 for Detroit and 0.64 for New Orleans). The ratios indicate that elevated PAH concentrations found in urban soils are attributable to contamination by pyrolytic sources, mainly automobile exhaust.

## 2072 DIOXINS AND DIOXIN LIKE COMPOUNDS FROM AGENT ORANGE AND OTHER SOURCES IN VIETNAM FROM THE 1970S TO THE PRESENT.

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The 2, 3, 7, 8-TCDD contaminant of Agent Orange (2, 4-D and 2, 4, 5-T phenoxyherbicides) which was heavily sprayed in parts of the south of Vietnam during wartime as a defoliant during 1962-71 was found in Vietnamese humans, wildlife and food from the 1970s to the present (2004). Levels as high at 1, 850 ppt lipid were found in milk collected in 1970 from heavily sprayed villages and as high as 400+ in blood in 2002. Very high levels were found in fish and other food in 1970s and 2004, the latter demonstrating new contamination 30-40 years after initial dioxin contamination. Elevated TCDD, as high as 1, 100, 000 ppt was found in some soil and elevation of sediment was found also. The 12 plus regions in Vietnam identified to date with elevated TCDD primarily from Agent Orange are referred to as Agent Orange hot spots. Marked variation in human and food TCDD levels are found in the same geographical areas documenting the usefulness of gc-ms analyses compared to Agent Orange spray records alone for exposure assessment. In most of Vietnam, especially in the non-sprayed north, no elevated TCDD from Agent Orange has been found. But areas with PCBs, polychlorinated dibenzofurans, HCH, and HCB in food or humans have been found. These chemicals and others such as organophosphates used in agriculture, complicate human health studies on the effects of Agent Orange (dioxin) on humans in Vietnam. New hot spots with elevated blood TCDD, Tra Noc and Binh My, will be compared to others previously discovered.

## 2073 DEVELOPMENT OF FLORIDA-SPECIFIC RISK-BASED SOIL AND GROUNDWATER CLEANUP TARGETS FOR VOLATILIZATION OF CHEMICALS INTO INDOOR AIR.

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Volatile chemicals beneath buildings can diffuse through soil air-spaces and enter building through foundation cracks. The vapor intrusion pathway accounts for the migration of chemicals from subsurface soils and groundwater into overlying buildings. Vapor intrusion is influenced by factors that vary regionally, such as soil moisture content, depth to groundwater, soil temperature, and soil type. Florida may pose a higher concern for vapor intrusion as the State has many conditions favorable for higher vapor intrusion rates (shallow groundwater table, warm average groundwater temperature, and sandy soils). We report the development of soil and groundwater levels protective of the vapor intrusion pathway for chemicals on the Florida's cleanup targets levels (CTLs) list that meet a primary screening criterion for potential volatility and toxicity specified by the USEPA (i.e., Henry's Law constants  $>1E-5$  and vapor concentration of pure phase chemical that poses excess lifetime cancer risk  $>1E-6$  or a hazard quotient  $>1.0$ ). The EPA's version of the Johnson and Ettinger Model was used to evaluate the vapor intrusion pathway. Florida-specific inputs were developed from a soils database for soil bulk density, moisture content, and soil hydraulic conductivity. State-specific information on groundwater table depth, soil temperature, and building characteristics were also used. Our analysis demonstrates that several of chemicals evaluated have CTLs based on the vapor intrusion pathway that are significantly lower than the default residential CTLs for soil and groundwater based on direct exposure. The difference in CTLs is even more significant when vapor intrusion CTLs are compared to Florida's natural attention values, which are based on 10- or 100-fold multipliers of the default CTLs. Vapor intrusion may be an important consideration when contemplating cleanup of contaminated soils beneath buildings at sites in Florida.

## 2074 A ROBUST ALGORITHM FOR CALCULATING OPTIMAL 95% UCLS ON THE MEAN FOR ENVIRONMENTAL DATASETS.

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The upper 95% confidence limit on the mean (95% UCL) is often used as an upper-bound estimate of average contaminant concentration for risk assessments at hazardous waste sites. Numerous statistical methods are available for the calculation of 95% UCLs, however, the choice of the most appropriate statistical test to use in specific situations is not straightforward. Also complicating the decision are sample data that contain a number of values below the detection limit. In the present analysis, we have systematically evaluated the performance of several 95% UCL and censoring techniques using simulated distributions with known population parameters. The UCL methods evaluated were: central limit theorem (CLT), standard bootstrap, bootstrap-t, Students-t, H-statistic (Land's method), MVUE and MLE based, and Chebyshev's methods. The effects of left censoring were evaluated by replacement, parameter estimation, bounding and regression techniques. For each method, 95% UCLs were calculated for simulated datasets of different sample sizes, skewness, and extent of left censoring. By comparing the 95% UCLs with the underlying simulation expected value, the coverage of each technique (i.e., the proportion of times the true population mean lies at or below the 95% UCL) was evaluated. The extent to which the true mean was overestimated by the 95% UCL for each technique was also determined. Based on the results of this analysis, a software tool (FLUCL - Florida UCL Calculator) was created that includes a decision matrix that uses information on sample size, skewness, and degree of censoring to select the optimal UCL technique for site-specific datasets.

## 2075 THE EFFECT OF USING MULTIPLE CONTAMINANT 95% UCLS ON CUMMULATIVE RISK ESTIMATES.

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Soil risk assessment guidance from the USEPA and many States recommend the calculation of exposure point concentrations (EPCs) for chemicals of concern based on 95% upper confidence limits (95% UCLs) of the average concentrations within specified exposure units. These EPCs are used to calculate cancer and non-cancer risk estimates for each chemical of concern, which are summed to provide a cumulative excess lifetime cancer risk for carcinogens and a cumulative hazard index (HI) for non-carcinogens for each receptor. A potential flaw in this methodology is that 95% UCLs are not strictly additive in this fashion. As a result, this practice may yield cumulative risk and hazard estimates that are more conservative than the regulatory risk goals (i.e., 1E-6 or HI of 1.0) when multiple chemicals of concern are

present. Similarly, some states require that default risk-based cleanup criteria that are individually based on a defined risk target (i.e., 1E-6), be apportioned (i.e., lowered) when multiple chemicals of concern are present, such that the cumulative risk and/or hazard do not exceed the defined targets. We investigated this scenario by analyzing the effect of combining 95% UCLs from various synthetic lognormally distributed datasets of sizes from 10 to 30 with levels of correlation among the data of 0, 25, 50, and 75%. The simulations (10,000 per run) were performed in Crystal Ball for combinations of 1 to 10 chemicals. The input chemical concentrations were adjusted so that the expected risk levels for each combination of chemicals were held constant. Results indicate that combining upper confidence limits, even for highly correlated data, introduces additional conservatism in the final risk estimates. Similar issues exist for the summation of risks calculated from cancer slope factors, which are also upper percentile estimates. These observations suggest that methodology for summing chemical-specific risks should account for the number of chemicals present and the degree of correlation among the chemical concentrations.

## 2076

### EVALUATION OF THE PREDICTIVITY OF A FISH UPTAKE MODEL FOR MERCURY USING EMPIRICAL DATA.

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An ecological risk assessment was conducted for a former industrial facility located on a coastal estuary where mercury associated with historical industrial operations was released to environmental media including surface water and sediment. The viability of finfishes utilizing the estuarine system was identified as an assessment endpoint for the ERA. The body burden of methyl mercury in Red Drum (*Sciaenops ocellatus*) was identified as a specific measurement endpoint. During the field investigation, no Red Drum were collected from areas of the estuary most impacted by mercury contamination. As a result, regulators required a modeling approach based on the "Lavaca Bay" model (Evans and Engel, 1994) to address this measurement endpoint. This model can be used to estimate the body burden of methyl mercury in Red Drum based on concentrations of total mercury in sediment and surface water. The primary pathway for methylmercury exposure in the model is transfer and bioaccumulation in the aquatic food web. The model calculates a bioaccumulation factor (BAF) that represents the ratio of the uptake of methyl mercury from prey and its reduction via excretion and growth. This BAF is multiplied by the methyl mercury concentration in the diet to yield a concentration of methyl mercury in the Red Drum. For application at this site, the original model was modified so that the modeled concentrations of methyl mercury in prey items were replaced by measured concentrations of methylmercury in killifish, fiddler crabs, and blue crabs collected from the site. The following year Red Drum, Black Drum, and several other species of finfishes were collected and analyzed for methyl mercury. Measured concentrations of methyl mercury in the tissues of red and black drum were approximately 3 to 5 fold lower than modeled estimates using measured prey concentrations. The same measured concentrations were approximately 20 to 35-fold lower than the modeled estimates based on total mercury concentrations in sediment and surface water.

## 2077

### RETROSPECTIVE SCREENING-LEVEL HUMAN HEALTH RISK ASSESSMENT FOR RECREATIONAL EXPOSURE TO CONTAMINATED SEDIMENT.

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A retrospective screening-level human health risk assessment (SL-HHRA) was conducted to evaluate past exposure and assess potential risk to sensitive receptors from recreational exposure to contaminated sediment in a medium sized river. The purpose of this SL-HHRA was to determine if there are any potential human health concerns resulting from past or present exposure to contaminated sediment at this site. The SL-HHRA did not address future conditions as remediation activities are planned that will disrupt viable exposure pathways. The area of interest is characterized by two large floating docks projecting away from the shoreline into an inner harbour region of the river. These docks are primarily used for boat access by rowers associated with a local rowing club. Club members are generally adult and teen rowers, but also include youth aged 10 and over. Swimming and other types of recreational water activities (e.g., wading, fishing) are rare. Contaminants of concern (COCs) were identified by comparing maximum concentrations measured in the sediment to generic soil screening guidelines. PCBs, Sb, As, Be, Fe, Pb, and benzo(a)pyrene were identified as COCs. Exposure to COCs was estimated for model receptors, representing avid club members and summer youth camp members, for the entire period during which the rowing club has been in operation (26

years). Dermal contact with contaminated sediment and incidental ingestion of sediment suspended in water were identified as relevant exposure pathways. Potential health effects were determined by comparing the predicted intake of COCs to appropriate toxicity reference values developed by health-based regulatory agencies for oral exposure (e.g., USEPA, RIVM). Dermal intake was evaluated by correcting for relative absorption via the dermal route. Predicted exposure to COCs from dermal contact and ingestion of contaminated sediment was relatively low and unlikely to result in any adverse health effects (HQ < 1; negligible excess cancer risk).

## 2078

### PROVISIONAL REFERENCE DOSE FOR THE AROMATIC FRACTION OF JET FUEL: REASSESSMENT OF FRACTION RISK AND IMPACT ON RISK BASED CLEANUP LEVELS.

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In 1993, the Total Petroleum Hydrocarbon Criteria Working Group (TPHCWG) began developing a practical alternative to non-risk based cleanup standards for petroleum hydrocarbon contaminated sites. The TPHCWG defined all petroleum mixtures as thirteen effective carbon (EC) number range fractions based on expected environmental transport characteristics. EC numbers are derived from relative n-alkane gas chromatograph retention times. By 1997, the TPHCWG had developed preliminary toxicity criteria for each fraction using all available data, prioritizing mixture toxicity information. However, acceptance of such fraction-based approaches for petroleum risk assessment was hampered by the general lack of toxicity data, especially for some fractions. Specifically, few toxicity data were available for the EC<sub>>8</sub> - EC<sub>16</sub> aromatic fraction, which is likely one of the more toxic fractions. To address this data gap, a 90-day oral gavage toxicity study was conducted in female Sprague-Dawley rats and male C57BL/6 mice to characterize the potential toxic effects of the EC<sub>>8</sub> - EC<sub>16</sub> aromatic fraction of Jet Fuel A. Animals were dosed at 0, 20, 100 and 500 mg fraction/kg daily. The no observed adverse effect level (NOAEL) for the mixture was 20 mg/kg/day, based on blood chemistry and behavioral changes at middle and high dose groups in both rats and mice, and enlarged livers in the high dose groups of rats. This NOAEL was used as the point of departure for estimating a preliminary reference dose of 0.02 mg/kg/day for the fraction. When considered with the weight of evidence of toxicity information for individual compounds and mixtures in the fraction, this fraction study and resulting preliminary reference dose help strengthen confidence in the database for the EC<sub>>8</sub> - EC<sub>16</sub> aromatic fraction. This reanalysis should provide a greater acceptance of Tier 1 risk based cleanup levels at petroleum hydrocarbon contaminated sites.

## 2079

### DESIGNATION OF NAPHTHALENE AS A CARCINOGEN: RISK ASSESSMENT FOR INHALATION EXPOSURE PATHWAYS AT HAZARDOUS WASTE SITES.

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California EPA has designated naphthalene as a carcinogenic toxic air contaminant with a unit risk factor of 3.4 E-5 (ug/m<sup>3</sup>)-1. Naphthalene is a common pollutant in urban outdoor air. It is found in a number of products, notably diesel and other petroleum-based fuels. We present here a comparison of the impacts of naphthalene and benzene at a hazardous waste site (Site A) in California, where shallow groundwater is contaminated with wastes from refining petroleum and producing manufactured gas. Groundwater at 6-8 ft below ground surface contains benzene and naphthalene at concentrations up to 300 and 3,000 ug/L, respectively. The Johnson & Ettinger vapor intrusion screening model, using groundwater as a source and assuming a vadose zone of coarse sandy soil, predicts indoor air concentrations of 30 ug/m<sup>3</sup> for benzene and 17.4 ug/m<sup>3</sup> for naphthalene. The somewhat lower modeled mobility of naphthalene into indoor air is due to its lower vapor pressure and water solubility, compared to benzene. The Cal/EPA unit risk factor for benzene is 2.9 5 (ug/m<sup>3</sup>)-1, similar to that of naphthalene. Thus, concentrations of naphthalene ten times those of benzene in shallow groundwater yield similar estimates of cancer risk in indoor air in a residential setting (3.6 E-4 vs. 2.4 E-4). The USEPA Reference Concentration (RFC) for benzene is ten times higher than that of naphthalene (30 ug/m<sup>3</sup> vs. 3 ug/m<sup>3</sup>), which yields hazard quotients for non-carcinogenic toxicity of 1 for benzene and 6 for naphthalene for exposures to modeled concentrations in indoor air above Site A. Although naphthalene is less easily mobilized than benzene into indoor air than monoaromatic compounds such as benzene, the relatively high unit risk factor and RFC for naphthalene will probably increase its significance as a groundwater contaminant.

## INORGANIC CHEMICALS IN GROUND WATER AND SOIL: BACKGROUND CONCENTRATIONS AT CALIFORNIA AIR FORCE BASES.

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Inorganic chemicals have widespread industrial use and are significant contaminants. Risk assessment and risk management must differentiate between background (naturally occurring) and anthropogenic inorganic chemicals. This is important for site characterization, determining chemicals of concern, establishing cleanup levels, and long-term monitoring programs. This paper is an update of our 2001 report on background. The Air Force ERPIMS database was searched for uncontaminated sample locations for soil and ground water at 12 Air Force installations in 10 California counties. Background data for 27 inorganic constituents from 1300 monitoring well locations yielded as many as 4890 ground water samples for individual chemicals, while 3850 boreholes yielded as many as 12,000 soil samples. Medians, 95th, and 99th percentiles are reported for each chemical. Since statistical analysis of soil data indicated that background levels differed significantly with depth, separate background calculations for soil are presented for three depths (less than 3 feet, between 3 and 15 feet, and greater than 15 feet). For groundwater, background statistics for each constituent are given without regard to sampling depth. Some inorganic constituents were detected frequently and at levels that exceed important environmental thresholds such as Maximum Contaminant Levels (MCLs) or Action Levels for drinking water. Background 95th percentile levels equal or exceed federal and/or California MCLs for aluminum, antimony, arsenic, cadmium, chromium, and nickel. The 95th percentile level for lead exceeds the Action Level of 0.015 mg/L for drinking water measured at the tap. This analysis provides background levels that are representative of California Air Force Bases as a group. The background data in this presentation should not be used to replace local background data, but rather provide important benchmarks by which the adequacy of local data can be judged.

## SCREENING FOR POTENTIAL HEALTH RISKS FOLLOWING ACUTE EXPOSURE TO HEAVY METALS IN SOIL AT A FORMER MINING SITE.

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A screening-level health risk assessment (SLHRA) was conducted to evaluate potential health risks to visitors acutely exposed to inorganic chemicals in soil at a former mining site. While SLHRAs are generally used to indicate the absence of potential health problems, in the present study this model was used 1) to qualify the likelihood of potential health risks and 2) to support the development of risk management strategies without resorting to a comprehensive and resource-intensive site-specific health risk assessment. Since soil concentrations of various contaminants were substantially higher than current soil guidelines, the SLHRA was conducted using the contaminant presenting the greatest potential for acute health effects. Arsenic (As) was selected based on the highest "soil concentration to soil guideline" ratios at most sampling locations. Two acute reference doses were derived for As in order to assess potential acute health effects to the general population (based on acute human poisoning data) and to pregnant women (based on animal embryotoxicity data). Central tendency (CT) and reasonable maximum exposure (RME) scenarios were developed for receptors of various age groups and pregnant women. Hazard Indexes (HI) were then computed for various As soil concentrations. The SLHRA identified potential effects from acute exposure to As at most sampling locations (RME: HI=1-10 for soil As of 20-200 ppm; CT: HI=1-10 for soil As of 100-1000 ppm). Pregnant women and toddlers (7 mo. - 4 yr.) were identified as the most sensitive receptors. Incidental ingestion and dermal contact were the most relevant exposure routes/pathways. Although numerous uncertainties are generally associated with screening approaches, integration of additional parameters in the current study provides a reasonable degree of confidence that acute health effects would be experienced by visitors at the former mining site, lending support to the development of risk management strategies.

## RISK ASSESSMENT FOR IRON: USING THE INSTITUTE OF MEDICINE'S TOLERABLE UPPER INTAKE LEVEL AS A SURROGATE TOXICITY VALUE FOR IRON.

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The USEPA has not prepared an iron toxicity value for risk assessment in the Integrated Risk Information System (IRIS). Iron is a frequent cause of acute pediatric poisonings caused by overdoses of iron supplements. Chronic health effects from iron are less well established, but may include an increased risk of cardiovas-

cular disease. As an essential nutrient, iron risks must be assessed for both sufficiency and toxicity. Based on an analysis of NHANES III, iron deficiency affects 10-20% of US reproductive-aged women, with deficiencies twice as severe in blacks and Mexican-Americans compared with white, non-Hispanics. In setting the dietary reference intakes for iron, the Institute of Medicine (IOM) has examined the nutritional needs and toxicity of iron and has derived age, gender, and life-stage specific recommended daily allowances (RDAs) and tolerable upper intake level (ULs). The IOM defines a UL as "The highest average daily nutrient intake level that is likely to pose no risk of adverse health effects to almost all individuals in the general population." The adult UL of 45 mg per day was based on gastro-intestinal upset following daily administration of a 60 mg iron fumarate supplement, plus 10 mg from diet, divided by an uncertainty factor of 1.5. In the same study, heme iron supplements caused GI symptoms similar to the placebo controls. The UL is the basis for a risk assessment dose of 0.6 mg/kg-day. This dose meets the RDA for iron for all groups (RDA range is: 0.1-0.6 mg/kg-day) and protects against acute gastrointestinal symptoms. An intake of 0.6 mg/kg-day corresponds approximately to the 50<sup>th</sup> percentile children aged 1-5 and the 95<sup>th</sup> percentile of adults based on an analysis of NHANES III and FDA TDS. This dose may not be protective for individuals suffering from congenital iron metabolism disorders, such as hemochromatosis. This recommendation is consistent with the USEPA toxicity hierarchy which recommends using publicly available, peer-reviewed studies if an IRIS value is not available.

## DETERMINING SOIL REMEDIAL ACTION CRITERIA FOR ACUTE EFFECTS: THE CHALLENGE OF COPPER.

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Soil in the vicinity of copper smelters may contain copper at concentrations that are sufficiently elevated to pose a potential health risk, particularly to young children. Nausea, the primary acute effect of copper, paradoxically occurs at lower exposure levels than hepatotoxicity, the primary chronic effect. Therefore, our aim was to develop a method to determine a soil remedial action criterion (RAC) for copper that would protect against nausea for children living nearby a copper smelter. Nausea due to copper ingestion appears to be primarily a function of copper concentration in the stomach, and generally subsides within an hour. We used Monte Carlo analysis to generate a distribution of hourly RACs for copper based on the amount of soil ingested per hour, the volume of liquid and food in the stomach, and site-specific bioaccessibility. Using this distribution, we identified RACs associated with a high probability of not having nausea in an hour. However, it is more relevant to determine a RAC protective of nausea for a longer time period, such as a year. We therefore generated a new distribution of daily RACs, selected as the minimum hourly RAC constrained by total daily soil ingestion for each day. We then used combinatoric analysis to identify a percentile of the distribution of daily RACs, and the associated RAC, that would result in a high probability of having a minimal number of nausea episodes in a year. Selection of the actual RAC is a risk management decision, based on the number of nausea episodes considered acceptable, and the desired probability that individuals would not exceed that number of episodes. For example, considering the reversibility and relatively mild nature of nausea, it might be acceptable to set a cleanup level based on a 95% probability of having fewer than 10 episodes of nausea per year. Assuming that a child would be exposed to soil for 6 months of the year, as would be typical in a cold climate, our analysis indicates a copper concentration of 4,700 mg/kg would result in a 95% probability of having fewer than 10 episodes of nausea per year.

## PERCUTANEOUS ABSORPTION OF ARSENIC FROM ENVIRONMENTAL MEDIA.

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Current knowledge of percutaneous absorption of arsenic is based on studies of Rhesus monkeys using soluble arsenic in aqueous solution, and soluble arsenic mixed with soil (Wester et al. 1993). These studies produced mean dermal absorption rates in the range of 2.0% to 6.4% of the applied dose. Subsequently, questions arose as to whether these results represent arsenic absorption from environmental media. Factors such as chemical interactions, the presence of other metals, and the effects of weathering on environmental media all can affect the nature of arsenic and its potential for percutaneous absorption. This *in vivo* study used the monkey model and methods similar to those of Wester et al. to assess dermal arsenic absorption from environmental media; specifically, environmental soil, and residue on the surface of wood treated with chromated copper arsenate (CCA). Study design considerations included particle size, application rates, means of ensuring skin contact, and appropriate statistical evaluation of the data. This study also included an appli-

cation of arsenic in solution to allow calibration of the model against prior research and to compare to absorption of arsenic from the environmental media. The potential for background exposure to arsenic in the diet possibly obscuring a signal from a dermally applied dose of arsenic was also addressed. Results indicate that the urinary arsenic excretion levels in the animals exposed to arsenic in soil or CCA residue are not elevated above background. Conversely, urinary arsenic excretion in animals exposed to soluble arsenic in solution is significantly greater than background, and greater than the soil and residue exposure groups. We conclude that percutaneous absorption of arsenic from environmental media is negligible compared to soluble arsenic and soluble arsenic mixed with soil. This study is consistent with previous work and suggests that dermal arsenic absorption is not a risk factor for the media tested.

**2085**

### THE ROLE OF METHYLATED METABOLITES IN INORGANIC ARSENIC-INDUCED CANCER: A SYNTHESIS OF INFORMATION FROM *IN VITRO* AND HUMAN BIOMONITORING STUDIES.

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The precise mechanisms by which inorganic arsenic induces cancer in humans are not well established. As part of efforts to identify a carcinogenic moiety for inorganic arsenic, recent research has focused on the methylated metabolites of inorganic arsenic. Pentavalent methylated metabolites of inorganic arsenic, monomethylarsonic acid (MMAV) and dimethylarsinic acid (DMAV), are relatively non-toxic. Recent *in vitro* research suggests that methylated trivalent arsenic metabolites (i.e., monomethylarsonous acid [MMAIII] and dimethylarsinous acid [DMAIII]) induce comparable or greater cytotoxicity than inorganic arsenic, and may be indirect-acting genotoxins. Using available literature, we analyzed the potential role for methylated arsenic compounds in inorganic arsenic human cancers. This analysis considered results from human epidemiological and biomonitoring studies as well as results from *in vitro* studies. Our evaluation revealed that relatively high concentrations of urinary DMAV (compared to urinary MMAV) were associated with lower disease incidence in populations exposed to inorganic arsenic in drinking water. Due to limitations in study designs and uncertainties in analytical methods for the trivalent metabolites, it is difficult to draw a firm conclusion regarding the relationship between levels of trivalent methylated metabolites in urine and cancer. *In vitro* studies conducted in human and animal cell lines do not support an association between methylation capacity and inorganic arsenic-induced cytotoxicity and transformation. For example, UROtsa cells, a human urothelial cell line that lacks the capacity to methylate arsenic, transforms after incubation with inorganic arsenic. In addition, inorganic arsenic, but not methylated arsenic compounds, induce carcinogenic changes in human keratinocytes and osteosarcoma cells. Our analysis suggests that while the trivalent methylated arsenic compounds are of toxicological interest, there is sufficient evidence to conclude that these compounds do not fully explain the carcinogenicity of inorganic arsenic.

**2086**

### TOXICOLOGICAL EVALUATION OF 1, 1, 1-TRICHLOROETHANE AND RESULTING MINIMAL RISK LEVELS.

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1, 1, 1-Trichloroethane has been used in many industrial and household applications. Although regulatory actions are in place for an eventual phase-out, production was as high as 500 million pounds in 2002. The central nervous system (CNS) is the most sensitive target for trichloroethane toxicity. Clinical signs of toxicity following high-level exposures include CNS depression, hypotension, cardiac arrhythmia, diarrhea, vomiting, mild hepatic effects, and dermal and ocular irritation. Lower-dose exposures result in more subtle neurological effects such as impaired performance in tests designed to measure variables like manual dexterity, eye-hand coordination, perceptual speed, and reaction time. An acute-duration (? 14 days) inhalation minimal risk level (MRL) of 2 parts per million (ppm) was derived from a study by Mackay et al. (1987) that identified reduced psychomotor performance in humans exposed to 175 ppm for 3.5 hours. Observed performance changes correlated with 1, 1, 1-trichloroethane blood levels. A review of the intermediate-duration (15-364 days) database resulted in the selection of a gerbil study (Rosengren et al. 1985) for MRL development. Rosengren et al. (1985) observed astrogliosis in gerbils exposed to 210 and 1, 000 ppm; an inhalation MRL of 0.7 ppm was derived based on the no-observed-adverse-effect level of 70 ppm. The database for oral exposure to 1, 1, 1-trichloroethane is more limited. A National Toxicology Program feeding study was used to derive an intermediate-duration oral MRL of 20 mg/kg/day for decreased final body weight in B6C3F1 mice exposed for 13 weeks. While body weight is not an optimal endpoint, it is supported by a number of

other studies. These MRLs are also presented and discussed in the Agency for Toxic Substances and Disease Registry's toxicological profile for 1, 1, 1-trichloroethane that was released for public comment in October 2004.

**2087**

### PEAK EXPOSURE AND KINETICS OF TOLUENE IN MAN INCLUDING PBTK MODELLING EVALUATION.

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Occupational exposure to VOC (volatile organic compounds) is often characterised by alternating periods of low and high concentrations (even up to tenfold the TWA-TLV value). Exposure peaks may be reflected in dosimetry and in hazardous effects of VOC. If so, both should be taken into account in determination of a STEL (short-term exposure limit). To assess this, male volunteers were exposed by inhalation to 40 ppm toluene constant (Netherlands TWA-TLV) for 4 h (CONS) and to three peaks of 110 ppm for 0.5 h within 4 h (PEAK), such as that the external dose was similar for both exposure scenarios. Blood toluene concentrations (BTOL) and urinary *o*-cresol excretion rates (OCR) as well as some neurobehavioral parameters were measured. PBTK (physiologically based toxicokinetic) modelling was assessed as to the potential to predict effects of peaks and interindividual variation on dosimetry and as potential tool for incorporating these in STEL setting methodology. In CONS, BTOL increased with time whereas in PEAK, external peaks were reflected by fluctuating BTOL, the highest BTOL being measured at the end of the third peak. Both exhibited washout in approximately 2 h. The average  $AUC_{app}$  of toluene in blood was rather similar in both scenarios, the average  $C_{max}$  value was about 50% higher in PEAK. OCR were substantially higher in PEAK only in the urine excreted directly after exposure, not during exposure. In addition, more than 2-fold and 5-fold interindividual differences in BTOL and OCR were observed, respectively. Some neurobehavioral differences were observed between PEAK and CONS, but their toxicological significance is doubted. Average BTOL were well predicted by PBTK simulations. The model appeared mainly sensitive to changes in  $V_{max}$  and  $K_M$  as well to changes in blood flow to fat and liver. Their heterogeneity in the human population is often known and can be modelled easily. Conclusively, PBTK modelling may be a useful tool in evaluating the value of the toxicokinetic component of the intraspecies uncertainty factor as well as in improved, data-based determination of a STEL.

**2088**

### A META-ANALYSIS OF NEUROBEHAVIORAL DEFICITS FROM LONG-TERM EXPOSURE TO STYRENE.

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Long-term exposure to styrene is associated with a variety of effects on the nervous system in individual reports from the scientific literature. We have pooled the suitable data from these reports and conducted a meta-analysis in order to evaluate dose-effect relationships across the body of available information. Exposure levels were estimated from a meta-analysis of information relating the concentration of urinary biomarkers to air styrene concentrations. Neurobehavioral outcomes providing a sufficient number of studies for evaluation included simple reaction time (SRT), choice reaction time (CRT) and color perception expressed as the color confusion index (CCI). The data from individual reports were pooled into a single database for each endpoint. The outcome measurements were transformed to a common metric (percent of baseline). Dose-effect relationships were modeled with linear least-squares equations relating styrene exposure to each outcome variable. Statistically-significant relationships were observed between cumulative styrene exposure (expressed as ppm-years) and increased CRT, and also between cumulative styrene exposure and increased CCI. Eight work-years of exposure to 20 ppm styrene was estimated to produce a 6.5% increase in CRT, a slowing of reaction time which has been shown to significantly increase the probability of automobile accidents. The same exposure history was predicted to produce an increase in CCI equivalent to 1.7 additional years of age in men. (This abstract does not necessarily reflect EPA policy).

**2089**

### ROUTE-DEPENDENT EFFECTS OF TOLUENE ON SIGNAL DETECTION BEHAVIOR IN RATS.

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The acute effects of toluene and other solvents on behavior are thought to depend upon their concentration in the brain. We have shown previously that inhaled toluene and trichloroethylene disrupt sustained attention in rats as assessed with a

visual signal detection task (SDT). To test the hypothesis that acute toxicity of solvents is mediated exclusively by their internal dose, and to develop a dosing regimen not requiring inhalation equipment, we explored the efficacy of toluene given by the oral route on this task. Toluene was dissolved in corn oil and administered to 25 adult male Long-Evans rats by gavage at doses of 0, 400, 800, 1200, and 1600 mg/kg in two separate studies. Two rats in each study failed to work at the highest dose; toluene did not significantly affect performance of the other rats at any dose. Internal doses of toluene (concentrations in blood and brain) at the times of behavioral assessment were estimated for both exposure routes. Concentrations of toluene in the blood after oral dosing were estimated from published data in the literature, and concentrations in blood and brain during inhalation were estimated using a physiologically-based toxicokinetic (PBTK) model developed in our laboratory. Whereas the effects of inhaled toluene on the SDT (accuracy of signal detection and response time) were monotonically related to the concentration of toluene in blood and in brain, SDT performance was not affected by oral toluene, despite nearly-complete overlap in the range of internal doses produced by the two routes of administration (10 to 50  $\mu$ g/mL in blood). These observations indicate that the momentary concentration of toluene in the brain is an insufficient dose metric for explaining its effects on signal detection behavior, and complicate route extrapolations based on this dose metric. Hypotheses regarding these route-dependent effects are explored in an accompanying poster (Samsam et al.) [This abstract of a proposed presentation does not reflect EPA policy.]

## 2090 WHY DO THE ACUTE BEHAVIORAL EFFECTS OF TOLUENE IN RATS DEPEND ON THE ROUTE OF EXPOSURE?

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Despite evidence suggesting that the acute effects of organic solvents are related to their concentration in the brain, we have observed route-dependent differences in the acute behavioral effects of toluene. Whereas inhaled toluene disrupts the performance of rats on a visual signal detection task (SDT) that assesses sustained attention, toluene given orally in corn oil does not, even with similar concentrations in the blood (see Bushnell et al.). Several studies in adult male Long-Evans rats were conducted to determine the source of these route differences. Exp. 1 showed that the time course of the rise in toluene concentrations in blood and brain of rats inhaling 2000 ppm toluene (an effective treatment) matched that of rats given 800 mg/kg of toluene in corn oil by gavage (an ineffective treatment). Further experiments used rats previously trained to perform the SDT. Exp. 2 showed that the pre-test exposure interval (10 m vs. 30 m) did not account for the route difference, and Exp. 3 showed that co-administration of oral corn oil and inhaled toluene did not reduce the effect of toluene. Exp. 4 showed that oral administration of sodium benzoate, a toluene metabolite, did not reduce the effect of inhaled toluene. Exp. 5 showed that oral trichloroethylene (TCE) in corn oil (1200 mg/kg), which yields concentrations of TCE in blood of 10 to 30  $\mu$ g/mL (Prout et al., 1985), caused slight impairment on the SDT, relative to previous observations of robust effects of inhaled TCE with blood concentrations of 50 to 125  $\mu$ g/mL. Exp. 6 examined the effects of oral toluene given in a lipophilic but non-absorbable sucrose-esterified fatty acid complex (SEFA soyate). Further experiments will look at the role of physical activity on the kinetics of inhaled and orally-delivered toluene and on the generality of this route dependence across functional endpoints. These findings address the appropriateness of route-route extrapolations in setting reference toxicity values. [This abstract of a proposed presentation does not necessarily reflect EPA policy.]

## 2091 ASSESSING THE IMPORTANCE OF THE BEHAVIORAL EFFECT OF ACUTE EXPOSURE TO TOLUENE IN HUMANS.

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There is increasing interest in being able to evaluate potential benefit-cost relationships of controlling exposure to toxic substances. Behavioral effects of acute toluene exposure could be subjected to benefit-cost analysis if its effects were quantitatively compared to those of ethanol ingestion, which have already been so analyzed. Behavioral effects of inhaled toluene and ingested ethanol were quantified by meta-analysis of studies from the peer-reviewed literature describing their effects on choice reaction time (reaction time in a test requiring a subject to choose among two or more alternatives before responding). The internal doses of these compounds were estimated by a general physiological and toxicokinetic simulation from exposure parameters provided in the reports. The reported effects were converted to a common metric (proportion of baseline) and related to the estimated internal doses of toluene and ethanol, from which dose-effect equations were fitted. Next, a dose-equivalence equation was derived to express, within confidence limits, the dose of toluene as an equivalent dose of ethanol on the basis of equal magnitude

of effect. Finally, a nomogram was constructed to relate the inhaled concentration of toluene to equivalent effects of ethanol internal doses. The resulting function can be used to estimate the monetary value of behavioral deficits caused by a range of exposures to toluene using existing information about monetary consequences of ethanol ingestion. (This abstract does not reflect EPA policy.)

## 2092 CARDIOVASCULAR AND THERMOREGULATORY RESPONSE TO ORAL TOLUENE IN THE RAT.

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Toluene and other volatile organic compounds have often been shown to affect behavior in animals when given by inhalation, and less effective when given orally. Previous work showed that toluene increased heart rate (HR) and motor activity (MA), and reduced core temperature (Tc) in rats inhaling toluene for 1 hour while performing an operant task. It is important to assess the effect of oral toluene on autonomic and motor conditions under resting conditions. To this end, the effects of oral toluene on HR, Tc, and MA in resting, unrestrained rats were determined. Adult rats (male, Long-Evans) were surgically implanted with radiotransmitters to monitor HR, Tc, and MA. Toluene was administered by gavage in corn oil at doses of 0, 0.4, 0.8, and 1.2 g/kg at 11:30 hr while the telemetry data were monitored over 48 hours at 22 °C. Dosing with corn oil led to transient elevations in HR, Tc, and MA, a response attributed to the stress of handling, that recovered within 2 hr. All doses of toluene led to transient increases in HR that exceeded the control response during the first 0.5 hr after dosing. The higher doses of toluene elevated HR for more than 6 hr after dosing. HR remained elevated above controls by approximately 25 and 50 b/min in the 0.8 and 1.2 g/kg groups, respectively. Tc increased above controls for several hours after 0.8 g/kg toluene. There was a transient reduction in Tc below controls following 1.2 g/kg toluene during the first hour followed by a progressive elevation for 4 hr after dosing. MA of the 0.8 and 1.2 g/kg groups increased transiently above controls then recovered. There was a secondary rise in MA in the 1.2 g/kg group persisting for several hours after dosing. Toluene at doses of 0.8 and 1.2 g/kg induced a marked tachycardia, hyperactivity, and hyperthermia. These effects developed in spite of a lack of effect of the same oral doses of toluene on operant behavior. It will be important to compare oral and inhaled toluene exposure in rats maintained under rest and working conditions. This abstract does not reflect EPA policy.

## 2093 BENCHMARK DOSE ANALYSIS BASED ON STYRENE NEUROBEHAVIORAL META-DATA.

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There are a substantial number of research articles on the neurological effects of styrene (CAS 100-42-5) in humans. A meta-analysis of human neurological endpoints was undertaken and is described more fully elsewhere (Benignus et al., Environmental Health Perspectives, In Press; Boyes et al., Toxicologist, 2005); however, this meta-analysis did not directly provide a point-of-departure (POD) suitable for developing a reference concentration (RfC). Of the endpoints considered for analysis, three were judged to have adequate dose-response data on which an analysis could be performed: (1) color confusion index (CCI), (2) simple reaction time (SRT) and (3) choice reaction time (CRT). The analysis described here demonstrates an approach to using data from several studies on a single endpoint in deriving a POD that may be appropriate for the development of an RfC. The Benchmark Dose Software (BMDS, beta version 1.4) was used for that purpose on meta-data obtained from the previously described study. The lower-bound confidence limit of the Benchmark Dose (BMDL) was derived separately for two of the three neurological endpoints included in the meta-analysis (CCI, and CRT), using several approaches for each endpoint; the effect of styrene on SRT did not show a dose-related response and was therefore not evaluated further. The resulting BMDL values were then judged for appropriateness as the POD for deriving an RfC. The results indicate that either endpoint (CCI or CRT) provides similar POD values, and are supportive of the other as the basis for derivation of an RfC. The derivation of a POD using meta-data is rare but not unique in the IRIS database. (This abstract does not necessarily reflect EPA policy.)

## 2094 ACETYLCHOLINESTERASE AS THE CRITICAL EFFECT WHEN ESTIMATING THE SAFE DOSE FOR CHLORPYRIFOS?

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Chlorpyrifos is an irreversible inhibitor of cholinesterase (ChE), and inhibition of ChE is believed to be the most sensitive effect in all animal species evaluated and in humans. Recent epidemiology studies reported associations between umbilical

cord plasma chlорpyrifos levels and fetal birth weight decreases among minority women living in New York City during pregnancy. These associations raise questions whether impaired fetal development is the critical effect rather than the inhibition of ChE as is currently believed so. We analyze the available information from epidemiology studies and animal studies in order to identify the relative sensitivity of decreased birth weight and inhibition of ChE from exposure to chlорpyrifos. We find that the positive associations from some epidemiology studies are different from other epidemiology investigations. Moreover, a direct comparison of experimental animal neonatal information shows that cholinesterase inhibition is a more sensitive indicator of adverse effect than reduced body weight, and that neonates are equally, or perhaps less sensitive to cholinesterase inhibition than their maternal parent. We also briefly discuss items relevant to the establishment of a safe dose, such as the choice of appropriate species and study and selection of uncertainty factors.

## 2095

### CHARACTERIZATION OF DELTAMETHRIN (DLT) METABOLISM IN ADULT MALE SPRAGUE-DAWLEY RATS.

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DLT, a widely-used type II pyrethroid, is a relatively potent neurotoxicant. While the toxicity of DLT is extensively examined, studies of its toxicokinetics are limited. It is necessary to characterize the major detoxification pathways, as well as their relative importance, in order to construct a physiologically-based toxicokinetic model for DLT. The aim of this study was to characterize the enzymes responsible for DLT metabolism by measuring DLT disappearance following incubation of various concentrations of DLT (2 to 400  $\mu$ M) in plasma (esterases) and liver microsomes (esterases and CYP450s) prepared from 90-day-old male Sprague-Dawley rats (n=4). In plasma, isoOMPA, a known inhibitor of carboxylesterases (CaE), completely inhibited DLT metabolism at low concentrations (2 and 20  $\mu$ M, higher than peak *in vivo* blood levels in intoxicated rats). For CaE-mediated DLT metabolism in plasma,  $V_{max}$ = 275 $\pm$ 53 nmoles/h/ml and  $K_m$ =140 $\pm$ 34  $\mu$ M. In liver microsomes (without CYP450 cofactors), DLT metabolism was completely inhibited by isoOMPA, confirming the role of CaE in DLT metabolism in the liver;  $V_{max}$ =2567 $\pm$ 265 nmoles/h/g liver and  $K_m$ = 244 $\pm$ 22  $\mu$ M. The metabolic rate constants for liver microsomal CYP450-mediated metabolism of DLT were  $V_{max}$ =2040 $\pm$ 120 nmoles/h/ liver and  $K_m$  = 84 $\pm$ 4  $\mu$ M. While plasma and liver CaEs have similar affinity, the liver CaE has higher metabolic capacity. Liver microsomal P450 rate constants, however, show  $V_{max}$  levels similar to the liver CaE and higher than plasma CaE, but the affinity for DLT is greater than liver CaE and similar to plasma CaE. Therefore, the intrinsic clearance ( $V_{max}/K_m$ ) of the liver CaE is higher than plasma CaE; and the intrinsic clearance of liver CYP450 metabolism is greater than either liver or plasma CaE (Supported by EPA STAR Grant R830800). This is an abstract of a proposed presentation and does not imply Agency policy.

## 2096

### RISK OF TERATOGENICITY FROM TRICHLOROETHYLENE (TCE) AND DICHLOROETHYLENE (DCE) IN DRINKING WATER.

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Formal assessment of risk requires hazard identification, dose-response and exposure assessments, and risk characterization. "Hazard" in the USA refers to intrinsic toxic properties and "risk" refers to the probability of an adverse outcome. Without hazard, there is no risk. TCE and DCE are sometimes found in drinking water as well as occupational settings where the exposure is primarily by inhalation and secondarily via the skin. Epidemiologic studies of environmental or occupational exposure to TCE do not find an increase in developmental effects in humans. Experimental studies from multiple laboratories, employing many routes of exposure in mice, rats, and rabbits, find no adverse reproductive effects in the absence of maternal toxicity and no teratogenicity even in the presence of maternal toxicity. Only one group of investigators claims exposure to TCE causes developmental toxicity, specifically cardiac malformations. A single epidemiologic study from this group is sometimes cited as demonstrating that TCE in drinking water caused an increase in congenital malformations. Although the authors' confirmed their a priori impression that the prevalence of cardiac defects was elevated, no causal relationship between contamination of water and cardiac defects can be inferred due to inadequacy of exposure data and weaknesses of study design, many of them noted by the authors themselves. This same group published animal studies contending that TCE and DCE are specific cardiac teratogens, producing no other adverse de-

velopmental response. Part of what appears to be replication of studies actually is republication and the remainder rely on inappropriate 1) control groups, 2) experimental models (chick embryo), 3) dosing (*in utero*), and 4) over-diagnosis of variations as malformations. Furthermore, an independent laboratory using that group's microdissection technique found no treatment-related increase in cardiac malformations. Based on a review of these studies, the weight of evidence is that TCE and DCE are not teratogenic and are not specific cardiac teratogens.

## 2097

### DOES TRICHLOROETHYLENE CONTRIBUTE TO CONGENITAL HEART DEFECTS?

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Trichloroethylene (TCE) is an organic solvent currently used as a metal degreasing agent. TCE is also a common contaminant in the water supply, with levels often exceeding the EPA-defined maximum contaminant level of 5 ppb. Therefore, it is important to identify any risks to human health associated with TCE. Several epidemiological studies have reported links between TCE exposure during pregnancy and health problems including, but not limited to, congenital heart defects. Unfortunately, due in part to the complications involved with conducting retrospective epidemiological studies, several confounding factors make the results difficult to interpret, and epidemiological reports of the pre-natal risks of TCE have been contradictory. Similarly, the results of animal studies examining the potential of TCE to elicit cardiac anomalies have been inconsistent, and have often been performed at doses far exceeding levels found in the water. Nonetheless, the public perception in contaminated areas is that TCE causes heart defects. To determine specifically what is known about the relationship between TCE to the incidence of heart defects, a comprehensive analysis of all available epidemiological data and animal studies was performed. Hill's causality criteria were applied to the epidemiology data. Additionally, *in vivo* and *in vitro* studies examining possible mechanisms of action for TCE were evaluated. In the case of *in vitro* studies, a pharmacokinetic model was used to determine the equivalent *in vivo* doses. Taking all aforementioned data into consideration, there are no indications of a causal link between TCE exposure during pregnancy and heart defects. As a final evaluation, the heart defects reported in animal and human epidemiology studies as being associated with TCE were categorized by the morphogenetic process responsible for the defect. The analysis revealed no single mechanism as being predominantly affected by TCE, thereby providing no support for a likely risk of congenital heart malformation due to TCE exposure during gestation.

## 2098

### HUMAN HEALTH RISK ASSESSMENT FOR 4-CHLORO-1, 2-BENZENEDIAMINE.

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A risk assessment to determine acceptable levels of 4-chloro-1, 2-benzenediamine in drinking water was conducted according to Annex A of NSF International/American National Standards Institute (NSF/ANSI) Standard 60/61. No human data were identified in the scientific literature. Hepatic focal hyperplasia, renal pyelonephritis, and urinary bladder tumors and precursor effects were observed in rats at statistically increased incidences compared to controls after dietary administration of 4-chloro-1, 2-benzenediamine for 78 weeks. Uterine/endometrial hyperplasia was observed in female mice at an increased incidence compared to controls after chronic dietary administration of 4-chloro-1, 2-benzenediamine for 78 weeks, and combined hepatocellular adenomas and carcinomas were observed in male and female mice at statistically increased incidences compared to controls. The weight of evidence suggests that 4-chloro-1, 2-benzenediamine is genotoxic *in vivo* and *in vitro*. Based on the increased tumor incidences in rats and mice fed 4-chloro-1, 2-benzenediamine for two years, and its positive genotoxicity data, the weight of evidence supports that 4-chloro-1, 2-benzenediamine is likely to be carcinogenic to humans. Thus, a 10-5 cancer risk level was extrapolated from the chronic feeding BMCL10 of 45 mg/kg-day, which was based on the combined incidence of urinary bladder tumors and precursors in rats. A unit risk of  $6.3 \times 10^{-8}$  (mg/L) $^{-1}$  was calculated from the slope factor using the default 70 kg body weight and 2 L/day drinking water consumption of an adult. Thus, drinking water containing 16 mg/L of 4-chloro-1, 2-benzenediamine consumed for a lifetime was estimated to result in development of 1 excess tumor per 1,000,000 people. The long-term exposure levels derived include a Total Allowable Concentration of 0.2 mg/L, and a Single Product Allowable Concentration of 0.02 mg/L. A higher Short-Term Exposure Level was not derived, since 4-chloro-1, 2-benzenediamine was assumed to be a linear carcinogen.

## ADVERSE EFFECTS OF CYANOBACTERIAL PEPTIDES ON HUMAN HEALTH.

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Cyanobacteria (blue-green algae) occur in almost all fresh- or brackish waters worldwide. Certain species of these microorganisms are capable of producing a variety of non-ribosomally synthesized peptides, including the microcystins (MCs). These toxins have caused mortality in animals and are suspected to be responsible for illness in humans. The major health problems with MCs are most likely those associated with chronic intoxications with low MC concentrations via drinking water. However, the consumption of "blue-green algae food supplements" (BGAS), especially those produced from *Aphanizomenon flos-aquae*, is becoming common in the industrialized countries. Four studies on the MC-concentrations of BGAS have been carried out in the last six years. The MC amounts found varied from 0-35,000 ng/g DW. In the study presented here, BGAS from the European market contained distinctly more than 1000 ng MC-LR equivalents/g DW (determined by anti-ADD-ELISA and colorimetric proteinphosphatase assay). In the past years, a broad variety of other cyanobacterial peptides were discovered, suggesting that not only MCs may have an adverse impact on human health. To determine the potential cytotoxicity, peptides of some of these cyanobacterial peptide families were incubated with the HepG2 and the CaCo2 cell line and primary rat hepatocytes. Cell viability was assessed by the MTT-reduction and LDH release assay after various incubation times and peptide concentrations. Preliminary results suggest an effect in primary rat hepatocytes, whereas no effect was observed in continuous cell lines. In light of the high degree of MC contamination of BGAS, the known variability in BGAS consumption, the known risk of contamination of drinking water with low levels of MCs and the currently incalculable toxicological potential of other cyanobacterial peptides, the guideline values for MC and other toxic peptides should be critically reviewed and revised. Furthermore, the data strongly suggest that an increased routine control of water bodies and food supplements for toxin contamination is advisable.

## EXAMINING POTENCY FOR DEVELOPMENT OF THE PRELIMINARY CONTAMINANT CANDIDATE LIST (PRE-CCL).

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The 1996 Safe Drinking Water Act requires EPA to develop a list of contaminants with potential for regulation on a five-year cycle, and to make regulatory determinations from each list three years after its publication. EPA requested assistance from the National Research Council (NRC) of the National Academy of Sciences (NAS) and a Work Group from the National Drinking Water Advisory Council (NDWAC) in providing recommendations for developing the CCL. The NRC recommendations were published in 2001 and those from the NDWAC Work Group in 2004. Both groups supported a framework in which a Pre-CCL is initially selected from universe of contaminants of concern using screening data on health effects and potential occurrence in drinking water. The CCL is chosen from the Pre-CCL using additional data on attributes for health effects and occurrence. In order to screen contaminants from the Universe for inclusion on the Pre-CCL, the NDWAC Work Group recommended that EPA use measured or QSAR-generated LOAELs, LD50s, and cancer classifications as measures of potency. In conjunction with this recommendation, USEPA assembled a screening data set that included 825 measured and/or modeled LOAEL values, 1, 549 measured and/or modeled LD50 values and the cancer classifications for 745 contaminants. This data set was used to evaluate a variety of Pre-CCL health effects screening approaches. As part of the evaluation, USEPA compared measured with modeled data for LOAEL and LD50 values and examined the impact of a variety of selection criteria on whether or not individual contaminants would be included or excluded from the Pre-CCL based on health effects data. The inclusion of data-rich, regulated contaminants in the data set provided an opportunity to evaluate the effectiveness of various screening criteria in capturing contaminants of known health concern. [The opinions expressed are those of the authors and do not necessarily reflect the opinions of the USEPA.]

## CLASS-BASED DRINKING WATER ACTION LEVEL FOR ALKYL SUBSTITUTED NAPHTHALENES.

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Mono-, Di-, Tri-, and Tetra-C1-C4 alkyl substituted naphthalenes extract into potable water from pipe and tank coatings. When such products are evaluated to a national health effects standard for use with drinking water, chemicals extracting

from these products must be below appropriate action levels. As defined by Standard 61, a Total Allowable Concentration (TAC) is the maximum concentration of a nonregulated contaminant allowed in a public drinking water supply, and the Single Product Allowable Concentration (SPAC) is 10% of the TAC. C1-C4 alkyl substituted naphthalenes comprise a class of chemicals that feature a naphthyl ring with alkyl side chain substitutions, where the substituted group(s) can be any alkyl chain from 1 to 4 carbons. Data on these chemicals comprise genotoxicity, carcinogenicity, and pharmacokinetic data. These data indicate that the lung is a target organ. Genotoxicity assays are consistently negative and indicate that alkyl substituted naphthalenes pose a small risk of genotoxicity. The effects of chronic ingestion of 1- and 2-methylnaphthalene have been evaluated in B6C3F1 mice. These studies identified dose-related pulmonary alveolar proteinosis. Toxicity data on this class of compounds indicates that the smallest chemical (i.e., 2-methylnaphthalene) is the most toxic, and can be considered worst-case. The USEPA derived a reference dose (RfD) of 0.004 mg/kg-day for 2-methylnaphthalene, which was used to evaluate the health hazards of C1-C4 alkyl substituted naphthalenes. The RfD is based on a chronic mouse study that identified dose-related, significant increases in pulmonary alveolar proteinosis. A TAC of 30 µg/L for this class of chemicals was derived by multiplying the USEPA 2-methylnaphthalene RfD by a default 70 kg body weight, a default daily water consumption (2L/Day), and a default 20% relative source contribution factor. Because empirical data relating to the number of drinking water sources containing C1-C4 alkyl substituted naphthalenes were not identified, the SPAC was set at 10% of the TAC (3 µg/L).

## COMPARATIVE RISK ASSESSMENT OF MULTIMEDIA EXPOSURE TO PERCHLORATE AND OTHER AGENTS THAT INHIBIT IODIDE UPTAKE INTO THE THYROID GLAND.

