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Metabolism and Biomarkers of Heterocyclic Aromatic Amines in Molecular Epidemiology Studies: Lessons Learned from Aromatic Amines

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Abstract

Aromatic amines and heterocyclic aromatic amines (HAAs) are structurally related classes of carcinogens that are formed during the combustion of tobacco or during the high-temperature cooking of meats. Both classes of procarcinogens undergo metabolic activation by *N*-hydroxylation of the exocyclic amine group, to produce a common proposed intermediate, the arylnitrenium ion, which is the critical metabolite implicated in toxicity and DNA damage. However, the biochemistry and chemical properties of these compounds are distinct and different biomarkers of aromatic amines and HAAs have been developed for human biomonitoring studies. Hemoglobin adducts have been extensively used as biomarkers to monitor occupational and environmental exposures to a number of aromatic amines; however, HAAs do not form hemoglobin adducts at appreciable levels and other biomarkers have been sought. A number of epidemiologic studies that have investigated dietary consumption of well-done meat in relation to various tumor sites reported a positive association between cancer risk and well-done meat consumption, although some studies have shown no associations between well-done meat and cancer risk. A major limiting factor in most epidemiological studies is the uncertainty in quantitative estimates of chronic exposure to HAAs and, thus, the association of HAAs formed in cooked meat and cancer risk has been difficult to establish. There is a critical need to establish long-term biomarkers of HAAs that can be implemented in molecular epidemioIogy studies. In this review article, we highlight and contrast the biochemistry of several prototypical carcinogenic aromatic amines and HAAs to which humans are chronically exposed. The biochemical properties and the impact of polymorphisms of the major xenobiotic-metabolizing enzymes on the biological effects of these chemicals are examined. Lastly, the analytical approaches that have been successfully employed to biomonitor aromatic amines and HAAs, and emerging biomarkers of HAAs that may be implemented in molecular epidemiology studies are discussed.

Introduction

Historically, the exposure to carcinogenic aromatic amines occurred during the production of dyes and other complex chemicals, and by their use as antioxidants in rubber-manufacturing processes *(1,2)*. A number of aromatic amines arise during the combustion of tobacco *(3,4)* and occur in the emissions of cooking oils *(5)*. Several heterocyclic aromatic amines (HAAs) are also produced during the high-temperature burning of tobacco *(6,7)*; however, the principal source of exposure to many HAAs occurs by consumption of well-done cooked meats *(8-10)*. HAAs are also present in pan-fried residues used for gravies

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(11,12), and arise in fumes of cooking oils *(13)* and the airborne particulates generated by the frying or grilling of meats *(14)*. Chemicals from both classes of compounds induce tumors at multiple sites in experimental laboratory animals during long-term carcinogen bioassays (see Figure 1 for chemical structures). Certain aromatic amines are classified as human carcinogens (Group 1), and several prevalent HAAs have been listed as probable or possible human carcinogens (Group 2A and 2B), based on toxicity data reviewed by the International Agency for Research on Cancer *(3,15)*. The *Report on Carcinogens*, 11th edition, of the National Toxicology Program, also concluded that prevalent HAAs are "reasonably anticipated" to be human carcinogens *(16)*. Thus, there is much concern about the health risk associated with the exposure to these structurally related classes of chemicals.

Aromatic amines and HAAs undergo metabolic activation by *N*-hydroxylation of the exocyclic amine group, to form the proposed arylnitrenium ion, which is the critical metabolite implicated in toxicity and DNA damage *(17,18)*. However, the biochemistry and chemical properties of aromatic amines and HAAs and their metabolites are distinct and different biomarkers of these carcinogens have been employed in human biomonitoring studies. The term biomarker has varied meanings that comprise: markers of susceptibility; makers of the internal dose; markers of the biologically effective dose; markers of early biological effects; markers of altered function; and markers of clinical disease *(19,20)*. In the context used here, the biomarkers are defined as markers of exposure and the biologically effective dose, and are representative early biomarkers of cancer risk. Some of the biomarkers include the unaltered compounds or metabolites in bodily fluids, or protein and DNA adducts derived from the genotoxic metabolites. The characterization of the urinary metabolic profiles of the genotoxicants can provide an estimate of the relative extent of bioactivation, as opposed to detoxification, undergone by the chemicals in vivo *(21)*. These measurements can also reveal interindividual differences in metabolism due to polymorphisms that encode for enzymes involved in xenobiotic metabolism; such differences can affect the genotoxic potency of procarcinogens *(22)*. However, urinary biomarkers of many carcinogens, including HAAs, are transient and only capture the last 24 hours of exposure. For individuals who chronically but intermittently consume grilled meats, urinary HAA biomarkers may go undetected. Longer-lived biomarkers of HAA exposure and genetic damage are required for epidemiological investigations. Certain drugs and carcinogens, including some HAAs, bind with high affinity to proteins and pigments in the hair follicle and become entrapped in the hair-shaft during hair growth *(23-25)*. The biomonitoring of HAAs in hair may provide a more accurate estimate of chronic exposure than the inferences obtained from food frequency questionnaires that are often used in molecular epidemiology studies *(19)*. However, the identification and measurement of chemical specific DNA adducts in the target tissue are the most relevant findings for risk assessment *(20,26)*. Unfortunately, DNA adduct measurements in tissue are often precluded by the unavailability of biopsy samples, which restricts the usage of this biomarker in large scale human studies. Accessible biological fluids, such as blood *(27)*, urine *(21)*, exfoliated bladder epithelial cells in urine *(28)*, or exfoliated mammary epithelial cells in milk of lactating women *(29,30)*, have served as surrogate matrices in which to assess exposure to chemicals or their metabolites or the formation of protein or DNA adducts. The identification of protein or DNA carcinogen adducts clearly demonstrates exposure to the biologically active metabolite, but the adduct must correlate with cancer risk, if it is considered valid as a biomarker of health risk *(31,32)*. The levels of macromolecular carcinogen adduct formation also should be influenced by polymorphisms in genes that encode enzymes involved in the bioactivation and/or detoxication of these chemicals *(22)*.

2-Aminofluorene (AF) and *N*-acetyl-2-aminofluorene (AAF) are perhaps the most well-studied among the aromatic amines *(33)*. AF and AAF were originally developed as pesticides but never used as intended because they were discovered to be animal carcinogens

(34). The pioneering research conducted on the metabolic fate of AF, AAF, and other prototypical arylamines, and the interactions of their metabolites with nucleic acids and proteins *(33,35,36)* have served as a foundation of knowledge for the development of human biomarkers towards aromatic amines as well as HAAs *(31,37,38)*. Many of the salient studies on the metabolism and biochemical toxicology of aromatic amines are summarized in review articles by Kiese *(39)*; Irving *(40)*, the Millers *(35,36,41)*, Hoffmann and Fuchs *(42)*; Neumann *(43)*; Gorrod and Manson *(44)*; and Kadlubar and Beland *(45)*. The impact of occuptational and tobacco exposures to aromatic amines and cancer risk is summarized by Clayson *(34)*, the Weisburgers *(46)*, and reviewed in the IARC Monographs *(1-3,47)*. The interested reader will find the historical perspectives of aromatic amine carcinogenesis and many citations of the original research in these reviews. More recent reviews on the implementation of biomarkers to monitor human exposure to aromatic amines are highlighted in articles by Neumann *(38,48)*, Skipper and Tannenbaum *(31,49)*, Yu and colleagues *(50)*; Sabbioni and Jones *(51)*, Talaska and Al-Zoughool *(52)*, and Richter and Branner *(53)*.

The research on HAAs commenced in 1977, when this class of genotoxicants was discovered *(8)*. The identification of HAAs in cooked foods is highlighted by Sugimura, Nagao, Wakabayashi, and colleagues *(8)*; Felton, Knize, and colleagues *(10)*; and by others *(54-57)*; mechanisms of HAA formation *(58,59)*; metabolism and genotoxicity *(60-70)*; genetic changes involved tumor genes of HAA carcinogenicity *(9,71,72)*; use of transgenic and mutant animal models for investigations of HAA-induced mutagenesis and carcinogenesis *(73,74)*; earlier reviews on aproaches for human biomonitoring of HAAs and their metabolites *(24,75)*; and the toxicological evaluation of HAAs by IARC *(15)* and the National Toxicology Program *(16)* are also cited.