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Interest has increased in how the thyroid gland might be affected by environmental exposure to perchlorate, which is increasingly being detected in drinking water sources throughout the United States. In addition, several organizations have recently begun to collect data on perchlorate concentrations in a wide variety of food and beverage items, including produce and milk. Perchlorate acts through inhibition of iodide uptake at the sodium-iodide symporter (NIS). This effect is an essential precursor to any other thyroid effects that could occur at much higher doses, including effects on thyroid hormone production. Of particular concern is whether perchlorate could be present during pregnancy at sufficient doses to cause adverse effects to the fetus, especially the nervous system. To address health concerns, a number of agencies, including USEPA, have proposed drinking water guidelines for perchlorate that are well below the threshold level at which perchlorate first begins to cause iodide uptake inhibition (IUI) into the thyroid gland. However, typical American diets and other common sources contain other natural and anthropogenically-added agents, including nitrate and thiocyanate, that act through the same mechanism of action as perchlorate. While these agents are less potent IUI agents than perchlorate, their concentrations in typical diets are substantially greater. To estimate the relative contribution of perchlorate, thiocyanate, and nitrate to total IUI, daily intake rates of these agents were calculated using reported media concentrations, with average daily intake rates from USDA. After adjusting for the relative potency of each agent using *in vitro* and *in vivo* bioassay data, the total IUI contributed by perchlorate is estimated to be a very small fraction of that contributed by other agents. These comparisons suggest that IUI is a mundane, nonadverse event, and indicate a contradiction with assertions that levels of perchlorate currently being detected in the environment pose a health risk.

## HUMAN HEALTH RISK ASSESSMENT FOR THIOCYANATE.

J. Russell, C. McLellan, G. Ball and V. Bhat. *NSF International, Ann Arbor, MI*. Sponsor: M. Dourous.

A risk assessment to determine acceptable levels of thiocyanate in drinking water was conducted according to Annex A of NSF International/American National Standards Institute (NSF/ANSI) Standard 60/61. Data on thiocyanate toxicity were identified in humans and laboratory animals. Thyroid effects were seen in some patients taking therapeutic doses of thiocyanate, but death was also observed without reported thyroid effects. Although most of the animal studies focused on thyroid effects, the doses used were higher than those causing deaths in humans, making them unsuitable for human health risk assessment. A chronic study found an increased incidence of liver tumors, but not thyroid tumors, in rats administered thiocyanate in drinking water, but a subsequent study at higher doses found no increase in liver tumors. No genotoxicity data were located for thiocyanate. The key study followed 246 patients on thiocyanate therapy for hypertension for 2-10 years. Doses ranged from 0.2-0.6 g/day, with the standard dose starting at 0.3 g/day and adjusted depending on therapeutic effectiveness and blood thiocyanate levels. The

LOAEL was considered 0.3 g/day (or 4.3 mg/kg-day), based on transient muscle fatigue, nausea, and vomiting in some patients on initiation of thiocyanate therapy, and lowering of blood pressure in humans taking thiocyanate for hypertension. A total uncertainty factor of 100x was applied to the LOAEL to determine the reference dose (RfD) of 0.04 mg/kg-day. The 100x uncertainty factor included 3x each for intraspecies and LOAEL to NOAEL extrapolation, study duration, and database deficiencies. There is some uncertainty in this risk assessment, because the mechanism of action of thiocyanate-induced toxicity is not fully determined. The long-term exposure levels derived include a Total Allowable Concentration of 0.2 mg/L, and a Single Product Allowable Concentration of 0.02 mg/L. A Short-Term Exposure Level of 0.9 mg/L was also derived. These drinking water levels should be applied as a total for the sum of sodium, potassium, and ammonium salts. Other thiocyanates require separate risk assessments.

## 2104 DERIVATION OF A DRINKING WATER ACTION LEVEL FOR TRIBUTYL PHOSPHATE.

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Tributyl phosphate (TBP) is used as a plasticizer in the manufacturing of cellulose esters, lacquers, plastics, and vinyl resins that come into contact with potable drinking water. When such products are evaluated to a national health effects standard for use with drinking water, any chemical extracting from these products must be below an appropriate action level. As defined by Standard 61, a Total allowable Concentration (TAC) is the maximum concentration of a nonregulated contaminant allowed in a public drinking water supply, and the default Single Product Allowable Concentration (SPAC) is 10% of the TAC. In order to determine an action level for TBP, a comprehensive health effects evaluation was performed. There are no epidemiological data indicating that occupational exposure to TBP results in adverse human health effects other than headache, nausea, and symptoms of skin, eye, and mucous membrane irritation. TBP is not mutagenic nor does it promote chromosomal aberrations in cytogenetic assays performed *in vitro* or *in vivo*. A two-year dietary study in male and female Sprague-Dawley rats identified dose-related increases in the incidence of urinary bladder hyperplasia and neoplastic lesions (comprising urinary bladder papillomas and transitional cells carcinomas) in rats. Two subchronic studies have confirmed the linkage between TBP treatment and the induction of urinary bladder hyperplasia in rats. A review of the toxicological dataset for TBP indicates that urinary bladder hyperplasia and neoplastic lesions are the most sensitive health effects endpoints upon which to base a TAC and SPAC. A multistage model was used to extrapolate to low-dose TBP exposure due to an unknown mode of action for TBP. A TAC of 30 µg/L was derived for TBP, and is based upon an LED10 of 9.35241 mg/kg/day. A SPAC of 3 µg/L was derived by multiplying the TAC by 0.10. These action levels are based upon the most sensitive health effects endpoint, as well as current risk assessment methodologies.

## 2105 CONSIDERATION OF SENSITIVE POPULATIONS FOR RISK ASSESSMENT OF CHEMICALS IN DRINKING WATER.

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In consideration of special sensitive populations, public health goals (PHGs) for chemicals in drinking water were developed for five chemicals based on infants or children as the sensitive population; these include copper (170 ppb), fluoride (1 ppm), nitrate (45 ppm), nitrite (10 ppm) and oxamyl (50 ppb). Three are based on female/pregnant women; these are dinoseb (14 ppb), methoxychlor (30 ppb) and perchlorate (6 ppb), the last of which also include the fetus and infants as sensitive subgroups. The risk assessment procedure evaluates cancer and non-cancer endpoints, animal and human data, and specific parameters for infants, children and pregnant women, such as relevant body weight and water intake rate for these groups, where appropriate. Recent passage of AB 2342 requires new risk assessments that involve infants and children to assess the following based on available information: 1) The possibility of disproportionately high exposure of infants and children relative to the general population, such as may occur in the case of bottle-fed infants. 2) Special susceptibility of infants and children to the contaminant relative to the general population. 3) The effects on infants and children of the contaminant and other substances that have similar effects. 4) The interaction of multiple contaminants on infants and children. For the above chemicals, the possible biologic basis for sensitivity is explored, and oral reference values of 0.21 mg/day (total), 0.25 mg/kg-d, 0.01 mg/kg-d, .005 mg/kg-d, and .000037 mg/kg-d were developed for copper, oxamyl, dinoseb, methoxychlor and perchlorate, respectively, for the specific subpopulations. The reference values for nitrate, nitrite and fluoride are the same as the PHGs, based on the concentrations in drinking water that produced no adverse effects involving infants and children, and no further modifications were made to adjust the values.

## 2106 INHALATION EXPOSURE TO NONVOLATILE CHEMICALS DURING SHOWERING D M MANGANARO M S HUTCHESON T ZEWDIE, MASSACHUSETTS DEPARTMENT OF ENVIRONMENTAL PROTECTION, BOSTON MA, USA.

T. Zewdie, D. M. Manganaro and M. S. Hutcheson, *Office of Research and Standards, Massachusetts Department of Environmental Protection, Boston, MA*.

The literature is replete with data on risk assessment of inhalation exposures of volatile chemicals. The typical scenario assumes that volatile chemicals that are present in heated water used for showering volatilize and are inhaled by the individual taking that shower. Risk assessors have historically assumed that inhalation exposure to non-volatile chemicals does not occur. In the last decade, however, a number of studies have emerged in the literature that address the potential for inhalation of aerosols generated by the shower spray. The major topics discussed in these studies include mechanisms of aerosol formation, removal, shifts in size distribution, and amounts of respirable aerosol particles formed and inhaled during showering. Inhaled aerosols are most frequently of various sizes and deposit in the different regions of the respiratory tract based on their sizes. In general a large number of large water droplets (>10 µm) and small aerosols (<10 µm) are produced from shower spray. Aerosols less than 7 µm may reach the gas exchange (alveolar) region and, therefore, contribute significantly to inhalation exposure. The respirable aerosol concentrations generated during showering and the dose delivered through inhalation exposure can be determined from these studies. Based on the above information, this report will identify circumstances under which showering inhalation exposures to nonvolatile chemicals can be of concern. Included will be water concentration limits to protect from inhalation exposures for a variety of nonvolatile chemicals.

## 2107 MEASUREMENT OF AIR CONCENTRATION OF D CIS/TRANS ALLETHRIN GENERATED BY A MOSQUITO REPELLENT LAMP IN AN OUTDOOR ENVIRONMENT.

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OFF!® Mosquito Lamps were introduced in the market as an outdoor mosquito area repellent product providing up to 4 hours of protection for a 4.5 m x 4.5 m area downwind from the lamp. This product contains a mat impregnated with the active d cis/trans allethrin (355 mg) placed at the top of the lamp using a candle as the heat source to volatize the active. This study determined the air concentration of active that would be generated as a result of using the OFF!® Mosquito Lamp in an outdoor location of a private residence, simulating actual use conditions. Air samples were collected from 12 locations at three different heights downwind from a lit lamp on a table. A fan was placed on the opposite side of the sample tubes to direct the active emission towards the collection tubes. Samples were collected during the life of the repellent mat (4 hours) and for 2 hours afterwards (post burn). This scenario was to simulate potential high exposure scenario for a child or adult during typical activities, e.g. sitting, standing, playing. The results of the study clearly showed that air concentrations around a mosquito repellent lamp placed outdoors is very low with most of the values below the LOQ (0.2 ng/l of air). Residues of allethrin were detected downwind from the lamp along the wind vector at a distance of 1 m (max. active air conc. 0.9 ng/l) and 2 m (max. active air conc. 0.7 ng/l). Active residues were detected only during the product life phase and not for the post burn phase. This exposure information was used in a product risk assessment demonstrating large safety margins from product use thus far exceeding minimum safety standards.

## 2108 MECHANOTRANSDUCTION IN PARTICLE TOXICOLOGY.

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Exposure to fiber-like particulate materials has been associated with lung injury; however, the mechanisms involved are not fully understood. It has been proposed that as a particle transverses from an isotropic material to an acicular one, the toxic response resulting from exposure to that material increases. These effects are thought to manifest from a mechanical aggravation of the cells (poking, rubbing, etc.) and/or difficulty in phagocytosis (clearance hindrance, *in vivo*). *In vitro* studies investigating the toxicity of particulate systems in the presence of cyclic mechanical motion have been limited, and these issues ultimately remain unsolved. We have initiated a program investigating the impact of mechanotransduction on particle toxicity. Well-characterized fiber-like (amorphous silica nanorods and asbestos fibers), platy (Talc, Bryan Co.) and isotropic (amorphous silica spheres) have been

exposed to normal mesothelial cells (MET-5A; ATCC, Manassas, VA) at concentrations ranging from 25 to 250 mg/cm<sup>2</sup>. To modulate particle-integrin interactions, the identical exposures were performed with the exception that the particles of interest were either preconditioned with fibronectin or coated with polyethylene glycol (PEG) groups to respectively enhance and mitigate integrin interactions. The cells were incubated for 24hrs at prescribed dosages and pretreatment in Bio-Flex plates under a constant strain of 5% at 1 Hz (Flexercell Strain Unit FX-4000T, FlexCell\* International). Particle cytotoxicity and IL-8 release were monitored via LDH release (Roche Diagnostics) and ELISA (Quantikine). Cyclic stretching in the absence of particles was not shown to lead to a significant increase in LDH or IL-8 release. Preliminary evidence suggests that cell death and IL-8 release increase in the presence of particles with cyclic mechanical cell stretching and that this may be further augmented with enhanced particle-integrin interactions.

**2109**

#### A QUANTITATIVE CANCER RISK ASSESSMENT FOR AIRBORNE ASBESTOS GENERATED BY VEHICULAR TRAFFIC ON A ROAD PAVED WITH ASBESTOS-CONTAINING SERPENTINE ROCK.

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The California Department of Toxic Substances Control (DTSC) performed a roadside asbestos air monitoring study at Garden Valley (El Dorado County), CA in 2002-2003. The study was designed to address DTSC's concern regarding potential exposure of communities to airborne asbestos fibers resulting from vehicular traffic on unpaved roadways known to contain asbestos. Within Garden Valley, the study site was Slodusty Road, an unpaved road which was surfaced with asbestos-containing serpentine rock. Air samples were collected at distances ranging from 5 to 300 feet away from the edge of the road, on both sides of the road. Two traffic scenarios were utilized: 1) 30 vehicles/hour (vph) driven at a speed of 25 miles per hour (mph); 2) 10 vph at 10 mph. Airborne asbestos was detectable by the air monitor farthest from the road in both the 30 vph/25 mph and the 10 vph/10 mph test scenarios (190 and 130 feet, respectively; asbestos concentrations were  $2.5 \times 10^2$  -  $3.0 \times 10^4$  and  $2.9 \times 10^1$  -  $3.1 \times 10^3$  PCM-equivalent fibers/m<sup>3</sup>, respectively). Time-adjusted airborne asbestos cancer risk estimates for lifetime exposure using those two scenarios ranged from  $2.7 \times 10^{-4}$  to  $3.2 \times 10^{-2}$  and  $3.1 \times 10^{-5}$  to  $3.3 \times 10^{-3}$ , respectively. These data suggest that the airborne asbestos cancer risks for the two vehicle frequency and speed scenarios probably bound the high-end estimated cancer risks to Slodusty Road residents. Airborne asbestos concentrations were resampled after the road was resurfaced to remove serpentine aggregate as a potential asbestos source. Time-adjusted airborne asbestos cancer risks using the 10 vph/10 mph and 30 vph/25 mph scenarios ranged from  $5.3 \times 10^{-6}$  to  $1.5 \times 10^{-4}$  and  $1.3 \times 10^{-5}$  to  $2.9 \times 10^{-4}$ , respectively. The cancer risks associated with the postresurfacing 10 vph/10 mph and 30 vph/25 mph scenarios were 1-2 and 1-3 orders of magnitude less than those of the corresponding preresurfacing scenarios, respectively.

**2110**

#### IMPROVED DOSIMETRIC ADJUSTMENT FACTORS FOR INTERSPECIES EXTRAPOLATION OF INHALED, POORLY SOLUBLE PARTICLES.

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Risk assessment of inhalation exposures to inhaled poorly soluble particles (PSP) frequently relies on the extrapolation of dose-response data obtained from toxicological studies in laboratory animals to human exposure scenarios. Accurate extrapolation requires dosimetry adjustment to account for differences in delivered dose due to differences in airway geometry and ventilation. Default approaches currently rely on empirical models that describe average particle deposition for each lung region. A default duration adjustment is also typically applied to extrapolate the experimental regimen to an equivalent, continuous human exposure scenario. Mechanistic models that account for anatomical and physiological determinants of the spatial heterogeneity explicitly address time-dependent parameters and delivered dose and are expected to increase the accuracy of dose-response analysis and interspecies extrapolation. Airflow distribution models based on lung compliance and airway resistance in humans and uniform lobar expansion in rats were used in a multiple-path, particle dosimetry model (MPPD) (CIIT Centers for Health Research, Research Triangle Park, NC) to calculate particle deposition, clearance, and retention in rats and humans. Realistic asymmetric lung geometries based on airway morphometric measurements were used in the calculations. Human equivalent concentration (HEC) estimates were obtained for various plausible dose met-

rics of PSP toxicity. Each metric was constructed as an expression of an internal dose measure (e.g., particle number) and a normalizing factor (e.g., number of alveolar macrophages). In general, those based on mass per unit area yielded the highest resultant HEC estimates, while a dose metric based on retained mass led to the lowest value of HEC, dependent on particle size. Results obtained from this study will aid in interspecies data extrapolation of data on inhaled PSP and improve the accuracy of health risk assessments.

**2111**

#### DEVELOPMENT OF A CHRONIC INHALATION REFERENCE EXPOSURE LEVEL FOR RESPIRABLE CRYSTALLINE SILICA.

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Chronic crystalline silica exposure to workers can result in silicosis, characterized by histologically unique silicotic nodules and by fibrotic scarring of the lung. In California silica is a chemical subject to the Air Toxics Hot Spots Act. We have calculated an inhalation Chronic Reference Exposure Level (cREL) for silica in humans, a level below which no adverse effects would be expected, using a Benchmark Concentration approach with epidemiological data from miners. In the key study Hnizdo and Sluis-Cremer (Am J Ind Med. 24(4):447-57, 1993) investigated silicosis risk retrospectively in a cohort of 2, 235 white male South African gold miners, of whom 313 (14%) developed radiographic signs of silicosis at an average age of 55.9 years. From the data on silicosis incidence in miners versus cumulative silica exposure, we estimated a NOAEL of 600 ( $\mu\text{g}/\text{m}^3$ )-years silica and a  $\text{BMC}_{01}$  of 636 ( $\mu\text{g}/\text{m}^3$ )-yr silica. After time-adjusting the  $\text{BMC}_{01}$  from occupational to equivalent continuous exposure (210  $\mu\text{g}/\text{m}^3$ -yr), dividing by the 24 years of mean occupational exposure, and applying an intraspecies uncertainty factor of 3, a cREL of 3  $\mu\text{g}/\text{m}^3$  for respirable silica (as defined occupationally) was obtained. Data from cohorts of South Dakota gold miners and Chinese tin miners gave similar results. Strengths of the cREL include: the availability of several long-term studies of inhalation in workers at varying exposure concentrations; adequate histopathological and radiologic analysis; adequate follow-up; a dose-response effect in several studies; observation of a NOAEL in the key study; and the power of the key study to detect a small effect. Uncertainties include the general underestimation of silicosis by radiography alone (with resulting higher, less health-protective cREL estimates) and the uncertainties in exposure estimation, especially when reconstructing historical levels of exposure.

**2112**

#### EFFECT OF ARSENIC AND DIELDRIN ON FEMALE RATS: PRELIMINARY DOSE RANGE STUDIES.

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This report represents phase one of a 5 year study to evaluate the effect of arsenic(As), dieldrin (Ded) and their mixture on female reproduction. Dose range studies were conducted in As and Ded to define the maximum tolerated dose (MTD) for use in the definitive reproductive study. Adult 60 day old Sprague-Dawley female rats were randomly assigned to groups (6-7/group) and orally gavaged daily for 30 consecutive days with 0, 1, 2, 4, 8, or 16mg/kg of either sodium arsenite or dieldrin. Daily clinical observations, biweekly body weight, and weekly food consumption were recorded. Other parameters including organ weights, pathology, hematology, clinical chemistry, reproductive hormones, T3 and T4, corticosterone, liver cytochrome P450 (1A1 & 2B1) and reactive oxygen species were determined. Arsenic caused mortality at 16mg and 8mg/kg (100% & 67% respectively). Clinical findings such as tremor and paresis were observed in 4 and 8mg/kg groups. Food consumption decreased during the 1st week in all As treated groups. Pathological lesions were noted in the stomach, liver and brain. Liver weight increased in 4mg/kg and higher doses. There were decreased RBC and lymphocyte counts, hemoglobin, hematocrit, and albumin levels in 4 and 8 mg/kg groups. The neutrophil count increased in both 4 and 8mg/kg groups. The GGT level increased only in the 8mg/kg As group. Dieldrin data is as follows. There was 100% mortality in 16 and 8mg/kg groups and 57% in the 4mg/kg group. Hyperactivity and aggressiveness were observed in 4mg/kg and above. Food consumption decreased during the 1st week followed by an increase during the 3rd and 4th weeks in the 4mg/kg group. Pathological lesions were noted in the brain and stomach of 4mg/kg and above. Liver weight increased in the 2 and 4mg/kg groups. Levels of CYP450 1A1 and 2B1 increased in all Ded treated groups but were significantly higher in 1 and 4mg/kg group. In conclusion, the MTD will be less than 4mg/kg body weight in both arsenic and dieldrin groups. (Supported by MHPF/ATSDR Cooperative Agreement # U50/ ATU473408-01)

**2113**

CHARACTERIZATION OF ISCHEMIA-REPERFUSION EFFECTS IN RENAL CELLS — A SCREENING APPROACH.

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Acute renal failure from ischemia-reperfusion (IR) injury remains a serious clinical problem. While various excellent animal models have been established in the last years, only few validated *in vitro* (human) models exist. Therefore, the aim of the presented study was to investigate the suitability of a human renal cell line (IHKE) and to compare the effects observed with the results from a well-established equivalent porcine renal cell line (LLC-PK1), which is frequently used for IR studies. For this comparison, the experiments with LLC-PK1 of various other authors were repeated under our standard conditions to eliminate confounding factors of the cell culture as handling differences, possible deviations from the original cell strain, etc. IR was simulated by adaptation of the mineral oil immersion method, described by Meldrum, K.K. et al., *J. Surg. Res.* 99 (2001). The effects of IR were investigated using a systematic screening approach according to DOE (design of experiment) recommendations for an inscribed central composite design, considering initial cell number, serum content, duration of ischemia and reperfusion and using several endpoints, i.e. cell number, Hoechst staining, TUNEL assay and MTT reduction assay. These data demonstrated that the IHKE cell line may be a suitable model for IR research.

**2114**

OCHRATOXIN A- INDUCED GENE EXPRESSION DEREGULATIONS IN THE KIDNEY OF EKER RATS ANALYZED ON AFFYMETRIX CHIPS.

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Hereditary renal cell carcinomas (RCC) in the Eker rat model are due to a dominant germline mutation in the tuberous sclerosis 2 (Tsc2) tumor suppressor gene. As Eker rats still harbour one normal copy of the Tsc2 gene, a second somatic mutation might be the rate limiting step for the development of RCC. As previous studies have shown a clear susceptibility of Tsc2 mutant rats to RCC induction by chemical carcinogens, the Eker rat may represent a useful model to study the action of renal carcinogens. As Ochratoxin A is suggested to be a human renal carcinogen based on sufficient evidence of carcinogenicity in experimental animals, Eker rats were used to assess novel early markers of OTA induced carcinogenesis. In order to examine these events, male rats were treated for up to 14 days with a daily oral dose of 210 µg OTA/kg BW shown previously to induce kidney tumors in chronic study. After 1, 3, 7 and 14 days, groups of three rats were sacrificed, RNA from the kidney cortex was isolated and gene expression patterns were analyzed on Affymetrix RAE-230A chips. Using statistical and clustering tools provided by GeneData Expressionist Analyst software, characteristically deregulated genes were extracted and then functionally annotated. Those genes with altered expression belonged, amongst others, to the functional categories of amino acid and protein metabolism, membrane transport and oxidative stress. At the dose used, DNA damage response genes were not found to be deregulated. In conclusion, even low doses of OTA have a clear influence on gene expression within the first two weeks of toxin exposure. Preliminary functional analysis of the deregulated genes suggests an epigenetic rather than a genotoxic mechanism of action at the low dose.

**2115**

CHARACTERIZATION OF OCHRATOXIN A- TRANSPORTING ORGANIC ANION TRANSPORTERS IN RENAL CELLS.

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Ochratoxin A (OTA) is a toxic metabolite produced by several fungal species. It is found with disturbing regularity in foodstuffs and fodder, therefore OTA is consumed by humans and animals. OTA is associated with the development of kidney carcinomas in laboratory animals and with nephropathies in pigs, as well as with two human renal diseases, namely Balkan endemic nephropathy (BEN) and urothelial tumors. The mechanism underlying its toxicity is still unknown. One plausible explanation for the obvious renal specificity of OTA could be accumulation in proximal tubular cells during the course of excretion (transport and/or reabsorption) thereby reaching toxic concentration within the cells. Several functional expression studies with *Xenopus laevis* oocytes have indicated OTA to be transported by organic anion transporters (Oats). In the present study the expression of Oat1, Oat2 and Oat3 in proximal tubular cells were investigated. All three transporter isoforms could be detected in porcine renal cortical tissue at RNA-level via RT-PCR, using human OAT1/2/3 primers. Sequence analysis and comparison of human and pig Oat-sequences showed high similarity. However, the expression

of these transporters was lost during preparation/ passaging of primary porcine kidney cells (PKC). Optimization of preparation and culture conditions is currently being undertaken with the aim of maintaining stable expression and functionality of Oats in PKC.

**2116**

CYTOTOXICITY OF THE NEPHROTOXINS ARISTOLOCHIC ACID AND MAM-ACETATE ON HUMAN AND PORCINE KIDNEY CELL LINES AND PRIMARY KIDNEY CORTEX CELLS.

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The nitrophenanthrene derivatives aristolochic acid I and II (AA), are the major components of the plant extract of *Aristolochia* spp. In 1982 it was observed that AA is a potent carcinogen in rats, causing a high incidence of tumours in the forestomach as well as primary tumours in the renal cortex, renal pelvis and urinary bladder. AA-exposed humans presented with a rapidly progressive renal fibrosis followed by a high prevalence of urothelial cancer. Cycasin is contained in cycad plants used by some populations as food. It is carcinogenic in several animal species, inducing tumours in various organs including the kidney. However, little is known about the toxic effects in human kidney. Methylazoxymethanol (MAM), the aglycone of cycasin is assumed to be the metabolite responsible for toxicity. The closely related synthetic substance methylazoxymethanol acetate (MAM-Ac) is more stable and was therefore used in this study. The cytotoxic effects of AA and MAM-Ac on human and porcine cell lines (HEK293, IHKE, LLC-PK1) as well as on human and porcine primary kidney cortex cells (HKC, PKC) were investigated. Exposure times were 24, 48, and 72h and the endpoints chosen were MTT reduction and cell number. Differences in sensitivity between species, sex and cell type were investigated. Preliminary data suggest distinct differences in sensitivity between species and between primary cells and cell lines. For example pig primary cells were approximately 36 times more sensitive to AA than their human counterparts.

**2117**

EFFECT OF ETHYLENE GLYCOL METABOLITES ON VARIOUS CYTOTOXICITY PARAMETERS IN HUMAN PROXIMAL TUBULE CELLS.

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Ethylene glycol (EG) is nephrotoxic due to its metabolism. Most studies suggest that the toxicity is due to oxalate accumulation, but some have suggested that toxicity results from effects of metabolites such as glycoaldehyde (GLA) or glyoxylic acid (GXA) on proximal tubule cells. Initial studies showed that calcium oxalate monohydrate (COM), the form of oxalate *in vivo*, but not glycolic acid (GA) or GXA increases ethidium dimer uptake and decreases calcein AM fluorescence, suggesting cytotoxicity of COM. The present studies were designed to re-examine these results using other measures of cell death, such as lactate dehydrogenase (LDH) release and the conversion of the tetrazolium salt XTT to a colorimetric dye. Human proximal tubule cells in culture were incubated in physiologic buffers for 6 h at 37°C with COM (1-10 mM), GA (5-25 mM), GXA (0.2-5 mM) and GLA (0.2-2 mM). To assess effect of acidity on the cytotoxicity, all incubations were carried out at pH 6, 6.5, 7 or 7.4. The results show that COM dose-dependently increased LDH release, while GA, GXA and GLA did not increase the release. Conversely, COM had no effect on the XTT assay, while GA and GXA decreased XTT activity only at acidic pH. GLA decreased XTT activity at 1-2 mM and this effect was exacerbated at pH 6. The correlation between the uptake of ethidium monomer and the release of LDH suggest that COM is cytotoxic to human kidney cells in culture, while none of the other metabolites are toxic at relevant concentrations. The data also suggest that the XTT assay is not a valid measure of cytotoxicity in this system.

**2118**

PEROXIREDOXINS ARE MODIFIED BY QUINONE- GENERATED REACTIVE OXYGEN SPECIES.

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2, 3, 5-Tris-(glutathion-S-yl)hydroquinone (TGHQ), a metabolite of hydroquinone, induces ROS-dependent DNA damage and cell death in renal proximal tubule epithelial cells (LLC-PK1). 2-D PAGE coupled to MALDI-TOF peptide mass mapping and post source decay revealed that TGHQ altered the relative intensity of three spots, each identified as peroxiredoxin 3 (Prx3), in LLC-PK1 cells.

After TGHQ treatment, the intensity of the spot with a more acidic pI increased, whereas intensity of the spot with more basic pI decreased. Non-denaturing western blot analysis using a specific antibody against Prx3, revealed an additional band after TGHQ treatment, migrating faster than the equivalent band in the untreated sample, possibly due to oxidation. Interestingly, Prxs behave as antioxidative enzymes to reduce peroxides, and regulate MAPK activation. Prxs can be hyperoxidized and are inactivated by oxidation of the reactive cysteine residue to either a sulfenic or sulfonic acid, in the presence of large amount of ROS. Because TGHQ induces ROS-dependent MAPK and histone H3 phosphorylation precede cell death, the post-translational modification of Prxs, especially as a consequence of oxidation, may play an important role in ROS-induced cell death. Attempts to identify the Prx3-associated proteins are under way in LLC-PK1 cells. Our data suggest that TGHQ generated ROS induce a post-translational modification within Prx3, which likely plays an important role in TGHQ-mediated renal cell death. Prx3 oxidation in ROS-treated renal cells is also highly reproducible, and can therefore be used as a biomarker for ROS-induced renal cell damage. (Grants: ES07784, DK59491, GM39338, P30ES-07784, P30ES-06694)

**2119** UP-REGULATION OF HEAT SHOCK PROTEIN 27 AND RETINOL BINDING PROTEIN CONTRIBUTES TO 11-DEOXY-16, 16-DIMETHYL PROSTAGLANDIN E2 MEDIATED CYTOPROTECTION.

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11-Deoxy-16, 16-dimethyl prostaglandin E2 (DDM-PGE2) protects renal proximal tubular epithelial cells (LLC-PK1) against oncotic cell death via the activation of a thromboxane A2 (TP) receptor. Cytoprotection is associated with the up-regulation of several proteins, including ER stress proteins, elongation factors, and several cytoskeletal proteins. 2-D gel electrophoresis coupled with MALDI-TOF peptide mass mapping revealed heat shock protein 27 (HSP27) was also selectively induced by DDM-PGE2. HSP27 is known to regulate actin filament dynamics and to participate in the stabilization of the actin cytoskeleton. Consistent with this, DDM-PGE2 prevented actin depolymerization induced by Cytochalasin D (CD), an actin filament disrupting agent, and HSP27 co-localized with disrupted actin after CD treatment. Moreover, HSP27 translocated into the nucleus, and co-localized with actin in close proximity to the cell membrane following treatment with 2, 3, 5-(trisglutathion-S-yl)-hydroquinone (TGHQ). HSP27 is a molecular chaperone that is phosphorylated in response to reactive oxygen species. Following TGHQ treatment, 2-D PAGE and western blot confirmed the phosphorylation of HSP27, correlating with its role in compensatory cytoprotection. DDM-PGE2 also induced the synthesis of retinol binding protein (RBP). The TP agonist, U46619, recapitulated the effects of DDM-PGE2, and the TP antagonist, SQ29, 845, abolished the induction of RBP in parallel with a loss in cytoprotection. The retinoic acid nuclear receptor agonist, all-trans retinoic acid, induced RBP with similar kinetics as DDM-PGE2 and U46619, with maximum induction occurring at 12 hours. All-trans retinoic acid also recapitulated the cytoprotective effects of DDM-PGE2 in LLC-PK1 cells, suggesting the possible involvement of retinoid signaling in the cytoprotective response. (GM56321, ES06694).

**2120** ASSESSMENT OF *IN VITRO* TOXICITY OF SELECTED CHEMO-THERAPEUTIC DRUGS USING HUMAN PRIMARY RENAL CELLS.

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Chemo-therapeutic drugs often cause organ toxicity due to non-specific effects on cancerous and normal cells. Improving safety or reducing toxicity presents a critical challenge for drug discovery. In the present study, effects of selected anti-cancer drugs (camptothecin, doxorubicin, colchicine, paclitaxel, cisplatin, and carboplatin) on cell function/viability and proliferation mechanisms were investigated. Potency or anti-proliferation activity of each drug on cancer cells (hepatoma HepG2) and human primary renal proximal tubule cells (hRPTECs) were determined with the [<sup>3</sup>H]thymidine incorporation assay and results indicated all six drugs blocked cell proliferation of both cell types. EC<sub>50</sub> values for anti-proliferation activity suggested that camptothecin is most potent, followed by doxorubicin, paclitaxel, colchicine, cisplatin and carboplatin in hRPTECs. Cytotoxicity to hRPTECs was assessed with the ATP bioluminescence assay that monitors changes in cellular ATP level. Camptothecin, doxorubicin and cisplatin induced cytotoxicity in hRPTECs with EC<sub>50</sub> for cytotoxicity at 0.4, 11.5 and 39.3  $\mu$ M, respectively. The tested drugs displayed wide dosage separation between potency threshold and cytotoxicity based on EC<sub>50</sub> ratios. These findings using hRPTECs correlated well

with reported human nephrotoxicity. Therefore *in vitro* hRPTEC system provides a useful tool for understanding drug properties and for future safety testing of new chemo-therapeutic drugs.

**2121** NEPHROTOXICITY OF N-(3, 5-DICHLOROPHENYL)-3-HYDROXYSUCCINAMIC ACID IN MALE AND FEMALE FISCHER 344 RATS.

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Previous studies demonstrated that heated solutions of N-(3, 5-dichlorophenyl)-3-hydroxysuccinamic acid (3-NDHSA), a metabolite of the agricultural fungicide N-(3, 5-dichlorophenyl)succinimide (NDPS), induced nephrotoxicity in rats. However, the possibility existed that warming 3-NDHSA cyclized it to the nephrotoxic metabolite N-(3, 5-dichlorophenyl)-2-hydroxysuccinimide. The purpose of this study was to examine the nephrotoxic potential of non-heated solutions of 3-NDHSA in male and female Fisher 344 rats to determine the effects of heating on nephrotoxic potential and gender differences in toxic potential. Rats (4/group) were administered a single injection of 3-NDHSA (0.1, 0.2 or 0.4 mmol/kg, ip) or vehicle, and renal function was monitored at 24 and 48 hr. In male rats, 3-NDHSA 0.2 mmol/kg induced mild nephrotoxicity seen as diuresis and transient, mild proteinuria. However, 3-NDHSA 0.4 mmol/kg induced marked nephrotoxicity. In female rats, 3-NDHSA 0.1 mmol/kg did not affect renal function, while 3-NDHSA 0.2 mmol/kg induced mild nephrotoxicity as evidenced by transient diuresis and proteinuria. As in males, 3-NDHSA 0.4 mmol/kg induced marked nephrotoxicity. These results indicate that 3-NDHSA is equipotent to NDPS in inducing nephrotoxicity, but unlike NDPS and other nephrotoxic NDPS metabolites, 3-NDHSA does not exhibit gender differences in nephrotoxic potential. Supported in part by NIH grant DK31210.

**2122** COMPARISON OF THE *IN VITRO* NEPHROTOXIC POTENTIAL OF DICLOFENAC AND ACETAMINOPHEN IN ISOLATED RENAL PROXIMAL TUBULES FROM MALE FISCHER 344 RATS.

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Acetaminophen (APAP) and diclofenac (DC) which are frequently used as analgesics have each been associated with causing adverse renal effects. While nephrotoxic events associated with APAP are generally due to drug overdose, diclofenac has been shown to cause nephrotoxicity at therapeutic doses. The purpose of this study was to assess and compare the nephrotoxic potential of low concentrations of APAP and DC. To assess nephrotoxicity, the effects of APAP and DC on cellular lactate dehydrogenase (LDH) release and nucleotide levels (ATP, ADP, AMP) were investigated in isolated renal proximal tubules (IRPT) from male Fischer 344 rats. IRPTs were incubated for 30 or 60 minutes in the presence of APAP (200 or 500  $\mu$ M), and for 15 or 30 minutes with DC (200 or 500  $\mu$ M). APAP was not nephrotoxic at the tested concentrations and time points in IRPTs. However, acute DC-induced toxicity in IRPTs as demonstrated by increased LDH release was preceded by a decrease in ATP levels. ATP levels were found to be significantly depressed by both concentrations of DC at 15 minutes, while LDH release was not increased. By 30 minutes, DC treatment caused a significant increase in LDH release, and ATP and ADP levels were both significantly decreased. This data indicates that while APAP does not appear to have any nephrotoxic potential at these low concentrations, early events in DC toxicity appear to result in the alteration of mitochondrial production of ATP, or, due to a toxic cellular reaction, the cellular needs for ATP exceeds the cells ability to produce ATP. This toxic event (alterations in ATP levels) occurs at an earlier time point than general cellular toxicity as demonstrated by LDH release and is in agreement with the potential for DC to induce nephrotoxicity at therapeutic doses.

**2123** *IN VITRO* GSH ADDUCT FORMATION: EVIDENCE FOR BIOACTIVATION OF THE NEPHROTOXICANT N-(3, 5-DICHLOROPHENYL)SUCCINIMIDE THROUGH ALCOHOL-O-GLUCURONIDATION AND SULFATION.

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The agricultural fungicide, N-(3, 5-dichlorophenyl)succinimide (NDPS), is nephrotoxic in rats. Previous studies indicated that metabolism of NDPS (both Phase I and Phase II) contributes to the mechanism of toxicity. Small amounts of

Phase II metabolites, including alcohol-O-linked glucuronides and sulfates, have been identified *in vivo*. These conjugates were also produced *in vitro* from N-(3, 5-dichlorophenyl)-2-hydroxysuccinimide (NDHS) an oxidative metabolite of NDPS. In the current study, we investigated the chemical reactivity of the NDHS glucuronide and sulfate conjugates and compared their *in vitro* GSH adduct formation with NDHS. Reactivity of the synthetic NDHS-O-sulfate (NSC), a putative reactive metabolite, was also studied. NDHS (0.1 mM) was incubated with rat liver S9 in the presence or absence of co-factors for conjugations (UDPGA or PAPS). GSH (5 mM) was added in all the reactions, and the mixtures were incubated for 40 min at 37 °C. In a separate experiment, aliquots of the incubates were collected at different time point (5-160 min) after GSH addition. Samples were analyzed using LC-MS/MS. Six GSH adducts were formed in incubations where UDPGA/PAPS were added, and they were identified as three pairs of diastereomers of GS-NDHS, and 2-/3-GS-N-(3, 5-dichlorophenyl)-2-/3-hydroxysuccinamic acids (2-/3-GS-NDHSA). Compared to NDHS, NSC was ~500-fold more reactive towards GSH conjugation. Fortifying Phase II cofactors also increased the formation of GSH adducts by ~8-fold. Results from these experiments demonstrate that formation of the alcohol-O-glucuronide and O-sulfate conjugates in NDPS metabolism may represent bioactivation pathways, and may potentially contribute to the mechanism of nephrotoxicity.

## 2124

### EFFECT OF ENANTIOMER-BASED INHIBITORS OF $\text{Ca}^{2+}$ -INDEPENDENT PHOSPHOLIPASE A<sub>2</sub> ON RENAL AND CANCER CELL DEATH.

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Recent studies suggest that human embryonic kidney cells (HEK293), kidney (Caki-1), lung (A549), prostate (PC-3) carcinomas, and brain glioblastomas (A172) express both the cytosolic (iPLA<sub>2</sub>β) and microsomal (iPLA<sub>2</sub>γ) isoforms of  $\text{Ca}^{2+}$ -independent phospholipase A<sub>2</sub> (iPLA<sub>2</sub>), and that iPLA<sub>2</sub> inhibition decreased chemotherapeutic-induced apoptosis in rabbit renal proximal tubules cells (RPTC). However, the effect of iPLA<sub>2</sub> inhibitors on chemotherapeutic-induced cell death in human renal and cancer cells is unknown. Treatment of PC-3 and A172 cells with racemic BEL (0-5.0  $\mu\text{M}$ ) increased annexin V (apoptotic cell marker) and propidium iodide (PI, oncotic cell marker) staining. In contrast, treatment of RPTC, HEK293, Caki-1 or A549 cells with BEL for 24 h did not. BEL treatment prior to cisplatin (0-100  $\mu\text{M}$ ) or vincristine (0-2  $\mu\text{M}$ ) exposure reduced apoptosis 30-50% in all kidney cells tested (RPTC, HEK293 and Caki-1 cells). In contrast, BEL had no effect on cisplatin- or vincristine-induced cell death in PC-3 or A172 cells, or cisplatin-induced apoptosis in A549 cells. Interestingly, pre-treatment of A549 cells with BEL altered the mechanism of vincristine-induced cell death from apoptosis to oncosis. Because racemic BEL inhibits both cytosolic iPLA<sub>2</sub>β and microsomal iPLA<sub>2</sub>γ, more enantiomeric selective inhibitors were studied. R- and S-enantiomers of BEL were separated using high performance liquid chromatography and the selectivity of S-BEL for cytosolic iPLA<sub>2</sub>β and microsomal iPLA<sub>2</sub>γ was verified in RPTC. Following verification of selectivity the effect of the two enantiomers on cell death was studied in Caki-1, HEK293, PC-3, A172 and A549 cells using 3-(4, dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT). Treatment with S-BEL alone caused greater decreases in MTT staining compared to cells treated with either R-BEL or racemic-BEL in all cell lines, suggesting that selective inhibition of iPLA<sub>2</sub>β may induce cell death. Collectively, these data suggest that iPLA<sub>2</sub> isoforms mediate the mechanisms of cell death in both human renal and cancer cells.

## 2125

### IDENTIFICATION AND CHARACTERIZATION OF iPLA<sub>2</sub>γ IN MITOCHONDRIA.

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We have shown that primary cultures of rabbit renal proximal tubular cells (RPTC) express  $\text{Ca}^{2+}$ -independent phospholipase A<sub>2</sub>γ (iPLA<sub>2</sub>γ) in the endoplasmic reticulum (ER). Inhibition of RPTC iPLA<sub>2</sub>γ with bromoenol lactone (BEL) potentiated lipid peroxidation and necrotic cell death induced by oxidants. While two recent reports described iPLA<sub>2</sub> activity in mitochondria, the specific isoform(s) of iPLA<sub>2</sub> responsible for this activity were not determined. The goals of the present study were to determine 1) whether RPTC possess mitochondrial iPLA<sub>2</sub> activity 2) the characteristics and identity of this iPLA<sub>2</sub> and 3) whether human kidney cells possess similar ER and mitochondrial activity. Mitochondria were isolated from rabbit kidney cortex, RPTC and human embryonic kidney (HEK293) cells and iPLA<sub>2</sub> activity measured as the cleavage of 16:0, [<sup>3</sup>H]18:1 phosphatidylcholine or plasmalogen substrates in the absence of  $\text{Ca}^{2+}$  (4 mM EGTA). iPLA<sub>2</sub> activity was detected in rabbit kidney and RPTC mitochondrial inner membranes. Three isoforms of iPLA<sub>2</sub> could be responsible for the mitochondrial activity, cPLA<sub>2</sub>γ, iPLA<sub>2</sub>β

or iPLA<sub>2</sub>γ. Using the differential sensitivity to iPLA<sub>2</sub> inhibitors among iPLA<sub>2</sub> isoforms, we determined that rabbit kidney mitochondrial iPLA<sub>2</sub> was inhibited by racemic BEL and R-BEL, but not methyl arachidonyl fluorophosphonate (MAFP) or S-BEL. Immunoblot analyses using an anti-peptide antibody to iPLA<sub>2</sub>γ revealed an 88 kDa immunoreactive protein in rabbit kidney ER and mitochondria. These results show that rabbit kidney mitochondrial iPLA<sub>2</sub> is iPLA<sub>2</sub>γ. We also identified iPLA<sub>2</sub>γ, but not cPLA<sub>2</sub>γ or iPLA<sub>2</sub>β, in the ER and mitochondria of HEK293 cells. In summary, iPLA<sub>2</sub>γ is found in two subcellular membranes that are subjected to oxidative stress and vital for cell function and survival. This is consistent with the hypothesis that iPLA<sub>2</sub>γ protects cells from oxidative stress by inhibiting and/or repairing lipid peroxidation.

## 2126

### ISCHEMIA-INDUCED CLEAVAGE OF CADHERINS IN NRK CELLS: EVIDENCE FOR A ROLE OF MT1-MMP.

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Ischemia is the leading cause of acute renal failure (ARF), a disease associated with high morbidity and mortality. Disruption of intercellular adhesion in the proximal tubules is linked to ARF, although the molecular mechanism(s) remain unclear. In these studies, we adapted a model of ischemia to normal rat kidney (NRK) cells and examined the cadherin/catenin complex, the predominant regulator of cell-cell adhesion. Ischemia was induced by applying a thin layer of phosphate buffered saline (PBS) solution supplemented with calcium and magnesium and a layer of mineral oil, restricting exposure to oxygen. Hypoxia was detected by an increase in pimonidazole adducts, as well as an increase in GLUT-1 protein levels. Ischemia did not decrease cell number, but there was a decrease in ATP levels. In addition, there was no evidence of cleaved caspase 3 or 9 during 6 hr of ischemia. Ischemic cells exhibited an extracellular 80 kDa and intracellular 40 kDa E-cadherin fragment after 4-6 hr of ischemia. While no fragments of N-cadherin were observed at any time point, the detectable levels of this protein decreased during ischemia. E-cadherin cleavage and loss of N-cadherin was associated with decreased cadherin function as revealed by cell aggregation. The effects of ischemia on E- and N-cadherin were independent of mRNA and protein synthesis. The MMP inhibitors, GM6001 and TAPI-O, inhibited cleavage and/or loss of E- and N-cadherin protein expression. TIMP-3 and to a lesser extent TIMP-2, but not TIMP-1, inhibits ischemic cleavage and/or loss of E- and N-cadherin, suggesting the involvement of a membrane-bound MMP. Blocking MT1-MMP activity fully protects full-length E- and N-cadherin. These results demonstrate that ischemia induces activation of latent MT1-MMP and subsequent disruption of cadherin/catenin complexes, implying that MT1-MMP plays a role in ischemia-induced ARF.

## 2127

### ROLE OF HEAT SHOCK PROTEINS AND SIGNALING PATHWAYS IN DETERMINING RESPONSE OF HUMAN PROXIMAL TUBULAR CELLS TO S-(1, 2-DICHLOROVINYL)-L-CYSTEINE (DCVC).

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Confluent, primary cultures of human proximal tubular (hPT) cells were used as a model system to determine time and dose dependence of toxic responses to DCVC, a nephrotoxic metabolite of the environmental contaminant and putative renal carcinogen trichloroethylene (TCE). Previous studies showed that DCVC causes cellular necrosis only at relatively high doses (> 100  $\mu\text{M}$ ) and long incubation times (> 24 hr) whereas both apoptosis and enhanced cellular proliferation occur at relatively low doses (10-100  $\mu\text{M}$ ) and early incubation times (2-8 hr). To investigate whether differential effects on expression of regulatory proteins were associated with these responses, we performed Western blots on hPT cells treated for up to 24 hr with a range of concentrations of DCVC. Exposure of hPT cells to concentrations of DCVC that are associated with apoptosis and enhanced cell proliferation caused marked increases (1.5- to 10-fold) in expression of several proteins that regulate apoptosis (Bcl-2, Bax, Apaf-1, caspase-9 and PARP cleavage products) and cellular growth and differentiation (c-Jun, p53, Hsp27). Effects on c-Jun, p53, and Hsp27 implicate function of the mitogen activated protein kinase pathway and changes in the cellular cytoskeleton in determining the response of the cell to DCVC exposure. DCVC exposure is also known to activate protein kinase C. Higher concentrations of DCVC caused an increase in expression of NF- $\kappa$ B p65 subunit, consistent with oxidative stress. Hence, we conclude that exposure of hPT cells to DCVC induces several proteins and activates multiple signaling pathways. Depending on dose and time of exposure, these signaling pathways can lead to either cell death by necrosis or apoptosis, repair, or enhanced cell proliferation. The precise pattern of expression of these and other proteins may serve as molecular

markers for TRI or DCVC exposure and effect in human kidney. Identification of these pathways may also suggest novel therapeutic approaches. (Supported by NIH Grant ES08828.)

## 2128

### IDENTIFICATION AND LOCALIZATION OF CALPAIN 10 TO MITOCHONDRIA.

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Calpains,  $\text{Ca}^{2+}$ -activated cysteine proteases, are a family of 14 proteases that can be sub-divided into two classes based on the presence (*typical*) or absence (*atypical*) of domain IV (the primary  $\text{Ca}^{2+}$ -binding domain). While calpains are largely cytosolic, we showed that the mitochondrial matrix contained calpain activity and that inhibition of this activity blocked  $\text{Ca}^{2+}$ -induced mitochondrial dysfunction. The goal of this study was to identify the mitochondrial matrix calpain. The calpain inhibitor PD150606 inhibits calpain activity by blocking  $\text{Ca}^{2+}$  binding in domain IV and, thus, can be used to differentiate between *typical* and *atypical* calpains. PD150606 did not inhibit matrix calpain activity. Subsequently, we examined all calpain sequences for a mitochondrial targeting sequence using the mitochondrial protein prediction algorithm MITOP2 (<http://ihg.gsf.de/mitop2/start.jsp>). Only calpain 10, an *atypical* calpain, received a positive score using this algorithm. Using immunoblot analysis and two different antibodies directed against calpain 10, a 75 kDa immunoreactive band was identified, consistent with the molecular weight predicted by the calpain 10 amino acid sequence. Finally, we expressed a calpain 10-GFP fusion construct in NIH-3T3 fibroblasts and determined that calpain 10 localized to the mitochondria. Thus, using a variety of approaches, we have shown that the mitochondrial matrix calpain is calpain isoform 10. The mitochondrial localization of calpain 10 has important implications for mitochondrial injury.

## 2129

### EPITHELIAL BARRIER CHARACTERISTICS AND EXPRESSION OF CELL ADHESION MOLECULES IN PROXIMAL TUBULE-DERIVED CELL LINES COMMONLY USED FOR *IN VITRO* NEPHROTOXICITY TESTING.

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During the course of studies examining the effects of nephrotoxic metals on proximal tubule derived cell lines in culture, we noticed that the cell lines seemed to vary markedly in their epithelial barrier characteristics. To examine this phenomenon in more detail, we monitored the development of transepithelial electrical resistance (TER) and the expression of a panel of cell adhesion molecules that are involved in maintaining epithelial barrier function in several proximal tubule-derived cell lines that have been commonly used as model systems for *in vitro* toxicity studies. The specific cell lines that were studied included: LLC-PK<sub>1</sub>, OK, NRK52E and HK-2, along with commercially available primary cultures of human proximal tubule epithelial cells (HTPE). The results showed that the LLC-PK1 cell line developed the highest TER (> 200 ohms $\bullet$ cm<sup>2</sup>) followed by the OK cell line (~50 ohms $\bullet$ cm<sup>2</sup>). All of the other cell lines failed to develop significant TER even after 2 weeks in culture. Immunofluorescence labeling studies showed that all of the cell lines, except HK-2 and OK, expressed high levels of the calcium-dependent cell adhesion molecule, E-cadherin; the OK cells expressed high levels of N-cadherin and the HK-2 cells expressed lower levels. All of the cell lines expressed moderate-high levels of the tight-junction scaffolding protein ZO-1. In addition, the LLC-PK<sub>1</sub> and OK cells expressed high levels of the tight-junction barrier protein occludin, which was co-localized with ZO-1. By contrast, the other cell lines exhibited only weak occludin labeling. These findings indicate that the OK cell line exhibits characteristics that most closely resemble those of proximal tubule epithelial cell *in vivo*, and they suggest that the differences in the barrier characteristics of renal epithelial cell lines may be related to differences in the expression of the tight-junction barrier protein occludin. Supported by NIH Grant R01 ES006478.

## 2130

### CYTOTOXICITY AND METABOLISM OF S-1, 2-DICHLOROVINYL-GLUTATHIONE (DCV-GSH) AND CHLORAL (CH) BY HUMAN PROXIMAL TUBULE CELLS (HPTC) IN CULTURE.

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Trichloroethylene (TRI) is an important industrial solvent that causes toxic nephrosis in rats and mice following chronic exposure. TRI is metabolised by CYP 450 to CH, as a major pathway, and via GSH conjugation to DCV-GSH as a minor pathway. We have examined the effect of daily exposure to CH and DCV-GSH for 10

days on primary HPTC in culture from 4 different donors. Confluent cells, 8-10 days after seeding, were exposed daily to CH at 0.03 to 3mM or DCV-GSH at 0.05 to 5 $\mu$ M. Cytotoxicity was determined using MTT following 1, 3, 7 and 10 days exposure. Respiration in the presence and absence of FCCP (1 $\mu$ M) was measured after 10 days exposure to DCV-GSH. The extent of CH metabolism to trichloroethanol (TCE-OH) and trichloroacetic acid (TCA) was determined in the media by gas chromatography, while the presence of formic acid in the media was measured by 1H-NMR spectroscopy. DCV-GSH was cytotoxic to HPTC with an IC50 value of about 5  $\mu$ M/day after 10 days exposure with no effect at 0.5  $\mu$ M. Treatment with DCV-GSH inhibited both resting and uncoupled mitochondrial respiration after 10 daily exposures at concentrations > 0.5  $\mu$ M DCV-GSH. CH was not cytotoxic at doses up to 1mM/day for 10 days to HPTC. The cells readily metabolized CH at this dose to TCE-OH and TCA with about 50% being converted to TCE-OH and 2.5% to TCA each day for 10 days. Exposure to CH (3mM) also lead to a 3-fold increase in the concentration of formic acid in the media (control 0.065 $\pm$ 0.021mM; treated 0.18mM $\pm$ 0.037mM). Low daily doses of DCV-GSH lead to progressive cytotoxicity indicating that the enzymes metabolizing GSH conjugates and activating the cysteine conjugate ( $\beta$ -lyase) were present in these HPTC. CH was also readily metabolized over 10 days and we provide preliminary evidence for an increased excretion of formic acid. Microarray analysis is being conducted to look for gene changes in HPTC following subchronic exposure to non-cytotoxic doses of the GSH-derived metabolites of TRI.