Arylamine-hemoglobin adducts have been extensively used as biomarkers to monitor occupational and environmental exposures to aromatic amines, and to assess the risk of urinary bladder cancer, a target organ of some aromatic amines *(34,46,76-78)*. The biochemistry of arylamine-induced toxicity and methemoglobinemia are well documented *(39,79)*. The arylhydroxylamine metabolites, produced by cytochrome P450s, can penetrate the erythrocyte and undergo a co-oxidation reaction with oxy-hemoglobin (oxy-Hb), to form the arylnitroso intermediates and methemoglobin (met-Hb). The arylnitroso compounds can undergo enzymatic redox cycling within the erythrocyte to reform the aryhydroxylamine and commence another round of co-oxidation with oxy-Hb, ultimately resulting in methemoglobinemia (Figure 2). The arylnitroso intermediate can also react with the Cys^{93} residue of the human β-Hb chain to form a sulfinamide adduct *(79)*. Many aromatic amines undergo the metabolic pathway of *N*-oxidation and form the arylamine-Hb sulfinamide adduct *(38)*. In the case of 4-aminobiphenyl (4-ABP), the site of adduction at the Hb-$Cys^{93\beta}$ chain was proven by xray crystallography *(80,81)*. Arylamine-Hb sulfinamide adducts appear to be fairly stable in vivo *(80)*, but upon acid or base treatment, the adducts undergo hydrolysis to yield the parent amine and the Hb-$Cys^{93\beta}$ sulfinic acid *(31,82)*. The released aromatic amine can be readily measured by mass spectrometry (MS) methods *(31,83)*. HAAs undergo metabolic activation by *N*-oxidation *(60)*, but the covalent binding of the *N*-hydroxy-HAA metabolites to Hb in rodents *(84-88)* and in humans *(89-92)* is very low and the HAA-Hb sulfinamide adduct does not appear to be a promising biomarker to assess human exposure. Alternative biomarkers of HAAs have been sought: some of these biomarkers include urinary metabolites, DNA adducts, serum albumin (SA) adducts, and HAA residues in hair *(24,63,67,93-95)*.

The measurement of HAA biomarkers in humans is a difficult analytical task, because usually only ~1 μg to several micrograms of each compound is consumed per day, for individuals eating well-done cooked meat *(96)*. This level of exposure is considerably lower

than the levels of occupational exposure to many arylamines. Thus, the concentrations of HAA biomarkers in biological fluids or tissues are often below the part per billion (ppb) level. Many HAA biomarkers are polar and thermally labile molecules, which precludes the employment of gas chromatography (GC) methods for chemical analysis. During the past decade, highly sensitive electrospray ionization (ESI) techniques *(97)* combined with liquid chromatography (LC) have been developed to detect non-volatile and thermally labile compounds, including several different types of HAA biomarkers *(98-103)*. The challenge remains to establish rapid and robust analytical methods that can be used to measure HAA biomarkers in large scale molecular epidemiological studies. Such biomarkers would permit an accurate measure of HAA exposure, and their inter-relationships with metabolic phenotypes/genotypes involved in HAA genotoxicity and disease risk.

Aromatic Amine and HAA Exposure and Carcinogenesis

Some aromatic amines are known human urinary bladder carcinogens *(1-3,34,47)*. The occurrence of urinary bladder tumors among workers in dyestuff factories was first reported by Rehn in 1895 *(104)*, who attributed these cancers to the patients' occupation, from which evolved the term aniline cancer *(46)*. The textile dye, chemical, and rubber-manufacturing industries were major sources of occupational exposure to AAs, such as aniline, 4-ABP, 2-naphthylamine (2-NA), benzidine (Bz), and methylenebis-2-chloroaniline (MOCA) (Figure 1), up through much of the first half of the 20th century *(1)*. During that time, epidemiological data emerged, which demonstrated that workers occupationally exposed to these aromatic amines had elevated incidences of bladder cancer *(105,106)*. Aniline is a key intermediate in the manufacturing of dyes. Aniline, however, was not carcinogenic in experimental animals, but 4-ABP, 2-NA, and Bz, contaminants in aniline dyes, were shown to be carcinogenic *(1-3,34,46)*. Hueper established the first successful model for human bladder cancer by demonstrating that dogs exposed to 2-NA developed bladder tumors *(107)*. Thereafter, Radomski and Brill showed that *N*-oxidation of 2-NA played a critical role in the initiation of bladder cancer in the same animal model *(108)*. The urinary bladder, as well as the liver, intestine, and female mammary gland are among the target organs of cancer development in rodents exposed to aromatic amines *(34,46,109)*.