## 2131

### PROTEOMIC CHARACTERIZATION OF THE EFFECTS OF A KINASE INHIBITOR ON PROTEIN EXPRESSION IN RAT KIDNEY.

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In a 14-day oral toxicity study performed in male Sprague-Dawley rats, a kinase inhibitor compound X, that has been developed for the treatment of asthma, induced kidney toxicity at a dose of 200 mg/kg, but not at a pharmacological dose of 10 mg/kg. A proteomic study was initiated to elucidate the molecular mechanisms underlying the observed drug-induced nephrotoxicity. After protein extraction, 2D gel electrophoresis was performed using differential gel electrophoresis technology. Protein identification of the up- and down-regulated spots was performed by mass spectrometry. Results showed that compound X, at the low dose of 10 mg/kg, induced over-expression of 5 proteins and down-regulation of 8 proteins, whereas at 200 mg/kg it gave an up-regulation of 29 proteins and a down-regulation of 20 proteins. Data analysis showed that a number of biochemical pathways were modulated such as amino acid, urea cycle and protein metabolism, as well as carbohydrate metabolism. Proteins involved in anti-oxidant defence and xenobiotic metabolism were also found to be affected by treatment, giving clues to the mechanism of toxicity of compound X in kidney. Among the proteins of interest, kynurenine aminotransferase, glycine amidinotransferase and phosphotriesterase related protein, known to be involved in kidney impairment and/or xenobiotic detoxification, were also modulated and corroborated the histopathological observations. In addition, mass spectrometry results showed that proteins like ornithine aminotransferase and glycine amidinotransferase were detected in 2D gels as several spots corresponding to different isoelectric points or molecular weights; this suggests that drug-induced modulation of protein isoforms could be potentially used as a toxicity-specific signature of compound X in the kidney.

## 2132

### REGULATION OF GRP78 IN THE GLOMERULAR MESANGIAL CELL STRESS RESPONSE.

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Chemical injury is associated with endoplasmic reticulum (ER) stress, a reaction characterized by activation of the unfolded protein response and proteasomal degradation of proteins expelled into the cytoplasm. The ER stress response may be caused by dysregulation of intra-ER  $\text{Ca}^{2+}$ , by oxidative stress, or buildup of misfolded proteins. Grp78 plays a central role in the ER stress response in various cell types, including neuronal and renal cells, and therefore, studies were conducted to evaluate mechanisms of the ER stress response in rat glomerular mesangial cells or kidney embryonic cells challenged with toxic metals (Mercury chloride ( $\text{HgCl}_2$ ) or lead ( $\text{Pb}$ ) acetate) or aromatic hydrocarbons (BaP), respectively. Challenge of mesangial cells with 1 or 10  $\mu\text{M}$   $\text{HgCl}_2$  or lead acetate was associated with concentration-dependent increases in grp78 mRNA and protein. CAT reporter assays

completed using constructs containing promoter sequences responsible for thapsigargin inducibility did not show trans-activation by either heavy metals or BaP. Co-treatment of metal-treated cells with actinomycin D (a transcriptional inhibitor) or cycloheximide (a protein synthesis inhibitor) ablated the induction response, suggesting that regulation of Grp78 by metals involves a transcriptional mechanism. Immunocytochemical analysis of kidney cells challenged with 3  $\mu$ M BaP revealed a 2-fold increase in Grp78 expression. Collectively, these data suggest that Grp78 is involved in the stress response of renal cells to different forms of chemical injury.

### 2133 REDUCTION OF CYCLOSPORIN A NEPHROTOXICITY BY GADOLINIUM CHLORIDE.

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The immunosuppressant cyclosporin A (CsA) causes both hepato- and nephrotoxicity in patients. It is known that CsA activates Kupffer cells in the liver. In addition, liver regulation of renal function accounts for, at least partly, impaired urinary excretion in a variety of diseases. Therefore, these experiments investigated if gadolinium chloride ( $GdCl_3$ ), which selectively destroys Kupffer cells, would affect nephrotoxicity due to CsA. Rats were treated with CsA (25mg/kg, i.g.) or an equivalent volume of vehicle for 5 days. Intracellular calcium in Kupffer cells isolated from vehicle-treated rats was not altered by acute CsA addition (1  $\mu$  g/mL); however, in Kupffer cells isolated from CsA-treated rats, CsA addition increased intracellular calcium about 15-fold, indicating that CsA sensitizes and activates Kupffer cells. Kupffer cells isolated from the liver decreased about 70% after  $GdCl_3$  (20 mg/kg, i.v.) treatment. However,  $GdCl_3$  did not significantly alter monocytes/macrophages in the kidney as determined by ED1 and ED2 immunohistochemical staining. Glomerular filtration rates (GFR) and serum creatinine were about 0.6 ml/min/100 g body weight and 0.45 mg/dL, respectively, in vehicle-treated rats. CsA decreased GFR by 70% and doubled serum creatinine levels.  $GdCl_3$  blunted CsA-induced decreases in GFR and increases of serum creatinine by about 50%. Taken together, CsA inhibits renal function, at least in part, in a Kupffer cell-dependent manner. These results suggest that hepatic-renal interaction probably plays a role in CsA nephrotoxicity.

### 2134 THE EFFECT OF S-ADENOSYL-L-METHIONINE (SAMe) AND ASCORBIC ACID ON P-AMINOPHENOL TOXICITY IN RENAL SLICES.

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p-Aminophenol is a known renal toxicant. The purpose of this study was to investigate the effect of the nutraceutical S-adenosyl-l-methionine (SAMe) and ascorbic acid on PAP *in vitro* nephrotoxicity. Renal cortical slices were prepared from kidneys obtained from Fischer 344 (F344) rats weighing 200-250 g. Renal slices were washed at 25°C in Krebs buffer. Renal slices were pretreated for 15 min with 2 mM ascorbic acid or 30-60 min with 0.2 mM SAMe. Slices were then incubated with 0, 0.1, or 0.25 5 mM PAP for up to 120 min at 37°C and constant oxygen. Renal slices exposed to 0.25 mM PAP showed toxicity as measured by increased lactate dehydrogenase (LDH) leakage and decreased pyruvate-stimulated gluconeogenesis. PAP decreased ( $p<0.05$ ) both total glutathione (GSH) and glutathione disulfide (GSSG) within 30 min when compared to vehicle controls. SAMe pretreatment did not reduce PAP toxicity as indicated by a comparable rise in LDH leakage. Pretreatment with ascorbic acid reduced PAP toxicity. Further studies examined the effect of ascorbic acid pretreatment on PAP associated depletion of glutathione. Pretreatment with ascorbic acid prevented PAP depletion of total glutathione. Total glutathione levels were diminished to 53% and 6% of control values following exposure to 0.1 and 0.25 mM PAP, resp. Tissues treated with 0.1 and 0.25 mM PAP pretreated with ascorbic acid had glutathione levels that were 111% and 91%, resp., of control. These data show that SAMe did not alter PAP toxicity. Furthermore, ascorbic acid pretreatment reduced toxicity of PAP in renal slices and this attenuation may be mediated by preventing glutathione depletion.

### 2135 HUMAN PRECISION-CUT KIDNEY-CORTEX SLICES AS A TOXICOLOGY MODEL FOR EVALUATING THE HYPERAMMONEMIC EFFECT OF VALPROATE: A CARBON 13 NMR STUDY.

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The aim of the present study was to evaluate the suitability of human precision-cut kidney-cortex slices as a model to study the mechanism whereby valproate stimulates renal ammoniogenesis and leads to hyperammonemia. The metabolism of ei-

ther unlabeled or 13C-labeled glutamine was studied in human kidney-cortex slices incubated either in Krebs-Henseleit medium for 4 hours or in William's medium E for up to 48 hours. After incubation, substrate removal and product formation were measured by enzymatic and NMR spectroscopy methods. Glutamate accumulation tended to plateau but glutamine removal and ammonia, alanine and lactate accumulation as well as flux through glutamate dehydrogenase increased to various extents with time for up to 4 hours of incubation indicating the metabolic viability of the slices. Valproate, a widely used antiepileptic drug and a hyperammonemic compound, markedly and in a dose-dependent manner stimulated ammonia production. With 3-13C-glutamine as substrate, and in the absence and the presence of valproate, 13C-glutamate, 13C-alanine and 13C-lactate accounted for 81% and 96%, 34% and 63%, 30% and 46% of the glutamate, alanine and lactate accumulations measured enzymatically, respectively. Combination of the 13C NMR results obtained with a mathematical model of the metabolic pathways involved revealed that valproate markedly stimulated fluxes through the ammoniagenic enzymes (glutaminase and glutamate dehydrogenase) and through alanine aminotransferase, phosphoenolpyruvate carboxykinase, pyruvate kinase and lactate dehydrogenase. The slices also metabolized glutamine and retained their reactivity to valproate during incubations lasting for up to 48 hours. These results demonstrate that, although endogenous metabolism substantially operates in the presence of glutamine, human precision-cut kidney-cortex slices are metabolically viable and respond to the ammoniagenic effect of valproate. Thus, this model is suitable for metabolic and pharmacological studies.

### 2136 UTILITY OF A SUBCLINICAL RENAL INJURY MODEL IN RATS FOR DETECTION OF INCREASED SENSITIVITY TO SITE-SPECIFIC NEPHROTOXIC METALS.

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Subclinical renal injury (SRI) can predispose individuals to adverse effects of nephrotoxic metals. The objective of this study was to determine if kidneys in rats with SRI are more sensitive than healthy control animals to nephrotoxic metals that exert their effect on different segments of the nephron. To induce SRI, male Sprague-Dawley rats were dosed (sc) daily for 3 days with 250 mg/kg gentamicin, an aminoglycoside that primarily injures the proximal convoluted tubules ( $S_1/S_2$  segments). Twenty-four hr after the third injection, SRI- and saline-pretreated rats were injected with either saline or  $K_2Cr_2O_7$  (5.0 mg Cr/kg, sc).  $HgCl_2$  (0.25 mg Hg/kg, iv), or  $LiCl_2$  (5.0 mEq Li/kg, ip), compounds generally believed to exert effects primarily on the proximal convoluted tubule ( $S_1/S_2$  segments), proximal straight tubule ( $S_3$  segment), and distal tubule, respectively. Blood urea nitrogen (BUN) and creatinine, and urinary N-acetyl- $\beta$ -glucosaminidase/creatinine ratio (NAG/Cr), specific gravity (SG) and total protein (TP) were assessed 24 hours after metal injection. BUN levels were increased by 1.5-, 1.4-, 1.7-fold and NAG/Cr levels were elevated by 3.4-, 5.7-, and 2.2-fold in SRI rats challenged with Cr, Hg, and Li, respectively, compared to saline-pretreated rats challenged with metals. Similar fold-increases in BUN and NAG/Cr levels were observed in SRI rats challenged with metals compared to SRI rats challenged with saline. Slight increases in blood creatinine were observed in SRI rats challenged with metals compared to all other treatment groups. Urine specific gravity decreased in SRI rats challenged metals compared to metal treatment alone, but values were similar to SRI rats challenged with saline. Urinary TP was not altered in any treatment group. These results suggest that the gentamicin-induced model of SRI is sensitive to the effects of compounds that exert effects on specific nephron sites, thereby increasing the utility of the model for preclinical screening of compounds that may injure various nephron segments.

### 2137 PREPLACED RENAL CELL DIVISION IS THE CRITICAL MECHANISM OF PROTECTION IN MICE AGAINST S-1, 2-DICHLOROVINYL-L-CYSTEINE-INDUCED ACUTE RENAL FAILURE AND DEATH.

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We have shown that mice pretreated with a low dose of S-1, 2-dichlorovinyl-L-cysteine (DCVC) protects them from death caused by a normally lethal dose of DCVC (autoprotection). Priming with low dose of DCVC increases sustained  $G_1$  to S-phase clearance due to the upregulated expression of phospho- retinoblastoma protein (pRB) that persists even after the lethal dose. Objective of the study was to test if the administration of colchicine (CLC), abolishes protection via down regulation of pRB leading to progression of injury in the absence of newly dividing/divided cells. First group of mice received a priming dose of DCVC (15 mg/kg) at 0

h followed by vehicle treatment (distilled water, 10 ml/kg) at 42 & 66 h prior to receiving a lethal dose (75 mg/kg) at 72 h. The second group of mice received CLC (1.5 mg/kg ip) instead of distilled water at 42 & 66 h, all other treatments remaining the same. Mice receiving low dose + CLC, DW + CLC and DW alone were maintained as the controls. CLC intervention abolished autoprotection and resulted in progression of injury leading to death. CLC abolished the promotogenic effect of the low dose of DCVC by down regulating cyclin D1/cdk4-mediated phosphorylation of retinoblastoma protein. S-phase stimulation was markedly inhibited as assessed by BrdU pulse and PCNA labeling. These mice suffered severe renal damage as assessed by light microscopy of H&E stained kidney sections. Plasma BUN and creatinine were significantly increased after CLC intervention. These findings suggest the protective dose-stimulated renal tissue repair and recovery from acute renal failure. Higher G<sub>1</sub> to S phase clearance by increased pRB is the key mechanism behind sustained stimulation of renal tissue repair in autoprotection. These findings raise the possibility that pRB-mediated mechanism may be availed for potential pharmacotherapeutic modulation to restore lost proximal tubular structure and function. (Supported by DK 61650).

**2138**

INHIBITION OF TISSUE REPAIR LEADS TO PROGRESSION OF INJURY, ACUTE RENAL FAILURE AND DEATH IN MICE.

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The objective of the present study was to test the hypothesis that inhibition of tissue repair by colchicine (CLC), an antimitotic agent, leads to progression of injury and animal death after exposure to a nonlethal dose of DCVC. Male Swiss Webster (SW) mice were administered a normally nonlethal dose of DCVC (30 mg/kg, i.p.) on day 0 and CLC (2 mg/kg, i.p.) at 42 h and 66 h after administration of DCVC and observed for mortality. BUN, serum creatinine, urinary volume, pH, specific gravity, glucose, protein, creatinine, N-acetyl  $\beta$ -D-glucosaminidase (NAG), calpain immunohistochemistry were assessed as markers of injury during a time course of 0 to 14 days. Immunohistochemistry and Western blotting for aquaporin 1 was also carried out. Tissue repair was assessed by <sup>3</sup>H-thymidine pulse labeling and PCNA immunohistochemistry. There was 100 % mortality by 120 h in the group of mice receiving CLC after DCVC as against 0 % mortality in mice receiving either DCVC or CLC alone. BUN and serum creatinine elevations peaked on days 2 and 3 in the group receiving DCVC and CLC but on days 5 and 6 in mice receiving DCVC alone which decreased later. Polyuria on days 1 and 2 in DCVC mice and oliguria occurred on days 4, 5 and 6 in the CLC control group. Urinary pH increased to 8 by day 1 in DCVC treated groups and to 9 in CLC treated group and remained so during 14 days in all the treated groups. Urinary glucose and NAG:creatinine ratio increased on day 1 in both the DCVC groups and was found unaltered in CLC treated group. CLC intervention decreased S phase stimulation by DCVC indicating inhibition of tissue repair. Calpain immunohistochemistry revealed higher extracellular calpain co-incident with progression of injury in the DCVC treated mice receiving CLC intervention. Neither DCVC with or without CLC nor CLC alone affected aquaporin 1 expression in the descending thin limbs of loops of Henle of the kidneys. The results from the present study indicate that tissue repair is essential for the survival of the mice (DK 61650).

**2139**

ROLE OF LOWER PROGRESSION OF DCVC-INITIATED RENAL INJURY IN DIABETES-INDUCED PROTECTION FROM NEPHROTOXICITY.

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Our previous work showed that streptozotocin (STZ)-induced type 1 diabetic (DB) mice exhibit significantly lower progression of renal injury and higher survival induced by augmented nephrogenic tissue repair after challenge with a normally LD<sub>90</sub> dose (75 mg/kg, i.p.) of S-1, 2-dichlorovinyl-L-cysteine (DCVC). Objective of the present work is to investigate the mechanisms of lower progression of DCVC-initiated renal injury in diabetes. Progression of renal injury is thought to be mediated via the destructive actions of cysteine protease calpain, which leaks out of dying cells. Formalin fixed kidney sections from DB and nondiabetic (NDB) mice were immunohistochemically stained for localization of calpain following treatment with the lethal dose of DCVC. Significantly higher number of necrotic proximal tubules stained positive for calpain and these cases were associated with acute renal failure (ARF) in NDB kidneys from 12 to 36 h after DCVC treatment. In stark contrast, the DB kidneys revealed fewer calpain-positive tubules commensurate with the lower progression of DCVC-initiated tubular necrosis (12 to 72 h) in these mice. Calpastatin, the endogenous inhibitor of calpain is known to be over-

expressed in dividing/newly divided cells, thereby making them resistant to the progression of injury. Since DB kidneys have 4-fold higher number of cells advancing in S-phase even before DCVC treatment, we hypothesized that overexpression of calpastatin in DB kidney may explain the lower progression of DCVC-initiated renal injury. Immunohistochemistry revealed higher expression of calpastatin in DB kidney as compared to the NDB kidney before DCVC treatment. These data suggest that overexpression of calpastatin in cells advancing to S-phase in DB kidney imparts resistance to destruction by calpain, thereby explaining the markedly lower progression of DCVC-initiated renal injury, recovery from ARF-bound injury, and survival in DB mice. (Supported by DK 61650)

**2140**

TISSUE DISTRIBUTION AND RENAL ONTOGENY OF ORGANIC CATION TRANSPORTERS IN MICE.

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Organic cation transporters (Octs) are primarily responsible for the elimination of cationic endobiotics and xenobiotics from the body. Organic carnitine transporters (Octns) represent a subfamily of Octs that are responsible for carnitine disposition. Understanding the tissue distribution of Octs may be important in elucidating their role in absorption, distribution, elimination and organ-specific toxicity of xenobiotics. Similarly, ontogenetic expression may indicate a role for Octs in age-specific sensitivity to xenobiotic toxicity. Therefore, the purpose of this study was to characterize the tissue distribution and renal ontogenetic expression of Oct1, Oct2, Oct3, Octn1, Octn2 and Octn3 mRNA in male and female mice. mRNA expression was determined using the branched DNA signal amplification assay (bDNA). The tissues examined were liver, kidney, stomach, duodenum, jejunum, ileum, large intestine, heart, brain, lung, testes, ovary, placenta and uterus. Oct1, Oct2, Octn1 and Octn2 mRNA expression was highest in kidney. Oct1 was also moderately expressed in liver. Octn1 and Octn2 were also moderately expressed in duodenum, jejunum and ileum. Oct3 mRNA levels were highest in placenta and ovary, whereas Octn3 was exclusively expressed in testes. Renal expression of Oct2 mRNA was female-predominant. However, this gender difference in Oct2 kidney expression was not observed until 30 days after birth. Expression of Oct1, Oct2, Octn1 and Octn2 in kidney increased gradually from prenatal day -2 through day 45 in both genders. The kidney-predominant expression of Octs implicates Octs as key mediators in renal excretion of cationic xenobiotics. Furthermore, the elevated expression of Oct3 in ovary and placenta and the exclusive expression of Octn3 in testes might suggest a critical functional role for these transporters in the sex organs. (Supported by NIH Grants ES09649, ES-09716 and ES-07079).

**2141**

DIETARY MODULATION OF THE RENAL TOXICITY OF P-NONYLPHENOL (NP) IN SPRAGUE-DAWLEY RATS.

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We previously demonstrated that NP at 1000 and 2000 ppm fed in a soy- and alfalfa-free diet (5K96) from gestation day (GD) 7 to postnatal day (PND) 50 induced polycystic kidney disease in male and female rats (Latendresse et al., *Toxicol. Sciences*. 62: 140, 2001). To test our hypothesis that the soy-free diet had exacerbated this toxicity, male and female weanling rats were assigned to one of four diets, two of which were soy free (5K-96 and AIN-93G) and two of which contained soy (Purina 5001 and AIN-93G with soy protein isolate as the protein source (AIN-SPI)). Rats were mated within diet groups at approximately 11 weeks of age. On GD7, half of the vaginal plug-positive dams in each diet group were placed on the same diet containing 2000 ppm NP while the other half continued on control diet. Exposure continued through gestation and lactation, and pups were continued on the same diet as their dam until termination at PND 50. Neither diet nor NP treatment had significant effects on the proportion of dams producing litters, litter size or weight. Body, liver, and kidney weights were collected at necropsy of male and female pups on PND 2, 14, and 21, male pups at PND 50, and dams. Portions of the kidneys were fixed for later microscopic evaluation and frozen for biochemical assays. Body weights were generally significantly higher in animals fed Purina 5001 than those fed the other diets. In the PND 50 animals, NP significantly lowered terminal body weight in all diet groups. Liver to body weight ratios were significantly elevated by NP in all diet groups except 5K96. Kidney to body weight ratios were significantly elevated by approximately 2-fold by NP in the 5K96 and AIN-93G diet groups, but not in the Purina 5001 group. Relative kidney weight was slightly elevated (approximately 30%) in the AIN-SPI diet group. These organ weight differences were not observed in pups at other ages or in dams. While histological evaluation of the kidneys remains to be completed, these results confirm a significant effect of diet on the renal effects of NP.

## ETIOLOGY OF HYDRONEPHROSIS WITHOUT OBSTRUCTION IN THE MOUSE KIDNEY BY LACTATIONAL EXPOSURE TO DIOXIN.

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The hydronephrosis is defined as a disease that has dilatation of the pelvis and calices proximal to the point of obstruction due to backpressure following the accumulation of urine. Prenatal exposure to teratogenic dose of 2, 3, 7, 8- tetrachlorodibenzo-p-dioxin (TCDD) has been established to cause this malformation in mice with hyperplasia of the ureteral epithelium. To elucidate the etiology of hydronephrosis by TCDD exposure, C57Bl/6 dams were given a single oral dose of 10g TCDD/kg on postnatal day (PND) 1 after the normal delivery, and pups were euthanized on specified times for pathophysiological and molecular analyses. Histopathological examinations on the hydronephrotic kidney from pups on PNDs 7, 14 and 21 showed that there is no apparent obstruction due to hyperplasia, and that immunostaining for cytochrome P450(CYP)1A1 is confined to the distal tubular epithelium. Pups that developed hydronephrosis manifested polyuria with elevated levels of Na and K in urine, and decreased concentrations of Na and K in the blood circulation. Microarray analysis with subsequent analysis by real-time RT-PCR on kidney tissues from pups showed gene expression of Na, K channel transporter genes are altered at least as early as PND7. The present results not only demonstrate a new finding on hydronephrosis without morphological obstruction, but also suggest a novel molecular mechanism to explain the etiology of this malformation that may be driven in an XRE-dependent fashion.

## EFFECTS OF CONAZOLE FUNGICIDES ON DEVELOPMENT AND PARTURITION IN THE RAT.

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Conazoles are fungicides used extensively in agriculture and as pharmaceuticals. As part of an effort to evaluate the changes in gene expression corresponding to reproductive toxicity, we examined the effects of three conazoles on pregnancy and neonates. Wistar Han rats were exposed via feed from gestation day 6 to the weaning of their litters to propiconazole (P) at 100, 500, or 2500 ppm; myclobutanil (M) at 100, 500, or 2000 ppm; triadimefon (T) at 100, 500, or 1800 ppm; or vehicle control. Litters were examined on postnatal days 0 and 8. The highest concentration of each of the three chemicals caused delayed parturition. T1800 had particularly severe effects, with 19% of the dams unable to successfully deliver live pups; in some cases dystocia led to maternal death. Increased pre- and postnatal mortality at M2000 and T1800 were attributed to effects on parturition. In addition, prenatal loss at P2500 was marginally increased. Pup weights were increased at M100 on day 0. Marginal increases in pup weight were noted on day 0 at M2000 and P2500, and on day 8 at M500 and P500. In contrast, T1800 pup weights were decreased on days 0 and 8. Anogenital distance (on day 0), analyzed with pup weight as a covariate, was increased in females at M2000, and in males at M2000, P2500, and T1800. We conclude that exposure to the highest concentrations of M, P, and T adversely affected parturition and consequently impacted perinatal survival. The effects on parturition and anogenital distance may be related to aromatase inhibition, a known property of conazole compounds. [This abstract does not necessarily reflect EPA policy.]

## TRIADIMEFON INDUCES RAT THYROID TUMORS THROUGH A NON-TSH MEDIATED MODE OF ACTION.

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Conazoles are a class of fungicides used as agricultural and pharmaceutical products which inhibit ergosterol biosynthesis. Members of this class are hepatotoxic and cause mouse hepatocellular tumors and/or rat thyroid follicular cell tumors. Triadimefon-induced rat thyroid tumors may arise through increased TSH secretion after induction of thyroxine metabolizing enzymes. Male Wistar rats treated with triadimefon (TRID, 100, 500, 1800 ppm), propiconazole (PROP, 100, 500, 2500 ppm), or myclobutanil (MYCL, 100, 500, 2000 ppm) in feed for 4, 30, or

90d were evaluated for clinical signs, body and liver wt, histopathology of thyroid and liver, metabolizing enzyme activity, and serum T3, T4, and TSH levels. There was a dose-dependent increase in liver wt but body wts were not different from control for all treatments. Pentoxyresorufin O-dealkylation (PROD) activities had a dose-related increase at all time points for all conazoles. UGT activities were induced to the same extent after 30 and 90d for all three conazoles. Livers from all high dose treated rats had centrilobular hepatocyte hypertrophy after 4d, while only TRID and PROP treated rats had hepatocyte hypertrophy after 30d, and only TRID treated rats had hepatocyte hypertrophy after 90d. Thyroid follicular cell hypertrophy and colloid depletion was present only after 30d TRID treatment. A dose-dependent decrease in T4 was present after 4d with all 3 compounds but only the high doses of PROP and TRID after 30d. T3 was decreased in the high dose group of TRID after 4d and in a dose-dependent manner for all compounds after 30d. Thyroid hormone levels did not differ from control values after 90d and TSH was not different from control in any exposure group. Although this group of conazoles does transiently perturb thyroid function, these data suggest that thyroid tumors arising after TRID treatment are not a result of persistent increases in TSH. [This abstract does not reflect EPA policy.]

## MYCLOBUTANIL AND TRIADIMEFON METABOLISM BY RAT CYP ISOFORMS AND LIVER MICROSONES.

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The mode of action of conazole fungicides is to inhibit cytochrome P450 (CYP) 51 activity and thus the biosynthesis of ergosterol by fungi. Conazoles can also modulate other CYP activities in vertebrate species including humans. Myclobutanil (MCL) and triadimefon (TRD) are agricultural conazoles with similar chemical structures. Laboratory animal studies have shown MCL and TRD have reproductive toxicity and carcinogenic activity, respectively. It has been proposed that modulation of CYP activity may be the common mechanism underlying their toxic effects. This study was designed to investigate 1) whether MCL and TRD modulated rat CYP activities that results in changes to conazole metabolism, and 2) which rat CYP isoforms were responsible for conazole metabolism. Liver microsomes were prepared from adult male Sprague-Dawley rats exposed orally to vehicle, MCL (10 or 150 mg/kg), or TRD (5 or 115 mg/kg) for 14 days. Total P450 concentrations in both high dosage groups were significantly higher ( $P < 0.01$ ) than vehicle control. The rate of MCL disappearance when incubated with microsomes was much more rapid than for TRD, and the disappearance rate for both chemicals increased with microsomes from rats exposed to higher dose of either chemical versus control. Incubation of MCL with recombinant rat CYP isoforms indicated that CYP2C6, 2C11, 3A1, and 3A2 had metabolized myclobutanil, but not CYP2B1, 1A1, 1A2, 2A1, 2A2, 2C12, 2C13, 2D1, 2D2, and 2E1. CYP2C6 and 3A1 were the only isoforms responsible for TRD metabolism but at a much lower rate. These results indicate that both MCL and TRD induce CYP activities that led to modest increases in their own metabolism. (This abstract does not reflect EPA policy. JT is supported by EPA/UNC Toxicology Training Agreement # CT827206. RM is supported by EPA/NCSU Training Agreement # CT826512010)

## COMPARATIVE LIVER P450 ENZYME ACTIVITY AND HISTOPATHOLOGY IN MICE TREATED WITH THE CONAZOLE FUNGICIDES: MYCLOBUTANIL, PROPICONAZOLE AND TRIADIMEFON.

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Conazoles are fungicides used in agriculture and pharmaceutical products and comprise a class of chemicals which inhibit ergosterol biosynthesis. Both propiconazole and triadimefon are hepatotoxic and hepatotumorigenic in mice, while myclobutanil is not a mouse liver tumorigen. The tumorigenic activities of these conazoles are thought to be mediated through increased liver cytochrome P450 enzyme activity leading to oxidative stress, mitogenesis and altered foci development. We have assessed how P450 enzyme activity and liver pathology correlate with known hepatotumorigenic responses to conazoles. Male CD-1 mice were treated in the feed for 4, 30 and 90 days with triadimefon (0, 100, 500 or 1800 ppm), propiconazole (0, 100, 500 or 2500 ppm) or myclobutanil (0, 100, 500 or 2000 ppm). Alkoxyresorufin O-dealkylation assays indicated that all 3 chemicals induced similar patterns of dose-related increases in pentoxyresorufin O-dealkylation (PROD), and to a lesser extent, ethoxyresorufin O-dealkylation and/or methoxyresorufin O-dealkylation. Propiconazole high-dose exposures over 30 and 90 days induced 36 to 38-fold higher levels of PROD. Myclobutanil and triadimefon at high dose exposures also induced PROD 10 to 15-fold over controls at all time points. All treatments gave dose-dependent increases in liver weights. Liver histopathology revealed

similar dose-dependent hypertrophy following exposure to all 3 conazoles. There were no significant treatment effects on body weight. Overall, with the exception of some high dose effects observed for propiconazole, a similar induction pattern for these specific AROD-assayed liver enzymes and histopathologies was observed for the 3 conazoles, regardless of their tumorigenic potential. [This abstract does not necessarily reflect EPA policy.]

**2147** COMPARISON OF HEPATIC GENE EXPRESSION PROFILES FROM MICE EXPOSED TO THREE TOXICOLOGICALLY DIFFERENT CONAZOLES.

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Conazoles comprise a chemical class of fungicides used as agricultural and pharmaceutical products. Both propiconazole and triadimefon are hepatotoxic and hepatotumorigenic in mice, while myclobutanil is not a mouse liver tumorigen. The tumorigenic activities of these conazoles are thought to be mediated through increased liver cytochrome P450 enzyme activity leading to oxidative stress, mitogenesis, and altered foci development. Our goal was to assess correlations of global gene expression in liver with the hepatocarcinogenic responses in mice. Male CD-1 mice were treated for 4 days with feed incorporated with triadimefon (0, 500 or 1800 ppm), propiconazole (0, 500 or 2500 ppm) or myclobutanil (0, 500 or 2000 ppm). At high dose exposures, propiconazole induced more than twice the number of differentially expressed genes (Affymetrix 430 2.0 gene array) compared to triadimefon or myclobutanil. 121 genes were upregulated by 50% or more in liver tissue from mice treated with any of the three conazoles; 87 genes were upregulated in both triadimefon and propiconazole; 102 genes were upregulated exclusively in triadimefon and 328 genes were upregulated exclusively in propiconazole. All three conazoles increased the gene expression of a series of cytochrome P450s including, cyp2b20, cyp2c37, and cyp2c55. These P450 increases were dose responsive with more than twenty-fold induction at the high dose in some cases. Effects of global gene expression at the lower doses are also being investigated. [This abstract does not necessarily reflect EPA policy.]

**2148** REPRODUCTIVE EFFECTS OF EXPOSURE TO CONAZOLE FUNGICIDES IN THE MALE RAT.

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Conazoles are a class of fungicides used in pharmaceutical and agricultural applications. Three agricultural conazoles were selected, representing a range of reported cancer and reproductive effects, for a study exposing litters (16-20 per dose group) from gestation day 6 to postnatal day (PND) 99. Wistar Han rats were exposed via feed to vehicle, myclobutanil (M, 100, 500, or 2000 ppm), propiconazole (P, 100, 500, or 2500 ppm), or triadimefon (T, 100, 500, or 1800 ppm). One male per litter was sacrificed at PND 1, 22, 50, 92 or 99. Measurements included anogenital distance (AGD) at PND 0, body weight (BW), organ weights, serum hormone levels, and age at preputial separation (PPS). Mating assays were conducted with adult males and untreated females. AGD was increased by the high concentration of all 3 conazoles. T1800 delayed PPS. Liver weights increased at PND50 by M2000 and P2500, and at PND92 by P2500 and T1800. Testis weights increased at PND22 by M2000, at PND50 by M2000 and T500, and at PND92-99 by T1800. Epididymal weights increased at PND92-99 by T1800, and ventral prostate weights increased at PND92 by M500, M2000 and T500. Serum testosterone increased at PND92-99 by M2000, P2500, T500 and T1800. Thyroid weights increased at PND92 by T1800, and total serum thyroxine levels decreased at PND92 by T1800. Mating assays identified impaired fertility by M2000 and T1800, apparently due to ejaculation failure. Effects on AGD, PPS, testis and accessory sex gland weights, serum hormones and fertility represent adverse reproductive outcomes following long-term exposures to conazoles. Future efforts will focus on elucidating mechanisms underlying these effects. [Funded by USEPA Cooperative Research Agreement No. CT826512010 with North Carolina State University. This abstract does not necessarily reflect EPA policy.]

**2149** COMPARISON OF GENE EXPRESSION PROFILES FROM RATS FED THREE TOXICOLOGICALLY DIFFERENT CONAZOLES.

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Conazoles are a class of fungicides used as pharmaceutical and agricultural products. In chronic bioassays, triadimefon was hepatotoxic and induced transitional cell adenomas in the thyroid gland. Both propiconazole and myclobutanil were he-

patotoxic but had no effect on the thyroid gland. It has been proposed that triadimefon induces thyroid tumors by altering the levels of thyroid hormone and TSH as a result of induction of xenobiotic metabolizing enzymes. Genomic profiling was utilized to investigate whether transcriptional alterations were associated with their toxic mode of action. Male Wistar rats (3 per group) were exposed to conazoles in the feed for 4 days: triadimefon (1800 ppm), propiconazole (2500 ppm), myclobutanil (2000 ppm). Hepatic gene expression was performed using Affymetrix GeneChips (Rat 230\_2). Differential gene expression was assessed at the probe level using Robust Multichip Average Analysis (RMA). De Novo gene expression between treatment groups was analyzed using hierarchical clustering, K-Nearest Neighbor, and PCA. All 3 conazoles could be discriminated from controls with propiconazole and myclobutanil clustering together and distinct from triadimefon. Statistical analyses showed 150 genes were in common among the 3 conazoles including several cytochromes P450s, GSTs, and one UDP-glucuronosyltransferase. In contrast, 500 genes were unique to triadimefon. These genes were mapped to several gene networks including steroid biosynthesis, cell proliferation, and DNA repair pathways. Future analyses will focus on associating these alterations with triadimefon's toxicological effects. [This abstract does not necessarily reflect EPA policy.]

**2150** PROFILING GENE EXPRESSION IN HUMAN H295R ADRENOCORTICAL CARCINOMA CELLS AND RAT TESTES TO IDENTIFY PATHWAYS OF TOXICITY FOR CONAZOLE FUNGICIDES.

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Conazoles are widely used agricultural and pharmaceutical fungicides which inhibit sterol and steroid biosynthesis. Studies with 3 agricultural conazoles were conducted by *in vitro* exposure of H295R cells and *in vivo* exposure of Wistar-Han rats. H295R cells express key steroidogenic enzymes and represent an alternative to the rat testis for assessing endocrine disruption. We used quantitative PCR (qPCR) and DNA microarrays to profile gene expression and identify common effects and toxicity pathways. H295R cells were cultured with 1, 3, 10, 30 or 100  $\mu$ M of each conazole for 48-72 hours. Adult rats were fed myclobutanil (100, 500, 2000 ppm), triadimefon (100, 500, 1800 ppm), propiconazole (100, 500, 2500 ppm), or vehicle-added rodent chow for four days. Expression of 11 steroidogenic genes was measured by qPCR for all H295R dose groups. In addition, Affymetrix Human U133plus2.0 GeneChips were used to profile gene expression for the 3 higher doses of each conazole. Rat testis gene expression for all dose groups was profiled using Affymetrix Rat 230 2.0 GeneChips. Resulting data were log-transformed and analyzed by multi-way ANOVA to determine interactions between the chemicals and the *in vitro* and *in vivo* systems. Comparison of results for steroidogenic genes between the H295R cells and rat testis, and between qPCR and microarray are underway. Microarray results in H295R cells and rat testis identified many orthologous genes and common biological pathways, including programmed cell death, cell cycle, lipid metabolism and steroid metabolism. [This abstract does not necessarily reflect EPA policy]

**2151** REPRODUCTIVE TOXICITY OF EXPOSURE TO CONAZOLE FUNGICIDES IN THE FEMALE RAT.

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Conazole fungicides are used widely in pharmaceutical and agricultural applications. Although some conazoles have been investigated extensively for toxicological effects, there is little published information on the reproductive toxicity of many of the agriculturally important conazoles. In this study, three agricultural conazoles were evaluated for effects on female reproductive development. Wistar-Han rats were exposed via feed to vehicle control, propiconazole (P) (100, 500, or 2500 ppm), myclobutanil (M) (100, 500, or 2000 ppm), or triadimefon (T) (100, 500, or 1800 ppm) from gestation day 6 to the first proestrus after post natal day (PND) 98. Body weight (BW) and anogenital distance (AGD) at PND0, age and BW at vaginal opening (VO), and BW and organ weight at termination were measured. Histological examination of ovaries was conducted. Female pup weights at PND0 were unaffected by treatment. AGD at PND0 was increased by M2000. Age of puberty (VO) was delayed by T1800 independent of BW. At PND99 there was a decrease in BW by T1800, an increase in relative liver weight by P2500 and

T1800, and an increase in relative ovarian weight by M2000 and T1800. All ovaries contained follicles at all stages of development and had normal appearing corpora lutea. We conclude that exposure to the high concentrations of M and T adversely impacted female reproductive development. The observed changes suggest a mechanism involving inhibition of estrogen synthesis or function. Ongoing analysis of estrous cyclicity, serum hormone levels, and ovarian pathology will serve to further characterize the effects of these exposures and help clarify mechanism. This abstract does not necessarily reflect EPA policy.

## 2152 A RISK ASSESSMENT OF THE TRIAZINE HERBICIDE ATRAZINE.

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Toxicity was assessed in animal studies and exposure estimated from occupational and dietary sources. The endpoints were: rabbit developmental toxicity (acute NOEL 5 mg/kg/day); dog cardiotoxicity (chronic NOEL 0.5 mg/kg/day); mammary tumors resulted from lifetime exposure. These occurred only in female SD rats, were malignant, dose-dependent and were also observed with other, related triazines. Evidence for a genotoxic basis for these tumors was either equivocal or negative. An endocrine basis for the mammary tumors, involving premature aging of the female SD rat reproductive system, has been proposed. There is evidence for the mechanism including one or more of the following: the induction of aromatase (CYP19) and/or other P450 oxygenases, an (antagonist) action at the estrogen feedback receptor in the hypothalamus, an (agonist) action at the mammary gland estrogen receptor or an effect on adrenergic neurons in the hypothalamic-pituitary pathway. The potential occupational exposure to atrazine was assessed during mixing, loading, and application. ADD values were 1.8 to 6.1  $\mu\text{g}/\text{kg}/\text{day}$ . The MOS values for short-term exposure were 820 to 2,800. Theoretical calculations of acute dietary exposure used tolerance levels, along with secondary residues, and water. Atrazine plus the (3) main chlorotriazine metabolites were combined. MOS values were above 2000, for all population subgroups. Dietary exposure to atrazine is therefore extremely unlikely to result in human health hazard. Possible ecotoxicity includes the feminization of frogs/toads, measured in laboratory and field studies. In the latter, the water bodies with the greatest numbers of deformed frogs sometimes had the lowest concentrations of atrazine. Other studies have also cast doubt on the feminization theory, except perhaps at very high levels of atrazine. Epidemiology studies have investigated atrazine's ability to cause adverse effects in humans. Although some studies suggest that atrazine elevates the risk of prostate cancer, the published literature is inconclusive with respect to cancer incidence.

## 2153 METHOXYCHLOR INHIBITS EXPRESSION OF ANTIOXIDANT ENZYMES IN THE MOUSE OVARY.

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Females are born with a finite number of primordial follicles, of which a small fraction reaches the antral stage. Antral follicles are responsible for releasing an egg for fertilization and maintaining cyclicity. *In vivo* studies with the organochlorine pesticide methoxychlor (MXC) have shown that antral follicles are the primary targets of MXC exposure. Specifically, MXC exposure decreases the number of antral follicles and increases the percentage of antral follicles undergoing atresia (cell death via apoptosis). While different pathways lead to toxicant-induced cell death, oxidative stress is known to cause apoptosis in non-reproductive and reproductive tissues. Certain toxicants produce reactive oxygen species, which are detoxified by antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT). Thus, this work tested the hypothesis that MXC inhibits the expression of selected antioxidant enzymes in antral follicles. To test this hypothesis, 39-day old CD-1 mice were dosed with either sesame oil (control) or MXC (32 or 64 mg/kg/day) for 20 days. After treatment, ovaries were collected and antral follicles were isolated from the ovaries and subjected to real time polymerase chain reaction for measurement of mRNA levels of SOD, GPX, and CAT. The results indicate that MXC significantly decreases mRNA expression as compared to controls of SOD (control=2.98  $\pm$  0.30 genomic equivalents (ge); MXC 32 mg/kg/day=0.94  $\pm$  0.08 ge; MXC 64 mg/kg/day=1.28  $\pm$  0.16 ge; n=3; p  $\leq$  0.003), GPX (control=2.36  $\pm$  0.48 ge; MXC 32 mg/kg/day=0.90  $\pm$  0.03 ge; MXC 64 mg/kg/day=1.09  $\pm$  0.10 ge; n=3; p  $\leq$  0.05), and CAT (control=2.02  $\pm$  0.24 ge; MXC 32 mg/kg/day=0.98  $\pm$  0.05 ge; MXC 64 mg/kg/day=1.13  $\pm$  0.07 ge; n=3; p  $\leq$  0.01). Collectively, these data indicate that MXC inhibits the expression of SOD, GPX, and CAT in antral follicles. Therefore, it is possible that MXC may cause atresia of ovarian antral follicles by inducing oxidative stress through inhibition of SOD, GPX, and CAT detoxifying pathways. (Supported by NIH HD38955, T32 ES07263, and Colgate Palmolive Fellowship)

## 2154 TRANSIENT ACTIVATION OF ACETYLCHOLINESTERASE BY PARAOXON.

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Previous studies with certain organophosphates such as paraoxon (*O, O*-diethyl *O*-(*p*-nitrophenyl) phosphate) and chlorpyrifos oxon (*O, O*-diethyl *O*-(3, 5, 6-trichloro-2-pyridyl) phosphate), have shown that their interactions with acetylcholinesterase are more complex than originally thought. The present study extends these observations by documenting conditions under which paraoxon causes a transient increase in hydrolysis of acetylthiocholine by human recombinant acetylcholinesterase that precedes the phosphorylation of Ser-203 of the catalytic triad. Co-incubation of 10 nM paraoxon with 0.4 mM acetylthiocholine and 0.4 pM enzyme active site yielded an initial velocity of  $0.0721 \pm 0.0242$  (mean  $\pm$  SD) OD units per min, compared to  $0.0165 \pm 0.004$  OD units per minute without paraoxon. The increased hydrolysis was apparent for up to about 3 min, after which time activity decreased as phosphorylation of the active site proceeded. Higher concentrations of paraoxon produced less measurable enhanced activity for shorter periods of time, as the phosphorylation reaction proceeded at a faster rate, until 100 nM paraoxon only inhibited activity. These results confirm that the interactions of some organophosphates with acetylcholinesterase are more complex than is often thought. Secondly, these studies suggest that paraoxon might bind to a site on acetylcholinesterase distinct from the active site, and that binding can modulate catalytic activity.

## 2155 EVALUATION FOR THE ADDITIVE TOXIC INFLUENCE OF ORGANOPHOSPHORUS PESTICIDES.

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Organophosphorus pesticides (OPs) are abundantly produced and used widely all over the world. OPs have been detected from samples prepared from food and the environmental materials. Although it has been concluded each OP hardly shows the adverse effect in the detected concentration level, the co-effect of multiple OP is not sufficiently considered. It is necessary to evaluate hazard assessment of multiple coexisted OPs, which may especially occur in the environment. Therefore, it is important to establish the testing method to evaluate the toxicity for multiple OPs. It is known that its and its oxide have the toxicity by the inhibition of acetylcholinesterase activity, an enzyme is received the crucial problem to nerve transmission. We established the testing method by measuring of cholinesterase (ChE) activity using 5-methyl-2-thienylthiocholine-iodide as substrate. We examined the inhibition of ChE activity for fifteen OPs by this *in vitro* method. Consequently, these OPs were classified into three groups according to the degree of inhibition, the OPs which inhibit ChE activity strongly at low concentration, the OPs which inhibit slightly, and the others. From comparison between the degree of inhibition and the value of acceptable daily intake, there was a relationship. It is indicated that the toxicity of OPs was simply evaluated by the degree of inhibition of ChE activity with this *in vitro* method. As results under coexisting condition of a few OPs in the combination of three groups, the additive action of the inhibition was found. In the present work, we could demonstrate the simple *in vitro* method to evaluate for the toxicity of coexisted OPs by degree of inhibition of ChE activity at low levels. Using this method, it is possible to evaluate the adverse load by coexisted OPs in the environment.

## 2156 BIOLOGICAL MONITORING OF EXPOSURE TO ORGANOPHOSPHORUS INSECTICIDES IN GREENHOUSE WORKERS.

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Exposure to selected organophosphorus insecticides (OPs), malathion, diazinon and acephate, was evaluated in greenhouse workers through measurements of the urinary excretion time courses of specific and non-specific biomarkers. For the study of each OP, the participation of eight individuals was obtained: two applicators, five workers manipulating treated plants and one office worker. Each participant provided all micturitions (in separate bottles) during a 24-h period following

the onset of an exposure episode to either malathion, diazinon or acephate. For several workers, the urinary excretion of malathion specific metabolites, mono- and di-carboxylic acids (MCA and DCA), increased following the onset of an exposure to malathion. Maximum values of MCA and DCA were observed between 8 and 12 h but concentrations had returned to baseline values by 24 h following the onset of exposure. Excretion patterns of methyl phosphoric derivatives (the sum of DMTP, DMTP and DMP) usually differed from that of MCA and DCA and concentration values were in general much higher than those of MCA and DCA. The 24-h cumulative excretion values of malathion biomarkers were however far below biological reference values proposed in a previous work. In all of the urine samples provided by the participants, urinary concentrations of 2-isopropyl-4-methyl-6-hydroxypyrimidine, a specific metabolite of diazinon, as well as acephate and methamidophos were below or equal to the analytical limit of detection (1, 0.8 and 0.2  $\mu$ g/L, respectively) except for acephate in one individual (concentrations between 1.4 and 3.5  $\mu$ g/L). There was also no marked increase in the urinary excretion of the non-specific metabolites of diazinon, ethyl phosphoric derivatives (the sum of DETP and DEP), following the onset of an exposure to diazinon. These results show that the greenhouse workers under study were poorly exposed to OPs.

**2157**

KINETIC DATA ON PESTICIDE METABOLISM IN HUMANS TO ALLOW PBPK/PD MODELS FOR PARATHION AND CHLORPYRIFOS TO PREDICT SUSCEPTIBILITY.

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The ultimate utility of any physiologically based pharmacokinetic (PBPK) model is limited by the availability of accurate kinetic parameters that reflect the rate of biotransformation of xenobiotics in the population of interest. The primary objective of this study was to obtain kinetic parameters (Vmax, Km values) for the metabolism of model organophosphate pesticides (parathion and chlorpyrifos) in liver from humans of various ages. A sensitive and specific HPLC method was developed to quantify the products of biotransformation resulting from incubations of pesticides with human liver microsomal specimens and recombinant human CYPs. The kinetics of parathion metabolism (1 to 100  $\mu$ M) was initially assessed in six specimens of CYP characterized human liver microsomes from males and females of 19 to 54 years of age. The Km for the formation of paraoxon and p-nitrophenol ranged from 8.7 to 39.1 and from 15.2 to 55.9  $\mu$ M, respectively, while the Vmax values ranged from 589 to 1495 and from 588 to 1232 pmol/min/mg, respectively. These results support the need to include a range of kinetic parameters to reflect the inherent interindividual variability in biotransformation. The Vmax for transformation of parathion to paraoxon in the six human liver microsomal specimens correlates with the formation of 6-beta hydroxytestosterone from testosterone, a marker for CYP3A4 activity ( $r^2 = 0.96$ ). Studies with recombinant CYPs also indicate that CYP1A2 and CYP2B6 contribute substantially to paraoxon formation, while CYP1A2 also effectively catalyzes the formation of p-nitrophenol. Additional studies will provide estimates of the age-specific kinetics of biotransformation of selected pesticides. These data will then be utilized in the multi-route, multi-chemical PBPK/ pharmacodynamic (PD) model (ERDEM, Exposure Related Dose Estimating Model) (Blancato et al., 2000; Knaak et al., 2001) to estimate the sensitivity of individuals within various age groups. (Supported by USEPA STAR grant R-83068301)

**2158**

ORGANOPHOSPHORUS PESTICIDES INHIBIT PRENYLATED METHYLATED PROTEIN METHYL ESTERASE.

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Prenylation and subsequent S-adenosyl-L-methionine-dependent methylation are essential secondary modifications for the biological activity of a significant number of eucaryotic proteins. Prenylated proteins such as the G- $\gamma$  subunits of G-protein coupled receptors, nuclear lamins, guanine nucleotide binding proteins such as Ras and Rab are prenylated and undergo methylation. Prenylated methylated protein methyl esterase (PMPMEase) readily hydrolyses the prenylated protein methyl esters thus making this step reversible and possibly regulatory. Organophosphorus (OPs) are known to cause conditions such as Parkinsonism and neurodegeneration. Mutations of the gene for paraoxonase, a liver esterase, are a risk factor for Parkinson's disease. The effect of OPs on PMPMEase was thus investigated. Benzoyl-glycyl-farnesyl-cysteine methyl ester (BzGFCM) was developed as a specific PMPMEase substrate and characterized by electron-spray ionization mass spectrometry to be of the calculated molecular mass. Rat liver supernatant and brain membrane extracts hydrolyzed BzGFCM forming benzoyl-glycyl-farnesyl-

cysteine (BzGFC) in a time- and concentration-dependent manner. The liver and brain enzymes cleaved BzGFCM with Km values of  $4.58 \pm 0.30$  and  $25.57 \pm 2.36$   $\mu$ M and Vmax values of  $2.21 \pm 0.03$  and  $0.17 \pm 0.003$  nmol/min/mg, respectively. The liver enzyme eluted from a Sephadryl 200 HR gel-filtration column as a single peak with an apparent size of 89 kDa. The brain enzyme eluted as two peaks of 53 and 890 kDa. OPs inhibited the liver enzyme with IC50 values from 4.77  $\mu$ M for parathion to 0.04  $\mu$ M for paraoxon, respectively. Only about 50% of the brain activity was inhibited by 0.4 to 1 mM solutions of paraoxon. Mipafox did not significantly inhibit the brain enzyme. BzGFCM was not hydrolyzed by various cholinesterases indicating its specificity for PMPMEase. Perturbations in prenylated protein metabolism may play a role in non-cholinergic OPs-induced toxicity since prenylated proteins play such important roles in cell signaling, proliferation, differentiation and apoptosis.

**2159**

ASSESSING EXPOSURES TO FLEA CONTROL INSECTICIDES FROM THE FUR OF DOGS TREATED WITH FLEA COLLARS.

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There are reported insecticide residues present in food, water, and on surfaces such as carpets treated for flea control. However, no studies have quantified dislodgable flea control insecticide residues on pets that could be transferred to children. Previous studies conducted in our laboratory have indicated that these dislodgable residues may be in the ng range. This study involved the treatment of pet dogs with over-the-counter flea collars containing the insecticides chlorpyrifos or tetrachlorvinphos. Insecticide residues on cotton gloves used to pet the dogs for 5 minutes and cotton t-shirts worn by a child for a 4-hour period were obtained. First morning urine samples were obtained from one adult and one child for urinary metabolite quantification. Transferable residues of both insecticides to the gloves were highest on and near the collar and were lowest in areas distant from the collar. The 14 day mean residues for chlorpyrifos over the collar and in the tail region were  $503 \pm 84$  ng/glove and  $10 \pm 2$  ng/glove, respectively. Chlorpyrifos residues observed at 20 days post application were  $434 \pm 78$  ng/glove and  $7 \pm 2$  ng/glove, respectively. Tetrachlorvinphos residues at 14 days post treatment were 22,  $413 \pm 3042$  ng/glove over the collar and  $81 \pm 20$  ng/glove in the tail region. The mean residues at 20 days post treatment were 15,  $788 \pm 2195$  ng/glove and  $82 \pm 34$  ng/glove, respectively. Chlorpyrifos t-shirt residue levels varied from 121 ug/g to 201 ug/g and tetrachlorvinphos t-shirt residue levels were in a range from 923 ug/g to 3098 ug/g. There were no significant differences in the levels of 3, 5, 6-trichloropyridinol, the urinary metabolite of chlorpyrifos, in adults or children after the placement of the collar. However, children had higher levels of the metabolite than adults, 15-20 ng/ml compared to 5-10 ng/ml. (Supported by EPA Grant R828017)

**2160**

EFFECT OF PYRETHRINS ON CYTOCHROME P450 FORMS IN CULTURED RAT AND HUMAN HEPATOCYTES.

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High doses of Pyrethrins (PY) have been shown to produce liver and thyroid tumors in rats by a mode of action involving the induction of hepatic xenobiotic metabolising enzymes. The aim of this study was to compare the effects of PY with those of the rat liver and thyroid tumor promoter sodium phenobarbital (NaPB) on some cytochrome P450 (CYP) forms in cultured rat and human hepatocytes. Female Sprague-Dawley rat and human (both male and female) hepatocytes were cultured for 3 days with 0-1000  $\mu$ M PY or NaPB. Over this concentration range PY and NaPB were not markedly cytotoxic to either rat or human hepatocytes. Levels of CYP mRNAs were determined by real-time quantitative reverse transcription-polymerase chain reaction methodology. Enzyme activities were determined in intact monolayers. In rat hepatocytes, treatment with PY and NaPB increased levels of CYP2B1 mRNA up to 8 and 28 fold, respectively, and levels of CYP2B1/2 mRNA up to 5 and 30 fold, respectively. PY and NaPB increased 7-benzyloxy-4-trifluoromethylcoumarin O-debenzylase activity (a CYP1A/2B marker) up to 3 and 5 fold, respectively. Both PY and NaPB also increased CYP2B-dependent 7-pentoxyresorufin O-depetylase and CYP3A-dependent testosterone 6 $\beta$ -hydroxylase activities. In human hepatocytes, treatment with PY and NaPB increased levels of CYP3A4 mRNA up to 15 and 17 fold, respectively, and testosterone 6 $\beta$ -hydroxylase activity up to 2.4 and 4.2 fold, respectively. The effects of PY and NaPB were concentration-dependent and exhibited a threshold, with low concentrations hav-

ing no effect on the markers of hepatic xenobiotic metabolism studied. These results demonstrate that both PY and NaPB induce CYP2B and to a lesser extent CYP3A forms in cultured rat hepatocytes. Like NaPB, PY can also induce CYP forms in cultured human hepatocytes. (Supported by the Pyrethrin Joint Venture).

## 2161

### RELATIVE POTENCIES FOR ACUTE EFFECTS OF PYRETHROIDS ON MOTOR FUNCTION IN RATS.

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A proposed common mode-of-action for pyrethroid insecticides, includes alterations in sodium channel dynamics in nervous system tissues, consequent disturbance of neuronal membrane polarization, abnormal discharge in targeted neurons, and changes in nervous system function. The present work provides *in vivo* functional data for use in the cumulative risk assessment of pyrethroids. This work characterized dose-response curves and calculated relative potencies for motor dysfunction produced by 11 commonly used pyrethroids. Five Type I (bifenthrin, s-bioallethrin, permethrin, resmethrin, tefluthrin) and six Type II compounds (beta-cyfluthrin, lambda-cyhalothrin, cypermethrin, deltamethrin, esfenvalerate, fenpropathrin) were tested in adult male Long Evans rats. Acute oral dose-response curves for motor function were characterized for each chemical [n=7-12 per dose, 6-8 dose levels per chemical, 1 ml/kg corn oil vehicle]. Motor function was measured using figure-8 mazes. Animals were tested for one hr during the period of peak effects (between 1 and 4 hour after dosing, as determined in pilot studies). All pyrethroids, regardless of structural class, produced dosage-dependent decreases in motor activity. Individual chemical ED30s were calculated using a flexible single-chemicals-required method of analysis applicable to chemicals with differing maximum-effect asymptotes (Gennings et al., 2004). Relative potencies were calculated based as the ratio of each chemical's ED30 to the ED30 of the index chemical, deltamethrin (ED30 ~3 mg/kg). Preliminary data analyses revealed ED30s that ranged from 1.4 to 210 mg/kg and estimations of the relative potency ratios for the pyrethroids ranged from 0.5 to 70. These data suggest a common toxicity endpoint for effects on general motor function in rats that can be used for assessing cumulative risk. Future work will test the ability of this common endpoint to predict additivity of pyrethroid mixtures. (This is abstract does not necessarily reflect USEPA policy)

## 2162

### USING ARRAY TECHNOLOGY TO IDENTIFY POTENTIAL BIOMARKERS FOR PYRETHROID INSECTICIDES.

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Pyrethroid insecticides affect nervous system function by disruption of sodium channels in nerve membranes. FQPA requirements for assessing cumulative risk have increased the need for rapid and sensitive biomarkers of effect. This project aims to develop biochemical markers of neurotoxicity following acute exposure to pyrethroids. Gene arrays were used to determine candidate proteins to serve as biomarkers. Male rats were orally exposed to deltamethrin (1-6 mg/kg, DLT) or permethrin (50-200 mg/kg, PER) in corn oil vehicle. Tissue was collected at 2, 6, or 24 hr after exposure. Total RNA was isolated from whole brain or frontal cortex. RNA was analyzed using either ClonTech Rat Toxicology 1.2 arrays (whole brain) or Affymetrix Rat 230 2.0 arrays (frontal cortex). Data analyses revealed relatively few genes that were altered in both PER and DLT treated tissue. For PER, 115 transcripts responded in a dose-related manner (including at least a 3-fold increase at highest dose). For DLT, 126 genes responded in a dose-related manner. From these two sets, 13 transcripts were identified as responding similarly for both pyrethroids, including 7 genes with known identities: e.g. carboxylesterase 1, recoverin, phenylalanine hydroxylase, leucyl-specific aminopeptidase PILS. For DLT treatment using the ClonTech Rat Toxicology 1.2 array, 29 genes responded in a dose-dependent manner. Of the 13 transcripts common to the two pyrethroids in the Affymetrix array, 2 were up-regulated >3-fold in the ClonTech Rat Toxicology 1.2 array with the same dose of DLT at 24 hours. These differences may be due to the use of frontal cortex versus whole brain or variation in treatment times. Future work will involve confirming candidate genes from both technologies using qRT-PCR or protein activity assays. (This is abstract does not necessarily reflect USEPA policy)

## 2163

### ACTIVATION OF MITOGEN-ACTIVATED PROTEIN KINASE AND TRANSCRIPTIONAL FACTORS PRECEDES CYCLOOXYGENASE-2 GENE EXPRESSION IN MACROPHAGES EXPOSED TO THE O, P- DDT.

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Dichlorodiphenyltrichloroethane (DDT) is used as an insecticide and prevents many people in the tropical zone from devastating malaria. On the other hand, a number of reports have indicated that it may act as an endocrine disruptor and also has possible carcinogenic effects. The purpose of this study was to test the hypothesis that o, p-DDT induces cyclooxygenase-2 (COX-2) gene expression in macrophages and that this is regulated at the level of mitogen-activated protein kinases (MAPKs). Exposure of the murine macrophage cell line RAW 264.7 to o, p-DDT for 24 h markedly enhanced the production of prostaglandin E2 (PGE2), a major COX-2 metabolite. PGE2 elevation was preceded by increases in COX-2 mRNA and COX-2 protein in o, p-DDT-treated cells. o, p-DDT induced rapid phosphorylation of extracellular signal regulated protein kinases 1 and 2 (ERK1/2), p38 and c-Jun N-terminal kinases 1 and 2 (JNK1/2) phosphorylation. To investigate the significant cis-acting regions which COX-2 promoter, transient transfection experiments were carried out using reporter vectors harboring deleted COX-2 promoters. The transcriptional factor binding sites for activator protein 1 (AP-1) and NF- $\kappa$ B could be important for the induction of COX-2 by o, p-DDT. The results of these studies suggest that induction of transcriptional activation of COX-2 by o, p-DDT might be mediated through the AP-1 and NF- $\kappa$ B activation.

## 2164

### SUCCESSFUL ELIMINATION OF PARAQUAT BY HEMOPERFUSION USING PULSATILE EXTRACORPOREAL CIRCULATION.

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A 42-years old female was admitted to our emergency department for ingestion of paraquat as suicide attempt. She had no previous medical history or operation history. She ingested paraquat (Gramoxone<sup>®</sup>) and alcohol 3 hours before. She had a shallow ulceration on tongue. We began gastric lavage with distilled water via orogastric tube as much as 20, 000 ml. Then, we poured activated charcoal powder into her stomach via orogastric tube for reduction of paraquat absorption. Using qualification method, paraquat was identified in her urine. Qualification method that is 2N Sodium hydroxide 1ml was mixed with Sodium hydrosulfite 10mg, and mixed reagent was mixed with the patient's urine 10ml during 30 seconds. After 2 minutes, the color of urine was identified. The positive result was bluish or blue greenish change. Her vital sign was stable. Hemoperfusion with charcoal filter (Adsorba300<sup>®</sup>, Gambro) was initiated after insertion of double lumen central vein catheter with pulsatile extracorporeal circulation unit (T-PLS<sup>®</sup> New Heartbio, Korea) in intensive care unit. She was covered with Vinyl curtain for prevention of oxidation of already absorbed paraquat. After 90 minutes of hemoperfusion, qualification of urine and serum converted to negative. Then, after 3hrs of hemoperfusion, the same qualification shown to be negative. Hemoperfusion was stopped. She was received diuretics and antiemetics. After 2 days later, she was moved to general ward and started regular diet. At that times, qualification test was negative. We recommended the being consultation of psychiatrics for suicide attempt but, she refused. She was discharged on 5th hospital day without complication and was prescribed methylprednisolone for prevention of pulmonary fibrosis. She was observed at our patient department without any complications.

## 2165

### DDT-INDUCED AROMATASE AND CYCLOOXYGENASE-2 GENE EXPRESSION IN TESTICULAR LEYDIG R2C CELLS.

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Dichlorodiphenyltrichloroethane (DDT) is a widespread environmental pollutant. Aromatase (estrogen synthase) is the cytochrome P450 enzyme complex that converts C19 androgens to C18 estrogens. In this study, we investigated the effect of DDT on aromatase gene expression through cyclooxygenase-2 (COX-2) in testicular Leydig R2C cells. Exposure of the R2C cells to DDT markedly enhanced COX-2 gene expression. Enhanced COX-2 enzyme levels by DDT treatment results in

increased production of prostaglandin E2 (PGE2), a major COX-2 metabolite. COX-2 inhibitors decreased DDT-induced PGE2 production. Furthermore, PGE2 increased aromatase activity in leydig R2C cells. In addition, DDT was found to increase aromatase gene expression in R2C cell in a dose dependent manner. Our results indicated that the increase of aromatase gene expression by DDT in R2C cell is mediated through increased PGE2 production by DDT-induced COX-2 gene expression. Overall, elevated levels of these factors in R2C cells microenvironment can result in increased aromatase activity via autocrine mechanisms in R2C cells.

## 2166 INDUCTION OF CYP3A4 BY O, P'-DDT IN HEPG2 CELLS.

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For almost 50 years, million of Mexicans have been directly or indirectly exposed to DDT (1, 1, 1-trichloro-2, 2-bis(4-chlorophenyl) ethane). DDT a well known endocrine disruptor agent is an organochlorine pesticide still used in many developing countries for the control of vector-transmitted diseases. Although DDT was banned in many countries, is still present in the environment due to its resistant to degradation. Among DDT effects, it has been reported its capacity to induce rodent cytochrome P450 (CYP) 2B and 3A subfamilies. However, little information is available of its effects on the regulation of human CYPs. Technical grade DDT contains 80% of the *o*, *p*'-DDT and 20 % of the *o*, *p*-DDT isomers. The *o*, *p*'-DDT isomer exhibits estrogenic activity through its binding to the estrogen receptor (ER). The ER is a ligand-inducible transcription factor that modulates the expression of several genes including CYP3A4, which is the predominant CYP expressed in human liver. CYP3A4 contributes to the metabolism of approximately half the drugs in use today, and catalyzes the 6 $\beta$ -hydroxylation of a number of steroids including cortisol, testosterone and progesterone. The aim this study was to analyze the effects of *o*, *p*'-DDT on CYP3A4 gene expression in HepG2 cell cultures. HepG2 cells were exposed to up to 10  $\mu$ M of *o*, *p*'-DDT during 12 and 24 hours. After treatment, cells were harvested and cellular contents were extracted for mRNA and immunoreactive protein analysis. Treatment with *o*, *p*'-DDT resulted in a significantly increase in CYP3A4 mRNA and immunoreactive protein in a dose-dependent response, up to 13-fold and 3.8 fold, respectively with the highest dose compared to untreated cultures. Our results indicate that *o*, *p*'-DDT was able to activate transcriptionally CYP3A4 gene since treatment with actinomycin D blocked the CYP3A4 mRNA increase induced by *o*, *p*'-DDT. Finally, studies are in progress to define the mechanisms by which *o*, *p*'-DDT induce the expression of CYP3A4.

## 2167 N-DEALKYLATION IS A KEY DETERMINANT IN THE HEPATOTOXICITY OF CHLOROACETANILIDE HERBICIDE ALACHLOR.

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Chloroacetanilides alachlor, acetochlor, and metolachlor (*N*-methoxymethyl-, -ethoxymethyl-, and -methoxyisopropyl-, resp.) are widely used pre-emergent herbicides. Coleman et al. (Env. Hlth. Persp, 108: 1151, 2000) have shown rates of *N*-dealkylation of alachlor  $\gg$  acetochlor  $\gg$  metolachlor  $\sim$  0 with human and rat liver microsomes and alachlor *N*-dealkylation to 2-chloro-*N*(2, 6-diethylphenyl)acetamide (CDEPA) by CYP3A4. In our previous studies in SD rats, hepatotoxicity was alachlor  $\gg$  metolachlor (Miranda et al., Toxicol. Sciences, 66: 321, 2002). These results support the hypothesis that *N*-dealkylation by CYP3A1/2, rat ortholog of CYP3A4, plays a critical role in chloroacetanilide hepatotoxicity. The objectives of the present investigations were 1) to compare and evaluate hepatotoxicity of alachlor, acetochlor vs non-toxic metolachlor in isolated rat hepatocytes, and 2) to identify the possible role of the *N*-dealkylated CDEPA and CYP3A in the alachlor bioactivation pathway. Results of the present investigation indicate that indeed *N*-dealkylation is a determinant of hepatotoxicity of the chloroacetanilide herbicides in isolated rat hepatocytes as supported by EC50 values for alachlor (486 $\pm$ 43  $\mu$ M), acetochlor (465 $\pm$ 91  $\mu$ M) and metolachlor (708 $\pm$ 68  $\mu$ M). Rat hepatocytes catalyzed *N*-dealkylation of alachlor to CDEPA, and CDEPA (EC50 = 224  $\mu$ M) was much more toxic than parent compound in isolated rat hepatocytes. We also found that further metabolism of the CDEPA to DEA is a detoxification pathway as DEA EC50 is  $\gg$ 1600  $\mu$ M. However, our observation that CDEPA was still produced in the presence of CYP3A inhibitor clorotrimazole, which did not prevent alachlor cytotoxicity, implicates an enzyme other than CYP3A1/2. Preliminary studies on alachlor suggest the relevance of rat hepatocyte results with cryopreserved human hepatocytes (BD Gentest, Lot # 65, CYP3A4 activity 140 pmol/min/10e6 cells, EC50 ~700  $\mu$ M). Collectively these results support a critical role for *N*-dealkylation in chloroacetanilide hepatotoxicity catalyzed by an enzyme other than CYP3A. Support: LA Board of Regents.

## 2168

### TRIBUTYLtin REGULATED STEROIDOGENIC ENZYME GENES EXPRESSION AND CHANGES SEXUAL HORMONE IN MOUSE TESTICULAR LEYDIG CELLS.

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Tributyltin (TBT) is a widespread environmental pollutant and distributed hormonal functions in reproductive organs. In this study, we investigated that the regulation of steroidogenesis genes induced by TBT in the rat leydig cell line, R2C. As the results, TBT treatment stimulated testosterone synthesis gradually up in does dependent manner. Moreover, to confirm TBT-mediated testosterone synthesis, we investigated several kinds of steroidogenic enzyme expression in R2C cells. RT-PCR analysis revealed that TBT increased the level of aromatase mRNA in does dependent manner. In addition, TBT increased 3beta-hydroxysteroid dehydrogenase mRNA in does dependent manner. However, TBT did not significant effects on 17beta-hydroxysteroid dehydrogenase and cholesterol side chain cleavage enzyme (P450SCC) mRNA level. In the present study, we have demonstrated that TBT induces testosterone contents via inducing steroidogenic genes expression in rat testicular Leydig cells. Taken together, these results suggest that TBT may disrupt endocrine homeostasis not only by directly binding to aromatase as previously suggested but also by inducing expression of steroidogenic enzyme genes.

## 2169

### TRIBUTYLtin INDUCE CELL CYCLE ARREST IN RAT TESTICULAR LEYDIG CELLS.

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Tributyltin used world-wide in antifouling paints for ships is a widespread environmental pollutant and cause reproductive organs atrophy in rodents. We investigated the effects of tributyltin on rat testicular leydig cells cycle arrest to establishing induction of growth arrest and apoptosis of R2C, rat leydig cells. Our data show that the induction of cell apoptosis and cell cycle arrest at G0/G1 phase by tributyltin might be associated with the induction of p53 and p21, and mediated via the apoptosis-related bcl-2 family proteins. In this report, we further investigated the mechanism involved in the cell cycle arrest induced by tributyltin in R2C cells. The inhibitory effect of tributyltin on the cell cycle progression of R2C cells which arrested cells at the G0/G1 phase was associated with a marked decrease in the protein expression of p53 and an induction in the content of cyclin-dependent kinase (cdk) inhibitor p21 protein. Moreover, this effect was correlated with the elevation in Cyclin D/Cdk 4 complex declined, preventing the phosphorylation of retinoblastoma (Rb) and the subsequent dissociation of Rb/E2F complex. This provides strong evidence that the apoptotic effect of tributyltin, arresting cells at the G0/G1 phase, was exerted by inducing the expression of p21 that, in turn, repressed the activity of cyclin D1/cdk 2 and the phosphorylation of Rb.

## 2170

### THE BIPYRIDYL HERBICIDE PARAQUAT CAUSES OXIDATIVE STRESS-MEDIATED TOXICITY IN HUMAN NEUROBLASTOMA SH-SY5Y CELLS: RELEVANCE TO PARKINSON'S DISEASE.

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Paraquat (PQ) is a cationic non-selective bipyridyl herbicide widely used to control weeds and grasses in agriculture. Epidemiologic studies indicate that exposure to pesticides can be a risk factor in the incidence of Parkinson's disease (PD). A strong correlation has been reported between exposure to paraquat and PD incidence in Canada, Taiwan, and the United States. This correlation is supported by animal studies showing that paraquat causes toxicity in dopaminergic neurons of the rat and mouse brain. However, it is unclear how paraquat triggers toxicity in dopaminergic neurons. Based on the prooxidant properties of paraquat, we hypothesized that paraquat may cause oxidative stress-mediated toxicity in dopaminergic neurons. To explore this possibility, dopaminergic SH-SY5Y cells were treated with paraquat, and several biomarkers of oxidative stress were measured. First, paraquat increased intracellular levels of reactive oxygen species, but decreased the levels of glutathione. Second, paraquat inhibited glutathione peroxidase activity, but did not affect glutathione reductase activity. On the other hand, paraquat increased GST activity by 24 h, after which GST activity returned to the control value at 48 h. Third, paraquat dissipated mitochondrial membrane potential (MTP). Fourth, paraquat caused increases of malondialdehyde (MDA) and protein carbonyls, as well as DNA fragmentation, indicating oxidative damage to major cellular components. Taken together, current data verify our hypothesis that paraquat induces ox-

idative stress-mediated toxicity in dopaminergic SH-SY5Y cells. Thus, these results suggest that paraquat may cause oxidative stress in dopaminergic neurons of human brain, and support the hypothesis that paraquat is an environmental risk factor implicated in the incidence of PD.

2171

DIFFERENTIAL INDUCTION OF CYCLOOXYGENASE-2 (COX-2) AND HEME OXYGENASE-1 (HO-1) BY UVB LIGHT IN GROWING AND CALCIUM-DIFFERENTIATED PRIMARY CULTURES OF MOUSE KERATINOCYTES.

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Exposure to ultraviolet light as a component of sunlight has been demonstrated to be a major factor in the development of skin cancer. The high energy wavelengths of the ultraviolet B (UVB) spectra (290-320 nm) can cause damage to DNA and also initiate an inflammatory response as well as intracellular oxidative stress. Numerous studies have shown that the presence of inflammatory mediators and reactive oxygen species contribute to the development of cancer. COX-2, responsible for the formation of prostaglandins, is known to be induced by UVB in human skin. HO-1, known to be induced by reactive oxygen species, is an effective cellular antioxidant. In the present studies, we compared the effect of UVB on the expression of COX-2 and HO-1 in undifferentiated and calcium-differentiated primary mouse keratinocytes. Both cell types constitutively express small amounts of COX-2 and HO-1 mRNA as measured by real-time PCR. Differentiated cells, however, express 2-fold more HO-1 than undifferentiated cells. UVB light (2.5 to 25 mJ/cm<sup>2</sup>) caused a dose-dependent increase in expression of both COX-2 and HO-1 mRNA and protein as measured western blotting. Undifferentiated cells showed a greater response to UVB light than differentiated cells in expression of both COX-2 and HO-1, with approximately twice the induction at the maximal UVB dose. In contrast, the differentiated cells exhibited higher absolute induction at every UVB dose. Taken together, these data indicate that not only the dose of UVB but also the differentiation state of the keratinocytes is critical to expression of either a pro-inflammatory enzyme, COX-2, or an antioxidant and protective enzyme, HO-1. Supported by NIH grants ES05022, CA100994, CA093798 and ES011932

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DECREASING MALATHION APPLICATION TIME FOR LICE TREATMENT REDUCES TRANSDERMAL ABSORPTION.

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Background: Head lice are the most common parasitic infestation in the United States and Europe. Children are commonly infected with head lice requiring topical treatment with pediculicides. The instructions for the Ovide® 0.5% malathion formulation include placing formulation on dry hair and leaving it on for eight to twelve hours. Ovide®, however is effective at killing 100% of both lice and nits in ten minutes. Our concern of over exposing children to malathion has led us to examine whether significantly more malathion will penetrate transdermally when applied for the recommended eight hours than for a shorter but apparently equally effective period. Methods: *In vitro* absorption studies were performed to determine if reducing malathion application time decreases skin absorption. Ovide® was placed on either full thickness haired rat skin or human abdominal skin for 0.5, 2, 4, or 8 hours before removal, skin was thoroughly washed with shampoo and penetration was allowed to continue for either 24 (rat and human), 48 (rat) or 72 (rat) hours. Results: A 0.5 hour exposure caused 0.36 + 0.14 % of the donor malathion to penetrate through human skin after 24 hours and 2.1 + 0.6% remained in the skin after washing with shampoo. After 8 hours of topical applications penetration was approximately 3 fold greater (1.02 + 0.41) and 3.4 + 0.5% remained in the skin ( $p < 0.05$  vs 0.5 hr). The relationship between absorption and exposure time also occurred for haired rat skin ( $p < 0.05$ ) although the magnitude was smaller than for human skin. This differential continued for the full 72 hours studied. Conclusions: Reducing Ovide® application from the suggested 8-12 hours to 30 minutes can significantly reduce transdermal absorption of malathion, without decreasing the product's efficacy. Further clinical studies in children are warranted to confirm the efficacy of this shortened application time.

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DERMAL PENETRATION OF SODIUM LAURYL SULPHATE AND ITS EFFECT UPON THE ABSORPTION OF OTHER CHEMICALS *IN VITRO*.

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Sodium Lauryl Sulphate (SLS) (an anionic surfactant) is a known skin irritant. The irritation caused by SLS produces reversible changes in the barrier function of the skin. The objective was to investigate the dermal penetration and localisation of SLS through full-thickness and dermatomed human skin *in vitro* and its effect upon the dermal penetration of testosterone and water. Human breast skin was mounted in flow-through diffusion cells. Receptor fluid (MEM + 2%w/v BSA) was pumped at 1.5ml/hr (pH 7.4, 32°C). 2%w/v <sup>14</sup>C-SLS in water (25μl) was applied to the surface (0.64cm<sup>2</sup>) of full thickness (~1mm) and dermatomed (280μm) skin for 1hr, removed, and the distribution determined at 0, 3, 6, 12 and 24hrs after removal of the SLS. SLS (0, 1, 2, 4 and 10%w/v [100μl]) was applied to full-thickness skin for 1hr then rinsed off. <sup>3</sup>H-water (100μl) was applied to the treated surface for 11hrs. In a further study, <sup>14</sup>C-testosterone (10μl) was applied for 24hrs to full-thickness and dermatomed skin pre-treated with SLS. Following a 1hr application of SLS (2%w/v), 4-5% of the applied dose was absorbed into full-thickness skin, and 1-1.5% into dermatomed skin, with no significant change in distribution over time. SLS did not readily pass into the receptor fluid, remaining within the stratum corneum and dermis after 24hrs. The total amount of water penetrating to the receptor fluid increased with concentration of SLS pre-treatment. However, with increasing concentration of SLS, testosterone penetration into the receptor fluid, decreased through both full-thickness and dermatomed skin, remaining within the skin instead. It appears that SLS localisation affected the skin such, that the passage of a hydrophilic compound (water) into the receptor fluid was increased, whilst the penetration of a lipophilic compound (testosterone) was reduced. It is believed that SLS forms micelles within the stratum corneum and dermis, preventing lipophilic chemicals from passing into the receptor fluid *in vitro*.

2174

INVESTIGATION OF SKIN BARRIER CREAMS FOR LOWERING PENETRATION OF JP-8 JET FUEL THROUGH *IN VITRO* PIG SKIN.

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Skin irritation remains a concern for Air Force personnel exposed to JP-8 jet fuel. One manner to lower or eliminate skin and systemic JP-8 exposure is to use a skin-enhancement barrier cream. To determine inhibition of JP-8 penetration for a number of existing marketed products, we applied an even coverage of each barrier cream on a 10 cm<sup>2</sup> section of harvested dorsal pig skin (dermatome avg. 0.6mm). After application of even coverage of a barrier cream at room temp onto pig skin, and the skin was weighed, placed in static penetration cell at 37°C for 45 min, and exposed to JP-8 (2mL) for 4 hr. JP-8 chemical component penetration was captured in VOLPO-saline (20 mL) into the lower chamber. Samples (20μl) were withdrawn from the lower chamber sidearm and heated (140°C) to stable vapor phase using a headspace sampler for component separation on a non-polar SPB-1 column with FID detection. Total average area of eluted hydrocarbon vapor from samples (n=3) was compared between the coated and non-coated pig skin after the 4hr penetration study. Generally, products tested ranged from no barrier properties, to a 35% rate of the control. Also, after completion of the 4hr penetration experiment, the pig skin was wiped with water and paper towels, and skin punch samples (4mm diameter) were taken with a dermal biopsy punch for vapor headspace analysis. These skin punch results showed the best barrier creams for both barrier maintenance as well as those aiding surface removal of JP-8 from skin. The results varied from no benefit to 15% of the JP-8 amount in unprotected skin. The best barriers creams to JP-8 were pr88, Ply No. 9, Chimal, SERPACWA and Prolin skin guard. These results show that not all creams promoting non-polar barrier qualities would create a barrier to JP-8 penetration in this model. Further work will test the degree of protection against JP-8 irritation each cream offers with regards to skin exposure of JP-8 to New Zealand white rabbits.

2175

EFFECT OF ACUTE AND CHRONIC EXPOSURE TO THE CLEANSER, TRICHLOROETHYLENE, ON THE DERMAL ABSORPTION OF THE BIOCIDE TRIAZINE.

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Triazine is often added to cutting-fluid formulations in the metal machining industry as a preservative that inhibits the growth of biological material. Unfortunately, triazine has been shown to cause contact dermatitis in exposed workers.

Trichloroethylene, TCE, is a solvent used for cleaning and rinsing. The purpose of this study was to examine the effect of TCE on the dermal absorption of the biocide triazine. A porcine skin *in vitro* flow-through diffusion cell system was used for these studies. In one set of experiments, the diffusion cells were dosed topically with <sup>14</sup>C-Triazine mixtures containing TCE and the 3 most common cutting-fluid components as aqueous mineral oil (MO) or polyethylene glycol (PEG) mixtures. The cutting-fluid components were 5% linear alkylbenzene sulfonate (LAS), 5% triethanolamine (TEA), and 5% sulfated ricinoleic acid (SRA). In another set of experiments, the diffusion cells were dosed with <sup>14</sup>C-Triazine mixtures containing the cutting-fluid components in aqueous MO or PEG-based mixtures after having been pre-exposed *in situ* to TCE for 96 hours. In the MO-based formulations, the absorption of <sup>14</sup>C-Triazine ranged from 3.05 to 3.65% dose. In the PEG-based formulations, the absorption of <sup>14</sup>C-Triazine ranged from 2.70 to 4.06% dose. In both sets of experiments, the porcine skin that was pre-exposed *in situ* to TCE showed greater absorption of <sup>14</sup>C-Triazine. The chronic effects of TCE appear to be more important in the PEG-based mixtures than in the MO-based mixtures. Supported by NIOSH Grant R01-OH-03669

**2176**

EFFECT OF *IN VIVO* JET FUEL EXPOSURE ON SUBSEQUENT *IN VITRO* DERMAL ABSORPTION OF AROMATIC AND ALIPHATIC HYDROCARBONS.

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The percutaneous absorption of topically applied jet fuel hydrocarbons (HC) through skin previously exposed to fuel has not been studied, although this exposure is the occupational norm. Pigs were exposed to JP-8 fuel soaked cotton for 1 and 4 days with repeated daily exposures. Pre-exposed and unexposed skin was dermatomed and placed in *in-vitro* diffusion cells. Five cells with exposed and four cells with unexposed skin were dosed with a mixture of 14 HC consisting of nonane, decane, undecane, dodecane, tridecane, tetradecane, pentadecane, hexadecane, ethyl benzene, o-xylene, trimethyl benzene (TMB), cyclohexyl benzene (CHB), naphthalene, and dimethyl naphthalene (DMN) in water + ethanol (50:50) as diluent. Five cells containing JP-8 exposed skin were dosed solely with diluent to determine skin HC retention. Flux, diffusivity and permeability were calculated. There was a 2- and 4-fold increase in absorption of specific aromatic HC like ethyl benzene, o-xylene and TMB through 1 and 4-day JP-8 pre-exposed skin, respectively. Similarly, dodecane and tridecane were absorbed more in 4-day than 1-day JP-8 pre-exposed skin. Absorption of naphthalene and DMN was 1.5 times greater than controls in 1 and 4-day pre-exposures. CHB, naphthalene and DMN had significant skin retention in 4-day pre-exposures as compared to 1-day exposures that might be capable of further absorption days post exposure. The possible mechanism of an increase in HC absorption in fuel pre-exposed skin may be via lipid extraction from the stratum corneum as indicated by Fourier Transform Infrared (FTIR) spectroscopy. This study suggests that pre-exposure of skin to jet fuel enhances the subsequent *in vitro* absorption of HC. Single dose absorption data from naive skin may not be optimal to predict the toxic potential for repeated exposures. For certain compounds, persistent absorption may occur days after the initial exposure. Supported by USAFOSR F49620-01-1-0080

**2177**

ABSORPTION OF LAWSONE THROUGH HUMAN SKIN.

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Lawson (2-hydroxy-1, 4-naphthoquinone) is the principal color ingredient in henna, a color additive approved with limitations for coloring hair by the Food and Drug Administration under 21 CFR 73.2190. The safety of lawson as a coloring agent in hair dye products has recently been evaluated by the Scientific Committee on Cosmetics and Non-Food Products (SCCNFP) of the EU. The SCCNFP concluded that lawson was mutagenic and not suitable for use as a hair coloring agent. Studies were conducted to measure the extent of lawson absorption through human skin. Lawson skin absorption was determined from two hair coloring products and two shampoo products, all containing henna. [<sup>14</sup>C]-Lawson (Sp. Ac. 22.9 mCi/mmol) was added to each commercial product and applied to excised, non-viable human skin (approx. 240 microns thick) mounted in flow-through diffusion cells perfused with a physiological buffer (HEPES-buffered Hanks' balanced salt solution, pH 7.4). Products remained on the skin for 5 min (shampoos) and 1 h (hair color paste). For the henna hair paste products, 0.29 and 1.4% of the applied dose was absorbed into the receptor fluid in 24 h while 2.2 and 3.7% remained in the skin. For the henna shampoo products, 0.32 and 0.34% of the applied dose was absorbed into the receptor fluid at 24 h while 3.6 and 6.8% remained in the skin. For all products, most of the lawson applied was washed from the surface of the skin (83 - 102%) at the end of the exposure period.

Extended absorption studies were conducted for 72 h to determine if skin levels of lawson in the 24 h studies might eventually be percutaneously absorbed. These studies determined that the majority of the lawson remained in the skin with little diffusing out into the receptor fluid. For example, the 72 h receptor fluid values following administration of henna paste products were only 0.48 and 1.61%. Since most of the receptor fluid values did not increase significantly during the extended studies, the receptor fluid values at 24 h should be used in a subsequent exposure assessment.

**2178**

SKIN PENETRATION OF BREAK-FREE CLP IN THREE SPECIES; SPRAGUE DAWLEY RAT, CD-1 MOUSE, AND YORKSHIRE PIG.

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Break-Free CLP (a complex mixture of polyalphaolephin oil, proprietary ingredients and other hydrocarbons) is used by the US and some European military, police departments and private citizens to clean, lubricate and protect firearms. Skin exposures from this widespread use are likely, although it is unknown whether systemic toxicity can result from skin contact. The purpose of this study was to determine the penetration of Break-Free in laboratory animal skin so that the hazards to humans can be estimated. We used static diffusion cells and GC/MS headspace analysis of receptor and skin samples to estimate the flux of liquid Break-Free across the skin for up to six hours. The sum of the concentrations of six major constituents of Break-Free was used as a surrogate for the concentration of the mixture. The constituents were decane (RT = 2.86 min), butanedioic acid dimethyl ester (RT = 3.2 min), undecane (RT = 4.25 min), dodecane (RT = 6.9 min), hexanedioic acid dimethyl ester (RT = 8.8 min), and tridecane (RT = 11.4 min). Average total flux values were 9.78 ug/cm<sup>2</sup>/h for pig, 43.2 ug/cm<sup>2</sup>/h for rat, and 131 ug/cm<sup>2</sup>/h for mouse. Concentration of chemical in the skin following a 6 hour exposure was 0.949 ug/mg skin in pig, 9.78 ug/mg skin in rat, and 31.6 ug/mg skin in the mouse. As expected, different components of Break-Free were found in the receptor solution than were found in the skin following exposure. The major Break Free components found in the receptor solution of all three species were decane and undecane, while the skin had highest concentrations of butanedioic acid dimethyl ester and dodecane. All six measured components were present to some extent in both receptor solution and skin of all species examined. We conclude that penetration of Break-Free is measurable but low in these species. The potential for systemic toxicity from this mixture can be evaluated based on toxicity of the individual components. (Funded by Naval Health Research Center Detachment Environmental Effects Laboratory)

**2179**

CHANGES IN GENE EXPRESSION IN RAT EPIDERMIS AFTER JET FUEL (JP-8) EXPOSURE TO THE SKIN.

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An estimated 13 million US workers are potentially exposed to chemicals and as a result irritant contact dermatitis is a common occupational disease. Understanding the molecular mechanisms behind skin irritation may establish prophylactic and therapeutic interventions for jet fuel-induced skin disease. No previous studies have shown how JP-8 fuel affects gene expression of skin cells located in the epidermal layer. The purpose of our study was to identify genomic changes in rat epidermis resulting from jet fuel-induced skin irritation. Five hundred microliters of JP-8 was placed in a Hill Top Chamber and secured with a Lomir rodent jacket onto the shaved mid-scapular region of male Fisher 344 rats. Exposure time was one hour, and the skin was collected and observed at 1, 4, and 8 hours after the beginning of the exposure. Skin samples were compared with samples from sham-treated rats. At the conclusion of the experiment, skin was excised, and the epidermis was removed in 5 micron sections using a cryotome. Total RNA was isolated from the epidermal layer. We measured changes in gene expression using the Affymetrix gene array (Rat Genome U34A chip). This experiment showed that changes in gene transcripts occur as early as one hour and become progressively greater after initiation of treatment with JP-8. At one hour, 116 genes were up regulated and 14 were down regulated. At four hours, 175 gene transcripts were up regulated and 100 were down regulated. At eight hours, 327 transcripts were up regulated and 473 were down regulated. When transcripts showing the greatest changes were cross-referenced within the Simplified Gene Ontology database (GeneSpring), the molecular function categories associated with these transcripts differed over time. After one hour, the nucleic acid binding genes were up-regulated the most. Enzymes were the most changed category of genes at the two later time points. These studies illustrate the rapid response of gene expression and the sequence of gene induction immediately after JP-8 exposures to the skin. (Funded by Air Force Office of Scientific Research)

## PROTEOMIC ANALYSIS OF JP-8 JET FUEL EXPOSURE IN HUMAN KERATINOCYTE CELL CULTURE.

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Dermal exposure to jet fuel is a significant occupational hazard. Previous studies have investigated its absorption and disposition in skin, and the systemic biochemical and immunotoxicological sequelae to exposure. Despite studies of JP-8 jet fuel components in murine, porcine or human keratinocyte cell cultures, proteomic analysis of JP-8 exposure has not been investigated. This study was conducted to examine the effect of JP-8 administration on the human epidermal keratinocyte (HEK) proteome. Using a two-dimensional electrophoretic approach combined with mass spectrometric-based protein identification, we analyzed protein (2DE) expression in HEK exposed to 0.1% JP-8 in culture medium for 24 h. Using 1 mL medium, HEK reached 65% confluence, while in 2 mL they achieved 95% confluence. Nearly 1, 200 protein spots were resolved by 2DE and image analysis. In the 95% confluent group, 0.1% JP-8 exposure resulted in significant expression differences ( $P < .01$ ) in 32 proteins of the 929 proteins matched and analyzed. In the slow growing cells, 45 proteins were differentially expressed ( $P < .01$ ). In both groups, roughly a third of these alterations were increases in protein expression, two-thirds were declines with JP-8 exposure. 2-way ANOVA was unable to demonstrate any interaction between confluence and JP-8 effects. Although 13 more proteins were altered ( $P < .01$ ) by JP-8 in the 65% slow-growing group, all proteins common to both groups responded similarly to JP-8. Peptide mass fingerprint identification of effected proteins revealed a variety of functional implications. In general, up-regulated proteins involved endocytotic/exocytotic mechanisms, their cytoskeletal components, and cell stress. Down-regulated proteins generally included those involved in vesicular function. Supported by AFOSR Grants # F49620-03-1-0089 (FAW) & F49620-01-1-0080 (JER).

## EXPRESSION PROFILING OF HUMAN EPIDERMAL KERATINOCYTE RESPONSE FOLLOWING JP-8 EXPOSURE.

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Jet fuel exposure initiates and promotes many biological and toxicological changes in human epidermal keratinocytes (HEK). In order to elucidate the molecular mechanisms involved in jet fuel toxicity, cDNA microarray was performed to examine the changes in gene expression in HEK after neat JP-8 exposure. Total RNA of controlled HEK and HEK cells harvested at 12 and 24 h after dosing were extracted for microarray analysis of 9600 expressed sequence tags (EST) including cancer, apoptosis, inflammation and metabolism-related genes. One-way ANOVA was employed to identify statistically significant ( $p < 0.05$ ) genes. The results showed that a total of 150 genes (1.6%) were identified as JP-8 responsive and their expression profiles were classified into 9 clusters by self-organizing map (SOM). Among the significantly regulated genes, those involved in basal transcription and translation were up-regulated while genes related to DNA repair, metabolism and keratinization were mostly down-regulated. Genes encoded for growth factors, apoptosis, signal transduction and adhesion were also altered. These results indicate that HEK responds to a single dose of JP-8 insult. A cascade of change in gene expression follows and demonstrated a complex expression profile. The data also describes several cellular processes previously not associated with jet fuel exposure. Taken together, this initial microarray analysis could advance the understanding of HEK responses to jet fuel exposure and provides a platform from which further studies concerning mechanisms and pathways of jet fuel toxicity could be approached. Supported by National Science Committee, Taiwan NSC 92-2313-B-005-117

## JP-8 JET FUEL EXPOSURE INDUCES INFLAMMATORY CYTOKINES IN RAT SKIN.

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The DoD has identified that one of the main complaints of personnel exposed to JP-8 jet fuel is irritant dermatitis. The purpose of this investigation is to describe the JP-8 induced inflammatory cytokine response in skin. JP-8 jet fuel or acetone

control was applied to the denuded skin of rats once a day for seven days. Skin samples from the exposed area were collected 2 and 24 hours after the final exposure. Histological examination of skin biopsies showed neutrophilic inflammatory infiltrate. RT-PCR was performed utilizing skin total RNA to examine the expression of various inflammatory cytokines. The CXC chemokine GRO-alpha was significantly upregulated at both time points, whereas GRO-beta was only increased 2 hours post final exposure. The CC chemokines MCP-1, Mip-1alpha, and Eotaxin were induced at both time points, whereas Mip-1beta was induced only 24 hours post exposure. IL-1beta, and IL-6 mRNA were significantly induced at both time points, while TNF-alpha was not significantly different from control. ELISA of skin protein confirmed that MCP-1, TNF-alpha, and IL-1beta were modulated as indicated by PCR analysis. However, skin IL-6 protein content was not increased at either time point. Data from the present study indicates that repeated (seven day) JP-8 exposure induces numerous proinflammatory cytokines in skin. The increased expression of these cytokines and chemokines may lead to increased inflammatory infiltrate in exposed skin, resulting in JP-8 induced irritant dermatitis.

## PROTEOMIC ANALYSIS OF NANOPARTICLE EXPOSURE IN HUMAN KERATINOCYTE CELL CULTURE.

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There has been explosive growth in engineering disciplines using nanoparticles, structures with characteristic dimensions between 1 and 100nm. These range from applications in the fields of ceramics to microelectronics as well as many areas of drug delivery. Nanomaterials have shown that they can be incorporated into human keratinocytes (HEK) in cell culture. There is no information available on the protein expression in cultured HEKs exposed to nanomaterials. This study was conducted to determine the effect of multi-walled carbon nanotubes (MWCNT) on the HEK cell proteome. Using a two-dimensional electrophoresis-based proteomics approach, we analyzed protein expression in HEKs exposed to 0.4mg/ml of MWCNT in culture medium for 24 h. Approximately 970 protein spots were resolved by two-dimensional electrophoresis and their relative abundance compared using image analysis. The expression of 32 proteins was significantly altered ( $p < 0.05$ ) by the MWCNT. Peptide mass fingerprint identification of the effected proteins revealed the identities of 22 proteins, 15 of which were upregulated while 7 decreased in expression. The protein alterations suggest dysregulation of intermediate filament expression, cell cycle inhibition, effects on metabolism, increased lysosome/melosome biogenesis, altered vesicular trafficking/exocytosis, and membrane scaffold protein down-regulation. Supported by AFOSR Grant F49620-03-1-0089 (FAW) and Center for Chemical Toxicology Research and Pharmacokinetics (NAMR).

## NANOTUBE DISPERSAL IN HUMAN KERATINOCYTE CELL CULTURE USING SURFACTANTS.

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Nanoparticles exhibit a variety of unique chemical and physical properties. These unique properties have placed these particles in the forefront of emerging technologies and may result in similarly unique biological effects. We are currently studying the interactions of multi-walled carbon nanotubes (MWCNT) in cultured human keratinocytes. Many of these particles cannot enter the cell since substantial van der Waals attractions cause them to readily aggregate in the aqueous culture medium. Surfactants may be used to disperse these aggregates to increase the effective MWCNT concentration. However, these surfactants must not be toxic to the cells. HEKs were exposed to serial dilutions (10%, 5%, 1%, 0.5%, and 0.1%) of Pluronic® (L61, L92, and F127) and Tween (20 and 60) surfactants in media for 24 h in a humidified 5% CO<sub>2</sub> atmosphere at 37°C. HEK viability (neutral red uptake), proportional to surfactant concentration, ranged from 27.1% to 98.5% with Pluronic® F127; cell viability with the remaining surfactants was less than 10%. MWCNT (0.4mg/ml), vortexed and sonicated in media containing 5% or 1% Pluronic® F127, were incubated with HEKs under culture conditions for 24 h. Media was harvested at 1, 2, 4, 8, 12, and 24 h post exposure, quickly frozen, and stored at -80°C until IL-8 assay. The 24 h viability of HEKs exposed to both 5% F127 + MWCNT and 1% F127 + MWCNT was significantly less ( $p < 0.05$ ) than the corresponding media and surfactant controls. Normalized IL-8 release from the F127 and F127 + MWCNT treatments was significantly higher ( $p < 0.05$ ) than the

corresponding media controls. Preliminary data indicates that the surfactant Pluronic® F127 does reduce MWCNT aggregates in the aqueous medium without causing a significant decrease in HEK viability.

**2185**

LOCALIZATION OF INTRADERMALLY INJECTED QUANTUM DOT NANOPARTICLES IN REGIONAL LYMPH NODES.

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The *in vivo* disposition of nanoscale materials is of interest due to their potential inclusion in a large number of products. As part of a project to determine the dermal penetration of nanoscale material, we examined the disposition of nanoscale material injected in the dermis of mice. Quantum dots (QD) consisting of CdSe nanocrystals, encased in a ZnS shell and PEG outer coating were injected intradermally (ID) in female SKH-1 hairless mice and their disappearance from the injection site and disposition were determined using digital macrophotography and fluorescence microscopy. Mice were injected in each flank with 5  $\mu$ l of 5.5  $\mu$ M red QD (655 nm emission; 6 x 12 nm rods) and 7.5  $\mu$ M yellow QD (565 nm emission; 6 nm spheres), or sterile saline, imaged under UV illumination, and sacrificed at 0, 6, 12, 18, 24, and 48 hrs. Under UV illumination, the highly fluorescent QD could be observed and digitally imaged moving from the injection sites via subcutaneous lymphatic ducts to regional lymph nodes. The tracking of fluorescence in subcutaneous lymphatic ducts with accumulation in lymph nodes occurred minutes after injection in some animals and was no longer apparent after 6 hrs. Residual fluorescent QD remained at the site of injection until necropsy. Lymph nodes from QD injected animals exhibited fluorescence that was localized in subcapsular and trabecular lymphatic sinuses. QD fluorescence in lymph nodes was most abundant at 0 hr and 6 hr, but was detected at all time points. In conclusion, intradermally injected nanoscaled QD remained as a depot in skin, penetrated the surrounding viable subcutis and were visibly distributed to draining lymph nodes via subcutaneous lymphatics. These results are consistent with the previously reported use of near infrared emitting nanoscale particles for imaging sentinel lymph nodes.

**2186**

IMAGING THE PENETRATION OF RUBPY-DOPED SILICA NANOPARTICLES INTO HUMAN AND MOUSE SKIN WITH FLUORESCENT MICROSCOPY.

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Nano-sized particles have the potential to enter body through routes that are inaccessible to larger particulates. One such route is dermal absorption. To study the penetration of nano-particulates into mammalian skin, we exposed whole-thickness mouse and dermatomed (600  $\mu$ m) human skin *in vitro* to suspensions of RubPy doped 50-nm silica particles under static and dynamic conditions. For static samples, 100 x 300 mm skin sections were placed flat with the epidermis side up and a 5  $\mu$ L aliquot of a 20 mg/mL suspension of the dye doped silica particles was placed on the center of a section. Static samples were left undisturbed for 2 h. Dynamic samples were treated similarly, but placed upon a single-hinged flexing device, which flexed the upper half of the section between 0 and 45° above horizontal 30x/min for two hours. After the 2-h incubation, the skin sections were rinsed with PBS and gently blotted to remove excess particles. After fixing in formaldehyde, samples were cut from dermis side out in 20- $\mu$ m sections and examined by fluorescent microscopy. The silica particles were found to penetrate into the stratum corneum and epidermis of both skin types and under both treatments with a higher concentration of particles occurring in the tissues of flexed samples. Particle count in the epidermis was low compared to the stratum corneum and higher in the mouse than the human; presumably a result of the thinner stratum corneum of the mouse.

**2187**

MODULATORY EFFECTS OF SUBCHRONIC EXPOSURE TO SIMULATED SOLAR LIGHT ON TATTOOED SKIN IN SKH-1 MICE.

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Tattooing is the process of depositing pigment into the dermis of the skin with a needle producing an indelible decorative pattern. In an attempt to determine the phototoxicity of tattoo pigments, we have shown that tattooing induces an acute

inflammatory response in the skin with recovery within 13 days. The present study examines the effect of subchronic exposure of tattooed mice to simulated solar light (SSL). Female SKH-1(*br/br*) hairless mice were tattooed longitudinally on the dorsal side. The test mixtures consisted of vehicle (10% glycerol in water) or vehicle containing cadmium sulfide (CdS) or Pigment Red 22 (PR22). After 2 weeks to allow skin recovery, half of the mice were exposed to 1.4 Standard Erythema Dose/day SSL for 13 weeks and then euthanized. Minimal dermal inflammation was present in one-third of the non-tattooed and vehicle-tattooed skin not exposed to SSL. The incidence of dermal inflammation increased to 67% and 56%, respectively, upon exposure to SSL. In contrast, exposure to SSL did not increase the incidence of dermal inflammation in the CdS (100%) and PR22 (67%) tattooed skin. Acanthosis was present in all SSL-exposed animals except the CdS-tattooed group. Dermal necrosis and fibrosis, and epidermal hyperplasia were present exclusively in CdS-tattooed animals (100%) with or without SSL. Dermal histiocytic infiltration was noted only in the PR22-tattooed skin and increased in incidence from 33% to 100% in the presence of SSL. Western blotting and immunohistochemistry showed cutaneous COX-1, COX-2 and ODC proteins were significantly different in animals exposed to SSL when compared to non-exposed mice, notably in the CdS group. Real time PCR demonstrated differential expression of cyclin D1, HIF-1 $\alpha$  and cMyc in response to SSL in tattooed and non-tattooed mice. The results demonstrate the modulatory effects of subchronic exposure to SSL in the skin of tattooed mice.

**2188**

ROLE OF VITAMIN E IN THE ANTIOXIDANT DEFENSE SYSTEM OF SKIN IN YOUNG AND OLD MICE EXPOSED TO CUMENE HYDROPEROXIDE.

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The skin is exposed to numerous environmental, chemical, and physical stressors (UV-irradiation) whose injurious action is often associated with the development of oxidative stress. While the skin possesses an elaborate antioxidant (AO) defense system to prevent oxidative stress, excessive exposure to occupational and environmental insults can overwhelm the cutaneous AO capacity. Age-related decline of AO protection may further enhance sensitivity of skin to chemically induced oxidative damage. To evaluate whether aging affected the AO capabilities of the skin, we studied changes in vitamin E, glutathione (GSH), ascorbate, and total AO reserve levels in the skin of female mice from 4 to 32 weeks of age. Among studied AO, we observed the most significant and rapid reduction occurred in the vitamin E content. To study how topical exposure to cumene hydroperoxide (Cum-OOH) affected the AO status of the skin of young/old mice, two models were used: 1) mice given a diet deficient in vitamin E and 2) mice with a genetic deletion of the tocopherol transporter protein (Ttpa knockout). We found that oxidative DNA damage (8-oxo-2'-deoxyguanosine) in skin of old mice (32 weeks) occurred independently of vitamin E status while DNA damage in skin of young animals (13 weeks) exposed to Cum-OOH was dependent upon vitamin E. Cum-OOH induced oxidative stress in old mice as assessed by depletion of GSH, ascorbate, and total AO reserve. Cum-OOH induced morphological changes to a greater extent in the skin of old vitamin E deficient animals compared to young mice. Similar results were found in the Ttpa knockout mice exposed to Cum-OOH; however, the mice only had a 40% reduction in their vitamin E levels and the resulting changes were not as profound as in the mice given the vitamin E deficient diet. In conclusion, AO, in particular vitamin E, play a prominent role in the protection of skin against oxidative injury induced by Cum-OOH exposure *in vivo*.

**2189**

DETECTION OF BENZO(A)PYRENE-INDUCED DNA DAMAGE IN THE SKIN OF CD-1 MICE USING THE COMET ASSAY.

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The single cell gel electrophoresis assay (SCGE or "comet" assay) is a rapid, sensitive method used to detect DNA damage *in vivo* and *in vitro*. The principle of the assay is based on the ability of denatured, cleaved and unwound DNA to migrate out of the nucleus when an electric current is applied to the cell, whereas intact, undamaged DNA remains within the confines of the nucleus. While this assay has been successful in studies to predict potential clastogenic agents, there is little data published on the use of the comet assay to analyze these agents in intact mammalian skin. Since DNA strand breaks result in the formation of micronuclei, we hypothesized that the comet assay would provide a sensitive method for detecting DNA damage in the skin of CD-1 mice challenged topically with benzo(a)pyrene (BaP), a prototypical carcinogen and potent electrophilic agent. To test this hypothesis, BaP (2mg/ml), or acetone (vehicle), was painted onto the shaved back of CD-1 mice daily for 3, 6, and 10 days. At each time point, two mice were sacrificed

and skin samples were collected and processed for the comet assay. DNA single strand breaks were quantified by the differences observed in comet moment, percent (%) tail DNA, and comet tail length. These parameters were significantly increased by BaP compared to vehicle in all animals. When the level of DNA damage was analyzed using comet moment to compare the distribution of the skin cells in BaP- and vehicle-treated animals, BaP treatment resulted in a significant increase in the number of cells with DNA damage. This response elicited by BaP did not increase in severity beyond day 3. These findings indicate that a net balance of DNA damage and repair is likely achieved in the skin of BaP-treated animals over the course of 10 days of exposure. Furthermore, chemically-induced DNA damage can be detected using the comet assay in a short-term exposure model.