Historically, the levels of industrial exposure to some aromatic amines were elevated in many manufacturing and chemical plants. In one study, the airborne concentration of Bz in a manufacturing plant, producing 3,000 pounds per shift, was reported to range from <0.007 mg/m^3 to a maximum of 17.6 mg/m^3, at various locations within the factory *(110)*. This exposure resulted in levels of Bz present in urine at concentrations up to 159 μg/L, following the work shift *(110)*. In another chemical manufacturing plant, the concentrations of MOCA in urine from post-work shift workers were detected at levels ranging from 70 - 1500 μg/L, and the urinary levels of *o*-toluidine reached up to 132 μg/L from workers, following the work shift in another chemical production plant *(111)*. In the United States and many developed countries, strict federal regulations have drastically diminished the industrial usage of many carcinogenic aromatic amines. However, some aromatic amines, including 4-ABP and Bz, are still found as contaminants at the ppb concentration in color additives *(112,113)*, paints *(114)*, food colors *(115)*, leather and textile dyes *(116,117)*, fumes from heated cooking oils *(5)*, and fuels *(118)*. Cigarette smoking *(4)* is a prominent source of exposure to aromatic amines. 4-ABP and 2-NA occur in mainstream tobacco smoke at levels ranging from 0.3 - 4 and 2 – 14 ng per cigarette, respectively, whereas the amounts of *o*-toluidine range from 9 to 144 ng per cigarette *(4,119)*. Another potential source of exposure to some aromatic amines is through the usage of commercial hair dyes *(120,121)*. The exposure to a number of aromatic amines still continues via their oxidized nitroarene derivatives that are present in the atmosphere due to incomplete combustion of organic

materials *(51,122)*. There also appears to be considerable non-tobacco associated exposure to monocyclic alkylanilines; the source(s) of exposure remain to be determined *(123)*.

Carcinogenic HAAs were discovered nearly 35 years ago, when Professor Takashi Sugimura at the National Cancer Center in Tokyo, Japan, showed that the charred parts and smoke generated from broiled fish and beef contained substances that exhibited potent activities in *Salmonella typhiumurium*-based mutagenicity assays *(8)*. Since that hallmark study, more than 25 HAAs have been shown to form in meats, fish, and poultry prepared under common household cooking practices *(10,57)*. The concentrations of HAAs can range from less than 1 ppb to greater than 500 ppb *(9,10,124-126)*. The amounts of HAAs formed in meats are dependent upon the type of meat and the method of cooking; the HAA content generally increases as a function of temperature and the duration of cooking *(125,127)*. There are two major classes of HAAs (Figure 1). The “pyrolytic HAAs” arise during the high-temperature pyrolysis (>250 °C) of some individual amino acids, including glutamic acid and tryptophan, or during the pyrolysis of proteins *(6,9,128)*, but pyrolytic HAAs also can form, at the low ppb concentrations, in some cooked meats *(129)*. HAAs of the second class, aminoimidazoarenes (AIAs), are formed in meats that are cooked at lower temperatures (150 - 250 °C) more commonly used in household kitchens. The Maillard reaction is thought to play an important role in the formation of many AIAs *(10,58,130)*. The *N*-methyl-imidazole-2-yl-amine portion of the molecule is derived from creatine, and the remaining parts of the AIA skeleton are assumed to arise from Strecker degradation products (for example pyridines or pyrazines), formed in the Maillard reaction between hexoses and amino acids *(58,131)*. An aldol condensation is thought to link the two molecules through an aldehyde or related Schiff base, to form 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-3-methylimidazo[4,5-*f*]quinoxaline (IQx)-ring-structured HAAs *(132)*. 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) can form in a model system containing phenylalanine, creatinine, and glucose *(133)*; however, PhIP can also form in the absence of sugar *(10,132)*. PhIP is the most abundant of the carcinogenic AIAs formed in well-done cooked meats and poultry, where the concentration can reach up to 500 ppb *(10,125-127,129,130,134)*.