## 2190 EFFECT OF METHYL SUBSTITUTION OF BENZENE ON THE PERCUTANEOUS ABSORPTION AND SKIN IRRITATION HAIRLESS RATS.

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To study the structure activity relationship (SAR) between the methyl substitution of benzene and 1) permeation or retention in skin layers 2) their irritation potential in hairless rats after dermal exposures. Methods: The skin permeation or retention studies were carried out on freshly excised hairless rat skin mounted on Franz diffusion cells filled with 6% Brij 98 in normal saline. Benzene, dimethyl benzene (xylene) and tetramethyl benzene (isodurene) spiked with respective <sup>3</sup>H radiolabeled chemicals were applied to the epidermal surface. After defined exposure period, the receptor samples were collected; the exposed skin area was tape stripped for SC, and sectioned into epidermis and dermis with a cryotome. The permeation rate and retention in stratum corneum (SC), epidermis and dermis were determined by scintillation counting of the samples. The skin irritation of the chemicals was studied in hairless rats by occlusive (230  $\mu$ l for 1 h using Hill top chambers®) and unocclusive (15  $\mu$ l every 2 h for 8 h a day for 4 days) dermal exposures. The transepidermal water loss (TEWL) and erythema were measured at different time intervals after dermal exposures. Results: The permeation of benzene decreases with increase in methyl groups of benzene (2.5 and 80 times higher flux by xylene and isodurene). The retention of chemicals in SC showed a direct correlation between amount retained in SC and methyl substitution or log P of the chemical. The retention in epidermis and dermis of different chemicals were approximately 10-15 times higher than SC. The skin irritation (TEWL and Erythema) increases with methyl substitution of benzene (isodurene > xylene > benzene). The TEWL data correlated "very well with erythema scores of the chemicals. Conclusion: A clear relationship between the flux or skin retention and methyl substitution of the chemical was established. The skin irritation increases with methyl substitution of benzene under the experimental conditions of present study.

## 2191 LONG TERM REPRODUCIBILITY OF EPIDERM<sup>TM</sup>, AN EPIDERMAL MODEL FOR DERMAL TESTING AND RESEARCH.

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An *in vitro* model of human epidermis, EpiDerm (EPI-200), cultured from neonatal, normal human epidermal keratinocytes has been sold commercially by MatTek Corporation since 1993. Using serum free medium, weekly lots of EpiDerm are produced and shipped for dermal irritancy, product efficacy, percutaneous absorption, pharmacological, and basic skin research studies. In 2000 and 2002, respectively, European and US regulators approved the use of EpiDerm to assess the skin corrosivity of chemicals. Validation studies utilizing EpiDerm for phototoxicity and skin irritation are currently underway. For commercial and regulatory purposes, it is crucial that the model is reproducible both within a given lot and between lots, especially over extended periods. To address tissue reproducibility, quality control testing of each EpiDerm lot involves exposure to the surfactant, 1% Triton X-100 (TX), and a negative control, ultrapure water. Using the MTT assay, which historically has been the *in vitro* endpoint of choice for European and US regulators, a dose response curve is constructed and an exposure time which reduces the tissue viability to 50% (ET-50) is interpolated. The yearly average ET-50 since 1996 has varied from 6.5 hr (2000) to 7.5 hr (1998). The coefficients of variation (CV) for the negative control have averaged under 7% for every year since 1997 and the average CV for all tissues has never exceeded 13.2%. Using light microscopy, histological H&E cross-sections show an epidermis-like morphology that is reproducible both within and between lots. Hence, over the past 10+ years of commercial production, EpiDerm has remained a highly reproducible, stable toxicological model that is ideally suited for industrial and regulatory toxicology and other skin related studies.

## 2192 DETERMINING SKIN IRRITATION POTENTIAL OF INDUSTRIAL FLUIDS IN HUMANS USING TRANS-EPIDERMAL WATER LOSS (TEWL).

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Evaluating the skin irritation potential of industrial fluids is becoming increasingly important for downstream lubricant users. In the absence of validated alternative methods, animal models (eg. OECD method guideline 404) have been relied upon to provide a prediction of the irritant potential of a fluid in the workplace. It is suggested that evaluating the irritant potential of industrial fluids in human volunteers using trans-epidermal water loss (TEWL) and colorimetry measurements could offer a scientifically-valid alternative to established animal models, and provide greater predictive accuracy for estimating potential skin reactions in workers following occupational exposure. Several industrial fluids were evaluated for skin irritation potential using a 4-hour semi-occluded exposure in rabbits (OECD method 404). These same fluids; in addition to 0.2% SLS as a positive control, and negative control treatments including water, highly refined lubricant base oil and an untreated site; were also examined in a panel of 30 human volunteers using a 24-hour occluded exposure. Irritation was quantitatively scored using  $\Delta$ TEWL, colorimetry, and estimation of erythema/edema by a trained dermatologist. RESULTS: Three of the supplied fluids produced Primary Irritation Index (PII) scores in rabbits of >3.0 (erythema and edema values met the EU criteria for a skin irritant, R38), but were found to be unsuitable for investigation in volunteers based upon initial observations of severe irritation that exceeded ethical limits for testing in humans. Other fluids produced PII = 1.0 when tested in rabbits (did not meet the EU skin irritant criteria) were found using the TEWL model to have a slightly higher potential to cause skin irritation in the workplace than that of non-additized, highly-refined mineral oil. The results show that measurement of  $\Delta$ TEWL following 24-hour occluded exposure in human volunteers is sufficiently sensitive to identify industrial fluids that have the potential to cause an adverse skin reaction in the workplace under unprotected exposure conditions.

## 2193 EVALUATION OF HISTORICAL POSITIVE CONTROL DATA FOR RESPONSE CONSISTENCY AND REDUCTION IN ANIMAL USE IN PHOTOTOXICITY AND PHOTOALLERGY ASSESSMENTS.

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In phototoxicology, positive control agents (PCA) have been included in animal studies when assessing potential adverse effects of pharmaceuticals and chemicals. Whether to use PCA groups in all such assays is a decision based on regulatory requirements, the effort to reduce animal use and confidence in the consistency of the required response. In acute phototoxicity (PT) assays performed in our laboratory, 8-methoxysoralen (8-MOP) and lomefloxacin (LOM) are commonly used PCA as is 3, 3', 4', 5'-tetrachlorosalicylanilide (TCSA) for contact hypersensitivity (CH) and photoallergy (PA) assessments. Historical control data (HCD) were compiled from studies over the past 7 years for cutaneous PT induced by 8-MOP in hairless mice, haired (Long-Evans) rats, haired guinea pigs and hairless guinea pigs, by LOM in hairless mice and CH and PA induced by TCSA in guinea pigs. Ocular PT HCD using Long-Evans rats were also compiled. The routes of administration for PT included topical, oral (gavage) and intravenous while the topical route of administration was evaluated for CH and PA. In all studies, the light source was a filtered xenon arc solar simulator emitting environmentally-relevant ultraviolet, visible and infrared radiation. Review of the HCD revealed that erythema, edema and flaking assessed by visual observation were the most common signs of cutaneous PT, CH and PA. Corneal opacity and/or edema detected by ophthalmologic examination and substantiated by histopathological evaluation were the hallmarks of ocular PT. The incidences of the cutaneous and ocular findings were consistent and reproducible across studies and over time. Therefore, the HCD revealed that the *in vivo* phototoxicology assays are robust and these data, rather than positive control animals, can be used to demonstrate the validity of the assays and appropriately reduce the use of animals in these assessments.

## 2194 QUANTIFICATION OF MIXTURE INTERACTIONS ON DERMAL PERMEABILITY-A SOLVATOCHROMATIC APPROACH.

R. E. Baynes, B. M. Barlow, X. R. Xia, J. L. Yeatts, J. D. Brooks and J. E. Riviere. Center for Chemical Toxicology Research and Pharmacokinetics, North Carolina State University, Raleigh, NC.

The influences of mixture-mixture interactions, differing solvent systems, and surfactants on permeability and dermal absorption have not been well studied. Past research has focused on the toxicity of a single solute dosed in an organic solvent; unfortunately, these conditions do not generally represent real world scenarios of

occupational use. The scope of our study is to identify and quantify physicochemical interactions that drive dermal permeability using Linear Solvation Energy Relationships (Log permeability =  $c + r^*R_2 + s^*\pi_2^H + a^*\sum\alpha_2^H + b^*\sum\beta_2^H + v^*V_x$ ) during complex mixture exposure. Seven solutes (propylbenzene, biphenyl, naphthalene, methyl benzoate, 4-nitrotoluene, phenol, and 3-methyl phenol) with a wide range of known physicochemical properties (Log p, solubility, molecular weights, etc.) and LSER solvatochromatic parameters ( $R_2, \pi_2^H, \alpha_2^H, \beta_2^H, V_x$ ) were prepared in various mixtures (0-100% aqueous or ethanol solutions and/or 0.1%, 1% and 10% SLS), and applied to a porcine skin flow thru diffusion cell system. Dermal permeability was then calculated using the resultant perfusate and SPME GC/MS or GC/FID detection. The LSER system coefficients ( $r, s, a, b, v$ ) were compared to derive an interaction coefficient value ( $\Delta r, \Delta s, \Delta a, \Delta b, \Delta v$ ). Preliminarily results indicate that ethanol at 50% strength decreased both hydrogen bond acceptor activity ( $\Delta r - 2.010$ ) and McGowan molecular volume ( $\Delta v - 0.729$ ), while the presence of surfactants increased both hydrogen bond acceptor activity ( $\Delta b + 1.979$ ), and McGowan molecular volume ( $\Delta v + 1.305$ ). These results indicated that system basicity and lipophilicity play a significant role in dermal permeability of solutes after exposure to complex solvent and surfactant mixtures. Further studies are aimed at discerning quantitatively the biological and chemical contributions of these interactions. Supported by NIOSH Grant R01-OH-03669

## 2195

### PREDICTING HUMAN SKIN ABSORPTION OF CHEMICALS: DEVELOPMENT OF A NOVEL QUANTITATIVE STRUCTURE ACTIVITY RELATIONSHIP.

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The objective of this study was to construct and validate a simple model using over 340 chemicals of diverse structure for the quantitative prediction of percutaneous absorption based on their physicochemical properties. Such a model is a valuable tool for screening and prioritization in safety evaluation and risk assessment of chemicals. A number of models will be presented. Of particular interest is a simple three-parameter QSAR model to predict the permeability coefficient,  $K_p$ , with a training set of 306 chemicals and their physicochemical descriptors. In addition to the experimental  $K_{ow}$  values, over 300 2D and 3D atomic and molecular descriptors were analyzed using MDLs QSAR computer program (MDL Information Systems, Inc., San Leandro, CA 94577). All  $K_p$  values used in training and validation were experimental data obtained from various sources. Using the stepwise regression analysis, three molecular descriptors were determined to have significant statistical correlation with  $K_p$  ( $R^2 = 0.8225$ ):  $\log K_{ow}$ ,  $x_0$  (quantification of both molecular size and the degree of skeletal branching), and  $S_{SSS}CH$  (count of aromatic carbon groups). Subsequently, the model was validated using both internal (leave-one-out) and external validation (90/10 random split) procedures. The  $R^2$  from internal validation is 0.8108, and that from external validation is 0.5829,  $\log K_p$  ( $\text{cm}/\text{hr}$ ) =  $0.5892 * \log K_{ow} - 0.1006 * x_0 - 0.1617 * S_{SSS}CH - 2.62353$  ( $R^2 = 0.8225$ ) In conclusion, an algorithm to estimate human skin absorption was derived based on physicochemical properties of over 300 compounds. Compared to other skin absorption QSAR models in the literature, our model has incorporated more chemicals and explored more descriptors in addition to the commonly used MW and  $K_{ow}$ . Additionally, our model is reasonably predictive and has stood up to both internal and external statistical validations.

## 2196

### A NOVEL SYSTEM COEFFICIENT APPROACH FOR QUANTITATIVE ASSESSMENT OF DERMAL ABSORPTION FROM CHEMICAL MIXTURES BY USING THE MEMBRANE-COATED FIBER TECHNIQUE.

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Chemical exposure in most environmental and occupational settings involves complicated chemical mixtures, not individual chemicals. However, current health risk assessment of dermal absorption is based on permeation data for individual chemicals due to lack of available quantitative methods or experimental data from chemical mixtures. In the system coefficient approach, a set of solute descriptors, [ $R, \pi, \alpha, \beta, V$ ], represents the molecular interaction strengths of a chemical of interest, while a set of system coefficients, [ $r, s, a, b, v$ ], represents those of the skin/medium system. The permeation coefficient or partition coefficient ( $\log K$ ) is correlated with the system coefficients and the solute descriptors via Abraham's linear free energy relationship. The major components of the mixture determine the system coefficients. When the major components change in composition or proportion, the system coefficients will be changed. Therefore, the changes in the system coefficients can be used to study the changes of the chemical mixtures:  $\log K = c + (r + \delta r)R +$

$(s + \delta s)\pi + (a + \delta a)\alpha + (b + \delta b)\beta + (v + \delta v)V$ . When 25% of ethanol was added to the water solution of the 32 calibration compounds, the system coefficient changes of the polyacrylate/water system [ $\delta r, \delta s, \delta a, \delta b, \delta v$ ] were [-0.12, 0.17, 0.23, 0.33, -0.42], respectively. When 1% of sodium lauryl sulfate was added to the water solution, the system coefficient changes [ $\delta r, \delta s, \delta a, \delta b, \delta v$ ] were [-0.41, 0.34, 0.23, 0.74, -1.53], respectively. In practical risk assessments, the changes in system coefficients can be treated as sufficiently similar mixtures. If the system coefficients of the mixture of concern and the system coefficients of the sufficiently similar mixtures are determined, the permeability or partition coefficient of any chemical can be obtained by using the system coefficient approach. Supported by NIOSH R01-OH-07555 and OH-03369 and AFOSR F49620-01-0080

## 2197

### PREDICTION OF DERMAL ABSORPTION OF CHEMICAL MIXTURES USING BOTH PENETRANT AND MIXTURE COMPONENT PROPERTIES IN A HYBRID QUANTITATIVE STRUCTURE PERMEABILITY RELATIONSHIP (QSPR).

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Occupational and environmental exposure to topical chemicals is usually in the form of complex chemical mixtures, yet risk assessment is based on experimentally derived data from individual chemical exposures or computed physicochemical properties. We present an approach using modified QSPR models where absorption through two systems (porcine skin flow-through diffusion cells-PSFT and isolated perfused porcine skin flaps-IPPSF) are well predicted using a QSPR model describing the individual penetrants, coupled with a mixture/vehicle factor (MF) that accounts for physicochemical properties of the vehicle/mixture components. The baseline equation is  $\log K_p = c + mMF + rR + s\pi + a\alpha + b\beta + vV$  where  $R$  is molar refractivity,  $\pi$  is polarizability constant,  $\alpha$  is H-bonding acidity,  $\beta$  is H-bonding basicity and  $V$  the McGowan molecular volume of the penetrants of interest;  $c, m, r, s, a, b$  and  $v$  are strength coefficients coupling these descriptors to skin permeability ( $K_p$ ) in PSFT (12 penetrants in 24 mixtures) or to AUC of the absorption flux profile in IPPSF (8 penetrants in 4 mixtures). Mixtures consisted of different combinations of vehicles (water, ethanol, propylene glycol) and additives (sodium lauryl sulfate, methyl nicotinate). Across all exposures with no MF,  $R^2$  for absorption was 0.68 in IPPSF and 0.58 in PSFT. With the MF, correlations increased to 0.74 for IPPSF and 0.85 for PSFT. The initial MF is a function of the Henry Constant for the mixture components, although other factors are presently being investigated. The importance of these findings is that there is an approach whereby the effects of a mixture on absorption of a penetrant of interest can be quantitated in a standard QSPR model if physicochemical properties of the mixture are also taken into account. The good correlations obtained in the IPPSF, which has previously shown to be predictive of *in vivo* human absorption, suggests that this approach merits attention. Supported by NIOSH R01-OH-07555

## 2198

### COMPUTER-CONTROLLED SYSTEM FOR GENERATION OF CHEMICAL VAPORS IN *IN VITRO* DERMAL UPTAKE STUDIES.

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Recent work in our laboratory shows that dermal uptake of chemicals may be assessed *in vitro* by thermogravimetric analysis (TGA), i.e. by passing chemical vapor over a piece of skin while recording the weight increase under carefully controlled temperature and humidity conditions. This technique requires (1) a high-precision TGA balance and (2) a stable generation and supply of water and chemical vapors at known concentrations. For the latter purpose, we have developed a high precision computer-controlled exposure system. Clean, dry air flows at controlled rates (45 ml/min) through stainless steel gas impinger bottles containing the chemicals of interest. The impingers reside in a water bath maintained at exactly 25°C. To prevent retention and condensation of chemical vapor downstream, all tubing and connectors are made of stainless steel and insulated and kept at 30°C with a heat cable. Two parallel impinger systems with separate mass flow regulators allow for different exposure sequences, for example: 50% water + 50% chemical A for 1 hour, followed by 50% water only for 3 hours then 50% water + 50% chemical B etc. The airflow is redirected to the impingers by computer-controlled magnetic valves. A dedicated computer software allows execution of exposure sequences with millisecond precision. Measurements of dry air leaving an impinger containing cyclohexanone showed that the concentration in outlet air (measured by photoionization detection) rapidly reached a plateau (90% response in approx. 8 sec) and remained stable at a level corresponding to the theoretical saturation concentration. Cyclohexanone has a low vapor pressure compared to many other solvents, and we expect that chemicals with a higher vapor pressure behave similarly. In conclusion, this computerized system with automated, high-precision generation of sequences

of chemical vapour allows measurements of dermal uptake of chemical vapor with little manual effort. The system is closed, facilitating studies of toxic substances. This study was supported by the Swedish Council for Working Life and Social Research (FAS).

**2199**

CAN CHRONIC SKIN IRRITATION BE PREDICTED BY AN ACUTE TEST?

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A repeated sodium lauryl sulfate (SLS) exposure test is a model for chronic skin irritation. However this test is too time-consuming to be used as a screening tool for individual susceptibility. Our objective was to investigate whether an acute 24-h SLS patch test predicts the outcome of a repeated SLS patch test. Twenty healthy volunteers were exposed to SLS on the volar forearm. On one site 1% SLS (200 µl) for 24 h (acute patch test) was applied, on another site 0.1% SLS for 6 h, 4 days a week, during 3 weeks (repeated patch test). Skin barrier function (transepidermal water loss, TEWL) and skin redness (measured with an erythema meter) were measured at day 2 for the acute and at day 17 for the repeated SLS application. Exposure to SLS resulted in an increase of TEWL and erythema after both the acute and the repeated test. Large inter-individual differences in these responses were observed. A relation was found between the increase in skin redness after the acute and the repeated test ( $r = 0.72$ ,  $p = 0.001$ ). However, no correlation was found between skin barrier function in both tests ( $r = 0.06$ ). Skin barrier function and skin redness were significantly correlated after the acute test ( $r = 0.68$ ,  $p = 0.001$ ), but no correlation was found after the repeated test ( $r = -0.00$ ). Higher erythema values at day 10 correlated ( $r = -0.58$ ,  $p = 0.011$ ) with a stronger decrease in TEWL values from day 10 to day 17. For screening purposes in occupational medicine, skin redness after a single 24-h exposure to SLS still has insufficient predictive value for redness after a repeated exposure. The lack of predictive value of an acute test might be due to differences in barrier repair between subjects during repeated exposure. Additionally, our data suggests that the extent of erythema during the second week may predict the improvement of the skin barrier (skin hardening).

**2200**

ANTI-PSORIASIS MODEL IN THE MOUSE.

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Psoriasis is a chronic cutaneous disease affecting approximately 1-3% of the population. It is a relapsing / remitting disease of variable severity and in the absence of a cure, current treatments are aimed towards reducing the severity of the lesions. Although there are several animal models available for psoriasis research, no one model imitates the disease completely. One such model is the mouse tail model, which simplistic in its approach, can be useful in identifying potential anti-psoriasis compounds, which target the cell differentiation aspect of the disease. The mouse tail is considered to be histologically and biochemically similar to psoriatic skin found in humans. It is composed of parakeratotic (PK) scales (a common feature of the abnormal epidermis in psoriasis), with orthokeratotic (OK) differentiation at the neck of the hair follicle. Many anti-psoriasis drugs alleviate the condition by inhibiting parakertosis and restoring the granular (OK) cell layer of the epidermis. For validation purposes, 0.1mL of control, dithranol, calcipotriol or betamethasone valerate were applied topically to the tail of male CD-1 mice. Animals were treated once daily, five times per week, for two weeks. Approximately two hours following the final treatment the animals were killed and their tails prepared for histopathological assessment. Using the Seescan Image Analysis System the percentage of OK was determined and epidermal thickness measured. Dithranol produced a significant increase in OK and epidermal thickness when compared to the control group. The increase in epidermal thickness was considered to be due to the known irritant effect of dithranol. Calcipotriol produced a significant increase in OK and slight decrease epidermal thickness. Betamethasone valerate had little effect on OK restoration, but did cause significant thinning of the epidermis. In conclusion, the potential of compounds to restore the granular layer as an indicator of anti-psoriasis activity can be assessed using the mouse tail model, providing that the compounds target cell differentiation rather than the inflammatory aspect of the disease.

**2201**

EVALUATION OF THE TOXICITY OF MEDICAL COUNTERMEASURES FOR SULFUR MUSTARD.

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Since 1997, Battelle's Medical Research and Evaluation Facility has supported the US Army Medical Research Institute of Chemical Defense program in evaluating candidate drugs for protection from sulfur mustard (SM) injury using the mouse

ear vesicant model (MEVM). Several compounds have proven to reduce tissue damage from SM when tested as pre- and/or post-treatments. In order to gain information on the toxicity potential of four of these compounds, a study was conducted applying various doses of each compound to both ears and observing the mice for 14 days. Clinical observations and body weights over time were recorded. A simple motor incapacitation test also was administered. The motor incapacitation test consisted of placing a mouse on the top of a 5"x 5" screen that was subsequently inverted. Mice climbing back to the top of the screen grid within 1 minute of inversion passed the performance test. Mice which fell or remained on the bottom of the grid for the one minute test period failed. The four compounds tested were 4-methyl-2-mercaptopuridine-1-oxide (ICD# 1308), indomethacin (ICD# 2086), BAL (ICD# 2525), and 8-methyl-N-vanillyl-6-nonenamide or capsaicin (ICD# 3537). Motor incapacitation testing demonstrated no evidence of gross behavioral deficit for any of the compounds tested. Minor clinical signs resolved within a few days for ICD# 2525 and #3537. ICD #2086 proved highly toxic in the range of doses tested and only a dose-related reduction in body weight gains from days 0 to 14 was observed for the other three compounds. This work was supported by the US Army Medical Research and Materiel Command under Contract DAMD17-99-D-0010, Task Order 0002.

**2202**

MOUSE EAR VESICANT MODEL (MEVM) EVALUATION OF TREATMENT COMBINATIONS AGAINST TOPICAL SULFUR MUSTARD CHALLENGE.

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The mouse ear vesicant model (MEVM) has been used as a screening tool at Battelle to identify compounds that protect skin from sulfur mustard (SM)-induced injury. The original MEVM provided a quantitative edema response and histopathologic endpoints as measurements of inflammation and tissue damage at 24 h following topical exposure to SM (0.16 mg). Compound effectiveness was defined as a reduction in these endpoints. To further evaluate lead candidate compounds, the MEVM has been modified for use as a 7-day model and a modified Draize scoring system of 0-4 (no damage to extensive necrosis) was incorporated as the primary endpoint to quickly and easily evaluate the extent of ear tissue damage up to day 7 and/or day 14. Nine lead candidate compounds were selected to be evaluated as single 10 min post-treatments after a 0.08 mg SM challenge. These compounds were tested alone and in combination and ear tissue was scored on days 7 and 14. Compounds and/or combinations of compounds either passed or failed based on Draize ear tissue scores being statistically decreased from the SM no-compound treatment group. Draize scores were significantly reduced when 4 compounds (4-methyl-2-mercaptopuridine-1-oxide (ICD# 1308), BAL (ICD# 2525), octyl homovanillamide (ICD# 2980), and 8-methyl-N-vanillyl-6-nonenamide or capsaicin (ICD# 3537) were tested alone or in combination with 7 or 8 other compounds. For ICD# 1308, 2980 and 3537, none of the combination therapies provided significantly greater reduction in SM injury than did the compound alone. No compound or combination tested appeared more effective than #1308 alone. No combination therapy provided significantly greater reduction in SM injury than both individual components. This work was supported by the US Army Medical Research and Materiel Command under Contract DAMD17-99-D-0010, Task Order 0002.

**2203**

90-DAY SUBCHRONIC DERMAL TOXICITY OF INSECT REPELLENT SS-220i IN RATS.

J. T. Houpt and G. J. Leach. *US Army Center for Health Promotion and Preventive Medicine, Aberdeen Proving Ground, MD.*

John T. Houpt, Glenn J. Leach, U. S. Army Center for Health Promotion and Preventive Medicine, Aberdeen Proving Ground, MD, USA. A 90-day subchronic toxicity study was conducted to determine the dermal toxicity of the candidate insect repellents SS-220i (CAS# 69462-43-7). Entomological studies have demonstrated SS-220i to be as effective or better than N, N-diethyl-m-toluamide (DEET) in repelling various species of mosquitoes and biting flies. The entomological data supports continued development of SS-220i as a topical insect repellent. Information generated from this study, along with results from other laboratory animal toxicity studies, will be utilized to predict the potential risk to human health upon exposure to this compound. SS-220i was administered via the dermal route to young adult Sprague-Dawley rats five days per week for ninety days at dose levels of 0, 60, 175 or 350 mg/kg for females. Male dose levels were 0, 60, 250 or 500 mg/kg. The 21-day range-finding study, as well as the subsequent subchronic study, indicated that SS-220i demonstrated no systemic toxic effects. Skin irritation was evident in all exposure groups and lower body weights were present in mid and high dose groups of both sexes. These changes were all resolved following the 7 week recovery period. Female rats were more susceptible to the dermal irritating effects of

SS-220i than males. The No-Observed Effect Level (NOEL) for subchronic dermal exposure to SS-220i as determined from this study, is 60 mg/kg/day. (Abstract does not reflect US Army Policy)

**2204**

**DATABASE CONSOLIDATION BASED ON TOXML OF GENETIC TOXICITY DATA SUBMITTED TO CFSAN/OFAS AND CDER.**

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Structure searchable electronic databases are a valuable new tool that will assist the FDA in its mission to promptly and efficiently review incoming submissions. CFSAN/OFAS, in collaboration with Leadscape, Inc., is consolidating genetic toxicity data submitted in petitions from the 1960s to the present day. CDER/OPS/ICSAS is separately gathering similar information. Presently these data are distributed in various locations as paper files, microfiche, and non-standardized toxicology memoranda. The organization of the data into a consistent, searchable format should reduce paperwork, expedite the toxicology review process, and provide valuable information to industry that is currently available only to the FDA. Furthermore, by incorporating chemical structures with genetic toxicity information, biologically active moieties can be identified and used to develop QSAR modeling and testing guidelines. Additionally, chemicals devoid of data can be compared to known structures, allowing for improved safety review through the identification of analogs. Four database frameworks have been created: bacterial mutagenesis, *in vitro* chromosome aberration, *in vitro* mammalian mutagenesis, and *in vivo* micronucleus. Controlled vocabulary for these databases has been established. The separate databases are all tied together into an overall genetic toxicity database for standardization and easy accessibility. Beyond the genetic toxicity databases described here, additional databases for subchronic, chronic, and teratogenicity studies are in preparation.

**2205**

**INTER-LABORATORY EVALUATION OF BIOLUMINESCENT SALMONELLA REVERSE MUTATION ASSAY USING TEN (10) MODEL CHEMICALS.**

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We have developed the Bioluminescent Salmonella Reverse Mutation Assay as a tool for detecting mutagenicity applicable for high throughput screening of new chemicals. In this study, we report the inter-laboratory evaluation of the assay using 10 model chemicals in three independent laboratories located in the US and Japan. The chemicals were tested in at least three independent experiments using strains TA98-lux and TA100-lux in presence and absence of metabolic activation (rat S9). The results were statistically evaluated and compared to published results (NTP). Seven of ten compounds tested were positive to either TA98-lux and/or TA100-lux in presence or absence of S9 (nitrofurazone, o-dianisidine dihydrochloride, benzo-<a>pyrene, 1, 4-benzoquinone dioxime, 2-amino-5-nitrophenol, 2-bromo-4, 6-dinitroaniline, busulfan) and remaining 3 of 10 compounds were negative (anthracene, crystal violet, benzyl chloride). Final results of all three inter-laboratory evaluations yielded 100% concordance to published data. Results of all three inter-laboratory evaluations specifically from each 40 different condition (e.g. 98 +S9 for busulfan) obtained almost perfect concordance. We conclude that the Bioluminescent Salmonella reverse mutation assay is a robust, accurate and economical high throughput assay applicable for mutagenicity screening.

**2206**

**MEASUREMENT OF MUTANT FREQUENCY IN T-CELL RECEPTOR GENE BY FLOW CYTOMETRY ON EL-4 MOUSE LYMPHOMA CELLS.**

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It is well known that somatic mutations are induced by ionizing irradiation. Peripheral CD4<sup>+</sup> lymphocytes deficient in the expression of TCR/CD3 complex on the cell surface show CD3<sup>4+</sup> due to a somatic mutation at the T-cell receptor

(TCR) genes. We have previously reported the measurement of mutant frequency on the TCR gene of mice T-lymphocytes after irradiation by flow cytometry. In this study, we developed an *in vitro* experiment system using murine EL-4 lymphoma cells and observed mutant frequency of EL-4 cells defective in the TCR gene expression after irradiation by flow cytometry using an Epics XL. EL-4 cells were stained with fluorescein-labeled anti-CD4 and phycoerythrin-labeled anti-CD3 antibodies. They were analyzed with a flow cytometer to detect mutant EL-4 cells lacking surface expression of TCR/CD3 complexes which showed CD3<sup>4+</sup> T cells. Mutant T cells could be observed at 2 days after 3 Gy irradiation. Frequency of mutant EL-4 cells was  $4.0 \times 10^{-4}$  for 0Gy and the value increased to the maximum level of  $38 \times 10^{-4}$  between 4 and 8 days after 3 Gy irradiation and these data were found to be best fitted by a linear-quadratic dose-response model. After the peak value the TCR mutant frequency gradually decreased with a half-life of approximately 3.2 days. We also examined the hprt (hypoxanthine phosphoribosyltransferase) mutation assay at seven days after irradiation and the cytogenesis-blocked micronucleus assay at 20 hours after irradiation. The frequencies of hprt mutation and micronuclei were found to be best fitted by a linear-quadratic dose-response model and a linear dose-response model, respectively. The method to detect mutation on TCR gene is quick and easy in comparison with other methods and is considered useful for the mutagenicity test.

**2207**

**A HIGH-THROUGHPUT UNSCHEDULED DNA SYNTHESIS (UDS) ASSAY USING FLOW CYTOMETRY.**

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There is a growing need in industry and regulatory agencies for an inexpensive screening assay that can reliably determine the genotoxic potential of the large number of new chemical compounds synthesized each year. The Unscheduled DNA Synthesis (UDS) Assay is a proven method to identify and characterize genotoxic chemicals. It is based on the ability of damaged DNA to incorporate radiolabeled thymidine during the repair of various types of genetic lesions, which are subsequently detected by standard autoradiographic methods. However, several technical aspects of the standard UDS assay, including its use of radioactivity, have greatly limited its more widespread application. We have made modifications to the standard UDS assay through incorporation of BrdU incorporation and flow cytometry as a detection method. In addition, this is an *in vitro*, non-animal based test, using human hepatocyte cultures or avian embryonic hepatocytes, as well as less metabolically active human keratinocytes as the test system, therefore addressing all of the 3Rs of animal testing. We have also demonstrated the accuracy and sensitivity of this version of UDS, termed 'FLUDS', which allows higher throughput and much shorter study times (a few days versus weeks for the standard UDS assay). This new assay can discriminate between innate and biotransformed genotoxins, and is fully quantitative. It has reliably detected and characterized the UDS induced by ethylnitrosourea, aphidicolin, 2-acetylaminofluorene, and fluorene. Due to its high-throughput capability and other improvements over the standard UDS assay, the FLUDS assay now adds an important and humane tool of considerable value to regulatory agencies, academia as well as scientists in the pharmaceutical, chemical, cosmetic and consumer products industries.

**2208**

**MODIFIED BACTERIAL MUTATION TEST PROCEDURES FOR EVALUATION OF PEPTIDES AND AMINO ACID-CONTAINING MATERIAL.**

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Biological materials such as peptides can release amino acids either spontaneously or as a result of enzymatic degradation by S9 during the course of bacterial mutation testing. Low levels of released amino acids from soluble materials can cause moderate increases in the number of revertant (mutated) bacterial colonies on the plate, while higher levels lead to overgrowth of non-mutated bacteria, making counting of mutant colonies impossible. In the case of poorly soluble material, the released amino acids can be present at high levels in localized spots on the plate, which leads to growth of 'pseudo-revertant colonies'. The 'treat and wash' modified pre-incubation method is an adaptation of the treat and plate method used for evaluation of antibiotics and involves washing the bacteria free of test compound after a 90-minute exposure prior to plating out on agar plates. The MC overlay method is a modified version of the plate incorporation assay in which a top overlay containing 4% high viscosity methyl cellulose in place of agar is used to stabilize peptide in solution. Both modified methods have shown expected increases in revertant colony counts with standard positive control materials. Peptides that produced false positive results or could not be evaluated due to overgrowth of non-mutant bacteria using standard methods, showed no evidence of genotoxicity using the modified methods. The mean revertant colony counts of the untreated controls for both methods were within the laboratory historical control range. It is concluded that

the treat and wash and MC overlay methods are valid versions of the bacterial mutation test for avoiding complications associated with feeding effects due to released amino acids.

## 2209

### DEVELOPMENT OF A NOVEL MICRONUCLEUS ASSAY USING THE HUMAN 3-D SKIN MODEL, EPIDERM™

R. D. Curren<sup>1</sup>, G. Mun<sup>1</sup>, D. P. Gibson<sup>2</sup> and M. J. Aardema<sup>2</sup>. <sup>1</sup>*Institute for In Vitro Sciences, Inc., Gaithersburg, MD* and <sup>2</sup>*Procter & Gamble Co., Cincinnati, OH*. The rodent *in vivo* micronucleus assay is an important part of a tiered testing strategy in genetic toxicology. However, this assay, in general, only provides information about materials available systemically, not at the point of contact, e.g. skin. Although *in vivo* rodent skin micronucleus assays are being developed, the results will still require extrapolation to the human. Furthermore, to fully comply with recent European legislation such as the 7<sup>th</sup> Amendment to the Cosmetics Directive, non-animal test methods will be needed to assess new chemicals and ingredients. Therefore we have begun development of a micronucleus assay using a commercially available 3-D engineered skin model of human origin, EpiDerm™ (MatTek Corp, Ashland, MA). We first evaluated whether a population of binucleated cells sufficient for a micronucleus assay could be obtained by exposing the tissue to 1-3  $\mu$ g/ml cytochalasin B (Cyt B). The frequency of binucleated cells increased both with time (to at least 120 h) and with increasing concentration of Cyt B. Three  $\mu$ g/ml Cyt B allowed us to reliably obtain 40-50% binucleated cells at 48h. Mitomycin C (MMC) was then used (in the presence of 3  $\mu$ g/ml Cyt B) to investigate toxicity and micronuclei formation in EpiDerm™. Exposing the tissue directly through the growth medium for 48h gave a dose response for toxicity between 0.03 and 0.6  $\mu$ g/ml. Maximum micronuclei induction (~5%) occurred at 0.06-0.6  $\mu$ g/ml MMC. Experiments conducted with and without Cyt B indicated higher frequencies in the presence of Cyt B as expected. A topical application protocol was then developed using two 10  $\mu$ l (per 0.64  $\text{cm}^2$  tissue) applications of MMC in ethanol 24 and 48h prior to harvest. Maximum micronucleus response (~8%) and toxicity occurred with applications of 6-60  $\mu$ g/ml MMC. The background frequency of micronuclei was very low (~0.1%). These studies show that micronuclei can be reproducibly induced in a 3-D skin model and are the first steps in developing a routine “*in vivo*-like” assay for chromosomal damage in human tissue.

## 2210

### INCREASED MICRONUCLEI FOLLOWING OXYMORPHONE ADMINISTRATION ARE SECONDARY TO INCREASED BODY TEMPERATURE.

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Oxymorphone is a potent opioid analgesic. Oral administration of oxymorphone to rats at doses  $\geq$ 20 mg/kg and mice at 500 mg/kg produces an increase in bone marrow micronucleated polychromatic erythrocytes (MPCEs). Oxymorphone does not produce chromosome aberrations *in vitro*, suggesting that the increase in MPCEs may involve indirect mechanisms. Opioids are known to affect thermoregulatory mechanisms. Environmentally- or chemically-induced changes in body temperature can increase the incidence of MPCEs in rodents. Studies were conducted to examine the relationship between increased MPCEs in rats given oxymorphone and changes in body temperature. Single oral doses of oxymorphone previously shown to increase MPCEs (20, 40 mg/kg) also produced a marked, rapid increase in body temperature. Pretreatment of animals with sodium salicylate was effective in preventing the increase in body temperature after oxymorphone administration. MPCEs were evaluated in rats after administration of oxymorphone (40 mg/kg, PO) alone or preceded by an oral dose of sodium salicylate (300 mg/kg, 60 min or 500 mg/kg, 30 min). When animals were pretreated with sodium salicylate, peak body temperature was lower, and body temperature returned to baseline more quickly than when oxymorphone was given alone. Administration of oxymorphone alone produced a statistically significant increase in the incidence of MPCEs (3.6 per 1000 PCEs vs. 0.4 in controls). The number of MPCEs in animals pretreated with sodium salicylate was similar to the vehicle control group. Sodium salicylate alone had no effect on the number of MPCEs. Systemic oxymorphone exposure was not significantly impacted by sodium salicylate pretreatment; Cmax and AUC values were similar when oxymorphone was given alone or preceded by sodium salicylate. These results indicate that the increased incidence of MPCEs following oxymorphone administration is directly related to increased body temperature.

## 2211

### THE CONTRIBUTION OF NON-CHEMICALLY INDUCED HYPOTHERMIA IN THE FORMATION OF MICRONUCLEI (MN) IN THE MOUSE BONE MARROW MICRONUCLEUS TEST (MNT).

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Previous studies indicated certain chemicals induce substantial and prolonged body temperature (BT) changes accompanied by an increased incidence of micronuclei (MN). The contribution of hypothermia alone vis-à-vis direct effects of the chemi-

cal/its metabolites in the etiology of MN formation is unknown. The present study reports effects of non-chemically induced hypothermia on MN formation in CD-1 mice. Six males/group were restrained in plastic cone-type devices and housed at normal room temperature (RT = 21°C) or in an incubator maintained at a temperature of 12°C for 7h. Preliminary studies indicated restraint was necessary to induce hypothermia in mice maintained at lower ambient temperatures. BT of all mice was monitored at baseline and multiple times throughout the 7h cold exposure period using subcutaneous digital transponders. Bone marrow samples were evaluated for MN formation 24h after the 7h exposure period. Restrained mice housed at 12°C for 7h experienced a significant and prolonged hypothermia (~12°C decrease in BT) but recovered to near normal BT 1 hr after returning to standard housing. No changes in BT occurred in mice restrained but housed at RT. A small, but statistically significant increase in MN was observed in bone marrow of mice receiving a 7h cold exposure treatment (RT = 0.67 MN/1000 PCE vs. 12°C/7h = 2.17 MN/1000 PCE,  $\alpha \leq 0.05$ ). These results suggest a potentially confounding role for hypothermia in the mouse bone marrow MNT and the need to exercise caution in interpreting the biological significance of marginal increases in MN at doses inducing hypothermia.

## 2212

### IN VITRO MICRONUCLEUS SCORING BY FLOW CYTOMETRY: DIFFERENTIAL STAINING OF MICRONUCLEI AND APOPTOTIC CHROMATIN ENHANCES ASSAY RELIABILITY.

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The *in vitro* micronucleus test has received considerable attention in the areas of drug safety assessment and toxicological research. The less tedious nature of the assay relative to chromosome aberration analyses is a driving force, and explains why many drug screening programs have adopted the endpoint. High-throughput scoring of micronuclei (MN) would further enhance the utility of the assay for lead optimization and other early drug development work. Although a flow cytometric (FCM) method for scoring cell culture-derived MN has been described in the literature, it lacks the ability to distinguish these events from apoptotic chromatin (Nusse and Marx (1997) *Mutat. Res.* 392, 109-115). Here, we report advances to the previously described method whereby a sequential staining procedure is used to differentially label these two types of sub-2n particles. With this method, apoptotic cells are labeled with ethidium monoazide (EMA). After a photoactivation step which binds EMA to apoptotic chromatin, cells are stripped of their cytoplasmic membranes and incubated with RNase plus a pan-nucleic acid dye (SYTOX Green). This process provides a suspension of free nuclei and MN which are differentially stained relative to chromatin associated with dead/dying cells. Studies with mouse L5178Y cells demonstrate that: 1) EMA labels apoptotic and necrotic cells, and 2) the sequential labeling procedure provides reliable MN enumeration, even when cultures contain high percentages of dead cells. Data from FCM-based analysis following treatment with hydroxyurea, methyl methanesulfonate, benzo(a)pyrene, and etoposide are presented, and are shown to correspond closely with microscopy-based measurements. Collectively, these data suggest that this EMA/SYTOX staining procedure provides reliable, high-throughput enumeration of *in vitro* MN. Further collaborative investigations with additional cell lines and genotoxins are planned to test the robustness of this methodology. This work was supported by a grant from the National Cancer Institute, No. R44CA094493.

## 2213

### ESTABLISHMENT OF HUMANIZED IN VITRO GENOTOXICITY TEST SYSTEM.

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Genotoxicity tests have been generally used as hazard identification for mutagens or possible carcinogens. To pursue the possibility whether the genotoxicity tests can be applied for evaluating genetic (cancer) risk for human, we established a humanized *in vitro* genotoxicity test system comprised of human cells and human metabolic system. Human lymphoblastoid cell line WTK-1 and human liver S9, which is supplied from Human and Animal Bridge (HAB) Research Organization in Japan through National Disease Research Interchange (NDRI) in the USA, were used in this study. We optimized the protocol for micronuclei test (MN) and TK-gene mutation assay (TK). Carcinogens as well as non-carcinogens required for metabolic activation were examined by this system and their results were compared to those examined under rat S9 and other existing genotoxicity data. Human carcinogens (class 1 by IARC), benzidine, cyclophosphamide, and 2-naphthylamine clearly showed genotoxicity under human S9 as well as rat S9. Rodent carcinogens (2A or

2B), benzo(a)[a]pyrene, IQ, and N-nitroso-di-n-butylamine exhibited no or very weak responses under human S9 although they were extremely genotoxic under rat S9. On the other hand, 2-aminoanthracene, which is non-classified in IARC, yielded stronger genotoxicity under human S9 than under rat S9, suggesting that special attention should be taken for evaluating its human carcinogenicity. The humanized genotoxicity tests must be more advantageous to evaluate genotoxicity for human than other genotoxicity tests, and the data would be helpful for estimating human risk of carcinogens or non-carcinogens if the epidemiological evidence is not available.

## 2214

### DIFFERENTIAL S-9 ACTIVATION OF HYDROCODONE BITARTRATE AND NALTREXONE HYDROCHLORIDE WITH HUMAN LIVER S-9 AND RAT LIVER S-9 IN THE MOUSE LYMPHOMA FORWARD MUTATION ASSAY.

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*In vitro* genotoxicity assays routinely include induced rat liver S-9 as part of a metabolic activation system. However, the rat liver system may not accurately predict the risk for human exposure since P450 gene expression and enzyme activity profiles can differ dramatically between the two species. Hydrocodone bitartrate and naltrexone hydrochloride were both assayed in the mouse lymphoma forward mutation assay with and without rat liver activation or a human S-9 activation system. In the absence of rat liver S-9 activation, hydrocodone bitartrate and naltrexone hydrochloride were negative for mutagenicity over a wide range of cytotoxicity. When hydrocodone bitartrate was analyzed for mutant induction at concentrations from 9.85 to 375 µg/mL in the presence of induced rat liver S-9, dose related increases in mutant frequency were observed from 62.5 to 375 µg/mL. When naltrexone hydrochloride was analyzed for mutant induction at concentrations from 75 to 550 µg/mL in the presence of induced rat liver S-9, dose related increases in mutant frequency were observed from 300 to 550 µg/mL. In order to determine the human relevance of these results, both hydrocodone bitartrate and naltrexone hydrochloride were tested in the presence of a human S-9 activation system. Hydrocodone bitartrate and naltrexone hydrochloride were tested in the presence of a pooled human S-9 metabolic activation system with concentrations ranging from 9.85 to 1600 µg/mL and 31.3 to 1200 µg/mL, respectively. In each of these tests, no dose-related increases in mutant frequency were observed in two trials over a wide range of cytotoxicity. These data demonstrate that the positive responses observed with hydrocodone bitartrate and naltrexone hydrochloride in the presence of induced rat liver S-9 metabolic activation could not be repeated with a human S-9 system, suggesting that the *in vitro* genotoxicity assay using the rat liver S-9 activation may not be predictive of genotoxicity in humans.

## 2215

### APPLICATION OF HUMAN EXOGENOUS METABOLIC SYSTEM IN GENOTOXICITY EVALUATION.

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Species-differences in drug metabolism is a well-established phenomenon, believed to be a key determinant of species-differences in drug toxicity. However, current genotoxicity assay involves mainly exogenous activating systems derived from rodents, with the most commonly used being Aroclor 1254-induced rat liver postmitochondrial supernatant (S-9). A drawback with the use of rat liver S-9 is that genotoxicity involving human metabolites would be missed, thereby leading to false negative results. Conversely, genotoxicity involving rat-specific metabolites would lead to false positive results. We believe that genotoxicity studies should also involve human liver-derived exogenous metabolic activation systems. Using Chinese hamster ovary cells (CHO) and measuring cytotoxicity and genotoxicity, we evaluated the ability of two human liver-derived exogenous activating systems: human hepatocytes and human liver S-9, in the activation of promutagens cyclophosphamide (CP), dimethylnitrosamine (DMN) and dimethylbenzanthracene (DMBA). Our results show that all three promutagens were genotoxic in the presence of the human exogenous activating systems, with human S9 apparently more potent than human hepatocytes. Results with human S9 is comparable to that obtained with rat liver S9, except that with rat S9, DMBA was the most mutagenic of the three promutagens, while DMN was the most mutagenic with human S9. Results of the study suggest that human liver-derived exogenous metabolic systems such as human hepatocytes and human S9 can be applied in routine *in vitro* genotoxicity assays and may provide results more relevant to human than that obtained with nonhuman systems.

## 2216

### MOLECULAR ANALYSIS OF SPONTANEOUS AND 3'-AZIDO-3'-DEOXYTHYMIDINE-INDUCED L5178Y *TK*<sup>+/−</sup> MOUSE LYMPHOMA CELL MUTANTS.

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The aim of this study was to investigate the types of mutational damage induced by the clastogen 3'-Azido-3'-deoxythymidine (AZT). AZT induced a significant dose-related increase in the thymidine kinase (*Tk*) mutant frequency (MF) in L5178Y/ *Tk*<sup>+/−</sup> 3.7.2C mouse lymphoma cells. At the highest dose (1 mg/ml), AZT induced approximately a 5-fold increase in MF over that of the control. 65% of the mutants were small colonies whereas 40% of the mutants in the control were small colonies. 150 AZT-induced mutants and 69 spontaneous mutants were isolated and analyzed. Loss of heterozygosity (LOH) analysis using a heteromorphic microsatellite locus within the *Tk* gene was performed to determine the status of the *Tk*<sup>+</sup> allele. In addition, gene dosage analysis was conducted using a Real-time PCR method to elucidate the number of *Tk* alleles in the mutants showed LOH at the *Tk* locus. One *Tk* allele would indicate deletion while two alleles would be consistent with a recombination event. Among the AZT-induced *Tk* mutants, the percentage of point mutation, deletion and recombination events were 0%, 43% and 57% in the small colonies and 25%, 24% and 51% in the large colonies, respectively. Among the spontaneous mutants, they were 3%, 30% and 67% in the small colonies and 58%, 0% and 42% in the large colonies. These results indicate that more deletions and fewer point mutations occurred in the small colonies than large colonies, both in AZT-induced and spontaneous mutants. More deletions and fewer point mutations were observed in the AZT-induced mutants than the spontaneous mutants. These results are consistent with the fact that AZT induces mutations via a clastogenic mechanism.

## 2217

### GENOTOXIC EFFECTS OF 4-HYDROXYNONENAL TRIACETATE, A CHEMICALLY PROTECTED FORM OF THE LIPID PEROXIDATION PRODUCT 4-HYDROXYNONENAL, AS ASSAYED IN L5178Y MOUSE LYMPHOMA CELLS.

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The lipid peroxidation product 4-hydroxy-2-enal (4-HNE) is cytotoxic and mutagenic at superphysiological concentrations. To characterize the mechanism of action of 4-HNE, we assessed genomic damage using the mouse lymphoma assay (MLA) which measures the frequency of mutations in the *Tk* gene. As a strong electrophile, 4-HNE reacts readily with nucleophilic centers on cellular components. When added extracellularly, it may therefore react preferentially with proteins close to the cell surface and not reach deeper targets such as DNA in the cell nucleus. We therefore tested the hypothesis that 4-HNE triacetate (4-HNE(Ac)3), a protected form of 4-HNE which is metabolically converted to 4-HNE (Neely, M.D., et al., J. Neurochem. 72: 2323-2333, 1999) is a more effective mutagen than 4-HNE. When added in serum-containing medium which simulates extracellular generation in an organism, 4-HNE was not mutagenic in the MLA system up to 38 mM. In contrast, exposure to 4-HNE(Ac)3, which mimics intracellular formation of 4-HNE, resulted in dose-dependent mutagenesis. At 17 mM 4-HNE(Ac)3, the mutant frequency was  $7.2 \times 10^{-4}$  which was >7-fold higher than the spontaneous mutant frequency in mouse lymphoma cells. The majority of mutations induced by 4-HNE(Ac)3 were clastogenic events affecting a large segment of the chromosome (up to at least 40 cM from the *Tk* gene). The results indicate that, in the presence of serum which approximates physiological conditions, intracellularly generated but not external 4-HNE is strongly genotoxic at concentrations which may occur during oxidative stress.

## 2218

### PHOTOMUTAGENICITY OF RETINYL PALMITATE AND ANHYDRORETINOL IN MOUSE LYMPHOMA CELLS.

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Retinyl palmitate (RP), a storage form of Vitamin A, is frequently used as an active cosmetic ingredient. However, when exposed to sunlight, RP is facilely decomposed to photodecomposition products, including anhydroretinol (AR). In this study, we evaluated the mutagenicity of RP and AR in mouse lymphoma cells when concomitantly exposed to UVA light irradiation. The L5178Y/ *Tk*<sup>+/−</sup> mouse lymphoma cells were treated with different doses of RP and AR alone/or under UVA

light (320-400 nm). While exposure of cells to UVA light alone at 0.122 J/cm<sup>2</sup>/min for 5-45 min displayed a dose-response for mutation induction in the Tk gene, with a induced mutant frequency (MF) of 136 10-6 after 30 min of light irradiation, cells received RP alone at 25-250  $\mu$ g/ml for 4 hrs did not increase MF. Treatment of cells with 0.5-25  $\mu$ g/ml RP under UVA light for 30 min produced a synergistic mutagenicity effect. At 25  $\mu$ g/ml RP with UVA exposure (12% of relative total growth), the induced MF was about 3-fold higher than that for UVA exposure alone. Similar results were obtained from AR treatment. While AR itself was not mutagenic, it induced mutation induction in the cells when exposed concomitantly to UVA light. To elucidate the underlying photomutagenic mechanism of RP, we determined the loss of heterozygosity (LOH) at four microsatellite loci spanning the entire chromosome 11 for RP/UVA-induced mutants. Ninety three percent of the mutants lost the Tk+ allele at Tk locus, and 84% of the damage extended to 6cM in chromosome length. These results suggest that RP and AR are photomutagenic in mouse lymphoma cells through a clastogenic action.