Several of the pyrolytic HAAs also are produced during the burning of tobacco. These HAAs induce *lacI* transgene mutations and aberrant crypt foci in the colon of mice *(135,136)*, and cancer of the liver and/or gastrointestinal tract of rodents *(9,137-139)*. 2-Amino-9*H*-pyrido[2,3-*b*]indole (AαC) occurs in mainstream tobacco smoke at levels up to 258 ng/cig *(140-142)*. The amounts of AαC formed in tobacco smoke are ~25 to 100-fold higher than those of 4-ABP *(4)* or benzo(a)pyrene *(143)*, and comparable to the levels of the tobacco-specific nitrosamine 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone *(144)*; these latter compounds are human carcinogens *(145)*. Other HAAs occur at lower quantities in tobacco smoke: 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC) forms at 10-fold lower amounts than AαC *(6,7,140)*, the glutamic acid and pyrolysate mutagens, 2-amino-6-methyldipirido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-aminodipirido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2), and the tryptophan pyrolysate mutagens 2-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) and 2-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) occur at <1 ng/cig *(146,147)*. Several AIAs also arise in tobacco smoke: 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) occurs in mainstream smoke at levels up to 23 ng/cig *(7,144)*, while IQ *(148)* occurs at <1 ng/cig *(146)*. Creatine, a constituent of muscle, is thought to be an essential precursor for the formation of AIAs, on the basis of studies on AIA formation in model systems *(58)*. For that reason, the occurrence of AIAs in tobacco smoke is surprising, although creatinine is present in the soil and in plants *(149)*. PhIP has also been identified in incineration ash and in airborne and diesel-exhaust particles *(150)*. The mechanisms of AIA formation during combustion remain to be determined. The possible causal role of some HAAs in tobacco-associated cancers warrants investigation.

The β-carboline compounds 9*H*-pyrido[3,4-*b*]indole (norharman) and 1-methyl-9*H*-pyrido[3,4-*b*]indole (harman) are formed at considerably higher levels in tobacco condensates and in cooked foods than are other HAAs (Figure 1) *(141,151)*. Norharman and harman are not mutagenic in *S. typhimurium* in the presence or absence of liver S9 fraction mixture; however, a synergistic mutagenic effect is observed when these compounds are co-incubated with aniline or *o*-toluidine *(152)*. This co-mutagenic effect is attributed to the formation of novel, mutagenic HAAs *(153)*. The structures of the compounds formed are 9-(4′-aminophenyl)-9*H*-pyrido[3,4-*b*]indole (amino-phenylnorharman, APNH), 9-(4′-amino-3-methylphenyl)-9*H*-pyrido[3,4-*b*]indole (amino-methyl-phenylnorharman, AMPNH) and 9-(4′-aminophenyl)-1-methyl-9*H*-pyrido[3,4-*b*]indole (aminophenylharman, APH). APNH is a liver and colon carcinogen in F344 rats *(154)*.

The HAAs studied induce tumors at multiple sites in rodents during long-term feeding studies. The target organs include the oral cavity, liver, stomach, colon, pancreas, and the prostate gland in males, and the mammary gland in females *(9,155)*. The total dose required to induce tumor formation (TD_{50}) varies for each HAA and is host species-dependent. The TD_{50} values of the individual HAAs have been reported to range from 0.1 to 64.6 mg/kg/day in rodents *(9)*. The dose concentrations of HAAs used in these carcinogen bioassays were large: up to several hundred parts per million of HAA in the diet were given to rodents over a 2 year period *(9,156)*. However, the carcinogenic potency of some HAAs is markedly enhanced in experimental laboratory animals exposed to tumor promoters or agents that cause cell proliferation *(9,157-159)*. Moreover, only a fraction of the HAA doses employed during long-term feeding studies can efficiently induce aberrant colonic crypt foci, large intestinal tumors *(158,160,161)*, or mammary gland tumors *(157,162)*, when a diet that is high in fat is incorporated into the feeding regimen. IQ is also a powerful liver carcinogen in non-human primates, with a latent period of just 27 to 37 months, making this compound one of the most powerful carcinogens assayed in non-human primates *(163)*. Summaries of the genetic alterations of target genes of HAAs in experimental animal carcinogenicity studies are available *(9,72,73)*.