## 2219 GENETIC TOXICOLOGICAL STUDIES FOR LUPINUS TERMIS ALCOHOLIC EXTRACT.

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Lupin species have been widely cultivated for human consumption and used in traditional medicine. The Lupin species, Lupinus termis has been widely cultivated in Sudan and used as an aqueous extract to treat a variety of dermatological disorders. Lupinus termis seeds also have been used as a skin softener and in a variety of cosmetic preparations. Previous studies have shown the effectiveness of the alcoholic extract of Lupinus termis seeds in the treatment of chronic eczema. In the current study, the mutagenic and/or clastogenic potential of the alcoholic extract of Lupinus termis seeds was investigated using the Mouse Lymphoma Assay and the Micronucleus Test. In Mouse Lymphoma assay, L5178Y TK+/- cells were treated for 4 hrs with a range of concentrations of the alcoholic extract, in the presence and absence of S9. Benzo(a)pyrene and 4-Nitro-quinoline-1-oxide were the positive controls. In Micronucleus test, Balb/C male mice were treated with the 50% LD50 of the alcoholic extract. Mitomycin C (2 mg/Kg) was used as the positive. Mice were sacrificed 24 hrs and 48 hrs after treatment, the bone marrow was flushed and cell suspensions were prepared to determine the incidence of micronucleated polychromatic erythrocytes. The results obtained in these genetic toxicological assays showed that there were no differences between experimental and negative control samples. These results corroborate and complement the previously obtained results for the *Salmonella typhimurium* Reverse Mutation Assay. This genetic toxicological study suggests that under the experimental conditions, the alcoholic extract of Lupinus termis seeds does not have either mutagenic nor clastogenic potential.

## 2220 TAXOL INDUCES MUTATIONS IN THE TK GENE OF L5178Y/TK+/- MOUSE LYMPHOMA CELLS THROUGH A MITOTIC NON-DISJUNCTION MECHANISM.

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Taxol (paclitaxel) is a potent anti-cancer drug that binds to tubulin and inhibits cell division. Taxol has been shown to induce chromosome aberrations in human lymphocytes *in vitro* and to induce micronuclei in mice. However, Taxol is not mutagenic in the Ames test or the CHO/Hprt gene mutation assay. In this study, we evaluated the mutagenicity of taxol in the Tk+/- 3.7.2C mouse lymphoma assay (MLA). Three independent experiments were performed using 4-hour treatment. Taxol induced a dose-related increase in mutant frequency. The results were similar in the three experiments with a 0.75  $\mu$ g/ml Taxol-induced mutant frequency of 163 x 10<sup>-6</sup> at 30% relative total growth in one experiment. Forty two percent of the mutants from this treatment were small colony mutants. Loss of heterozygosity (LOH) analysis of 73 mutants from control cultures and 160 mutants (both small and large colony) from taxol-treated cultures was conducted using four microsatellite loci spanning the entire chromosome 11. Almost all (99%) of the taxol-induced mutants lost all 4 microsatellite markers, indicating the loss of the entire chromosome 11b containing the Tk+ allele. Only 22% of the mutants from untreated control cultures showed the same microsatellite pattern. These data suggest that taxol induces mutations in the MLA via a mitotic non-disjunction mechanism. This adds to the weight of evidence that the MLA can detect aneuploids.

## 2221 INCREASED GERMLINE MUTATION FREQUENCIES INDUCED BY ETHYLENE DICHLORIDE IN MALE MICE.

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The ability of genotoxic agents to disrupt or cause cessation of spermatogenesis in mammalian species including the human has been known for many years. This was generally the prediction (and diagnosis) of males undergoing vigorous chemotherapy and/or radiation therapy for cancer. However, in recent years it has become recognized that many of these individuals may become fertile again. The return to fertility after a genotoxic agent induced sterile period has not been well studied. We have developed animal models for the study of the sterilizing effects of ethylene dichloride (EDC) in male C57BL/6 mice. If a sterile period is produced in males by the killing of stem germ cells, then spermatogenesis cannot continue until a critical mass of stem germ cells is reached by the "clonal" expansion of the surviving stem germ cells. Based on this theory mutant clusters, sporadic clusters mutated stem cells, should be formed upon re-initiation of spermatogenesis. The formation of mutant clusters has been demonstrated in sperm from treated Drosophila, however due to limitations in methodologies, mutant clusters in the sperm of mammalian species, i.e. mice, has not been shown as of this date. To address this problem we used the ultra-sensitive molecular intragenic mutation assay (Needle-in-a-haystack PCR/RE/LCR assay) which is capable of one-in-a-million sensitivity. Sexually mature male C57BL/6 mice received EDC i.p. injections with dosages ranging from 5 to 40 mg/kg or vehicle and subsequently held for 45 days to allow for complete turn over of spermatogenesis. Males were then paired with females to determine fertility. Mice were sacrificed after siring two litters or determining that there was permanent sterility. The 5mg/kg male mice recovered to fertility and sired litters of pups after a sterile period of up to 5 weeks. Our results showed that there was an expansion of Bmp5 short ear mutant clusters following the return to fertility, to a frequency of one-in-ten thousand in mature sperm.

2222 MICROARRAY GENE EXPRESSION ANALYSIS REVEALS DIFFERENCES BETWEEN LARGE AND SMALL COLONY THYMIDINE KINASE MUTANTS OF L5178Y MOUSE LYMPHOMA CELLS.

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The L5178Y mouse lymphoma assay (MLA) is widely used to evaluate chemicals for their ability to induce mutation. The thymidine kinase (Tk) mutants detected in the assay fall into two categories: small colony and large colony Tk mutants. Small colony mutants grow slowly and their induction is associated with clastogenicity. Large colony mutants grow at normal rates and their induction is associated with point mutations. In order to investigate the molecular basis for differences between the large and small colony Tk mutants, microarray gene expression analysis was conducted on 4 small and 4 large colony Tk mutants. Long-oligonucleotide microarrays of 20, 000 mouse genes were used in 2-color hybridizations using a reference design. Correlation (R value) within the small and large colony groups ranged from 0.90 to 0.97. The raw data was normalized using locally weighted linear regression (LOWESS) in ArrayTrack, a database with software tools developed at the NCTR. Based on cluster analysis of the gene expression changes, the large colony mutants could be distinguished from the small colony mutants. In addition, the gene expression patterns in the large colony group of mutants were very homogeneous while the patterns from the small colony group of mutants were highly heterogeneous. The Significance Analysis of Microarray (SAM) program was then used for analysis of significant gene expression changes between the two groups (1.4-fold and false discovery rate <0.05). Genes whose expression was significantly different were mapped onto the mouse karyotype using ArrayTrack. Approximately 30% of the genes whose expression was significantly increased in the small colony mutants mapped to chromosome 11, where the Tk gene resides. These results suggest that altered expression of genes near the Tk gene is associated with the small colony phenotype.

## 2223 MICRONUCLEUS INDUCTION AND DNA DAMAGE IN V 79 CELLS *IN VITRO* BY DUSTS FROM HARD METAL SINTERING AND DETONATION COATING PROCESSES.

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ABSTRACT Hard metal bulk dusts from a molding-sintering process and from a detonation-coating process were characterized for their ability to induce micronuclei and DNA damage in cultured V 79 Chinese hamster lung fibroblast cells. The study included the precoating mixture and overspray material from a detonation coating process, an unsintered material from a molding-sintering operation, and the tungsten carbide and cobalt ingredients from the sintering process. Doses were selected after viability testing and ranged from 0 to 200  $\mu$ g/cm<sup>2</sup>. Results indicate that the unsintered sample and pre- and post-coating detonation coating mixtures showed a positive dose-response relationship for micronucleus induction; WC alone was weakly positive for a single dose only. The overspray material from the detonation-coating process showed greater micronucleus induction than any of the other materials. Results from the DNA damage assay indicate that the three hard

metal mixtures are all capable of DNA damage, with similar dose-response curves. The addition of 10 or 20 mM N-acetyl cysteine, a general antioxidant, significantly attenuated DNA damage by all three samples. The results indicate that materials from either conventional or detonation-coating processes are capable of genotoxicity in V 79 cells *in vitro*, including DNA damage and micronucleus induction, and DNA damage is at least partially through oxidative mechanisms. The post-detonation material is a more potent inducer of micronuclei than are the predetonation and unsintered dusts, but the DNA damage potential of all three dusts are quantitatively similar.

**2224**

TRICHLOROETHYLENE (TCE) INHALATION DID NOT INDUCE CYTOGENETIC DAMAGE IN THE RAT BONE MARROW MICRONUCLEUS TEST (MNT).

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TCE was previously reported to induce a small increase (2-3 fold) in micronucleated polychromatic erythrocytes (MN-PCEs) in the bone marrow of CD rats following a single 6h inhalation exposure. The current study attempted to replicate these findings and to correlate them to potential TCE-induced hypothermia. Male CD rats were exposed by inhalation to targeted concentrations 0, 50, 500 2500 or 5000 ppm TCE for 6h on a single day. Chamber concentrations of TCE were measured once/h. Relative body temperatures (BT) of 6 rats/group were measured pre-exposure and at 3, 19 & 48h post exposure using programmable transponders. BT was monitored at 5-min intervals 22h pre-exposure, during exposure and 19 h post-exposure in a single rat/group using radio-telemetry. Bone marrow samples were evaluated for MN formation at 19h & 48h post-exposure. Rats treated with cyclophosphamide monohydrate and sacrificed 19 hrs later served as positive controls. Actual mean chamber concentrations were 0, 53, 521, 2579 and 4677 ppm. A single 6h inhalation exposure to 5000 ppm TCE caused marked toxicity and deaths in male rats. A small, but notable drop in BT was identified via radio-telemetry at 5000 ppm during exposure but was considered inconsequential to the interpretation of the test results. No increase in MN-PCE or decrease in the relative proportion of PCE was observed in any of the TCE treatment groups. The positive control rats had significant increases in MN-PCE. In conclusion, results from the current study indicated that inhalation exposure of rats to TCE up to a maximum tolerated concentration did not induce cytogenetic damage in the bone marrow.

**2225**

ABILITY OF ALKYLTIN COMPOUNDS TO PENETRATE CELL MEMBRANES AND ITS RELATION TO INDUCTION OF GENOTOXIC EFFECTS IN CHINESE HAMSTER OVARY (CHO) CELLS.

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Sponsor: E. Nelson.

Organometals, such as tin derivatives are distributed in the environment. Human exposure to these chemicals can lead to acute and chronic intoxication. Following uptake de-/alkylation occurs that changes the bioavailability and toxicity of the tin species. In this study, the tin de-/alkylation products monomethyltin chloride (MMT), dimethyltin dichloride (DMT), trimethyltin chloride (TMT), and tetramethyltin (TetraMT) were investigated for cyto- and genotoxic effects in relation to the cellular uptake. To identify genotoxic effects micronucleus (MN) assay, chromosome analysis (CA) and sister chromatid exchange (SCE) test were used. The cellular uptake was assessed using ICP-MS analysis. The toxicity of DMT and TMT was evaluated also after forced uptake by electroporation. Our results show that the ability of organotin compounds to penetrate cell membranes modulates their genotoxicity. DMT and TMT did not induce elevated numbers of MN up to a concentration of 1 mM. Chromosome analysis and SCE revealed that DMT induced significantly elevated numbers of CA and SCE at concentration of > 1 mM. The cellular uptake of the organotin compounds was dose dependent whereas the percentage of the exposure substrate found intracellularly was always < 1%. The order of cellular uptake for the organotin compounds was: DMT > TMT > MMT > TetraMT. After forced uptake by electroporation DMT and TMT induced significantly elevated numbers of MN at concentrations of 5 and 50  $\mu$ M, respectively. Altogether, our results show that the methylated tin compounds have just weak genotoxic potential. This can be explained by the poor membrane permeability of the compounds. After forced uptake of DMT and TMT, the genotoxic effects are increased. These findings suggest that methylated tin compounds are able to induce genomic damage if the membrane permeability is increased.

**2226**

RAT ERYTHROCYTE MICRONUCLEUS TEST: ROLE OF ERYTHROPOESIS AND EFFECT OF CIGARETTE MAINSTREAM SMOKE.

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The genotoxicity of cigarette mainstream smoke (MS) has been investigated using the *in vivo* micronucleus test. According to guidelines (OECD 474), the highest dose at which chemicals are to be tested should be sublethal. Since MS from filter cigarettes at sublethal doses contains significant amounts of CO, and CO may enhance erythropoiesis, its effect on micronucleus formation was investigated. Erythropoiesis was also stimulated using erythropoietin (EPO). The MicroFlow kit (Litron Laboratories) was used for quantification of CD-71-positive reticulocytes (proportion of reticulocytes among total erythrocytes: RET) and micronucleated reticulocytes (proportion of micronucleated reticulocytes among total reticulocytes: MnRET) from rat blood. Sprague-Dawley rats (8/group) were nose-only exposed to MS from the Reference Cigarette 2R4F at concentrations up to 1250  $\mu$ g TPM/l (1400 ppm CO) for 2 x 1 h/day, 4 days. RET increased from 1.2% to 2.5% ( $p<0.05$ ) and MnRET from 0.12% to 0.28% ( $p<0.05$ ). The non-filter Reference Cigarette 2R1 failed to increase the RET and MnRET at 1250  $\mu$ g TPM/l (700 ppm CO), but supplementing this smoke with CO to 1400 ppm increased RET and MnRET ( $p<0.05$ ). CO alone (1400 ppm) increased RET ( $p<0.01$ ), but not the MnRET. When rats were treated with 0, 30, or 100 U/kg EPO in combination with 0-1200  $\mu$ g TPM/l from the 2R4F, the EPO-treatment increased the RET from 1.1% to 6.2%. MS exposure did not increase the MnRET, whereas the EPO-treatment did (to 0.38%). The RET and MnRET were increased after treatment with a non-cytotoxic dose of cyclophosphamide (1.5 mg/kg) in combination with EPO-treatment at 30 and 100 U/kg ( $p<0.05$ ). We have demonstrated that the proportion of MnRET correlates with the proportion of RET in rat blood, indicating that enhanced erythropoiesis influences the proportion of MnRET after exposure to MS. It is thus important to carefully examine the proportion of RET in the micronucleus test with MS. The MS-induced increase in RET (as a result of CO) may yield confounded genotoxicity results.

**2227**

THE EFFECT OF BREVENAL ON DNA DAMAGE IN HUMAN LYMPHOCYTES INDUCED BY BREVETOXINS 2 AND 3 (PBTX-2 OR 3).

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Brevinal is a nontoxic polyether that competes with brevetoxin for the active site on the voltage-sensitive sodium channel. Brevetoxins are polyether toxins from blooms of marine dinoflagellates and activate voltage-sensitive sodium channels and may induce fish kills, marine mammal poisoning and adverse human health effects such as respiratory irritation and airway constriction. The purpose here was to determine if brevetoxins could induce DNA damage in human lymphocytes, and if the damage could be antagonized by brevenal. Unrepaired or erroneously repaired DNA damage may result in gene mutation, chromosome aberration, and modulation of gene regulation, which have been associated with immunotoxicity and carcinogenesis. The single-cell gel electrophoresis assay, or comet assay, was used to determine and compare DNA damage following various treatments. The data shown is the tail moment which is the percentage of DNA in the tail multiplied by the length between the center of the head and tail (units of measure are arbitrary). The negative control tail moment was 28.4 (S.E.=1.5), whereas the positive control(hydrogen peroxide) was 71.8 (2.2). PbTx-2, 10-8 M was 49.4 (9.9) and PbTx-3, 10-8 M was 71.8 (11.9). Brevenal, 1  $\mu$ g/ml 1 hour before the brevetoxins protected the lymphocytes from DNA damage (PbTx-2; 33.9 (1.4) and PbTx-3; 42.4 (0.8)). The tail moment for brevenal alone was 30.8 (2.6). PbTx-2 and 3 are potent inducers of DNA damage in normal human lymphocytes, which is fully antagonized by brevenal. Brevenal also competitively displaces tritiated brevetoxin in a synaptosome receptor-binding assay. Similarly, brevetoxin-induced DNA damage at 10-8 M in human lymphocytes was antagonized by brevenal. (Supported by the North Carolina Agromedicine Center and USDA/CSREES; The brevetoxins and the brevenal were provided under NIEHS grant P01 ES10594).

**2228**

BISPHENOL A-INDUCED DNA DAMAGE IN C57BL/6N MICE: PRACTICAL *IN VIVO* APPLICATIONS OF THE COMET ASSAY.

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Bisphenol A (4, 4'-isopropylidene-2-diphenol; BPA) is an industrial monomer used for the synthesis of polycarbonate plastics and epoxy resins. Although there is no evidence that BPA poses any known health risk to humans, animal studies suggest that

BPA exposure may lead to chromosomal abnormalities. Previous studies in our laboratory found that BPA-induced DNA damage was detectable using the comet assay, however BPA exposure did not result in chromosomal aberrations or the formation of micronuclei in Chinese hamster ovary (CHO) cells. The current study was conducted to investigate the potential of BPA to induce DNA damage in male C57BL/6N mice using the comet assay, as well as determine the feasibility of screening chemicals using the comet assay *in vivo*. Animals were orally gavaged with a single dose of BPA (100mg/kg) or water. At 1, 3 and 6 hours post treatment, the animals were sacrificed and the testis, liver, lung, and brain were collected, flash frozen in liquid nitrogen, and stored at -80°C until processed for the comet assay. In addition, the comet assay was also performed using fresh organs collected at the time of harvest. BPA challenge resulted in a time-dependent effect on DNA damage in the testis, but not in other organs examined, which was comparable in both fresh and frozen tissue. Comet moment, tail length and %DNA in the comet tail, were not affected after 1 hour of BPA exposure, but significantly increased after 3 hours. This response was subsequently decreased by 6 hours post BPA-challenge. An increase in the number of cells with elevated DNA damage was also observed in testis after 3 hours of BPA administration. These results indicate that BPA exposure might result in transient DNA damage that is rapidly repaired in the testis, but not in other organs. This is consistent with reported findings of decreased sperm counts and increased oxidative stress in the testis of BPA-treated animals. These data also indicate that frozen tissue, in lieu of fresh tissue, may be used in the comet assay, providing a practical application for screening compounds with the comet assay *in vivo*.

**2229**

THE EFFECT OF PUFF VOLUME ON THE SPECIFIC ACTIVITY OF CIGARETTE SMOKE CONDENSATE AS MEASURED IN THE AMES ASSAY.

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Previous studies have shown that identical cigarettes smoked under different smoking regimes (i.e. standard ISO/FTC versus Massachusetts/Canadian intense smoking protocols) resulted in lower specific activities (revertants/mg condensate) in the Ames assay as smoking intensity increased. The smoking regimes specified by some regulatory authorities differ in more than one smoking parameter (e.g. puff volume, puff interval, ventilation blocking). Therefore, to examine the effects of only puff volume on specific activity we smoked PMM16 industry-monitor cigarettes (un-ventilated) under identical conditions of temperature and humidity with a 2 second draw and 1 minute puff interval at 5 different puff volumes (10, 25, 35, 45, 55 ml). Condensates from each puff volume were collected on Cambridge filter pads, extracted in DMSO and tested in the Ames (preincubation) assay using *Salmonella* strains TA98 and TA100 under S9+ conditions. The delivery of Wet Total Particulate Matter (WTPM) increased from 3.7 to 33.0 mg/cigarette as puff volume increased. Additionally, the water content of the WTPM increased from 0.3 to 9.0 mg/cigarette as puff volume increased. The specific activities for WTPM in strains TA98 and TA100 showed a decreasing trend as puff volume increased. However, this trend of decreasing specific activity was eliminated in strain TA98 when calculated for Dry Particulate Matter (DPM: WTPM minus water) and FTC Tar (WTPM minus water and nicotine). Interestingly, the trend of decreasing specific activity with increasing puff volume remained in strain TA100 when calculated for DPM and FTC Tar. In conclusion, it appears that the trend of decreasing specific activity measured in TA98 may be the result of the water in WTPM acting as a diluent.

**2230**

GENOTOXICITY PROFILES OF COMMON ALKYL HALIDES AND ALKYL ESTERS.

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Genotoxic impurities could be generated during the synthesis and/or formulation of drugs. For instance, alkylating agents such as alkyl halides and alkyl esters of alkyl sulfonic acids might occur as products of strong acid/alcohol interactions during the process of drug salt formation. Although the genotoxicity of classic alkylating agents such as methyl and ethyl methanesulfonate has been well characterized, the majority of compounds from this class has been tested only to assess their mutagenicity using the *Salmonella* reversion assay. Therefore, we set out to investigate genotoxic profiles of 22 halogenated alkanes and alkylesters of sulfuric and alkane-aryl-sulfonic acids using a battery of cellular and molecular assays. The mutagenic potential was evaluated using the Spiral modification of the *Salmonella* reversion assay in TA98 and TA100 tester strains. Clastogenicity was measured using the *in-vitro* micronucleus assay in CHO cells. The deletion recombination (DEL) assay in *Saccharomyces cerevisiae* was used to further investigate the potential of selected

compounds to cause DNA breaks and chromosome instability. The tested esters of alkyl- and aryl-sulfonic acids produced mutagenic and clastogenic responses. In contrast, the esters of sulfuric acid displayed mostly clastogenic activity. The majority of alkyl halides were not genotoxic. In summary, our study contributes to better understanding of the genotoxic properties of common alkyl halides and alkyl esters and might provide guidance to managing risk of genotoxic process-related impurities of drug substances and products.

**2231**

DETECTING ANTHRACYCLINE-INDUCED INTRACHROMOSOMAL RECOMBINBINATION IN *SACCHAROMYCES CEREVISIAE* USING A DEL ASSAY.

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For more than thirty years, anthracyclines (initially daunomycin and adriamycin and, more recently, idarubicin and epirubicin) have represented one of the most commonly used classes of anticancer drugs. It is known that anthracyclines interact with DNA in a very complex manner. Studies in our lab have shown that anthracyclines can induce base-substitution and frameshift mutations in the bacterium *Salmonella typhimurium*. Furthermore, our lab has demonstrated that these mutations can be recognized by the DNA repair protein O<sup>6</sup> methylguanine DNA repair methyltransferase (MGMT). The objective of this study is to accurately access the recombinogenic potential of anthracyclines in a unicellular eukaryotic organism. In the yeast deletion (DEL) assay, recombination is induced by the formation of DNA strand breaks which are a substrate for initiation of genetic repair in this organism. Using the DEL assay, our lab has examined the role of DNA recombination pathways in the recognition and removal of anthracycline-induced DNA adducts. Specifically, daunomycin (79-fold induction), adriamycin (491-fold induction), idarubicin (88-fold induction), and epirubicin (325-fold induction) tested positive in this assay. These results indicate that anthracyclines can act as genotoxins in eukaryotes.

**2232**

CARCINOGENIC CR(VI) AND THE NUTRITIONAL SUPPLEMENT CR(III) INDUCE DNA DELETIONS IN YEAST AND MICE.

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The high levels of Cr(VI) contamination in drinking water across the country has led to concerns as to whether or not Cr(VI) ingestion from drinking water can lead to cancer. An argument against the role of Cr(VI) as an oral carcinogen is that it is reduced to Cr(III) in biological systems. Cr(III) is a popular nutritional supplement. In order to establish effective regulatory measures that protect the general public, the potential of Cr(VI) and Cr(III) to cause cancer via ingestion must be examined. There exists a large body of evidence, which confirms that chromosomal rearrangements such as DNA deletions are an important intermediate step in carcinogenesis. In this study, two systems screening for DNA deletions were used to compare the ability of Cr(VI) and Cr(III) to induce chromosomal rearrangements in the yeast *S. cerevisiae* and *in vivo* in the C57BL/6J pun/un mice. The mice were exposed to Cr(VI) and Cr(III) via drinking water during gestation and 70 kb DNA deletions at the pink-eyed unstable (pun) allele were determined in the resulting offspring. Induction of DNA deletion in yeast was measured by growth on selective media. Both Cr(VI) and Cr(III) significantly induced DNA deletions in mice and yeast as compared to the spontaneous level of deletions. Quantification of intracellular concentrations of Cr after exposure of yeast cells to either Cr(III) or Cr(VI) revealed that Cr(III) is more genotoxic than Cr(VI) once it is inside the cell. The quantification of tissue Cr concentration in mice confirms this trend. Therefore, this study concludes that both, the environmental contaminant Cr(VI) and the nutritional supplement Cr(III), when ingested via drinking water, lead to a genotoxic endpoint which is an intermediate in the process of carcinogenesis.

**2233**

YEAST DEL ASSAY DETECTS CLASTOGENS.

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The DEL Assay for chromosomal rearrangement in the yeast *S. cerevisiae* is able to detect a wide range of carcinogens. In a study with approximately 60 compounds of known carcinogenic activity, the DEL assays detected 86% correctly whereas the Ames *Salmonella* assay detected only 30% correctly<sup>1</sup>. Since the DEL assay is highly inducible by DNA double strand breaks, this study examined the utility of the DEL assay for detecting clastogens. Ten model compounds, with varied genotoxic activity, were examined for their effect on the frequency of DNA deletions with the

DEL assay. The *in vitro* micronucleus assay in CHO cells, a commonly used tool for detection of clastogens, was performed on the same compounds and the results of the two assays were compared. The compounds tested were: Actinomycin D, Camptothecin, Methotrexate and 5-Fluorodeoxyuridine, which are anticancer agents, Noscapine and Furosemide are therapeutics, Acridine, Methyl Acrylate and Resorcinol are industrial chemicals and Diazinon is an insecticide. Our data indicate that the DEL assay provides highly concordant data with the standard *in vitro* MN assay. The DEL assay might have a potential application as a robust, fast, and economical screen for detecting clastogens *in vitro*.

**2234**

NUCLEAR LOCALISATION AND DNA REPAIR CAPACITY OF HOGG1 VARIANTS POLYMORPHIC AT RESIDUE 326 FOLLOWING TRANSFECTION OF OGG1<sup>-/-</sup> NULL MOUSE FIBROBLASTS.

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Cells are continuously exposed to damaging reactive oxygen species (ROS), which are produced from both endogenous and exogenous sources. 8-oxodeoxyguanosine (8-oxodG) is an abundant base lesion formed during oxidative stress which, if not repaired, can give rise to G:C → T:A transversion mutations in DNA. The DNA repair enzyme human 8-oxoguanine DNA glycosylase-1 (hOGG1) catalyses the removal of 8-oxodG from DNA and therefore acts to promote genome stability. hOGG1 deletion, mutation and polymorphism may result in a hypermutator phenotype and individual susceptibility to oxidative pathologies including cancer, diabetes and ageing. Limited and conflicting evidence exists regarding the repair capacity of a prevalent (approximately 40% of population) hOGG1 polymorphism, the Cys326-hOGG1 variant. In the current study, an *in vitro* cell culture model was developed to investigate the cellular localisation and DNA repair capacity of Ser326 (wild-type), Cys326- and Gly326 (artificial)-hOGG1 variants. mOGG1<sup>-/-</sup> null mouse embryonic fibroblasts were transiently transfected with mammalian expression vectors containing either Ser326-, Cys326- or Gly326-hOGG1 cDNA and treated with the glutathione (GSH)-depleting agent DL-buthionine-[S, R]-sulfoximine (BSO) (1 mM) for 24 hours as a means of oxidative stress. GFP-tagged hOGG1 variants were all shown by fluorescence microscopy to be of nuclear localisation irrespective of GSH status. All variants also detected and repaired 8-oxodG with similar activity in an oligonucleotide incision assay. Again, this was irrespective of the GSH status of the cells. These results indicate that the variant forms have normal nuclear location and are functional under the conditions employed. Current studies are aimed at assessing relative intracellular repair activities following treatment with DNA oxidants.

**2235**

A COMPARATIVE STUDY OF UVB AND UVC MUTAGENESIS IN DNA REPAIR PROFICIENT AND MISMATCH REPAIR DEFICIENT (*PMS2*) MOUSE CELLS.

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Ultraviolet light is a potent environmental mutagen via the production of both cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4 PP). UVC radiation (200-280 nm) is readily absorbed by DNA which makes it a convenient tool for studying UV mutagenesis. However, UVB (280-315 nm) is a better tool to use when considering mutagens of environmental concern because, unlike UVC, UVB rays are not deflected by ozone and are capable of reaching the earth's surface. We therefore performed a comparative study of UVB and UVC mutagenesis in both DNA repair proficient and mismatch repair deficient (*Pms2*) backgrounds. Using an *Apri* reversion assay designed to detect both C→T and CC→TT mutations, mutation frequencies and the spectrum of mutations induced by both wavelengths were compared. These mutations are considered the 'signatures' of UV mutagenesis. For this assay, a phenylalanine codon (TTC) in mouse *Apri* was changed to proline (CCC) and reversion mutations were detected by acquisition of *Apri* function. An advantageous feature of this reversion assay is that both C→T and CC→TT substitutions restore *Apri* function. Interestingly, more tandem mutations were induced in mismatch repair-deficient cells by both UVC and UVB than in wild type cells. Also, there was profound difference in which base was altered in wild type cells treated with UVB or UVC. UVB induced single base changes at both target cytosines (CCC, CCC), whereas UVC induced single base mutations at only the internal cytosine (CCC). Two conclusions may be made from this study: mismatch repair protects against tandem CC→TT mutations, and UVB induces DNA lesions that are processed differently than UVC in wild type cells.

**2236**

MODULATION OF NNK-INDUCED GENETIC DAMAGE BY POLYMORPHISM IN THE BASE EXCISION REPAIR GENE APE1 IN SMOKERS.

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Tobacco contains many potent carcinogens, including the tobacco-specific nitrosamine NNK. Metabolism of NNK generates methylating and free radical species that can methylate DNA and cause oxidative DNA damage. The base excision repair (BER) pathway is designed to remove non-bulky adducts, such as those produced by NNK. The AP endonuclease APE1 plays a critical role in the BER process by initiating the repair of the AP sites generated by the excision of damaged bases in the DNA. Polymorphisms in several DNA repair genes have been identified, and many have been associated with cancer risk. In the current study we tested the hypothesis that the inheritance of the Asp148Glu polymorphism in APE1 influences the level of genetic damage resulting from exposure to nitrosamines found in tobacco smoke. We used the mutagen sensitivity assay, with NNK as a model mutagen, to test this hypothesis. Lymphocytes from 106 healthy volunteers were exposed *in vitro* to NNK, and the genotoxic response was measured by assessing the increase in chromosome aberration (CA) frequency. Our data indicate that the inheritance of the variant Glu allele is protective against NNK-induced genetic damage. A significant (P=0.005) decrease in NNK-induced CA (total breaks/100 cells) was observed in cells from smokers with the Glu allele (2.65) compared to cells from smokers homozygous for the wild-type Asp allele (4.71). The protective effect was significantly (P=0.009) more pronounced in males (1.85) compared to females (3.38). These data suggest that the 148Glu allele may affect the repair efficiency of NNK-induced genetic damage and consequently, may play a significant role as a risk factor for smoking-related cancers (Supported by an External Research Program grant from Philip Morris Inc. and NIEHS T32ES07254).

**2237**

THE MECHANISMS OF DITHIOCARBAMATE DEVELOPMENTAL TOXICITY.

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We have previously reported that the dithiocarbamate pesticide, sodium metan (NaM), and its active ingredient, methylisothiocyanate (MITC) cause notochord malformations in the developing zebrafish during a sensitive developmental window which occurs > 6 hours before the visual manifestation of the malformation. Several dithiocarbamates (DTC), isothiocyanates (ITC) and carbon disulfide were investigated to determine the ultimate *in vivo* toxicant perturbing the pathway(s) leading to a deformed notochord. Among other observations, all the DTC and carbon disulfide elicited notochord malformations similar to those previously observed with NaM and MITC. Of the ITC tested only MITC exposure led to notochord malformations. It appears that in development DTC and/or carbon disulfide interact with the target leading to a malformed notochord. The potential involvement of cellular oxidative stress pathways and the role of metals were also investigated. Reduced glutathione (GSH) suppressed DTC induced notochord malformation with toxicant co-exposures however; differences in GSH protection/recovery were evident when the exposure windows were manipulated. Surprisingly, GSH failed to diminish MITC-induced malformations. Studies using Cu and Zn as well as their respective membrane permeable and impermeable chelators indicate a potential contribution from Cu. In order to identify the target causing perturbed notochord development; preliminary microarray experiments were undertaken encompassing the critical window of sensitivity to identify primary gene expression changes in response to chemical exposure. In conclusion, the underlying mechanism of DTC developmental toxicity appears to require *in vivo* glutathione depletion prior to DTC-target interaction. Furthermore, metals may play a role in the notochord response to DTC. Supported by NIEHS #ES00210, #ES03850, and #ES07060.

**2238**

TCDD ALTERS GENE EXPRESSION IN ZEBRAFISH OVARY: INSIGHT INTO THE MECHANISMS BY WHICH TCDD IMPACTS REPRODUCTION IN FISH.

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TCDD (2, 3, 7, 8-tetrachlorodibenzo-p-dioxin) is a reproductive toxicant and endocrine disruptor in nearly all vertebrates. Fish are among the most sensitive vertebrates to the toxic effects of TCDD, and exposure to TCDD modulates their reproductive success by inhibiting ovarian development and estradiol production,

and ultimately reducing egg production. The molecular mechanisms by which TCDD induces these reproductive alterations have not been fully characterized. The ovary is a major target organ for TCDD toxicity, and estrogens are required for normal growth and development of the ovary. Therefore, TCDD-induced effects on reproduction may reflect AHR mediated alterations in ovarian gene expression with specific impacts on steroidogenesis. This study was designed to investigate the impact of TCDD exposure on gene expression as they relate to the suppression of estradiol biosynthesis or the alteration of oogenesis or vitellogenesis. Adult female zebrafish were exposed to different concentrations of TCDD in the diet for a period of four weeks, after which total RNA was isolated from the ovary. We have previously shown that adult female zebrafish show a significant decrease in the ovosomatic index following chronic dietary exposure to TCDD and, at high doses, exhibit severe ovarian necrosis and decreased egg production. Genes and pathways whose expression in the ovary was affected by TCDD were identified by microarray analysis using a common reference experimental design. Several genes were identified as being dysregulated at least 2-fold following dietary TCDD exposure (approximately 4% of genes surveyed were upregulated and 1.4% downregulated) and on-going analyses will provide additional information regarding the cellular pathways that are affected. Identifying potential molecular targets of TCDD constitutes the first step in understanding the mechanisms that underlie the toxic effects of TCDD on the female reproductive system.

**2239**

MATRIX METALLOPROTEINASE INHIBITION DURING ZEBRAFISH EMBRYOGENESIS FOLLOWING EXPOSURE TO HYDROCORTISONE AND DEXAMETHASONE.

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Matrix metalloproteinases (MMPs) are a family of enzymes capable of digesting various components of the extracellular matrix (ECM). These degradative processes are essential for a number of biological and pathological processes, including embryogenesis, where MMPs participate in the normal ECM remodeling that occurs during tissue morphogenesis and development. Studies have demonstrated that MMP gene expression is inhibited by the action of glucocorticoids through mechanisms involving the glucocorticoid receptor. Further, glucocorticoids have been shown to alter developmental processes in several species, including mice and humans. Therefore, we propose that glucocorticoids alter normal development through inhibition of MMPs. Zebrafish (*Danio rerio*) were used as a model to study the effects of MMP-13 (Collagenase-3) inhibition during embryogenesis following acute exposure to the glucocorticoids hydrocortisone (HC) and dexamethasone (DX). Zebrafish eggs approximately 3 hours post-fertilization were exposed to 1, 10, or 100 ppm HC or DX for 24 hours. Total RNA was isolated from developing eggs and reverse transcription was performed to produce cDNA for quantitative PCR. Normalization of quantitative PCR data to  $\beta$ -actin revealed an inhibition of MMP-13 gene expression at 100 ppm when compared to negative and solvent controls. This effect was observed for both glucocorticoids, although DX was a more potent inhibitor of MMP-13 gene expression than HC. These results suggest that exposure to glucocorticoids during embryogenesis has the potential to affect normal development by preventing MMPs from performing essential ECM remodeling processes.

**2240**

EARLY EMBRYONIC RESPONSES OF ZEBRAFISH TO DIESEL PARTICULATE MATTER.

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Exposure to diesel particulate matter is reasonably anticipated to be a human carcinogen. An increased risk of lung cancer is found in the majority of human studies. Different toxicity tests have been conducted on carcinogens and the toxic effects they have on humans as well as other living organisms. Fish embryo assays have been conducted because they are more effective compared to short-term assays using juvenile and adult fish, and they offer more diverse endpoints for the evaluation of teratogenic effects (Sutter, 1987). Additionally, fish models serve as useful for the assessment of water-borne and sediment-deposited toxins because they provide advanced warnings of the potential danger of new chemicals and the possibility of environmental pollution. This study was conducted to determine the impact of diesel particulate matter (DPM) on the embryonic development of zebrafish from 12-240 hours post fertilization (hpf). Zebrafish (*Danio rerio*) embryos were exposed to different concentrations of DPM and early life stages were assessed for mortality, average time to hatch, and other endpoints. DPM did not increase mortality from (0-48 hpf), nor did it affect time to hatch (48-96 hpf). DPM doses of 25 $\mu$ g elicited toxic responses in zebrafish larvae. Pericardial edema and craniofacial malformations were first observed at 72 hpf, followed by an onset of spinal deformity (96hpf) and mortality (132 hpf). At 48, 72, 96, 120, and 144 hpf, the aver-

age heart rate decreased slightly with increased DPM concentration. At 24 and 48 hpf, spontaneous movement was reduced in DPM-treated zebrafish. This demonstration of zebrafish responsiveness to DPM early life toxicity coupled with the considerable information on developmental biology and genetics of zebrafish provides a foundation for future investigations into the mechanism of diesel particulate matter toxicity.

**2241**

ARYL HYDROCARBON RECEPTOR (AHR) ACTIVATION INHIBITS ZEBRAFISH EARLY LIFE STAGE CAUDAL FIN REGENERATION.

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Adult zebrafish have the remarkable ability to completely regenerate their caudal fins within two weeks following surgical amputation by a process that involves reactivation of early developmental signal transduction pathways. We have previously established the adult caudal fin regeneration model to investigate the molecular mechanism(s) of TCDD toxicity. When adult zebrafish are injected with TCDD, fin regeneration is significantly impaired at multiple regenerative stages, suggesting that outgrowth rather than re-differentiation is specifically disrupted by TCDD-mediated aryl hydrocarbon receptor (AHR) activation. Mechanistically, we propose that inappropriate activation of AHR by high affinity ligands such as TCDD interfere with growth-promoting signal transduction pathways. The key is to identify these pathways, and their down stream targets, in a whole animal model amenable to molecular and genetic manipulations. Because the true advantage of the zebrafish model is the numerous molecular and genetic tools available to manipulate and monitor development during early life stages, and because there are many technical limitations of the adult regeneration model, we sought to develop an embryonic model to test the hypothesis that AHR activation interferes with fibroblast growth factor (FGF) signaling *in vivo*. Here we report that when the embryonic caudal fin is surgically amputated at 2 days post fertilization, complete regeneration occurs by 5 days. However, when zebrafish embryos are waterborne exposed to TCDD for two hours immediately following amputation, larva fin regeneration is completely blocked. Importantly, the molecular responses to TCDD in the fin are similar to the responses following direct FGF receptor inactivation, suggesting commonality. This embryonic model will allow mechanistic identification of the interactions between FGF and AHR signal transduction pathways *in vivo*. Supported by NIEHS grants ES10820, ES00210, and #ES03850.

**2242**

TCDD IMPAIRS EXTRACELLULAR MATRIX REMODELING IN THE REGENERATING ZEBRAFISH CAUDAL FIN.

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Adult zebrafish can completely regenerate their caudal fin following amputation. Exposure to 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) inhibits this regenerative process at several stages. Proper regulation of transcription, nerve innervation and extracellular matrix composition is essential for complete fin regeneration. The process is initiated by the formation of an epithelial wound cap over the amputation site by 12 hours post amputation (hpa). Once the cap is formed, mesenchymal cells proliferate and migrate from sites distal to the wound plane and accumulate under the epithelial cap forming the blastemal structure within 48 hpa. Blastemal cells differentiate and proliferate, and the regenerate becomes innervated with nerves and blood vessels during the regenerative outgrowth phase which is completed at about 14 days post amputation (dpa). Regenerative outgrowth does not occur in TCDD-exposed zebrafish. To test the hypothesis that TCDD blocks regeneration by impairing extracellular matrix remodeling, male zebrafish were i.p. injected with 50 ng/g TCDD or vehicle and caudal fins were amputated. Collagen metabolism was impaired by TCDD exposure. Histological staining demonstrates that TCDD exposure leads to an accumulation of collagen just posterior to the amputation site by 3 dpa. Transgenic fish expressing green fluorescent protein in vascular endothelium were also used to visualize revascularization. By 3 dpa, the vascular network in the regenerating fin of TCDD-exposed fish is disorganized compared to vehicle exposed animals. Furthermore, immunohistochemical analysis revealed that axonal outgrowth and innervation was impacted by TCDD as early as 3 dpa. Quantitative real time PCR studies revealed that the aryl hydrocarbon pathway is active and that matrix remodeling genes are expressed in the regenerate following TCDD exposure. Supported by NIEHS grants ES10820, ES00210, and #ES03850.

2243

ETHANOL- AND ACETALDEHYDE-MEDIATED DEVELOPMENTAL TOXICITY IN ZEBRAFISH.

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Ethanol is a well-established developmental toxicant. However, the mechanism(s) of toxicity remains unclear. Zebrafish are becoming an important model system for the evaluation of chemical and drug toxicity. In this study zebrafish embryos were utilized to compare the developmental toxicity resulting from ethanol and acetaldehyde exposure. Embryos were exposed to waterborne ethanol and acetaldehyde concentrations at two different intervals up to 48 hours post-fertilization, encompassing the earliest stages of embryogenesis. The waterborne ethanol concentration that causes 50% mortality ( $LC_{50}$ ) and 50% malformations ( $EC_{50}$ ) following a 45-hour ethanol exposure was approximately 340 mM (1.98% v/v) and 138 mM (0.80% v/v), respectively. Reducing the exposure window to the first 24 hours of embryogenesis, similar signs of toxicity, such as pericardial edema and craniofacial malformations, were produced at nearly identical ethanol concentrations. The  $LC_{50}$  for acetaldehyde was 0.54 mM and the acetaldehyde exposed embryos developed similar, but not identical responses as those induced by ethanol. Ethanol associated craniofacial abnormalities observed in mammals have been suggested to be partly due to apoptotic cell death of neural crest cells that are critical for craniofacial morphogenesis. Using acridine orange and TUNEL assays to distinguish between necrotic and apoptotic cell death, we observed a marked increase in apoptotic cells in the brain of ethanol exposed embryos. To test the hypothesis that oxidative stress plays a role in ethanol toxicity, several antioxidants were used to alter ethanol-dependent responses. These results demonstrate that the zebrafish model will provide an opportunity to further evaluate the role of oxidative stress in mediating the action(s) of ethanol on vertebrate development. Supported by NIH grants AA12783, ES03850, and ES00210.

2244

EFFECTS OF ETOPOSIDE ON HUMAN FETAL HEMATOPOIETIC STEM CELLS.

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During fetal development, the liver serves as the primary hematopoietic organ in which hematopoietic stem cells (HSC) comprise a large proportion of hepatic cell populations. As HSC are pluripotent and are capable of initiating long-term hematopoiesis, injury to these cells during pregnancy may have ramifications with regard to the subsequent development of infant acute leukemias. In the current study, we used fetal HSC as a model to examine the effects of etoposide, a chemotherapeutic agent used in adults that can cause secondary leukemias containing genetic translocations similar to those in the infant acute leukemias. Exposure of HSC to a range of etoposide doses from 23 nM-5  $\mu$ M resulted in a dose-dependent inhibition of proliferation, with effects observed at as low as 23 nM. DNA damage, as detected by the COMET assay, was observed at 138 nM etoposide exposure, but not at lower concentrations. Exposure to 138 nM etoposide also stimulated an increase in the CD33 early myeloid and CD38 lymphoid lineages within HSC, but did not stimulate apoptosis in total HSC populations. Collectively, our data indicate that human fetal HSC are highly sensitive to physiological relevant concentrations of etoposide, as evidenced by a loss of proliferative capacity of the overall cell populations, alterations in differentiation, and induction of DNA damage. Supported by NIH ES09427 and USEPA STAR R827441.

2245

DOSE-DEPENDENT SURVIVAL DIFFERENCES IN CHICKENS DEVELOPMENTALLY CO-EXPOSED TO NEAR-INFRARED LIGHT THERAPY AND DIOXIN.

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Photobiomodulation by red to near-infrared (630—1000 nm) light has been shown to stimulate mitochondrial energy (ATP) production, accelerate wound healing, and promote cellular survival following a toxic insult. 2, 3, 7, 8 Tetrachlorodibenzo-p-dioxin (TCDD) increases late embryo mortality in chickens by causing biochemical changes that culminate in decreased available energy. We tested the hypothesis that *in ovo* treatment with a red light-emitting diode (LED) array would attenuate TCDD-related embryo mortality. Domestic chicken eggs (*Gallus gallus*) were divided into ten treatment groups (no-inject, safflower oil vehicle, and 2, 20, 200 ppt TCDD), all with or without LED treatment (670 nm at a fluence of 2 J/cm<sup>2</sup> at 24 h intervals). Surprisingly, we observed an LED-induced in-

crease in mortality in the vehicle control and 2 ppt treatment groups. In contrast, LED treatment decreased mortality in the 200 ppt dioxin treated group (36% decrease in mortality rate). When week-three mortality was analyzed by correcting for the death occurring within the first two weeks of incubation, there remained a 22% decrease in mortality rate for the LED versus non-light treated chicks exposed to 200 ppt dioxin. By comparison, there was an equivalent week-3 mortality rate in the LED treated and non-light treated chicks in the vehicle control and 2 ppt dose groups. Thus an oil-NIR light interaction appears to contribute to early death at sub-lethal doses of TCDD. However, at higher doses of TCDD, LED treatment significantly attenuates TCDD toxicity. We speculate that 670 nm LED treatment may act directly on the oil vehicle and generate reactive oxygen species or alternatively may activate enzymes which metabolize the vehicle to an epoxide. (Supported by DARPA N66001-04-1-8923).

2246

ALTERATION OF NORMAL CARDIAC DEVELOPMENT BY TRICHLOROETHYLENE AND ITS METABOLITE TRICHLOROACETIC ACID.

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Congenital heart defects (CHD) are common birth anomalies, yet little is known regarding the specific mechanisms that induce these developmental malformations. Epidemiological and animal model studies have established an association between CHD and gestational exposure to trichloroethylene (TCE) and trichloroacetic acid (TCA), a metabolite of TCE. Both of these halogenated hydrocarbons are common drinking water contaminants in the US. The reported cardiac defects consistently emphasize malformations of the valves and septa; however, next to nothing is known regarding the molecular and cellular basis for the cardiac teratogenicity caused by TCE or TCA. To study this, we developed a chick embryo model in which exposure to these halogenated hydrocarbons reproducibly alters heart development. We found that TCE exposure at various levels of 0.2, 4, and 200 nanomoles/egg during cardiac cushion (early valve) formation decreases embryonic survival and triggers a U-shaped dose response curve. The most potent TCE exposure of 4 nanomoles/egg was associated with a significantly increased proliferative index (as measured by bromodeoxyuridine incorporation) within the cells of the atrioventricular canal and outflow tract cushions; cardiac cushions ultimately form the mature heart valvuloseptal structures. Moreover, direct comparison of equimolar exposures (4 nanomoles/egg) of TCE, trichloroethanol (TCOH) and TCA resulted in the metabolite TCA increasing cushion cell proliferation to a similar level as the parent compound. Correspondingly, we found that TCA was the most potent at decreasing survival of all the TCE metabolites examined. Studies investigating the effect of TCE and TCA on molecular signals obligatory for cushion formation are in progress. Overall, our results show that the halogenated hydrocarbons TCE and TCA are cardiac teratogens at environmentally relevant exposure levels. (Supported by ES11738)

2247

TOXICOGENOMICS IN MALFORMATIONS INDUCED BY FUNGICIDE FLUCONAZOLE.

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Some case-reports associate the use of fluconazole (an antifungal triazole for human mycosis treatment) early before or during pregnancy with birth defects in conceptuses. Also other triazoles, used in agriculture as fungicides, showed a teratogenic effect in embryos developed *in vitro*. For triazoles, in addition of the induction of a pattern of malformations overlapping those induced by an excess of RA, it has been shown the inhibition of the metabolism of all-trans-retinoic acid as well as alterations of the hindbrain and of the migration of hindbrain neural crest cells (NCC), basic process for the craniofacial morphogenesis. The aim of the present study was to identify and characterise the composite changes in gene/protein expression that may occur at selected critical periods of development. CD:Crl rat embryos (9.5 d.p.c.) were exposed *in vitro* at the teratogenic concentration of Fluconazole (500 microM) dissolved in ethanol. After 20 hours in culture (WEC) the embryos were morphologically examined and processed for histologic and biochemical analysis. Total RNA was isolated from hindbrain of control and exposed embryos cultured for 20 h, which is critical in NCC migration from hindbrain to the embryonic cranio-facial primordia, using a single-step isolation. The identification of genes differentially expressed was performed using a commercially available rat cDNA expression macro-array. Of the 1200 genes analysed only 46 resulted expressed in embryo cultures. Of particular interest was the upregulation of integrin 1 alpha, neuro specific enolase, TIMP 1 and the down-regulation of several potassium channels, alcohol dehydrogenase, RET1 and 2, VEGFR1. We are currently evaluating the role of such genes in the embryonic processes giving rise to the ob-

served malformations. Overall, the use of genomic allows to disentangle mechanistic aspects of teratogenic relevance. Acknowledgement. This work was partially funded by MIUR (COFIN 2003)

**2248**

RELATIVE POTENCIES OF SELECTED DIHALOACETIC ACIDS AND THEIR MAJOR METABOLITES IN MOUSE WHOLE EMBRYO CULTURE.

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Haloacetic acids (HAA) are produced by disinfection and present in tap water. Several HAAs produce developmental effects when administered to rodents. To better understand the causes of HAA-induced effects *in vivo* we compared the time and concentration dependent effects of three developmentally toxic HAAs (bromo-chloro-(BCA), dibromo-(DBA), dichloro-(DCA) acetate) and their major metabolites (glyoxylate(GO), glycolate(G), oxalate(O)) in whole embryo culture. CD-1 mouse conceptuses (3-6 somite stage, GD8) were exposed to parent or metabolite for 1, 3, 6 or 24H and morphological development evaluated at 24H. A 24H exposure to each of the compounds produced dysmorphogenesis. To compare the potencies of these compounds an ED05 benchmark concentration (BMC) was calculated for induction of dysmorphogenesis. The relative potencies and BMC of these compounds are BCA(52 $\mu$ M) = DBA(69 $\mu$ M) > GO(442 $\mu$ M) > G(645 $\mu$ M) > O(792 $\mu$ M) > DCA(1792 $\mu$ M). Conceptuses were then exposed to these compounds for 1, 3 or 6H at concentrations that produced high rates of dysmorphogenesis with a 24H exposure. A 3 or 6H exposure to 300 $\mu$ M BCA produced prosencephalic and pharyngeal arch defects. A 6H exposure to 11000 $\mu$ M DCA produced severely malformed embryos. A 6H exposure to 300 $\mu$ M DBA altered somitogenesis but not morphogenesis. A 6H exposure to 1500 $\mu$ M GO, 3500 $\mu$ M G or 3500 $\mu$ M O did not produce defects. Therefore, BCA could be the proximate toxicant because it disrupts development with short-term exposures and at concentrations lower than the metabolites. DBA requires exposures longer than 6H to disrupt morphogenesis but is more potent than the metabolites. Exposure to DCA disrupts development, but much lower concentrations of the metabolites are sufficient to produce those effects. Therefore when administered *in vivo*, BCA is likely the proximate toxicant, and the metabolites are the likely toxicants when DCA is administered. More information relating time and concentration will be needed to determine if the effects of DBA administration are the result of DBA and/or its metabolites. This abstract does not represent EPA policy

**2249**

EVALUATION OF GLYCOLIC ACID IN RABBIT WHOLE EMBRYO CULTURE.

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The purpose of this study was to evaluate the development of rabbit post-implantation embryos exposed *in vitro* to glycolic acid (GA), the developmentally toxic metabolite of ethylene glycol (EG) in rodent species. This study was part of a larger effort to understand why high doses of EG are teratogenic in rodents, but not in rabbits, and to understand the relevance of the animal data to humans. Rabbit conceptuses were explanted from untreated pregnant New Zealand White rabbits on gestation day 9. The conceptuses were then exposed in culture to 0, 2.5, 6.0, or 12.5 mM of GA, or to the positive control, 5.0 mM methoxyacetic acid (MAA). The two highest GA concentrations had previously been shown to cause a high incidence of craniofacial and somite defects in rat whole embryo cultures (Carney *et al.*, *Teratology* 53:38-46, 1996; Klug *et al.*, *Toxicology in Vitro* 15:635-642, 2001). After approximately 48 hours in culture the rabbit embryos were evaluated for viability (heart beat), growth (somite number, head length, yolk sac protein content) and morphological development (quantitative morphology score, percent malformed embryos). There were no effects of GA on any parameter of development. This lack of embryotoxicity contrasted sharply with the teratogenic effects of MAA, which induced malformations in 83% of the embryos. These data suggest a fundamental difference in the sensitivity of rat and rabbit embryos to GA, but the relevance of this finding to humans is as yet unclear. Funded by the Ethylene Oxide and Derivatives Sector Group of CEFIC (Brussels, Belgium).

**2250**

ENDOGENOUS EMBRYONIC CATALASE ACTIVITY DURING ORGANOGENESIS IN EMBRYO CULTURE, *IN VIVO* AND AFTER EXPOSURE TO PHENYTOIN-ENHANCED OXIDATIVE STRESS.

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Reactive oxygen species (ROS) including hydrogen peroxide ( $H_2O_2$ ) have been implicated in the mechanism of birth defects, and the developing embryo is uniquely susceptible due to its low levels of most antioxidative enzymes. Exogenous adminis-

tration of the antioxidative enzyme catalase, which detoxifies  $H_2O_2$ , is known to be embryoprotective, but little is known about the embryonic expression, inter-individual variability or protective importance of the endogenous enzyme. Herein, we determined the endogenous activity of catalase in embryos in culture and *in vivo* during organogenesis. CD-1 mouse embryos were explanted on gestational day (GD) 9.5 (plug = GD 1) and cultured for 24 hr at 37°C in medium containing the ROS-initiating teratogen phenytoin (20  $\mu$ g/ml, 80  $\mu$ M) or its vehicle (0.002 N NaOH), followed by analysis of sonicated homogenates for catalase activity, which was compared to *in vivo* embryonic activities on GDs 9.5, 10.5 and 12.5. The mean catalase activity was similar in embryos *in vivo* and in culture (range: 30-55 U/mg protein), constituting about 6% of maternal hepatic activity, and was not increased by exposure in culture to phenytoin. Sonication revealed higher embryonic activity than previously reported, without altering its activity relative to maternal levels. Catalase activity among individual littermates on GD 12.5 varied at least 4-fold, suggesting that inter-individual differences may contribute to embryopathic risk. (Support: Canadian Institutes of Health Research)

**2251**

IN VITRO STUDY ON THE EMBRYOTOXIC POTENTIAL OF N-METHYL-PYRROLIDONE (NMP) AND ITS METABOLITES.

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INTRODUCTION: N-methyl-2-pyrrolidone (NMP) and its metabolites, 5-hydroxy-methyl-pyrrolidone (5H-NMP), N-methylsuccinimide (MSI) and 2 hydroxy-methyl-succinimide (2H-MSI), were compared *in vitro* for their embryotoxic potential in order to identify the agent(s) relevant to embryotoxicity under *in vivo* conditions. METHODS: Two *in vitro* test systems were used: 1. the whole embryo culture embryotoxicity test (WEC) using postimplantation embryos and 2. the balb/c 3T3 cytotoxicity test (CT) (balb/c 3T3 fibroblasts). In the WEC system the test substances were ranked according to growth and differentiation parameters, especially the frequency of dysmorphogenes. The NOEC WEC was defined as the maximum tested concentration showing no adverse effect on the total morphological score whereas ICMax WEC was the lowest tested concentration that showed a maximum rate of malformation. In the CT system IC50 3T3 was defined as the concentration leading to 50% growth inhibition. RESULTS: The rankings obtained in the WEC system (in terms of decreasing embryotoxicity) were: NMP (2, 500 and 6, 000  $\mu$ mol/l) > 5H-NMP (8, 000 and 12, 000  $\mu$ mol/l) > 2H-MSI (12, 000 and 24, 000  $\mu$ mol/l) > MSI (16, 000 and 44, 000  $\mu$ mol/l); (NOEC WEC and ICMax WEC). In the CT system the ranking of decreasing cytotoxicity was: MSI (4, 800  $\mu$ mol/l) > NMP (7, 300  $\mu$ mol/l) > 2H-MSI (8, 000  $\mu$ mol/l) > 5H-NMP (29, 000  $\mu$ mol/l); (IC50 3T3). Specific dysmorphogenes induced by NMP and 5H-NMP were observed in the head region of the embryos, abnormal development of the second visceral arches and open neural pores. CONCLUSIONS: Using the prediction model developed by the European Centre for the Validation of Alternative Methods (ECVAM) which is based on NOEC WEC, ICMax WEC and IC50 3T3, NMP and 5H-NMP were classified as weakly embryotoxic; MSI and 2H-MSI as non-embryotoxic. This *in vitro* study indicates that embryotoxicity previously observed after NMP administration to experimental animals is predominantly caused by the parent compound, NMP. + This work has been sponsored by the NMP Producers Group Inc. Washington/DC.

**2252**

IN VITRO STUDY ON THE EMBRYOTOXIC POTENTIAL OF BRANCHED-CHAIN CARBOXYLIC ACIDS.

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INTRODUCTION: Several branched-chain alcohols are important industrial products and may be regarded as metabolic precursors of corresponding branched-chain carboxylic acids. The developmental toxicity of certain branched-chain carboxylic acids such as valproic acid (VPA) or 2-ethylhexanoic acid (EHA) has been confirmed *in vivo*. 2-Propylheptanol (PHOH) is a branched-chain alcohol which is developmentally inactive in rats, but until now nothing is known about the embryotoxicity of the presumptive metabolite 2-propylheptanoic acid (PHA) and the metabolite of the major impurity in the commercial PHOH, 2-propyl-4-methylhexanoic acid (PMHA). Therefore, the embryotoxic potentials of these two carboxylic acids as compared to VPA and EHA as reference substances were determined *in vitro*. METHODS: Two *in vitro* test systems were used: 1. the whole embryo culture embryotoxicity test (WEC) using postimplantation rat embryos and 2. the balb/c 3T3 cytotoxicity test (CT). In the WEC system the test substances were ranked according to growth and differentiation parameters. RESULTS: The rankings obtained in the WEC system (in terms of decreasing embryotoxicity) were: PHA (17 and 150  $\mu$ g/ml) > VPA (17 and 150  $\mu$ g/ml) > EHA (100

and 300  $\mu$ g/ml) > PMHA (100 and 500  $\mu$ g/ml). In the CT system the ranking of decreasing cytotoxicity was: PHA (450  $\mu$ g/ml) > PMHA (1, 570  $\mu$ g/ml) > VPA (2, 010  $\mu$ g/ml) > EHA (3, 020  $\mu$ mol/l). Concentration given correspond to the NOECWEC, ICMMax WEC and IC50 3T3 of the WEC and CT, respectively. All test substances induced dysmorphogenes in the neural tube, otic and optic system. CONCLUSIONS: Using the prediction model developed by the European Centre for the Validation of Alternative Methods (ECVAM) which is based on NOECWEC and ICMMax-WEC and IC50-3T3, all tested chemicals were classified as weakly embryotoxic. In order to assess the relevance of these *in vitro* data for the situation *in vivo*, corresponding pharmacokinetic modelling is needed.

## 2253 CHARACTERIZATION OF ALCOHOL METABOLIZING ENZYMES IN JAPANESE MEDAKA.

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Cardiovascular defects in developmental ethanol exposure have been identified in both human and animal models and used as a phenotypic feature of fetal alcohol syndrome (FAS). The molecular mechanism of FAS has not yet been fully characterized. We used the Japanese medaka developmental model to evaluate FAS produced by acute ethanol exposure. Viable medaka eggs within 1h of fertilization were exposed to ethanol (0-400mM) in hatching solution for 48 h. Cardiovascular development was observed from 1-7 dpf. The embryos exposed to low ethanol concentrations (0-100mM) exhibited blood circulation at 2 dpf but active circulation was significantly delayed at higher ethanol doses (200-400mM). Moreover, embryos exposed to these ethanol concentrations (200-400mM) developed tube heart and blood clots in the Blood Island and in the circulatory vessels. We hypothesized that the cardiovascular abnormalities seen in developing medaka were due to one or more metabolites of ethanol metabolism. To examine this possibility, we have begun to characterize the genes for alcohol metabolizing enzymes in medaka. By applying PCR-based technologies, we previously cloned and characterized two alcohol dehydrogenase (ADH) enzyme cDNAs from the liver tissue of medaka. Additional work has yielded partial clones of two subtypes of aldehyde dehydrogenase cDNA (ALDH $\alpha$  and ALDH $\beta$ ) in adult medaka liver. ALDH $\alpha$  mRNA showed tissue specific expression, however ALDH $\beta$  was ubiquitously expressed (brain, eye, GI, Gill, heart, kidney, liver, muscle, skin, spleen and ovary). The expression patterns of ALDH $\alpha$  and  $\beta$  mRNA were different during development. ALDH $\alpha$  was first expressed at 2 dpf, but in contrast ALDH $\beta$  was expressed from fertilization onward. Our results indicate that ALDH $\beta$  is the major enzyme expressed during the early part of embryonic development and likely plays a significant role in the generation of ethanol- metabolites and the subsequent development of cardiovascular defects.

## 2254 VISUALIZATION OF TISSUE DISTRIBUTION AND METABOLISM OF BENZO(A)PYRENE IN EMBRYONIC MEDAKA (*ORYZIAS LATIPES*).

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The tissue distribution of chemical during critical developmental stages is one factor that can contribute to the sensitivity of fish early life stages to the toxic effects of persistent bioaccumulative toxicants (PBTs). A new tool to monitor the tissue distribution of fluorescent PBTs in small organisms is multiphoton laser scanning microscopy (MPLSM). Imaging fluorescent PBTs that are not metabolized is straightforward because the location of fluorescence indicates the distribution of the PBT. However, a PBT such as benzo[a]pyrene (BaP) presents challenges because of the formation of metabolites with similar emission spectra. In this study, differences in the excitation of BaP and metabolites at four diagnostic multiphoton excitation wavelengths were used to investigate the tissue distribution of BaP during fish early development. Within 2h of fertilization, medaka eggs were exposed for 6h to BaP, then rinsed and transferred to clean media from which they were sampled throughout embryonic stages and through 3d post-hatch. At 1d post-fertilization there was strong fluorescence in the coalesced oil globules in the yolk and moderate fluorescence in the yolk itself. Images, along with chemical analysis of whole embryo extracts, indicated that BaP was metabolized early in development. Metabolism occurred well before liver formation and changes in fluorescence suggested a conversion from hydroxylated BaP to a BaP-glucuronide. Metabolism appeared as an initial loss of fluorescence from the oil globule with a sequential increase in fluorescence in the yolk, gall bladder, and intestine. The increased fluorescence was due to the formation of more highly fluorescent BaP metabolites. These metabolites were rapidly eliminated soon after the embryos hatched. The ability to image the metabolism and redistribution of chemical provides insight into the early metabolic

competency of embryos and indicates that fish embryos can metabolize xenobiotics much earlier than previously demonstrated. This abstract does not necessarily reflect EPA policy.

## 2255 PFOA INDUCES DYSMORPHOGENESIS IN MOUSE WHOLE EMBRYO CULTURE.

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Perfluorooctanoate (PFOA) is a perfluoroalkyl acid (PFAA) found in numerous industrial and consumer products. Many PFAAs persist in the environment and are found in humans and animal tissues throughout the world. PFOA is a developmental toxicant *in vivo* producing embryonic, fetal and postnatal lethality, and altered fetal and neonatal growth. In order to determine if direct exposure of conceptuses to PFOA disrupts development, CD-1 mouse conceptuses (3-6 somite stage, GD8) were exposed to PFOA in whole embryo culture. At the end of a 24H culture period embryonic morphology was assessed and scored using a scoring system developed by our laboratory. Scores ranged from 0, for morphologically normal, to a maximum of 58 for severely affected embryos. In control medium, 82% of embryos (18/22) grew normally and the mean score was  $0.4 \pm 0.2$ . Exposure to 0.1 or 0.2mg/ml PFOA did not alter development (4/4 and 11/17, normal embryos respectively). However, dysmorphogenesis was induced by PFOA at 0.4 (82%), 0.6 (92%), 0.75 (100%) and 1.0 (100%) mg/ml. Exposure to 1.25mg/ml produced 100% embryo lethality. Prosencephalic and pharyngeal arch hypoplasia and abnormal heart outflow tract development were induced by  $>0.4$ mg/ml PFOA. Embryonic scores were increased at PFOA concentrations  $>0.4$ mg/ml and were  $0.0 \pm 0.0$ (0.1),  $1.7 \pm 0.7$ (0.2),  $11.3 \pm 2.2$ (0.4),  $11.3 \pm 2.0$ (0.6),  $23.5 \pm 1.5$ (0.75) and  $31.1 \pm 3.9$ (1.0). The benchmark concentration for a 5% increase in dysmorphic embryos by PFOA was  $0.04 \pm 0.01$ mg/ml. These studies demonstrate that a direct exposure to PFOA for 24H disrupts development and induces embryo lethality. This abstract does not represent EPA policy.

## 2256 THALIDOMIDE DEPLETES GLUTATHIONE AND INITIATES OXIDATIVE STRESS IN RAT NEURAL CREST CELLS.

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Thalidomide is believed to be bioactivated to free radicals and initiate oxidative stress in teratogen-sensitive and insensitive embryos. Thalidomide and other teratogens that produce reactive oxygen species may exert their teratogenic effects by mechanisms that involve glutathione (GSH) oxidation and alteration of redox-sensitive regulatory systems. Cultured rat neural crest cells, important for their roles in sensory and structural embryogenesis *in vivo*, were used as a model system to characterize alterations and responses caused by thalidomide. Neural crest cells were isolated from thalidomide-resistant Sprague-Dawley rats on gestational day 11 and grown in culture. Total reduced GSH and oxidized glutathione (GSSG) concentrations were measured using HPLC. Untreated rat neural crest cells were found to contain very low concentrations of GSH (1.3 pmol/ $\mu$ g protein) and GSSG (0.017 pmol/ $\mu$ g protein, even in the presence of measurable amounts of GCLc and GCLm; enzymes responsible for GSH synthesis. Treatment with 10  $\mu$ M or 50  $\mu$ M thalidomide for 1, 3, 6, or 24 hr produced time and dose-dependent decreases in GSH to 0.52 pmol/ $\mu$ g protein and increased GSSG to 0.23 pmol/ $\mu$ g protein. Based on these changes, 50  $\mu$ M thalidomide produced a positive shift in intracellular redox potential from -219 mv to -161 mv after 24 hr, creating an environment where most cells become susceptible to misregulation of transcription factors and apoptosis. Qualitative assessment of GSH depletion in neural crest cells using CMFDA fluorescence staining and confocal microscopy confirmed the loss of GSH during the first 6 hr of exposure to thalidomide. Our results indicate that thalidomide produces a significant depletion of GSH and an increase in GSSG, resulting in a shift of cellular redox potential towards a more oxidative environment. These observations confirm that thalidomide is capable of initiating oxidative stress in neural crest cells obtained from an insensitive rodent species and may be useful in characterizing specific redox-sensitive regulatory pathways that regulate differentiation and proliferation.

## 2257 EXAMINATION OF METAL-INDUCED CELL CYCLE ALTERATIONS AND APOPTOSIS IN C57BL/6 AND SWV MOUSE EMBRYONIC FIBROBLASTS.

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Neural tube defects (NTDs) are the most common of all serious human birth defects. Studies have identified genetic components that may confer increased susceptibility to environmentally-induced NTDs. The SWV and C57BL/6 mice strains

have shown a large disparity in sensitivity to environmentally-induced NTDs. Due to its complex status of proliferation, differentiation, and cell turnover, the developing embryo is highly susceptible to metal-induced cell cycle alterations and apoptosis. We hypothesize that differential metal-induced impacts within cell cycle and apoptosis pathways at the molecular level contribute to observed differing sensitivities between these two strains. A primary *in vitro* model was established by isolating murine embryonic fibroblasts (MEFs) at gestational day 14. Using the neutral red assay, a cytotoxicity profile was generated after a 24hr exposure to environmentally relevant concentrations of cadmium (Cd) and methyl mercury (MeHg). To characterize metal induced-alterations within cell cycle pathways, cell cycle kinetics were assessed using BrdU/Hoechst/PI and transcriptional changes of cyclin b1 were measured using RTPCR. Finally, to examine changes in apoptotic pathways, caspase 3/7 and 8 activities were measured. The SWV exhibited slightly higher sensitivity to Cd-induced cytotoxicity. For both metals, we observed inhibition of cell cycle progression, G2/M accumulation, and repressed cyclin b1 expression, but no differences were evident between strains. The C57BL/6 displayed a higher increase in caspase 3/7 and caspase 8 activity compared to the SWV with 10uM Cd. In summary, SWV and C57BL/6 MEFs exhibited dose dependent metal-induced alterations in cell cycle and apoptotic pathways. Since we found no differences in metal-induced cell cycle alterations, this suggests that differential sensitivity to metals between the SWV and C57BL/6 are possibly due to unique metal-induced cell death mechanisms. (Funded by EPA: R826886 and NIEHS: ES09601, ES07033, ES11387 and ES10613.)

**2258**

IDENTIFICATION OF GENE CLUSTERS AND SIGNALLING PATHWAYS AFFECTED BY DIBUTYL PHTHALATE - NUCLEAR RECEPTOR INTERACTIONS IN FOETAL RAT TESTES.

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Transcriptional profiling and immunohistochemical analysis in foetal testes of Wistar rats exposed *in utero* to di(n-butyl)phthalate (DBP) 500 mg/Kg were performed in order to identify signalling pathways associated with DBP-induced testicular dysgenesis. High density microarray analysis (22K array) in foetal testes at gestational days (GD) 15, 17 and 19 indicated DBP altered the expression of three functionally distinct gene clusters. A battery of genes involved in the synthesis of testosterone [including steroidogenic factor 1 (SF-1), steroidogenic acute regulatory protein (StAR), cytochrome P450 side chain cleavage (Cyp11a) and 17 $\alpha$ hydroxylase-17, 20-lyase (Cyp C17)] were down-regulated. This correlated with a 70% reduction in foetal testes testosterone levels. Insulin-like factor 3 (Insl3) and genes associated with cellular protection against oxidative stress were also down-regulated. SF-1 RNA levels in DBP-exposed foetal testes in individual litters measured by TaqMan correlated with changes in expression of the SF-1-regulated genes Insl3, StAR and Cyp11a. Immunohistochemical analysis of foetal testes indicated that down-regulation of the SF-1-regulated gene inhibin alpha was found in Leydig cells but not Sertoli cells, thus suggesting a cell type-specific effect. In adulthood, 65% of rats exposed *in utero* to DBP had either bilateral or unilateral cryptorchidism whereas there was no cryptorchidism in control animals. These results suggest that DBP-induced testicular dysgenesis involves effects on several different signalling pathways and is mediated in part through down-regulation of SF-1 together with a battery of genes regulated by this orphan nuclear receptor in a cell type specific manner. This studies were funded by the European Council for Plasticisers and Intermediates.

**2259**

VALPROIC ACID INCREASES CONSERVATIVE HOMOLOGOUS RECOMBINATION FREQUENCY: IMPLICATIONS FOR A MECHANISM OF VALPROIC ACID-INDUCED NEURAL TUBE DEFECTS.

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Valproic acid (VPA), a commonly used antiepileptic agent, is associated with a 1-2% incidence of neural tube defects (NTDs) when taken during pregnancy. However, the molecular mechanism by which VPA causes NTDs has not been elucidated. Previous research has shown that other teratogens exert their detrimental effects through oxidative stress, and an imbalance in the cellular redox state has also been noted after VPA exposure. Reactive oxygen species (ROS) can either directly cause DNA double strand breaks (DSBs) or oxidize DNA which can then lead to DSBs during repair. DSBs can be repaired through homologous recombination (HR), however HR is not error free and can result in detrimental genetic changes. Because the embryo requires tight regulation of gene expression in order to develop properly, we propose that the loss or dysfunction of critical genes involved in em-

bryonic development through aberrant HR may ultimately cause teratogenesis. To test our hypothesis that VPA causes an increase in DSBs that are repaired through HR, CHO 3-6 cells, which contain a neomycin direct repeat recombination substrate, were exposed to VPA (0.5, 1.0, 5.0 and 10.0 mM) for either 4 or 24 hours. A dose dependant increase in HR frequency was observed at 24 hours. Using the ROS sensitive dye 5-(and-6)-chloromethyl-2', 7'- dichlorodihydrofluorescein diacetate (CM-H2DCF), we have shown that exposure to VPA (5 and 10 mM) significantly increases intracellular ROS levels, suggesting that ROS may play a role in the increased HR frequency observed. HR can either occur with or without a loss of DNA. Through southern blot analysis, using neo cDNA as the probe, we have shown that VPA-induced (5 and 10 mM) HR occurs without a loss of DNA. This suggests that a loss of heterozygosity of genes critical to neural tube development may be a significant factor leading to NTDs. (Supported by CIHR)

**2260**

TERATOGENIC EFFECTS OF FORSKOLIN, CYCLOPAMINE AND ETHANOL ON EARLY BRAIN AND EYE DEVELOPMENT.

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Exposure of developing zebrafish (*Danio rerio*) to forskolin, cyclopamine, and ethanol has been shown to induce teratogenic effects affecting the patterning and formation of the brain and eyes. Many of these abnormalities are similar to some common human birth defects. Here, we compare and contrast the effects of these three toxins on developing embryos. Forskolin, cyclopamine and ethanol, all to differing extents, inhibit Sonic Hedgehog (Shh), a secreted protein with a signaling pathway essential for multiple events during embryogenesis, including the development of the brain and eyes. Shh protein and its receptors are also involved with signaling cascades that, when disrupted, cause developmental abnormalities. Cyclopia is a common phenotype seen in developing zebrafish embryos exposed to these three toxins, and resembles human SHH mutations. While these three toxins all act upon Shh, they differentially affect other genes important for brain formation. In our study, we have investigated several brain and eye patterning genes suspected to be affected by forskolin, cyclopamine and ethanol in developing zebrafish embryos. Zebrafish make an excellent animal model because of their high fecundity, external developmental growth, and extensive known genetic background. Embryos were exposed to each chemical starting at 30% epiboly (just prior to gastrulation) until they reached key developmental time points. Whole mount *in situ* hybridization for one forebrain gene, six3b, revealed dose-dependent effects with forskolin and ethanol which were not seen with cyclopamine. Morphological observation of body shape abnormalities demonstrated similarities between ethanol and forskolin but not cyclopamine. However, embryos exposed to cyclopamine had similar abnormalities to those seen with Smooth muscle omitted (smu) homozygous zebrafish, a smoothened (Shh receptor) mutant. A greater understanding of the mechanisms involved with forskolin, cyclopamine and ethanol treatments may shed some light on several human diseases that demonstrate similar malformations.

**2261**

PROTECTION AGAINST MNU-INDUCED FETAL MALFORMATIONS AND PLACENTAL MALDEVELOPMENT WITH DIETARY BUTYLATED HYDROXYTOLUENE SUPPLEMENTATION.

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Methylnitrosourea (MNU) is a multisystem carcinogen and teratogen formed in protein metabolism in the presence of nitrites and gastric acid. MNU damages proliferating tissues by generating reactive oxygen species (ROS). Murine dams given MNU mid-gestation produce fetuses with distal limb defects. Disrupted placental integrity by excessive ROS is thought to be a major contributing factor. Daily dam diet supplemented with antioxidant (butylated hydroxytoluene, BHT) may protect against development of fetal anomalies by abrogation of ROS activity in rapidly developing tissues. This study correlated placental integrity with fetal distal limb defects in MNU-exposed CD-1 mice fed BHT. BHT dams had no changed food preference over controls. MNU increased fetal resorptions, decreased fetal size, and increased fetal distal limb anomalies (syndactyly, oligodactyly, polydactyly, clubbing and webbing). BHT counteracted negative effects of MNU on fetal loss and development of distal limb defects (syndactyly in all limbs, oligodactyly and clubbing in hind limbs). However, MNU and MNU+BHT fetuses were smaller than control fetuses and demonstrated increased webbing in all limbs, suggesting maturation delay and placental disruption (cell death, inflammation); limb shortening was proportional to fetal size. MNU+BHT resulted in partial protection against placental toxicity of MNU, an effect that may relate to diminished fetal distal limb defects. Results suggest that: 1. damage to placenta by MNU is in part ROS-mediated and can be reduced by antioxidant therapy; and 2. reduced distal limb defects in

MNU+BHT fetal mice is likely related to improved placental integrity and function. These results provide a basis for future studies of cellular and molecular mechanisms involved in the therapeutic use of antioxidants in reproductive medicine. Supported by NIH #5K01-RR-17018-3

**2262**

*N*1- AND *N*7-DEOXYGUANOSINE ADDUCTS OF DIEPOXYBUTANE EXHIBIT DIFFERENT STABILITIES AND DECOMPOSITION PATTERNS UNDER PHYSIOLOGICAL CONDITIONS.

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1, 2, 3, 4-Diepoxybutane (DEB), an *in vivo* metabolite of 1, 3-butadiene, is a carcinogen and a potent mutagen. Previously, we have shown that DEB readily reacts with 2'-deoxyguanosine (dG) under physiological conditions to produce seven major nucleoside adducts resulting from alkylation at the *N*1- (P8, P9), *N*7- (P5, P5') and both the *N*1- and *N*2-positions of dG to form six- and seven-membered fused ring systems (P4-1, P4-2 and P6), respectively (Zhang and Elfarra, *Chem. Res. Toxicol.* 2003, 16, 1606; *ibid.* 2004, 17, 521). Because these adducts exhibited different stabilities and rates of formation under the reaction conditions, the goal of the present study was to characterize the stabilities and decomposition products of purified P8, P9, P5, P5', P4-1, P4-2 and P6 under *in vitro* physiological conditions (pH 7.4, 37 °C). The results show that whereas P4-1, P4-2 and P6 were stable for at least three weeks, P5, P5', P8 and P9 were labile with half-lives of 2.6, 2.7, 16 and 16 h, respectively. P5 and P5' decomposed initially by the loss of the sugar moiety to yield the corresponding guanine adducts, which exhibited a half-life of 33 h and decomposed by the addition of either water or HCl into the remaining oxirane ring. The decomposition of P8 and P9 yielded the stable nucleoside adducts P4-1, P4-2 and P6, in addition to nucleoside products resulting from the addition of water or HCl to the remaining oxirane ring. These results explain the formation of P4-1, P4-2 and P6 and provide a better understanding of the reaction of DEB with dG. The results also demonstrate significant differences in the stabilities and decomposition patterns of the dG-DEB adducts, which may facilitate the development of useful biomarkers of exposure to DEB (Supported by NIH Grant ES06841).

**2263**

DEPURINATION OF NUCLEOSIDES INDUCED BY HALOGENATED ALKANES IN THE PHYSIOLOGICAL CONDITION.

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2-Bromopropane,  $\text{CH}_3\text{CHBrCH}_3$ , is widely utilized as a cleansing solvent in electronic factories. It has been reported that a number of female workers, exposed occupationally to 2-bromopropane, were diagnosed with amenorrhea and male workers with oligospermia in Korea. 1-Bromopropane,  $\text{CH}_3\text{CH}_2\text{CH}_2\text{Br}$ , known as an alternative to ozone depleting solvents, which has structural similarity to 2-bromopropane, has been reported to be neurotoxic to rats in long-term inhalation exposure. 1- and 2-bromopropane were also reported as the causative agents for reproductive toxicity and immunotoxicity. We have been previously reported that the guanine adducts of 1-and 2-bromopropane was detected and quantitated by the treatment of 1- and 2-bromopropane to 2'-deoxyguanosine and calf thymus DNA at the physiological condition (pH 7.4, 37°C), as well as *in vivo* experiments, which indicated the possible mechanism of toxicity of 1- and 2-bromopropane. In this study, we observed the depurination of nucleosides by the treatment of 2-bromopropane and other halogenated alkanes at the physiological condition using HPLC and ESI LC/MS/MS. In addition, time-response and dose-response effects of depurination of nucleosides induced by halogenated alkanes at the physiological condition were investigated. The present results might explain the toxic effects of 2-bromopropane could be from the depurination of nucleosides as well as the adducts formation of the DNA.

**2264**

EXPOSURE-BASED SAFETY ASSESSMENT OF THE NATURALLY OCCURRING FLAVOURING METHYL EUGENOL.

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Methyl eugenol is a naturally occurring allylalkoxybenzene found in fruits, spices and essential oils that has been used as a food additive. It has structural similarities to the carcinogens safrole and estragole. Although high doses of methyl eugenol form DNA adducts and are hepatocarcinogenic in rodents (Abdo et al.

2001;Phillips et al. 1984;Randerath et al. 1984), it is generally regarded as safe (GRAS) at levels below 50ppm (Hall 1965). To investigate the potential toxicity of methyleugenol at doses more representative of human exposure, a 28 day feeding study with F344 rats was undertaken (1, 5 & 50mg/kg bw/day, 20 rats per group). Animals were monitored for signs of overt toxicity and necropsied at the end of the treatment period. Tissue samples were assessed for hepatic Proliferating Cell Nuclear Antigen (PCNA, a marker for increased cellular proliferation) serum gastrin (a marker of gastric damage) and DNA adducts (genotoxicity) using Nuclease P1 enhanced 32P-Postlabelling. Oral administration of methyl eugenol (daily gavage or continuous dietary admixture) for a period of 28 consecutive days did not induce signs of overt toxicity nor adversely affect weight gain or terminal liver weight. There was no dose-related macroscopic evidence of toxicity at necropsy, nor alteration of hepatic PCNA or serum gastrin. However, methyl eugenol-DNA adducts were detected in hepatic DNA from both male and female rats fed the dietary admixture ( $2.85 \pm 1.19$  adducts per  $10^8$  nucleotides) in the 50 mg/kg/day group. These data indicate that dietary exposure to methyl eugenol at doses of up to 50 mg/kg per day does not appear to induce overt toxicity, but is genotoxic. References Abdo, K. M. et al. *Food and Chemical Toxicology*, (2001), 39, 303-316. Hall, R. L. et al. *Food Technology*, (1965), 253, 151-197. Phillips, D. H. et al. *Carcinogenesis*, (1984), 5, 1623-1628. Randerath, K. et al. *Carcinogenesis*, (1984), 5, 1613-1622.

**2265**

DETECTION OF ELEVATED 8-HYDROXYDEOXYGUANOSINE IN FRESHLY ISOLATED MOUSE LUNG CELLS FOLLOWING *IN VIVO* TREATMENT WITH AFLATOXIN B<sub>1</sub>

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Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is a mycotoxin produced by some strains of *Aspergillus*, and is a recognized pulmonary and hepatic carcinogen. A well established mechanism of the carcinogenicity of AFB<sub>1</sub> is bioactivation to the AFB<sub>1</sub>-8, 9-exo-epoxide and binding to DNA to form AFB<sub>1</sub>-N<sup>7</sup>-Guanine. Another important mechanism may be the formation of reactive oxygen species during AFB<sub>1</sub> metabolism, leading to oxidative DNA damage. The objective of this study was to determine the ability of AFB<sub>1</sub> to cause oxidative DNA damage in different mouse lung cell types. The formation of 8OHdG in freshly isolated mouse lung cell digest, alveolar macrophages, alveolar type II cells, and Clara cells, resulting from *in vivo* AFB<sub>1</sub> treatment at various time points was assessed using HPLC with electrochemical detection. These cell fractions prepared by centrifugal elutriation were chosen as they have demonstrated involvement in the metabolism of AFB<sub>1</sub>. An ~6 fold increase in 8OHdG formation was demonstrated in alveolar macrophages isolated from mice treated with a single tumourigenic dose of 50 mg/kg AFB<sub>1</sub> ip and euthanized 2 h post-treatment (n=3, p<0.05). The trend observed (n=3) is an ~5 fold increase in 8OHdG formation in Clara cells and an ~4 fold increase in both alveolar type II cells and cell digest. No increase in 8OHdG formation was demonstrated at 12 h (n=3, p>0.05), 24 h (n=1), or at 48 h (n=1) post-treatment as compared to controls. These results suggest that oxidative DNA damage in mouse lung cells may contribute to the carcinogenicity of AFB<sub>1</sub>. (Supported by the Canadian Institutes of Health Research Grant No. MOP-10382)

**2266**

LIPID HYDROPEROXIDE-DERIVED DNA ADDUCTS GENERATED THROUGH REDOX CYCLING OF BENZO(A)PYRENE QUINONE.

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Polycyclic aromatic hydrocarbons(PAHs) are ubiquitous environmental pollutants found in tobacco smoke and car exhaust. PAHs are metabolized by cytochrome P-450s(CYPs) and epoxide hydrolases to catechols, which undergo further aldo/keto reductase-mediated oxidation to quinones such as benzo[a]pyrene quinone(BPQ). BPQ can enter a futile redox-cycle in the presence of NAD(P)H. Reactive oxygen species(ROS) including H<sub>2</sub>O<sub>2</sub> and superoxide are produced and amplified through the redox cycling. We hypothesized that in the presence of transition metal ions such as Cu<sup>2+</sup>, hydroxyl radicals would be generated from H<sub>2</sub>O<sub>2</sub> through Fenton chemistry. The hydroxyl radicals could then initiate lipid peroxidation of polyunsaturated fatty acids(PUFAs). Lipid-hydroperoxides undergo homolytic decomposition to give rise to bifunctional electrophiles including malondialdehyde(MDA), 4-hydroxy-2-nonenal(4-HNE), 4-oxo-2-nonenal(4-ONE) and 4, 5-epoxy-2(E)-decenal(4, 5-EDE). These endogenous bifunctional electrophiles can modify DNA bases such as 2'-deoxyguanosine(dGuo) to form M1G-2'-deoxyribose, 1, N<sup>2</sup>-propano-dGuo, heptanone-etheno-dGuo and etheno-dGuo, respectively. We have developed stable isotope dilution liquid chromatography(LC)/mass spectrometry(MS) to quantify these adducts in the selected reaction-monitoring(SRM) mode. These experiments demonstrated that redox cycling of BPQ in the presence of arachidonic acid promoted lipid peroxidation and elevated lipid hydroperoxide

derived dGuo adducts such as etheno-dGuo and heptanone-etheno-dGuo at low to moderate concentrations of BPQ(1-10  $\mu$ M). Adduct levels declined when a high concentration of BPQ(100  $\mu$ M) was used, which suggested that further oxidation of the initially formed adducts had occurred. Etheno-dGuo is mutagenic in mammalian cells and so this study raises the possibility that mutations can result through lipid hydroperoxide-mediated damage as well as through direct BPQ adduct formation or oxidative DNA damage. Therefore, it will be important in the future to assess the relative importance of these three pathways of DNA damage. Supported by NIH CA 92537.

**2267**

METABOLISM OF 7H-DIBENZO[C, G]CARBAZOLE (DBC) OR BENZO[*A*]PYRENE (BaP) IN *CYP1A2* OR *AHR* GENE KNOCKOUTS: IMPLICATIONS FOR CARCINOGENICITY.

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N-heterocyclic aromatics (NHA) are a subgroup of polycyclic aromatic hydrocarbons (PAH), which are environmentally important pollutants. *Cyp1a2* is one of several genes coding for metabolic enzymes that are regulated by the aryl hydrocarbon receptor (AHR). CYP1A2 is a major constitutive enzyme in the liver and possibly skin, capable of activating both NHA and PAH. The aim of this work is to elucidate the roles of the *Ahr* and *Cyp1a2* genes in the metabolism of the NHA DBC or the PAH BaP by studying how genetic damage is affected when *Cyp1a2* or *Ahr* is inactivated in mice treated with low doses of these carcinogens. Knockout mice (*Ahr*-/- and *Cyp1a2*-/-) were bred into a C57BL/6J (*wt*) genetic background for nine generations. Twenty-four hours after their backs were shaved, mice of the three strains were treated topically with DBC 8 mg/kg or BaP 33.3 mg/kg body weight (both in 25  $\mu$ l acetone), or acetone alone. The mice were euthanized 24 hours later and skin, liver and lung tissues were excised and stored at -80°C for DNA isolation.  $^{32}$ P-postlabeling was utilized to determine DNA adducts and to compare relative adduct levels. *Cyp1a2*-/- mice showed a 50% reduction of skin BaP-DNA adduct levels compared to *wt* controls. *Ahr*-/- mice had a 90% reduction of skin BaP-DNA adduct levels. These data suggest that CYP1A2 contributes significantly to the activation of BaP to a DNA-binding species in skin, and suggest that activation genes are rapidly induced in the skin and play a significant role in the metabolism of absorbed xenobiotics shortly after exposure. These phenomena were not observed in liver, where there was a 3-fold higher constitutive level of BaP-DNA adducts in *Ahr*-/- than in *wt* or *Cyp1a2*-/- mice. There was no significant effect of either gene knockout on the levels of DBC-DNA adducts in any of the tissues. These data indicate that metabolic activation of DBC occurs independently of either the *Cyp1a2* or *Ahr* gene. [NIH R01 ES04203-16A1, Warshawsky PI]

**2268**

CHARACTERIZATION OF STABLE BENZO[*A*]PYRENE-7, 8-QUINONE-DNA ADDUCTS IN CALF THYMUS DNA.

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Benzo[*a*]pyrene-7, 8-dione (BPQ) is a reactive aldo-keto reductase-mediated product of B[*a*]P-7, 8-diol, a major P450/epoxide hydrolase metabolite of the multi-species carcinogen, B[*a*]P. The role of BPQ in B[*a*]P's genotoxicity and carcinogenesis is evolving. Toxicity pathways involving BPQ include the formation of both stable and unstable DNA adducts and the generation of ROS via redox cycling. To investigate the stable DNA adduct pathway, we have synthesized nucleotide-3'-phosphate-BPQ DNA adduct standards and applied these to the characterization of BPQ-DNA adducts in calf thymus DNA (CT-DNA) using  $^{32}$ P-postlabeling analyses. Previously, we reported the full characterization of four BPQ-dGuo and two BPQ-dAdo nucleoside adducts [Balu *et al.* (2004) *Chem. Res. Toxicology*, 17, 827-838]. Using similar methods of syntheses and analyses, we prepared the analogous BPQ-nucleotide adducts. BPQ was incubated with CT-DNA for 24 h at 37°C.  $^{32}$ P-Postlabeling analysis showed the formation of 5 major and 7 minor DNA adducts. Adducts were also observed following the postlabeling of synthesized 3'-dGMP-BPQ and 3'-dAMP-BPQ standards. The 3'-dGMP-BPQ1, 2 standard exhibited 2 major adducts. The 3'-dGMP-BPQ3, 4 standard exhibited 2 major and one minor adduct. One major and one minor adduct were observed in both the 3'-dAMP-BPQ1 and the 3'-dAMP-BPQ2 standards. One adduct from the 3'-dGMP-BPQ3, 4 standard co-chromatographed with a major CT-DNA adduct. Adducts from the 3'-dAMP-BPQ1 and 3'-dAMP-BPQ2 standards appear to co-chromatograph with several of the minor CT-DNA adducts. This is the first reported example of the characterization of stable BPQ-DNA adducts in mammalian DNA and will serve as the foundation for future BPQ-DNA adduct studies *in vivo*. [This abstract does not necessarily reflect EPA policy.]

**2269**

INDUCTION AND PERSISTENCE OF BENZO(A)PYRENE- AND DIBENZO[*A, L*]PYRENE-DNA ADDUCTS IN DNA REPAIR-PROFICIENT AND DEFICIENT CELLS.

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Polycyclic aromatic hydrocarbons (PAHs), ubiquitous environmental contaminants found in automobile exhaust and tobacco smoke, are implicated in the etiology of lung and other human cancers. Benzo[*a*]pyrene (BP) and dibenzo[*a, l*]pyrene (DBP) are PAHs present in tobacco smoke and have been directly linked to skin, lung and mammary gland tumorigenesis in animal models. The ultimate carcinogens, the BP- and DBP-diol epoxides, are metabolites (generated by CYP1A1/CYP1B1 and epoxide hydrolase) that subsequently react with DNA to form guanine and adenine adducts. Ongoing metabolic activation, detoxification, adduct formation and removal by repair pathways affect DNA adduct levels. PAH adducts are generally removed by nucleotide excision repair (NER). To understand the dynamics of adduct formation and removal, BP- and DBP-DNA adduct levels were compared in NER-proficient (normal) and -deficient (xeroderma pigmentosum complementation group A) human fibroblasts designed with an inducible CYP1A1 gene.  $^{32}$ P-postlabeling experiments revealed formation of 1 major/2 minor and 3 major/several minor DNA adducts by BP- and DBP-dihydrodiols, respectively, in both cell lines. These adducts were only formed when CYP1A1 was induced and were chromatographically similar to those formed by direct treatment of dG and dA with BP- and DBP-diol epoxides. DNA adduct levels following DBP-dihydrodiol treatments (1-500 nM) increased in a dose-dependent manner, ranging from 42 to 1943 adducts/109 nucleotides and were significantly higher in NER-deficient cells, suggesting some adduct removal in NER-proficient cells during the 2 hr treatment interval. DBP- and BP-dihydrodiol treatments (at 100 nM) yielded similar total adduct levels. Following carcinogen removal, DBP adducts persisted in both cell lines while BP adducts decreased with time in the NER-proficient cells. Our results suggest that the strong carcinogenicity of DBP is due to poor repair of a subset of DBP adducts ultimately leading to mutations that increase the rate of cellular transformation.

**2270**

COMPARISON OF DNA ADDUCT FORMATION BETWEEN TISSUES FROM MICE TREATED WITH COMPLEX PAH MIXTURES.

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Contaminated wood preserving waste (WPW) facilities are found throughout the United States. Common contaminants at these sites include polycyclic aromatic hydrocarbons (PAHs) and pentachlorophenol (PCP). The purpose of this study was to compare DNA adduct formation from a complex mixture (CM) with those of chemical fractions isolated from the CM. The CM was isolated from oil collected as a non-aqueous phase liquid from a contaminated aquifer at an abandoned WPW facility. An aliquot of oil was fractionated using the EPA 3650B Method, liquid-liquid extraction, to obtain acid (AF), base (BF) and neutral (NF) fractions. The NF was enriched for chlorinated dioxins (PCDDs) and polycyclic aromatic hydrocarbons (PAHs) using a mixed bed silica column, and an alumina column, respectively, followed by a carbon column. A reconstituted mixture (RM) was prepared to mimic the levels of the 7 carcinogenic PAHs (cPAHs) and PCP found in the NF. All fractions were analyzed for chemical content using a GC/MS. The results indicate the AF contained mostly phenols, the BF contained a mixture of PAHs and phenols, the NF contained PAHs and PCDDs, the PCDD fraction contained hepta and octa PCDDs along with lower molecular weight PAHs, and the PAH fraction contained higher molecular weight PAHs. The fractions were applied topically to ICR female mice for a 24 hour exposure. DNA from harvested tissues were postlabeled with  $^{32}$ P to determine bulky adduct formation. The highest level of total DNA (tDNA) adducts was observed in skin, while the lung and liver tDNA adducts were not significantly different within treatment groups. Relatively low levels of DNA adducts were observed in tissues treated with the CM, while the PAH fraction produced the highest level of adducts. The data indicate that isolation of genotoxic components from complex mixtures allows for a more complete expression of component genotoxicity.

**2271**

METABOLIC ACTIVATION AND PAH-DNA ADDUCT FORMATION FROM URBAN DUST PARTICULATE MATTER (SRM 1649A) IN HUMAN MAMMARY EPITHELIAL CELLS IN CULTURE.

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Complex mixtures in the environment are the primary source in which humans are exposed to polycyclic aromatic hydrocarbons (PAH). It is well established that PAH such as benzo[*a*]pyrene (B[*a*]P), upon metabolic activation by cytochrome P450

(CYP) enzymes, bind to DNA and form covalent PAH-DNA adducts. These adducts can lead to mutations, which may ultimately result in cancer. In our study, the effect of complex PAH mixtures on the activation of carcinogenic PAH to DNA-binding derivatives was investigated in human mammary epithelial cells (MCF-10A). MCF-10A cells were treated for 24 and 48 h with Standard Reference Material (SRM) 1649a, a complex mixture derived from urban dust particulate matter obtained from the National Institute of Standards and Technology. Simultaneously, the cells were treated with two carcinogenic PAH, B[a]P, dibenzo[a, l]pyrene (DB[a, l]P) and an appropriate amounts of B[a]P\* (mg/ml, B[a]P present in 1 mg SRM). In addition, the individual carcinogenic PAH mentioned above were co-treated with SRM 1649a. CYP enzyme activity capable of activating B[a]P, DB[a, l]P and SRM 1649a to their reactive DNA binding derivatives were observed as measured by ethoxyresorufin-O-deethylase assay. CYP1A1 and CYP1B1 expression was confirmed by Western blot analysis. PAH-DNA adduct analysis by 33P-postlabeling and reversed-phase high-performance liquid chromatography revealed DNA adduct formation on exposure to all treatment groups. MCF-10A cells treated with SRM 1649a exhibited lower, but detectable DNA adducts in comparison with BP or BP\* at both 24 and 48h after exposure. An additive increase in DNA binding was evident with co-treatments of SRM 1649a with B[a]P, B[a]P\* or DB[a, l]P. These results suggest that components in environmental PAH mixtures can either promote or inhibit metabolic activation and DNA adduct formation, a finding relevant to assessing human cancer risk. This research was funded by NIH grant CA28825.

2272

DIESEL EXHAUST PARTICLES CAUSE INCREASED LEVELS OF DNA DELETIONS AND DNA ADDUCT FORMATION AFTER TRANSPLACENTAL EXPOSURE IN MICE.

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Diesel exhaust particles (DEP) are a major source of air-borne pollution and are linked to increased risk of lung cancer. Ambient air pollution to which the increasing use of diesel-powered engines contributes significantly has been linked to adverse pregnancy outcomes. Here we investigated genotoxic effects of DEP exposure, such as DNA deletions, oxidative DNA damage and DNA adduct formation, during embryonic development in mice. Pregnant dams were orally exposed to various doses of DEP (500, 250, 125, 62.5, 31.25 mg/kg/day) at embryonic days 10.5-15.5. In the offspring, the frequency of 70 kb DNA deletions spanning exons 6-18 at the pun allele that results in black-pigmented spots in the unpigmented retinal pigment epithelium of the eye of pun/pun mice was determined. A significant increase in the frequency of DNA deletions was observed in a dose-dependent manner. Levels of 8-OH deoxyguanosine indicating oxidative DNA damage were within the limits of the unexposed mouse embryos. 33P post-labeling analysis revealed low, but detectable levels of DNA adducts in the embryo tissue. The results from this study showed that transplacental exposure to DEP leading to DNA adduct formation resulted in a significant increase in the frequency of DNA deletions to the mouse fetus and such genetic alterations in the offspring may have pathological consequences later in life.

2273

MLH1-DEFICIENT MICE ARE HYPERSENSITIVE TO PHIP-INDUCED MUTATION AND ABERRANT CRYPT FOCI: EVIDENCE FOR DIFFERENTIAL PROCESSING OF PHIP ADDUCT MISMATCHES BY THE MISMATCH REPAIR PATHWAY.

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Disruption of the DNA mismatch repair (MMR) pathway results in elevated mutation rates and inappropriate survival of cells bearing DNA damage. Hereditary nonpolyposis colorectal cancer is an early onset predisposition for internal cancers caused by deficiency for one of several MMR genes. MMR-deficiency is also associated with sporadic cancer. The extent to which environmental factors influence the etiology of MMR-deficient cancers is unknown. 2-amino-1-methyl-6-phenylimidazo [4, 5-*b*] pyridine (PhIP) is a mutagen and carcinogen present in cooked meat that forms DNA adducts when metabolized. To determine whether PhIP represents a cancer risk to individuals with MMR-deficiency, *MLH1*<sup>-/-</sup> mice carrying the *cII* transgene were given 8 i.p. injections of 50 mg/kg PhIP. PhIP induced 3 times more mutations in the colons of *MLH1*<sup>-/-</sup> mice compared to their wildtype littermates, indicating that MMR normally suppresses PhIP-induced mutations. Analysis of mutational spectra in wildtype and *MLH1*<sup>-/-</sup> colon revealed that GC to TA transversions, the signature PhIP mutation, were similarly induced. In contrast, *MLH1*<sup>-/-</sup> mice were hypersensitive to -1 frameshifts, GC to AT transitions, and GC to CG transversions, indicating that both the level and types of mutation induced by PhIP are de-

termined by activity of the MMR system. In cancer studies, PhIP-treated *MLH1*<sup>-/-</sup> mice were hypersensitive to induction of colonic aberrant crypt foci. Adenomas of the small intestine were not induced, despite a similar induction of mutation in the small intestine as in the colon. Cell-turnover studies of colon and small intestine are in progress to determine the importance of MLH1-dependent apoptosis in PhIP-induced carcinogenicity. These data are consistent with the hypothesis that PhIP exposure increases mutagenesis and carcinogenesis in *MLH1*<sup>-/-</sup> mice, and support further evaluation of the risk that consumption of heterocyclic amines may impart to MMR-deficient individuals.

2274

OXIDATIVE METABOLISM OF THE SERM, RALOXIFENE LEADS TO PROTEIN COVALENT MODIFICATION.

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The selective estrogen receptor modulator (SERM), raloxifene is effective in the treatment of osteoporosis in postmenopausal women and is currently in clinical trials for chemoprevention of breast cancer. Similar to other SERMs, *in vitro* studies have shown that raloxifene can be metabolized to reactive intermediates by P4503A4 leading to enzyme inactivation. We have shown that raloxifene undergoes NADPH-dependent oxidation in liver microsomes to generate catechol and quinoid reactive intermediates, which form a variety of adducts with glutathione. In order to answer the question of whether raloxifene covalently modifies proteins, we have synthesized a raloxifene congener attached to a biotin tag via a linker moiety. This is a raloxifene covert oxidatively activated tag (COATag), that is unreactive towards isolated proteins and protein components of tissue homogenates. However, after incubation with rat liver microsomes in the presence of an NADPH generating system or incubation with glutathione-S-transferase in the presence of tyrosinase, the COATag covalently modified proteins. The COATagged proteins were quantified and identified by SDS-PAGE and Western blot analysis. Four major bands were observed in the blots from microsomal incubations, for which peptide mass maps were obtained using in-gel digestion with trypsin, followed by MALDI-TOF or ESI mass spectrometric analysis of the resulting peptide mixtures. Cytosolic glucose regulated protein (78 kDa, GRP78/BiP), protein disulfide isomerase isozyme A1 (57 kDa, PDIA1), and microsomal glutathione S-transferase, (17 kDa, mGST1) were identified. These data suggest that protein modification by raloxifene may contribute to its toxicity *in vivo*. This work was supported by NIH grant CA 79870.

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IDENTIFICATION OF ELECTROPHILE BINDING MOTIFS ON QUINONE-THIOETHER ADDUCTED RENAL PROTEINS.

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Glutathione conjugates of hydroquinone (HQGSH) produce severe renal proximal tubular necrosis and carcinogenicity in rats via a combination of protein arylation and the generation of ROS. To identify HQGSH-covalent protein adducts which may contribute to nephrotoxicity and nephrocarcinogenicity, renal proteins were subjected to SDS-PAGE, and adducted proteins detected by immunostaining with antibodies recognizing HQGSH adducted proteins. The corresponding SDS gel bands were excised, digested in gel with trypsin, and a total of 30 proteins were identified by MALDI-TOF and LC-MS/MS (Finnigan-MAT LCQ-SEQUEST and SALSA search programs). Several adducted proteins, including actin, serum albumin, transferin, glutamate-cysteine ligase catalytic subunit, moesin, and microsomal aminopeptidase N, were also identified in rat urine after HQGSH treatment. Using cytochrome c as a model protein we have previously identified the sequences GGKHKTG (23-29) and GIKKK (84-88) as binding sites for BQ, with the lysine residues participating in the formation of a novel cyclized diquinone adduct. To ascertain features that predispose certain proteins, or motifs within proteins ["electrophile binding motifs (EBM)", to chemical adduction, we searched the amino acid sequence of each adducted protein to determine for runs of lysine residues. Interestingly all 30 adducted proteins possess lysine rich sequences in two main contexts (i) lysines surrounding a likely nucleophilic amino acid (KXK), or (ii) two lysine residues preceded or followed by a nucleophilic amino acid (XKK or KKX). Such motifs are largely absent in randomly selected non-adducted proteins. In attempts to definitively identify chemically adducted EBMs within the adducted proteins we have synthesized biotinylated HQ. The biotinylated EBMs should be readily identifiable by MS-based methodologies. The potential biological/toxicological consequences of adduction to rationally selected "electrophilins" is currently under investigation. (GM39338, ES06694)

**2276**

## MOLECULAR MECHANISMS OF HYDROQUINONE INDUCED APOPTOSIS.

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The mechanisms by which the benzene metabolite hydroquinone(HQ) induces apoptosis are currently unclear and have therefore been investigated using Jurkat cells. HQ treatment of Jurkat cells resulted in apoptosis as assessed by loss of mitochondrial membrane potential, caspase-2 activation and cytochrome-c release after 4hr treatment. HQ-induced dissipation of mitochondrial membrane potential was inhibited by the pancaspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD.fmk) but not the specific caspase-2 inhibitor benzyloxycarbonyl-Val-Asp-Val-Ala-Asp-fluoromethyl ketone (zVDVAD.fmk). Whereas the proteolytic cleavage of caspase-2 as assessed by both the fluorogenic VDVAD.AFC substrate and western blotting was inhibited by zVAD.fmk and zVDVAD.fmk, release of mitochondrial cytochrome c still occurred suggesting that HQ might also target the mitochondrial independently from caspase activation. Furthermore, Bongkrekic acid, a permeability transition pore inhibitor, also failed to inhibit cytochrome c release from HQ treated cells. Estimation of Ca<sup>2+</sup> buffering capacity in digitonin-permeabilized cells treated with hydroquinone confirmed mitochondrial damage. However, benzoquinone, a metabolite of hydroquinone autoxidation, but not hydroquinone, added to isolated liver mitochondria resulted in a rapid decrease in Ca<sup>2+</sup> buffering capacity. These observations indicate that hydroquinone is not directly toxic to mitochondria but during apoptosis, mitochondrial damage could be due to formation of its autoxidation product benzoquinone resulting in a caspase-independent loss of cytochrome c release. These data provide insight into the mechanisms of hydroquinone induced apoptosis which may have important implications in the pathophysiology of benzene-induced myelotoxicity. (This work has been supported by NIH RO1 ES09554)

**2277**

## BIOCHEMICAL AND MICROARRAY ANALYSES OF BUPIVACAINE-INDUCED APOPTOSIS.

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It was recently reported that some of local anesthetics induce apoptosis in the cultured cells and that this apoptotic effect might be involved in the pathogenesis of neuro- or myotoxicity induced by local anesthetics, however, the detailed mechanism has not been clear. In this study, the mechanism by which apoptosis is induced by bupivacaine, a potent uncoupler of mitochondrial oxidative phosphorylation, was investigated using human promyelocytic leukemia cells, HL-60. Bupivacaine induced apoptotic bodies formation and DNA fragmentation in a time- and concentration-dependent manner in HL-60 cells. In good correlation with the degree of DNA fragmentation, caspase-3, -8 and -9, which play a pivotal role in the initiation and execution of receptor- or mitochondria-mediated apoptosis, were all clearly activated. However, bupivacaine induced neither mitochondrial permeability transition nor release of cytochrome c in experiments with isolated mitochondria. In microarray analysis, HSPs, c-jun and c-fos genes were up-regulated and c-myc and poly (ADP ribose) polymerase gene were down-regulated preferentially in bupivacaine-treated cells. These results suggest that direct action of bupivacaine on mitochondria does not occur and that the removal of c-myc mediated repression of apoptotic signals, decreased cellular resistance and attenuation in DNA repair may accelerate bupivacaine-induced apoptosis. These biochemical and microarray analyses are of value in developing a better understanding of the molecular mechanism of local anesthetics-induced apoptosis leading to neuro- or myotoxicity.