The average dietary HAA intake can range from less than 2 to >25 ng/kg per day *(96,164)*. This daily intake level is about one million to 10^5-fold lower than the TD_{50} values of individual HAAs to induce tumors in rodents during long-term carcinogen bioassays with standard feeding protocols *(9)*. Thus, the amounts of HAAs consumed may be too small to explain human carcinogenesis, assuming that the susceptibility of humans to HAAs is the same as that of rodents. However, the carcinogenic effects of chronic exposure to multiple HAAs could be additive or possibly synergistic in humans *(165)*. A linear relationship between DNA adduct formation and the HAA dose has been demonstrated in tissues of rodents treated over a wide range with MeIQx *(166)*, IQ *(167)*, and PhIP *(168)*. Moreover, several HAA-DNA adducts have been detected in human tissues *(90,169-178)*, demonstrating that even ppb concentrations of HAAs in the diet can damage DNA. HAAs may be implicated in the development of human cancer under conditions in which many other mutagens-carcinogens, tumor promoters, and factors stimulating tumor progression exist *(9,159)*. The colon, prostate, and female mammary gland are common sites of cancer in Western countries in which well-done cooked meats containing HAAs are frequently consumed *(96,179)*; and the rates of cancer in these organs are increasing in Japan and other countries that are adapting western dietary habits *(9)*. These findings have raised suspicion that HAAs may contribute to the incidences of these cancers and have led to a multitude of epidemiological studies guided by the understanding of HAA exposure and metabolism generated by the laboratory data.

Although the focal point of this review is on the metabolism and the implementation of biomarkers of HAAs for molecular epidemiology studies, the cooking of foods results in the

formation of other carcinogens, which include polycyclic aromatic hydrocarbons, furan, acrylamide, among other chemicals that may be harmful to human health. The fundamental question is: do individuals who eat small quantities of any of these carcinogens over a lifetime have an increased cancer risk? There has been debate about the relative level of concern regarding exposure to HAAs as opposed to other genotoxicants in the diet, such as acrylamide, which are present at higher levels than HAAs *(180)*. Risk assessment studies of dietary genotoxic carcinogens, including HAAs and acrylamide, have been reported *(179,181-185)*. The risk characterisation of some genotoxic carcinogens has been conducted by the method of margin of exposure (MOE), which is defined as the ratio between a dose leading to tumor formation in experimental animals and the human intake and can be used to indicate levels of concern and also the ranking between various exposures to genotoxic carcinogens *(184,186)*. The larger the MOE, the smaller the risk posed by exposure to the genotoxic carcinogen under consideration. The international mean intake of acrylamide, which is formed in heated starch-based foods, has been estimated to range from 0.3 to 2.0 μg/kg bw per day for the general population *(187)*. This amount of acrylamide is at least 10-fold greater than the daily HAA exposure. The MOE value for acrylamide was determined to be ~1000-fold lower than the MOE value estimated for PhIP *(184)*, which is the most mass-abundant HAA formed in cooked beef *(10)*. Recent risk assessment approaches have incorporated human exposure data combined with physiologically based pharmacokinetic/ pharmacodynamic (PBPK/PD) modeling, which are used to integrate rodent carcinogenicity data and reduce the uncertainty inherent in extrapolating toxicological findings across species and dose by employing common exposure biomarkers *(185)*. In one PBPK/PD modeling study, the risk estimates of population-based lifetime excess cancer risks, based on the average acrylamide consumption in the diet range, was estimated between 1 - 4 × 10^{-4} *(185)*. The human cancer risk factor estimates reported for HAAs have ranged widely *(179,181-183)*. An upper limit was estimated as ~1 cancer case per 10,000 individuals, when considering exposure to multiple HAAs *(181)*, and a lower limit was calculated at 50 cases per 10^6 individuals *(182)*. HAA biomarkers were not employed in these risk assessment studies. The wide spread among the risk estimates can be attributed to inter-study differences in the assumptions used to calculate risk factors, including differing estimates of daily individual HAA intake, which can vary by more than 100-fold *(10,125,126,134,188-190)*, different dose extrapolations from animal models using body weight versus surface area scalings, and the usage of TD_{50} values from various animal carcinogen bioassays, in which differences are seen in the HAA carcinogenic potency *(9,163,191)*. Moreover, pro- and anticarcinogenic dietary factors can affect the metabolism and biological potency of HAAs as well as other procarcinogens *(9,159,192)*. Biomarkers of early biological effects (i.e. macromolecular carcinogen adducts) that can be used in molecular epidemiology studies to assess the dietary exposure, absorption, as well as interspecies and interindividual differences in metabolism of procarcinogens may aid to advance our understanding of health risked posed by different environmental or dietary genotoxicants.

Enzymes of Metabolic Activation and Detoxication of Aromatic Amines and HAAs

The bioactivation of aromatic amines and HAAs, is largely carried out by cytochrome P450 (P450) enzymes *(35,36,60,193)*. Oxidation of the exocyclic amine group produces genotoxic arylhydroxylamin*e* and *N*-hydroxy-HAA metabolites, whereas oxidation of the aromatic and heterocylic aromatic ring systems produces detoxicated metabolites *(34,41,44,45,194-198)*. There are important differences in the biotransformation pathways of arylamines and HAAs, particularly, by *N*-acetyltransferases (NAT1 and NAT2), which are discussed below. The conversion of 2-acetylaminofluorene to *N*-hydroxy-2-acetylaminofluorene in the rat was the first unequivocal proof of *N*-hydroxylation of an aromatic amine in vivo *(199)*. The arylhydroxylamines, arylhydroxamic acids, and *N*-hydroxy-HAA metabolites are esterified

by *N*-acetyltransferases (NATs), sulfotransferases (SULTs), L-seryl-tRNA and L-prolyl-tRNA synthetases, and other ATP-dependent enzymes *(45,60,68,200-206)*. These esters are unstable and undergo heterolytic cleavage to produce the reactive nitrenium ion that binds to DNA *(37,45,63,194)* (Figure 3). In the case of monocyclic alkylanilines, oxidation of the aromatic ring produces phenols, which can can undergo spontaneous or peroxidase-catalyzed oxidation, to form the quinone imine, a highly reactive electrophile that can undergo redox cycling to produce reactive oxygen species *(123)*. This chemical reaction pathway may contribute to the DNA damage of monocyclic alkylanilines.

AIAs that contain the *N*-methyl-imidazole-2-yl-amine moiety, such as IQ and MeIQx, can undergo nitrosation with nitric oxide, under neutral pH conditions, to form 2-nitrosoamino-3-methylimidazo[4,5-*f*]quinoline and 2-nitrosoamino-3,8-dimethylimidazo[4,5-*f*]quinoxaline. These *N*-nitroso-AIA compounds are converted to reactive diazonium species that may form covalent DNA adducts *(207,208)*. A mechanism for the NAT2-catalyzed bioactivation of *N*-nitroso-MeIQx has been proposed (Figure 3) *(209)*. The bioactivation of AIAs via nitrosation may be an alternative mechanism to P450-mediated N-oxidation of AIAs and contribute to their genotoxicity, under inflammatory conditions, during which elevated levels of nitric oxide can arise *(209)*.

Cytochrome P450s

The mammalian *CYP1A1*, *CYP1A2*, and *CYP1B1* genes (http://drnelson.uthsc.edu/cytochromeP450.html), encoding cytochromes P450 1A1, 1A2, and 1B1, respectively, and several other xenobiotic metabolism enzyme genes, are regulated by the aromatic hydrocarbon receptor (AHR) *(210,211)*. These P450s are responsible for the metabolic activation of many aromatic amines, HAAs, and polycyclic aromatic hydrocarbons *(212-223)*. Cytochrome P450 1A2 accounts for approximately 15% of the P450 content in human liver *(224)*. The P450 1A1 and 1B1 isoforms are generally not expressed in liver but are present at variable levels in a number of extrahepatic tissues *(225-229)*. P450 1A2 catalyzes the oxidation of many clinically used drugs and alkaloids at appreciable levels including acetaminophen, imipramine, clozapine, caffeine, and theophylliine *(230)*. The 3-*N*-demethylation of caffeine is catalyzed by P450 1A2, and the urinary ratios among various caffeine metabolites following ingestion of this drug have been used to estimate individual P450 1A2 activity and its inducibility in vivo *(231,232)*. P450 1A2 catalyzes the *N*-oxidation of planar aromatic amines such as 4-ABP, 2-NA, AF, as well as many HAAs *(193,233)*, while P450 3A4, which is also prominently expressed in liver, catalyzes the *N*-oxidation of nonplanar aromatic amines such as MOCA *(234)*. P450 3A4 can activate other arylamines and HAAs *(235)*, but at considerably lower rates than P450s 1A1, 1A2, or 1B1 *(213,217,218)*. P450 2A6 was identified as the major P450 responsible for the *N*-oxidation of alkylanilines *(236)*. The rates of *N*-oxidation of 4-ABP, MOCA, 2-NA, and HAAs are similar with human liver microsomes *(212,215,216,222,233)*, and comparable steady-state enzyme kinetic parameters have been reported for the *N*-oxidation of 4-ABP and several HAAs with recombinant human P450 1A2 *(220-222,237)*. Human bladder microsomes also catalyze the *N*-oxidation of 4-ABP; some of this activity may be attributed to P450 2A13 *(238)*. In addition to *N*-oxidation, some P450s catalyze oxidation of the aromatic and heteroyclic aromatic ring systems *(44,195,239)*.