**2278**

## 1, 1-DICHLOROETHYLENE-INDUCED MITOCHONDRIAL DAMAGE PRECEDES APOPTOTIC CELL DEATH OF BRONCHIOLAR EPITHELIAL CELLS IN MURINE LUNG.

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1, 1-Dichloroethylene (DCE) causes pulmonary injury that is characterized by necrosis of bronchiolar Clara cells. Mitochondria have been identified as an early target in the toxic response. As mitochondria have been implicated in both necrotic and apoptotic cell death, we have undertaken studies to test the hypothesis that DCE induces apoptosis, in addition to necrosis, in murine lung. A primary objective is to identify the biochemical events associated with pulmonary apoptosis.

Groups of female CD-1 mice were treated with DCE (75 mg/kg, i.p.) or corn oil. Using an antibody directed against DCE-cysteine conjugates, protein adducts were detected primarily in association with mitochondria in the apices of bronchiolar Clara cells. Furthermore, morphological studies demonstrated early mitochondrial alterations in Clara cells that included severe swelling and disruption of cristae. Western blotting of lung cytosolic proteins showed greater immunoreactivity for cytochrome c in fractions from mice treated with DCE for 4 h than in controls. Immunohistochemical studies with an antibody to activated caspase-3 and TUNEL staining were used to detect apoptotic cells. In both experiments, positive reactivities were observed in the bronchiolar epithelium at 12 and 24 h after DCE treatment, whereas reactivities were absent in tissues from control animals. Finally, bronchiolar epithelial cells showing morphological criteria of apoptosis (chromatin condensation and margination) were observed at 24 h after DCE. Apoptotic-like cells were more abundant in larger bronchioles. These data suggested that DCE produces pulmonary bronchiolar apoptosis by inducing mitochondrial perturbations, causing release of cytochrome c into the cytosol and caspase activation. (Supported by Grant MOP 11706 from the Canadian Institutes of Health Research).

**2279**

## TMT TOXICITY IN HIPPOCAMPAL CELLS IS ASSOCIATED WITH INDUCTION OF BAX AND iNOS EXPRESSION.

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Trimethyltin (TMT) is an organotin that at low doses causes selective neuronal degeneration in the central nervous system, with the hippocampus being the most sensitive area. In turn TMT toxicity has been used as a model for neurodegeneration. In this study, primary cultured hippocampal neurons and an immortalized hippocampal cell line, HT-22, were exposed to TMT to assess the extent and type of cell death. The level of cell death was time and concentration dependent and HT-22 cells were more sensitive than primary neurons. TMT-treated cells exhibited apoptotic death as demonstrated by TUNEL staining, flow cytometry, DNA gel electrophoresis and increased caspase-3-like activity. The insult was abolished by the pan-caspase inhibitor z-VAD-FMK, indicating TMT-induced a caspase-dependent apoptosis. The cytotoxicity was characterized by an increase in reactive oxygen species (ROS) generation and reduction of mitochondria membrane potential ( $\Delta\Psi_m$ ). These actions were involved in the apoptosis since scavenging of ROS by free radical trapping agent, N-tert-butylnitron (PBN) or inhibition of the permeability transition pore opening with cyclosporin A, produced significant protection of HT-22 cells. TMT upregulated the expression of pro-apoptotic protein Bax and this was partly blocked by PBN. Also, immunoblot analysis showed an increased expression of iNOS after TMT treatment, which was associated with enhanced nitric oxide (NO) generation. Treatment with NG-monomethyl-L-arginine (L-NMMA), a specific inhibitor of iNOS, significantly decreased neuronal death and blocked the changes of Bax levels and caspase-3-like activity. It was concluded that ROS generation served as an initiator of the TMT-induced apoptotic cascade in which iNOS and Bax expression were upregulated and subsequent loss of  $\Delta\Psi_m$  led to caspase-executed cell death.

**2280**

## GENDER -SPECIFIC DIFFERENCES IN APOPTOTIC INDICES IN RAT LIVER AFTER CHRONIC ETHANOL CONSUMPTION.

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Chronic ethanol consumption causes liver injury and significant gender differences in susceptibility to liver damage have been demonstrated in males and females. Our aim was to determine whether the differential liver injury due to ethanol administration in males and females was due to enhanced apoptosis. We employed an oral ethanol feeding model in which fish oil was the major source of fat. Liver damage was evaluated in part by apoptotic indices, including the relative activities, in soluble fractions of the lysosomal cathepsins B and L, which indicate lysosomal leakage, which in turn, can initiate an apoptotic cascade. Male and female Wistar rats were pair-fed control and ethanol liquid diets for 8 weeks. At sacrifice, livers were homogenized. Subcellular fractions were prepared to determine the distribution of lysosomal cathepsins B and L in the particulate (mitochondrial/lysosomal) and soluble (cytosol) fractions. The content of cytochrome c, an indicator of mitochondrial damage and apoptosis was also assessed. Male and female ethanol-fed rats exhibited enlarged livers, and higher hepatic protein content than their controls. In both genders the total cathepsin B and L activities were the same in control and ethanol-fed rats. However the activities of these lysosomal enzymes in the cytosolic fraction (calculated as a percent of the total in lysosomes and cytosol) was 60% to two-fold higher in ethanol-fed females ( $p=0.03$ ) than their pair-fed controls, while no difference was detected in cathepsin B or L distribution in male rats. Similarly, cytochrome-c levels, calculated as a percent of that in the mitochondrial fraction, was

elevated three-fold in ethanol-fed female rats (compared with controls) while there was no significant increase in males. From these data we conclude that female rats consuming ethanol exhibited more severe liver damage than males and that part of this damage may have occurred due to enhanced apoptosis.

**2281** CALYCULIN A, A PP1/PP2A INHIBITOR, DELAYS APOPTOTIC SIGNALING IN TGHQ-TREATED HL-60 CELLS.

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2, 3, 5-tris-(Glutathion-S-yl)hydroquinone (TGHQ) is present in the bone marrow of rats and mice co-treated with hydroquinone and phenol, hematotoxic metabolites of benzene. TGHQ is also hematotoxic in rats, and induces DNA damage, growth arrest, and apoptosis in human promyelocytic leukemia (HL-60) cells. During TGHQ-induced apoptosis of HL-60 cells, reactive oxygen species (ROS) generation occurs and thioredoxin but not glutathione, is oxidized. Cytochrome c is released into cytosol from the mitochondria, concomitant with the dephosphorylation of Bcl-2 pS70 and Bax translocation into the mitochondria, in the absence of a disruption of mitochondrial inner membrane potential. We now show that secondary mitochondria-derived activator (Smac) is also released from mitochondria into the cytosol along with cytochrome c, and that X-linked inhibitor of apoptosis protein (XIAP) and pro-apoptotic Bid are cleaved by active effector caspases. Calyculin A, a PP1/PP2A inhibitor, delays the translocation of Bax to the mitochondria and enhances phosphorylation of Bcl-2 and Bad, leading to a delay in the release of cytochrome c and Smac into the cytosol. Subsequently the cleavage of initiator caspase 9 and the processing of effector caspase 3 are both delayed, resulting in a concomitant delay in the activation of caspase 3. The mechanism by which calyculin A delays the processing of caspase 3 is under investigation. Finally, phosphatidyl serine externalization is delayed in HL-60 cells treated with a combination of TGHQ and calyculin A. These data indicate that calyculin A protects cells from TGHQ-induced apoptosis via pre-mitochondrial-mediated effect. (ES09224 and ES06694).

**2282** SINGLE-WALLED CARBON NANOTUBES ACTIVATE RAW 264.7 MACROPHAGES: ROLE IN OXIDATIVE STRESS AND INFLAMMATORY RESPONSE.

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Single-walled carbon nanotubes (SWCNT) are an important member of nanomaterials with potentially broad range of revolutionary applications. Unprocessed SWCNT could become airborne and potentially reach lungs. Yet their pulmonary toxicity remains poorly characterized. The most commonly used technology in the manufacturing of SWCNT is catalytic disproportionation of gaseous CO on catalytic iron particles. Thus, SWCNT usually contain significant amounts (upto 40 wt%) of iron that may act as a catalyst of oxidative stress. Because inflammation induced by SWCNT provides a redox environment in which iron can fully realize their pro-oxidant potential, a combination of inflammatory response with catalytically-competent iron-containing SWCNT may synergistically enhance damage to cells and tissue. The primary responder that initiates inflammatory reaction to particle challenge is the pulmonary macrophages. Therefore, we used RAW 264.7 macrophages as a cell culture model to characterize the ability of these cells to respond to SWCNT with different levels of iron. Using EPR spectroscopy we found that non-purified in contrast to purified SWCNT displayed a broad signal attributable to high spin Fe<sup>3+</sup> in a distorted tetrahedral environment. Co-incubation of macrophages with SWCNT resulted in their engulfment detectable by TEM. Depletion of GSH and an increased number of apoptotic cells in response to SWCNT was observed. No intracellular production of superoxide (O<sub>2</sub><sup>·</sup>) or NO<sup>·</sup> was triggered by SWCNT as evidenced by flow cytometry. Iron-rich (but not iron-deplete) SWCNT effectively catalyzed conversion of extracellularly generated O<sub>2</sub><sup>·</sup> into hydroxyl radicals after zymosan activation of macrophages or in the presence of xanthine oxidase/xanthine as documented by EPR. Both iron-rich and iron-deplete SWCNT stimulated release of pro-inflammatory and fibrogenic cytokines IL-6 and TGF- $\beta$ , respectively. Supported by NIH HL70755.

**2283** PHYSICAL AND CHEMICAL INTERACTION BETWEEN CARDIOLIPIN AND CYTOCHROME C.

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Cytochrome C (Cyt c), an electron carrier between respiratory complexes III and IV of mitochondria, regulates apoptosis by triggering caspase cascades. Because release of Cyt c during mitochondrial membrane permeabilization requires oxidation

of cardiolipin (CL), we studied binding and redox interactions of CL with Cyt c. Incubation of Cyt c in the presence of different molecular species of CL (tetraoleoyl-, tetralinoleoyl- and bovine heart CL) yields complexes with peroxidase activity as evidenced by EPR spectroscopy of etoposide radicals formation and fluorescence responses of from oxidation of 2', 7'-dichlorofluoresceine, H2DCF to DCF. Neither saturated CL species (tetramyristoyl-CL) nor phosphatidylcholine elicited peroxidase activity of Cyt c. We further quantified binding of Cyt c with CL by monitoring the effects of acridine orange 10-nonyl bromide (nonyl acridine orange, NAO), a known binder of CL ( $K_b=2\cdot10^6$  M<sup>-1</sup>), on Cyt c-CL complexes formation detectable by non-denaturing PAGE as well as agarose electrophoresis. We found that Cyt c binds unsaturated CL with high affinity ( $K_b$  in the range of  $K_b=1\cdot7\cdot10^9$  M<sup>-1</sup>). Staining of the gels for protein (Coomassie Blue), lipid (Sudan black B) and peroxidase activity (3, 3'-diaminobenzidine) revealed that only lipid-containing Cyt c-CL complexes possessed peroxidase activity. Neither Cyt c alone nor CL liposomes exerted peroxidase activity. We conclude that high-affinity binding of CL with Cyt c forms a complex with potent peroxidase activity capable of regulating H<sub>2</sub>O<sub>2</sub> at the expense of oxidation of different reductants in mitochondria. Normally, the complex functions as an antioxidant protector and removes oxidants generated by electron transport. In apoptosis, excessive production of superoxide and H<sub>2</sub>O<sub>2</sub> causes oxidative stress, CL oxidation, thus facilitating membrane permeabilization. Supported by NIH HL70755.

**2284** CARDIOLIPIN DESTABILIZES CYTOCHROME C IN MITOCHONDRIA AND MAKES ITS CATALYTIC SITE ACCESSIBLE TO SMALL LIGANDS: ROLE IN APOPTOSIS.

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A small fraction of cyt c in mitochondria is tightly anchored to cardiolipin (CL) yielding conformationally destabilized cyt c. The latter acts as a CL-activated peroxidase that oxidizes polyunsaturated CL. Expression of peroxidase activity (PXA) of cyt c/CL complex (CCC) does not require the disruption of the hexa-coordinated form of cyt c and is evident before the characteristic absorbance band (700 nm) of Met80-heme disappears. Low temperature EPR spectroscopy revealed the formation of penta- and hexa-coordinated forms of CCC in the presence of Angel's salt. Heme-nitrosylated forms of CCC were also detectable after decay of its oxo-ferryl-associated tyrosyl radicals upon exposure to H<sub>2</sub>O<sub>2</sub> and then to Angel's salt. Conformational rearrangements and increased accessibility of the catalytic site to small ligands (H<sub>2</sub>O<sub>2</sub>, NO, NO<sup>·</sup>) is the major prerequisite for the CCC's PXA followed by the disruption of Met80-heme coordination bond. The latter was detectable by disappearance of its characteristic absorbance accompanied by heme degradation (accumulation of EPR signal with g=4.3, decrease of Soret band) dependent on the accessibility of heme to H<sub>2</sub>O<sub>2</sub>. One-electron reduction/oxidation of cyt c was not affected by CL. Unlike NO<sup>·</sup>, CO did not exert any significant binding with the reduced CCC and did not affect its PXA. Given that CL oxidation is critical for cyt c release, our results suggest that PXA of CCC is an important mechanism of mitochondrial apoptotic machinery and its modulation by small ligands such as NO<sup>·</sup> regulates apoptosis. Supported by NIH HL70755 and HL070807.

**2285** BLOCKADE OF N-METHYL-D-ASPARTATE RECEPTORS BY KETAMINE PRODUCES LOSS OF MONKEY FRONTAL CORTICAL NEURONS IN CULTURE.

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Ketamine, a noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist, has been used as a general pediatric anesthetic for surgical procedures in infants. Recent data suggest that anesthetic drugs may cause neurodegeneration, and there is also decreased expression of polysialic acid neural cell adhesion molecule (PSA-NCAM) in rodents treated with NMDA antagonists during development. The purpose of this study is to determine the robustness of ketamine-induced developmental neurotoxicity using newborn rhesus monkey frontal cortical culture and also to determine if dysregulation of NMDA receptor subunits promotes ketamine-induced apoptosis. Frontal cortical cultures were incubated for 24 hrs with 1, 10 or 20  $\mu$  M ketamine alone or co-incubated with ketamine and either NR1, NR2A, or NR2B antisense oligonucleotides (2  $\mu$ M). After washout of ketamine, cultures were kept in serum and glutamate-containing medium for 24 hrs. Ketamine (10 and 20

$\mu\text{M}$ ) caused a marked reduction in immunostaining for PSA-NCAM, a substantial increase in DNA fragmentation as measured by cell death ELISA, increased TUNEL-positive cells, a reduction in mitochondrial metabolism of MTT and a significantly increased release of lactate dehydrogenase. NR1 and NR2A, but not NR2B, antisenses protected the neurons from ketamine-induced degeneration. Western analysis showed that neurotoxic concentrations of ketamine resulted in a decrease in PSA-NCAM expression. NR1 and NR2B antisenses prevented this effect of ketamine, suggesting that ketamine-induced cell death is associated with a compensatory upregulation of the NMDA receptor. These data suggest that NR1 and NR2A antisenses offer neuroprotection from enhanced degeneration *in vitro*, and cortical cell death induced by ketamine negatively impacts cortical synaptogenesis, a process important in normal neural development. Supported by NCTR/FDA.

## 2286

### ACTIVATION OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ALPHA ENHANCES HEPATOCYTE APOPTOSIS.

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Chronic exposure to peroxisome proliferators (PP) leads to increased incidence of liver tumors in rodents. Liver tumor induction is thought to require increased hepatocyte proliferation and suppression of apoptosis. Transcript profiling showed increased expression of pro-apoptotic genes and decreased expression of anti-apoptotic genes in the livers of mice exposed to the PP WY-14, 643 (WY). We tested the hypothesis that prior exposure to WY would increase susceptibility to apoptosis inducers such as Jo2, an antibody which activates the Fas (Apo-1/CD95) death pathway. When compared to their untreated counterparts, wild-type mice pretreated with WY exhibited increased caspase-3 activation and hepatocyte apoptosis following challenge with Jo2. Livers from WY-treated PP-activated receptor alpha (PPARalpha)-null mice were resistant to the effects of Jo2. In the absence of Jo2, wild-type mice treated with WY exhibited increases in the activated form of caspase-9. As caspase-9 is a component of the apoptosome, we examined the expression of upstream effectors of apoptosome activity including members of the Bcl-2 family. Only the levels of the anti-apoptotic Mcl-1 transcript and protein were significantly altered by PP. The effects of PPARalpha activation on apoptosis were not specific to Jo2 as PPARalpha-null mice were also resistant to another treatment that induces hepatocyte apoptosis. These results 1) demonstrate that PPARalpha activation increases sensitivity of hepatocytes to apoptosis, 2) imply that PP exposure does not consistently lead to suppression of apoptosis in hepatocarcinogenesis and 3) identify a mechanism by which PPARalpha could serve as a pharmacological target in diseases where apoptosis is a contributing feature.

## 2287

### METABOLISM OF NITRO-PAHS ELICITS APOPTOTIC AND NECROTIC RESPONSES IN HEPA 1C1C7 CELLS.

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Polycyclic aromatic hydrocarbons (PAHs) and nitro-PAHs are widespread environmental pollutants formed during incomplete combustion of organic material. Some of these PAHs have been found to be mutagenic/ carcinogenic and implicated in the increased frequency of lung cancers in humans. We focused on elucidating the possible mechanisms involved in the metabolic activation and toxic effects of 1-nitropyrene (1-NP) and its amine metabolite 1-aminopyrene (1-AP), in comparison to the pneumotoxic antiandrogen, nilutamide (NIL) and its amine metabolite (NAM) in Hepa 1c1c7 cells. In cell-free systems 1-NP and NIL were nitro-reduced in a time, concentration and protein dependent fashion. The reaction was exclusively anaerobic and required both FMN and NADPH as co-factors. HPLC analyses revealed among others that the nitro moieties of 1-NP and NIL were reduced to the corresponding amine derivatives. Based on mass spectra, UV absorption and retention times, we show that NIL is metabolised to the N-hydroxy derivative as well. Other uncharacterised metabolites were also observed under aerobic conditions in whole cell cultures. Use of relevant inhibitors suggests NOS, but not CYP enzymes are involved in the metabolic activation of these nitroarenes. 1-NP appears to be slightly more potent than 1-AP with an average cell death of 35% at 60 $\mu\text{M}$  and a 24h exposure. In contrast, NAM was slightly more potent than the parent compound NIL; causing an average cell death of 70% at 10 $\mu\text{M}$ . The arenes also caused a significant decrease in G1 and doubling of cells at S-phase. Western analyses revealed that all the arenes increased the phosphorylation of p53, p38 and JNK. Interestingly, the levels of both pro-apoptotic Bax as well as anti-apoptotic Bcl-2/Bcl-xL were increased. Survival signals such as increased phosphorylation of Akt and Bad were also observed. Taken together, the relative potencies of the compounds to activate the studied apoptotic and anti-apoptotic signals could only partly explain their apoptotic effects.

## 2288

### THE ROLES OF MITOCHONDRIA AND CASPASE-6 IN 7,12-DIMETHYL-BENZ[ $\alpha$ ]ANTHRACENE-INDUCED BONE MARROW B CELL APOPTOSIS.

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Polycyclic aromatic hydrocarbons, common environmental pollutants, are known to cause immunosuppression and carcinogenesis. 7, 12-dimethylbenz[ $\alpha$ ]anthracene (DMBA) treatment induces bone marrow B cell apoptosis *in vitro* and reduced bone marrow cellularity *in vivo*. We have recently shown that caspase-8 plays a role in the DMBA-induced apoptosis of bone marrow pro-B cells and a non-transformed pro/pre-B cell line (BU-11), but it does not appear to be the initiator of the caspase cascade. Here we investigate the role of mitochondria in DMBA-induced apoptosis and how its activation leads to caspase-8 activation. Cytochrome c release from mitochondria begins within 6 hr of DMBA treatment concurrent with caspase-9 activation. 3, 3'-dihexyloxacarbocyanine iodide (DiOC<sub>6</sub>(3)) staining shows a loss of mitochondrial membrane potential; however this change is small, occurs after cytochrome c release, and is unaffected by caspase inhibitors. Consistent with an important role for caspase-9 is the significant suppression of apoptosis by the caspase-9 peptide inhibitor LEHD-FMK. Activation of caspase-9 is followed by formation of active caspase-3, -6 and -8 fragments, as well as cleavage of their respective substrates -fodrin, lamin A and Bid. IETD-FMK suppresses DMBA-induced apoptosis and this could be interpreted to suggest that caspase-8 is the apical caspase; however, VAD-FMK, DEVD-FMK and IETD-FMK all block the formation of active caspase-8 fragments, suggesting that caspase-8 activation occurs downstream. The fact that IETD-FMK blocks lamin A cleavage supports a role for caspase-6 in DMBA-induced apoptosis and suggests that caspase-8 is activated by caspase-6. We conclude that DMBA-induced pro/pre-B cell apoptosis is initiated by mitochondrial permeabilization but not membrane potential loss, is dependent upon caspase-9 activation, and is amplified by caspase-6-dependent caspase-8 activation. This work is supported by grants RO1-ES06086 and P01-ES11624.

## 2289

### AMELIORATION OF PARAQUAT AND CADMIUM CHLORIDE INDUCED APOPTOSIS BY $\kappa$ -CARRAGEENAN IN RAT PLEURAL MESOTHELIAL CELL CULTURES.

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Pulmonary fibrosis can be the end result of exposure to cadmium chloride and paraquat. It is known that apoptosis induced by such toxicants plays a key role in the pathogenesis of this disease. The hypothesis underlying the current investigation is that apoptosis induced by such toxicants can be ameliorated by  $\kappa$ -carrageenan (derived from Eucheuma cottonii). The working concentrations for both toxicants were established by MTT cytotoxicity assay in cultures of rat pleural mesothelial cells (RPMC). The protective effect of  $\kappa$ -carrageenan was assessed at 1, 5, 10  $\mu\text{g}/\text{ml}$  with 1 hour incubation prior to the addition of the toxicant. Induction of apoptosis was confirmed by assessing the increased activity of apoptotic markers using the caspase-3 enzyme activity assay, single stranded DNA (ssDNA) Apoptosis ELISA assay and TUNEL assay. Similarly, the protective effect of  $\kappa$ -carrageenan was assessed by such methods. 20 $\mu\text{M}$  of cadmium chloride and 10 $\mu\text{M}$  of paraquat resulted in approximately 50% cell viability following 24 hours incubation with the toxicants and thus these two concentrations were selected for the study. 5 $\mu\text{g}/\text{ml}$  of  $\kappa$ -carrageenan resulted in approximately 50% modulation of toxicity with respect to the treatment and this concentration was selected for the study. There was a significant increase in the concentration of DEVD-pNA cleavage products in cultures treated with toxicants as compared to control cultures. ssDNA analysis revealed a significant increase in single stranded DNA in toxicant treated cultures as compared to controls. TUNEL assay of toxicant treated cultures indicated a statistically significant increase in TUNEL positive cells as compared to control cultures. Treatment with  $\kappa$ -carrageenan followed by toxicant exposure reduced by approximately 50% caspase-3 enzyme activity, formation of ssDNA and TUNEL positive cells in cultures as compared to toxicant treated cultures. Cadmium chloride and paraquat induced apoptosis in RPMC and such apoptosis can be ameliorated by prior treatment with  $\kappa$ -carrageenan.

## 2290

### STUDY ON THE TOXICITY OF CADMIUM AND THE PROTECTIVE EFFECT OF FOOD COMPONENTS IN CELLS WITH HORMONE RECEPTORS.

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Cadmium, a human carcinogen, can induce toxicity in various cell lines and organs. Despite extensive research, the mechanisms of cadmium-induced apoptosis and cell arrest are poorly understood, and its toxicity and estrogenic potential in

human are not clear. This study was performed to investigate cadmium induced apoptosis and cell arrest on human breast cancer cells or human normal breast cells: MCF-7 cells, an estrogen receptor (ER) positive breast cancer cells, MDA-MB-231 cells, an ER negative breast cancer cells and MCF10A, human normal breast cells. MCF10A cells proved to be somewhat more sensitive (IC<sub>50</sub>= 100 uM at MCF-7 cells, 120 uM at MDA-MB-231 cells and 80 uM at MCF10A cells). All cells were treated with cadmium 100uM for 12hrs, and the apoptosis was determined by DNA fragmentation and DAPI staining. Our results showed that MCF-7 cells and MDA-MB-231 cells were arrested in S phase by flow cytometric analysis and G2/M phase and induced expression of p21 and p27 proteins by Western analysis after treated CdCl<sub>2</sub>. The expression of Cyclin A and Cdk2 proteins decreased in MCF-7 cells but no effect in MDA-MB-231 cells. The expression of Cyclin B1 and Cdc2 proteins decreased in MDA-MB-231 cells. ERK phosphorylation induced by cadmium (100uM). Co-treatment of zinc (100uM, 12hrs) recovered the cadmium-induced cell arrest in both cells. Also Our data showed that the cadmium induced apoptosis and cell arrest in human breast cancer cells by oxidative stress and antioxidants such as zinc inhibited cadmium-induced apoptosis and cell arrest.

**2291**

DIFFERENTIATION OF CISPLATIN-INDUCED DNA DAMAGE AND APOPTOSIS BY PHOSPHORYLATION OF HISTONE H2AX.

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The mechanisms of how cisplatin induced DNA damage leads toward cell death are beginning to be unravelled. Recent studies have shown that Histone H2AX, a universal component of chromatin structure in all eukaryotes, is phosphorylated on its Serine139 residue in response to different types of DNA damage such as DNA double strand breaks and hydroxyurea- or UV- induced replication blocks. In the present study, the phosphorylation of Histone H2AX induced by cisplatin and its clinically inactive isomer transplatin was compared by flow cytometric and immunoblotting methods. Both methods indicated that cisplatin is at least 100-fold more potent than transplatin in induction of phosphorylation of Histone H2AX. The time course study using Jurkat cells demonstrated that the significant elevation of phosphorylated Histone H2AX occurred about 4 hours after exposure to 30 mM cisplatin, earlier than cisplatin induced caspase activation, orientation change of phospholipids on cell membrane and DNA fragmentation during apoptosis. It is known that the tumor suppressor p53 protein plays an important role in response to DNA damage via a change of its intracellular level. Interestingly, after the treatment of cisplatin, the pattern of elevation of p53 is different from the formation of phosphorylated Histone H2AX. In response to cisplatin, the intracellular level of p53 was continuously elevated and peaked at 4 hours, whereas the increase of phosphorylated Histone H2AX was biphasic and reached its first peak about 1.5 hours followed by a decrease to basal level and initiation of a second increase. This study implies that, unlike the accumulation of p53, the change of phosphorylated Histone H2AX in the cells exposed to cisplatin reflects two different stages toward cell death. The first change might be an early indicator for the formation of the cisplatin-induced DNA lesion and the second increase might indicate activation of the apoptotic pathway in response to the cisplatin-induced damage.

**2292**

CISPLATIN-INDUCED APOPTOSIS OF GC1 TESTICULAR GERM CELLS MAY OCCUR BY AN AUTOCRINE MECHANISM INVOLVING FASL/FAS- OR TRAIL/DR5-MEDIATED SIGNALING.

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Male infertility is a concern for patients who undergo cancer chemotherapy because testicular germ cells appear to be as sensitive to elimination by chemotherapeutic drugs as tumor cells. Recent evidence indicates that the Fas-associated apoptotic-signaling pathway plays a role in cisplatin-induced testicular germ cell loss. Previously it has been suggested that Sertoli cells, through a paracrine interaction via their expression of FasL, mediate the apoptotic elimination of Fas-expressing germ cells. To investigate the participation of the Fas- and DR5-death receptor signaling pathways in cisplatin-induced germ cell apoptosis, we utilized the spermatogonia-derived cell line, GC1, as an *in vitro* model system. Cisplatin (0.1-10  $\mu$ M) caused time- and dose-dependent increases in apoptosis in GC1 cells with the highest amount of apoptosis observed with a dose of 10  $\mu$ M after 24 hours. Surprisingly, by immunohistochemical analysis, we found that GC1 cells not only express Fas and DR5, but they also express their cognate ligands; FasL and TRAIL. Western blot analysis revealed that exposure to 10  $\mu$ M cisplatin for 24 results in a 53% increase in FasL protein levels over control values whereas TRAIL levels did not change. In contrast, both Fas and DR5 protein levels were increased 2-fold by 24 h after exposure. In addition, as early as 12 hours after cisplatin (10  $\mu$ M) exposure, a

significant increase of cleaved caspase-3 could be measured. The robust up-regulation of these two death-receptor signaling pathways indicates their potential participation in triggering germ cell apoptosis after cisplatin treatment. Furthermore, the intriguing finding that both the death receptors investigated and their ligands are expressed in the same germ cell type points to the possibility that cisplatin-stimulated germ cell death is triggered by an autocrine mechanism. (Supported, in part, by grants from the Lance Armstrong Foundation, NIH/NIEHS ES09145 and ES07784).

**2293**

A DECREASE IN AKT LEVELS CORRELATES WITH SENSITIVITY TO ARSENIC-INDUCED GROWTH INHIBITION.

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Arsenic trioxide ( $As_2O_3$ ) is a highly toxic agent that has proven to be effective against acute promyelocytic leukemia (APL). The mechanisms of action of  $As_2O_3$  involve the induction of apoptosis through the stress-activated c-Jun N-terminal kinase (JNK) in NB4 cells (an arsenic-sensitive APL cell line). However, resistance arises after treatment with  $As_2O_3$ , and the mechanisms underlying this resistance are poorly understood. Herein, we investigated the role for the anti-apoptotic protein kinase, Akt, in the development of this resistance. Akt, a serine/threonine protein kinase, plays an important role in mediating cell survival signals coming through phosphoinositide 3-kinase (PI3K). It may also inhibit the JNK pathway. If the JNK pathway is activated to a pro-apoptotic state by arsenic, decreased Akt protein levels might further activate JNK and promote cell death. Our results show that  $As_2O_3$  decreases total Akt mRNA and protein expression in NB4 cells, but not in the  $As_2O_3$ -resistant cell lines. Kinase assays for Akt activity show a corresponding decrease in Akt activity in NB4 cells. Furthermore, when treated with  $As_2O_3$  in combination with wortmannin (WM), an inhibitor of PI3K and thus Akt, NB4 cells and resistant cells exhibit increased sensitivity to the cytotoxic effects of  $As_2O_3$ .  $As_2O_3$  treatment also affects Akt in other cell types. Indeed, in MDA-MB-468, a breast cancer cell line overexpressing activated Akt, we show a decrease in the protein levels following  $As_2O_3$  treatment, which correlates with an  $As_2O_3$ -dependent growth inhibition. However, in MDA-MB-231 cell, a breast cancer cell line expressing normal levels of activated Akt, these effects are not observed. Taken together, our results suggest that Akt protein is a pro-survival factor that plays a role in  $As_2O_3$  resistance.

**2294**

CARDIOLIPIN IS A PREFERRED SUBSTRATE FOR CYTOCHROME C INDUCED OXIDATION DURING INTRINSIC APOTOSIS.

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We used an oxidative lipidomics approach to assess oxidation of different lipid classes in cells during intrinsic apoptosis. The protocol is based on 2D-HPTLC separation of phospholipids (PLs), their hydrolysis by phospholipase A2 and analysis of fatty acid hydroperoxides (FA-OOHs). FA-OOHs are reacted with a fluorogenic substrate, Amplex Red, in the presence of microperoxidase 11 to stoichiometrically yield hydroxy fatty acids and resorufin. The latter is assayed by fluorescence HPLC according to our newly developed assay. ESI-MS analysis of oxygenated molecular species of PLs was performed using a triple-quadrupole tandem mass spectrometer equipped with an electro spray interface. In model experiments, we used tetralinoleoyl-cardiolipin, the most abundant molecular species of cardiolipin (CL) in mitochondria as a reactive substrate. When tetralinoleoyl-CL/cyt c complex was incubated with H<sub>2</sub>O<sub>2</sub>, several oxygenated species, including those containing mono-, di, and tri-hydroperoxides of CL and its hydroxy-derivatives, were detected. We further found that CL underwent a remarkable and selective oxidation after stimulation of human myelogenous leukemia HL-60 cells or mouse embryonic fibroblasts with pro-apoptotic stimuli, staurosporine and actinomycin D. More abundant and more unsaturated PLs, phosphatidylethanolamine and phosphatidylcholine, remained non-oxidized under the same conditions. In cyt c deficient mouse embryonic cells, pro-apoptotic stimuli (actinomycin D) did not cause oxidation of any of PLs. Selective and massive oxidation of CL was also detected in H<sub>2</sub>O<sub>2</sub>-treated HL-60 cells as well as in isolated mouse liver mitochondria. We conclude that cyt c acts as a catalyst of selective CL oxidation, a critical event in regulation of apoptotic signaling. Supported by NIH HL70755.

CYTOCHROME C CATALYZED CARDIOLIPIN  
OXIDATION IS ESSENTIAL FOR THE RELEASE OF  
PROAPOPTOTIC FACTORS FROM MITOCHONDRIA.

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Cardiolipin (CL) is ubiquitously present in the inner mitochondrial membrane and inter-membrane contact sites where it interacts with many proteins including cytochrome *c* (cyt *c*). CL has been suggested to play a critical role in the release of cyt *c* from mitochondria during apoptosis by either interacting with tBid or undergoing oxidative modifications. However, the mechanisms of CL involvement in mitochondrial membrane permeabilization remain elusive. In mitochondria, a small fraction of cyt *c* (5-10% Western Blot), likely CL-associated, remains membrane-bound after treatment with a channel-forming antibiotic, alamethicin, while the majority of electrostatically-bound cyt *c* is removed. Cyt *c* is activated to a peroxidase upon binding to CL as evaluated by oxidation of 2', 7'-dichlorodihydrofluorescein. Cyt *c*/CL complex catalyzes CL oxidation as determined by a newly developed fluorescence HPLC assay. We hypothesized that cyt *c* catalyzed CL oxidation is involved in the release of pro-apoptotic factors (e.g. cyt *c* and Smac/Diablo) during apoptosis. We utilized cyt *c* deficient mouse embryonic cells (Cyt *c*<sup>-/-</sup>) and wild type cells (Cyt *c*<sup>+/+</sup>) to investigate CL oxidation and release of cyt *c* and Smac/Diablo during apoptosis. Following actinomycin D (100ng/mL) treatment for 16 h, around 40% of Cyt *c*<sup>+/+</sup> cells were induced into apoptosis, while Cyt *c*<sup>-/-</sup> cells were resistant to this treatment. Both CL oxidation and release of cyt *c* and Smac/Diablo were observed in Cyt *c*<sup>+/+</sup> cells but not in Cyt *c*<sup>-/-</sup> cells. When isolated mouse liver mitochondria were incubated with oxidized tetralinoleoyl-CL containing liposomes, cyt *c* and Smac/Diablo were released to a greater extent as compared to incubation with tetralinoleoyl-CL containing liposome. Thus, catalysis by cyt *c* of CL oxidation is essential for the release of cyt *c* and other proapoptotic factors during apoptosis. Supported by NIH HL70755.

TWO FACETS OF ETOPOSIDE:PRO-APOPTOTIC  
AGENT AND ANTIOXIDANT.

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Etoposide, a phenolic DNA topoisomerase II inhibitor and antitumor agent, is known to induce intrinsic apoptosis accompanied by production of reactive oxygen species. It acts as a powerful lipid antioxidant and prevents phosphatidylserine (PS) oxidation and its externalization in apoptotic HL-60 cells, leading to failure in recognition and clearance by macrophages. Cytochrome *c* (cyt *c*) binds with high affinity with anionic phospholipids such as PS and cardiolipin (CL) to form a complex with peroxidase activity capable of oxidizing polyunsaturated phospholipids, including CL. Release of cyt *c* from mitochondria plays a pivotal role in triggering of caspase cascades whereby CL oxidation is involved in mitochondrial membrane permeabilization. Thus control of CL oxidation is critical in regulation of early stages of apoptosis. Given the lipid antioxidant potency of etoposide, we hypothesized that cyt *c* catalyzed CL oxidation during apoptosis can be sensitive to etoposide, hence affect execution of the apoptotic program. We found that CL was selectively oxidized in isolated mouse liver mitochondria as well in HL-60 cells treated with H2O2. In a model biochemical system, we showed that 160pmol lipid hydroperoxides per nmol CL were generated when 100μM liposomes containing a mixture of either tetralinoleoyl-CL (TLCL) or bovine heart CL with dioleoyl phosphatidylcholine (DOPC) were incubated with 4μM cyt *c* and 400μM H2O2. Oxidation of CL and production of hydroperoxides was also confirmed using ESI-MS. In the model system, etoposide inhibited CL hydroperoxide production in a concentration dependent manner. Complete protection of CL against oxidation occurred at the etoposide concentration of 50μM, by far higher than those minimally required for triggering apoptosis. Thus etoposide-dependent inhibition of CL oxidation is not likely to interfere with the execution of apoptotic program via prevention of mitochondrial membrane permeabilization. Supported by NIH HL70755, CA90787

HL-60 CELLS WITH METABOLICALLY  
BIOENGINEERED POLYUNSATURATED CARDIOLIPIN  
MOLECULAR SPECIES EXERT INCREASED  
SENSITIVITY TO APOPTOSIS.

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Recently we have shown that cardiolipin (CL)-bound cytochrome *c* (cyt *c*) functions as a peroxidase, catalyzing CL oxidation during apoptosis. Oxidized molecular species (MS) of CL were required for release of cyt *c* and other pro-apoptotic

factors from mitochondria. We hypothesized that MS of CL with highly unsaturated fatty acids such as C22:6 bound to cyt *c* are preferred oxidation substrates for peroxidase activity of the complex. Peroxidation of these MS of CL should enhance apoptotic responses of cells. Thus we attempted to manipulate intracellular levels of MS of CL enriched with C22:6 using its metabolic integration. Electro-spray ionization mass spectrometry revealed MS of CL with integrated C22:6 (m/z 1648 and 1656) in C22:6-treated cells. In addition, C22:4- and C22:5-CL species were present in the spectra. Control cells grown in standard medium contained C18:3-, C18:2-, and C18:1-fatty acids in CL (m/z 1472, 1474, and 1476, respectively) as a most abundant MS of CL. We found that CL underwent significant oxidation after STS stimulation in control cells. STS-induced oxidation of CL was more prominent in C22:6-treated cells compared to control cells. More abundant phospholipids such as phosphatidylcholine and phosphatidylethanolamine did not undergo any oxidation in both control and C22:6-treated cells under the same conditions of STS exposure. Notably, HL-60 cells grown in the presence of C22:6 showed an enhanced sensitivity to apoptosis induced by STS, as evidenced by caspase-3 activation and phosphatidylserine externalization. We conclude that MS of CL containing polyunsaturated longer chain fatty acids are prone to peroxidation during STS-triggered apoptosis due to their specific interactions with cyt *c* in the inner mitochondrial membrane. Our results demonstrate that dietary manipulations of CL fatty acid composition can be used as potent modulators of tumor cell sensitivity to apoptosis. Supported by NIH HL70755.

IDENTIFICATION OF NUR77 AGONISTS THAT  
INDUCE APOPTOSIS AND INHIBIT CANCER CELL  
SURVIVAL THROUGH NUCLEAR PATHWAYS.

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Nur77 is an orphan receptor and a member of the nerve growth factor-1-B (NGF1-B) subfamily of the nuclear receptor family of transcription factors. Ligand-dependent activation of Nur77 was investigated in Panc-28 pancreatic and other cancer cell lines transfected with either GAL4-Nur77 chimeras or a Nur response element. A series of 1, 1-bis(3'-indolyl)-1-(*p*-substitutedphenyl)methanes were screened in transactivation assays in pancreatic and other cancer cell lines, and for the first time we identified Nur77 agonists typified by 1, 1-bis(3'-indolyl)-1-(*p*-anisyl)methane that activated GAL4-Nur77 chimeras expressing wild-type or the ligand binding domain (E/F) of Nur77. In Panc-28 pancreatic cancer cells, Nur77 agonists activate the nuclear receptor and downstream responses including decreased cell survival, induction of cell death pathways including tumor necrosis factor related apoptosis-inducing ligand (TRAIL), and PARP cleavage. Moreover, the transactivation and apoptotic responses are also induced in other pancreatic, prostate and breast cancer cells that express Nur77. In Panc-28 cells, small inhibitory RNA for Nur77 not only reverses ligand-dependent transactivation but also induction of TRAIL and PARP cleavage. These results identify ligands that activate Nur77 through the ligand binding domain and show that ligand-dependent activation of Nur77 through nuclear pathways in cancer cells induce cell death pathways and are a potential new class of anticancer agents. (Supported by NIEHS ES09106 and M.D. Anderson Pancreatic Cancer Spore P20-CA10193)

THE IBM OF SMAC IS DISPENSABLE FOR APOPTOTIC  
FUNCTION.

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Smac is a proapoptotic protein that is activated by removal of the first 55 residues, which are a mitochondrial localization sequence. During apoptosis Smac is released by the mitochondria into the cytosol where it binds to X-linked inhibitor of apoptosis protein (XIAP) disrupting its inhibition of caspases. The first four amino acids of active Smac, A-V-P-I, are considered an IAP binding motif (IBM). The IBM is thought to be crucial for binding XIAP. Cytosolic expression of Smac beginning with Ala 56 was accomplished by a ubiquitin (Ub) fusion protein. The Ub-Smac fusion was deubiquitinated intracellularly, generating active Smac that was recovered from the cytosol of transfected cells. Expression of cytosolic Smac56 or mutants was used to test the idea that Smac antagonizes XIAP, a known E3 Ub ligase, by promoting its autoubiquitination and proteasomal degradation. Ub ligase activity depends on a functional ring domain of XIAP which docks an E2 ubiquitin conjugating enzymes. Cotransfection experiments showed that Smac interacted strongly with XIAP and markedly increased autoubiquitination of XIAP. Smac60, a splice variant lacking residues 62-105, interacted weakly with XIAP and failed to stimulate XIAP autoubiquitination. Smac60, which lacks residues 56-59 interacted strongly with XIAP and stimulated its autoubiquitination. Hence, the first four

residues of active Smac are not essential for XIAP binding and autoubiquitination. Conversely, XIAP also appears to increase the levels of Ub conjugated active Smac. Mutation H467A in the ring domain of XIAP ablated both autoubiquitination of XIAP and transubiquitination of Smac. Treatment with TNF $\alpha$  decreased endogenous XIAP levels in a cell line expressing Smac56. Proteasome blockade potentiated caspase-3 activation in cell lines expressing Smac56, 60, and  $\delta$ . Observations of cell rounding and detachment suggest that Smac56 and Smac60 potentiate the induction of apoptosis following proteasome inhibition. These findings suggest that the IBM of Smac is not essential for autoubiquitination of XIAP, caspase-3 activation, and apoptosis.

## 2300

### INVOLVEMENT OF THE AP-1 COMPLEX IN THE NEUROPROTECTIVE EFFECT OF PACAP AGAINST CERAMIDE-INDUCED TOXICITY.

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Ceramides, that are produced by sphingomyelin catabolism, constitute a novel class of second messengers. There is now clear evidence that ceramides play an important role in programmed cell death induced by cytotoxic agents. In particular, we have recently shown that ceramides activate c-Jun phosphorylation and stimulate the expression of the pro-apoptotic factor Bax. The neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) prevents ceramide-induced neurotoxicity and acts as a potent stimulator of the expression of both c-Fos and the anti-apoptotic factor Bcl2. As Bax and Bcl2 are known to be regulated by the transcription factor AP-1 (dimer of Fos/Jun members), the aim of the present study was to determine whether ceramides and PACAP differently affect the formation of AP-1 complexes. Gel shift experiments indicated that both PACAP and C2-ceramide stimulated AP-1 binding in a dose- and time-dependent manner. The effects of C2-ceramide (20  $\mu$ M) and PACAP (10-7 M) were maximum after 30 min of treatment. Co-incubation of granule neurons with PACAP and C2-ceramide resulted in an additive stimulation of AP-1 DNA binding activity. Characterization of the AP-1 complexes indicated that PACAP-induced AP-1 consisted in c-Fos/JunD heterodimers while C2-ceramide-induced AP-1 consisted in c-Jun homodimers. Western blot analysis showed that C2-ceramide increased p-c-Jun levels within 1 h of treatment and PACAP increased c-Fos expression within 30 min. The effect of C2-ceramide on c-Jun phosphorylation was blocked by PACAP, the JNK inhibitor and a PP2A blocker, while the action of PACAP on c-Fos was abrogated by C2-ceramide, an ERK blocker and a PKA inhibitor. These data suggest that the opposite effects of C2-ceramide and PACAP on Bax and Bcl2 expression may result from induction of different AP-1 dimers. This work was supported by INSERM, CIT, the LARC-Neuroscience network and the Conseil Regional de Haute Normandie.

## 2301

### ACTIVATION OF ENDONUCLEASE, OR CASPASE-ACTIVATED DNASE (CAD), AS A MARKER OF APOPTOSIS RATHER THAN NECROSIS IN DRUG- OR CHEMICAL-INDUCED ONCOSIS IN VIVO.

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The relative contribution and importance of apoptosis and necrosis to cell death in living model systems during drug- and chemical-induced target organ toxicity is intensely debated. It is difficult to extrapolate results from *in vitro* models to the *in vivo* setting, and *in vivo* conditions promote both death modes. Results showing that specific drugs produce necrosis are often based on late morphologic assessment. Adequate emphasis may not always be placed on pre-oncotic events, particularly when cells appear committed to apoptosis. CAD-independent DNA laddering has not been convincingly linked to necrosis *in vivo*. We hypothesize that a ladder of genomic DNA fragments, although controversial, remains a viable indicator of pre-oncotic apoptosis. To test this hypothesis, necrogenic doses (mg/kg) of acetaminophen (AP) 500, diclofenac (DCLF) 300, chloroform (CHL) 500, doxorubicin (DOX) 120, and furosemide (FUR) 500 were administered to 3 mo old male ICR mice. Analysis of serum chemistry (24-36h) revealed large increases in ALT activity (U/L, control 25 $\pm$ 2) for AP 19, 200 $\pm$ 895, CHL 3, 270 $\pm$ 286, DOX 20, 500 $\pm$ 651, and FUR 19, 600 $\pm$ 767, and elevated BUN (mg/dl, control 19 $\pm$ 2) for CHL 138 $\pm$ 12, DCLF 166 $\pm$ 6, DOX 112 $\pm$ 7, and FUR 93 $\pm$ 7. These agents induced frank toxicity resulting in cell death with the late morphology of oncosis in respective target organs. This was preceded by extensive genomic DNA fragmentation (> 300%

control) with DNA-laddering, consistent with an apoptotic phase of cell death for all agents. Such findings suggest that a preponderance of oncotic over apoptotic features should not lead to the conclusion that necrosis was the predominant mode leading to oncotic cell death. When CAD activation and clear-cut DNA laddering occur, it is possible for late morphologic evaluation documenting oncosis to bias interpretation of the means leading to cell death in favor of necrosis.

## 2302

### APOTOSE TRIGGERED MICRONUCLEI: THE ROLE OF APOPTOSIS ON THE OUTCOME OF MICRONUCLEUS ASSAY.

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The formation of micronuclei, aberrant mitoses, a mitotic arrest and other cellular perturbations may be apoptotic in nature rather than due to the genotoxic activity. Apoptosis is a highly regulated, controlled type of cell destruction programme that can be induced by a variety of physiological and pharmacological influences. It has been shown that histone deacetylase (HDAC) inhibitors an emerging new class of potential anticancer agents trigger the G2 checkpoint in normal cells. Loss of this G2 checkpoint can result in an aberrant mitosis and as a consequence in the induction of apoptosis and polyploid or micro-nucleated cells. To assess the potential of protease inhibitors for induction of micronuclei and to figure out the mechanism of interaction, we investigated the structurally different HDAC inhibitors Apicidin, Sodium Phenylbutyrate, Trichostatin A, Sodium Butyrate, Valproic acid and MS-275. To detect micronucleated cells in micronucleus test (MNT) *in vitro* L5178y mouse lymphoma cells were exposed to test compounds for 3 h (with S9) and 20 h (without S9). Following treatment 1000 cells per culture were evaluated for the induction of micronuclei. Appearance of apoptosis was monitored by measuring caspase-3/7 enzymatic activity and DNA fragmentation in cultures treated for 24h. The occurrence of micronucleated cells along with an induction of apoptosis was observed for Apicidin. This effect could not be observed for other tested compounds. However the results of Apicidin demonstrate that apoptosis can interfere with the induction of micronuclei in *in vitro* MNT tests. Further studies are needed to prove that apoptosis is the pre-dominant factor inducing micronucleated cells under treatment with Apicidin. The identification of possibly non-DNA targeting genotoxic mechanisms like apoptosis results in a different risk assessment.

## 2303

### ISOLATION OF THE APOPTOSIS INDUCING CONSTITUENTS OF *GUAIACUM SANCTUM* L. AND *GUAIACUM OFFICINALE* L. (ZYGOPHYLLACEAE) ON HUMAN BREAST CELLS *IN VITRO*.

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*Guaiacum sanctum* L. and *Guaiacum officinale* L. are Zygophyllaceae plant species used in South American folk medicine. Extracts of the plants have been shown to possess anti-inflammatory activity, including immunomodulatory and analgesic properties. However, to date no studies exist that investigate the direct effect on antitumor activity of the crude extracts. Recent studies show that the bark and heartwood of *Guaiacum sanctum* L. exerts a dose-dependent antiproliferative effect on the growth of human breast cancer with apoptotic morphologic features seen as low as 12.5  $\mu$ g/ml and as early as 6 hrs. The present investigation was initiated to isolate the active constituents from the bark and heartwood of *Guaiacum officinale* L. and to screen it via bio-assay guided fractionation for the induction of apoptosis. Extracts were fractionated with reverse phase HPLC chromatography and/or sephadex column chromatography. Active constituents were analyzed with LC-MS and NMR. The apoptotic index was calculated with fluorescence microscopy "double stained" with propidium iodide and Hoechst 33342 and via fluorescence-activated cell sorter (FACS) using Annexin+ /PI- and Annexin+ /PI+ cells. The data indicates that the active constituents belong to the class of lignans with molecular weights ranging from 325-410. Apoptosis for specific fractions was induced in a dose-dependent manner with the presence of morphologic features and an apoptotic index IC50 at 25 ppm for human breast cancer cell line MB-MDA-231. These findings suggest that further evaluation of *Guaiacum officinale* L. as an antitumor agent against human breast cancer is warranted. The study was supported by MIRT/NIH T37TW00076.

ESTIMATION OF AQUATIC SPECIES  
SENSITIVITY AND POPULATION-LEVEL  
RESPONSES

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Determining species sensitivity and population-level responses of aquatic organisms to contaminants are critical components of criteria development and ecological risk assessment. To address data gaps in species sensitivity, the U.S. EPA developed the Interspecies Correlation Estimation (ICE) program to predict acute toxicity to under-represented taxa, and the Acute to Chronic Estimation (ACE) program to predict chronic toxicity. ICE is based on existing acute toxicity data for 143 aquatic and terrestrial species, and estimates acute toxicity using least squares regression and over 4000 interspecies correlations. ACE uses linear regression and accelerated life testing to predict no-effect and low-effect concentrations for chronic mortality. Both ICE and ACE generally predict acute and chronic toxicity within two-fold of measured values and seldom exceeds four-fold. Population-level responses of aquatic species are estimated with matrix models using individual-level toxicity data, including altered survival and reproduction measured in life-cycle tests as input. Population modeling of pesticide toxicity to mysid shrimp has shown that population effects measured as changes in population growth rate can occur at both lethal and sublethal concentrations, and that population extinction correlates with median lethal concentrations. These modeling approaches provide useful tools for estimating the sensitivity of endangered species and other taxa with limited data, and for determining population-level responses from traditional organism-level toxicity test data.

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