The liver is the most active organ in the metabolism and bioactivation of many aromatic amines and HAAs *(60,66,197)*. The constitutive P450 1A2 mRNA expression levels can vary by as much as 15-fold in human liver *(240,241)*, and the expression of hepatic P450 1A2 protein ranges over 60-fold *(222,242)*. Varying levels of CpG methylation *(243)* and genetic polymorphisms of the upstream 5′-regulatory region of the P450 1A2 gene *(244,245)* alter the levels of P450 1A2 mRNA expression. Chemicals in the environment *(246)*, tobacco *(247,248)*, and diet, including constituents in cruciferous vegetables *(249,250)* and

grilled meat *(251,252)*, and medications *(248,253)* bind to the AHR and increase the rate of transcription of the *P450 1A2* gene, resulting in increased expression of P450 1A2 protein and other xenobiotic metabolism enzymes *(210,211)*. The interindividual variation in P450 1A2 activity is also observed in vivo for the metabolism of caffeine, a substrate for P450 1A2 *(233)*: more than a 70-fold range in P450 1A2 phenotype activity is observed in humans *(231,248,254)*. The genotype(s) responsible for the large range of interindividual differences in human hepatic P450 1A2 constitutive expression is still not well understood *(255)*. The large interindividual variation in expression of P450 1A2 may be an important determinant of individual susceptibility to aromatic amines and HAAs *(22,256)*.

There are also large interspecies differences in the metabolism of 4-ABP and HAA by P450s among mice, rats, and humans *(214,222,257-259)*, that are attributed to different levels of P450 expression, and differences in catalytic activities and regioselectivities of P450s towards these substrates. These interspecies distinctions in enzyme activities must be considered, when human risk assessments of genotoxicants are conducted from experimental animal toxicity data *(260)*. An example of the range in the amount of P450 1A2 protein expressed in human liver samples is shown in Figure 4. It is noteworthy that the expression of P450 1A2 is significantly greater in humans than in rodent strains that are used for carcinogen bioassays. Forty-three out of the 51 human liver microsomal samples contain higher P450 1A2 protein levels (5–250 pmol/mg microsomal protein, median 71 pmol/mg, N = 51) than liver microsomal samples of rats, where P450 1A2 content ranged from 5 to 35 pmol/mg microsomal protein, depending upon the strain, source, and diet *(222)*. The wide range in human P450 1A2 levels is paralleled by a large variation in the rates of *N*-oxidation of MeIQx and PhIP, which correlate well to the levels of P450 1A2. The rates of *N*-oxidation of MeIQx and PhIP are much lower in liver microsomal samples obtained from different strains of rat, which is reflective of the lower amounts of P450 1A2 protein expressed in rat liver.

There are important differences between human and rodent P450s in terms of catalytic activity and regioselectivity of HAA oxidation; these characteristics affect the toxicological properties of the molecules *(222,261)*. The catalytic efficiency of recombinant human P450 1A2 is superior to that of rat P450 1A2, in the *N*-oxidation of PhIP and MeIQx. Recombinant human P450 1A2 shows about a 1.5-fold greater k_{cat} (nmol product/nmol P450/min) and 13-fold lower K_m for PhIP *N*-oxidation compared to rat P450 1A2. In the case of *N*-oxidation of MeIQx, the K_m for recombinant human P450 1A2 and rat P450 1A2-mediated *N*-oxidation of MeIQx are similar, but the k_{cat} for recombinant human P450 1A2 was 16-fold greater than that of rat P450 1A2. The interspecies differences in the enzyme kinetic parameters for *N*-oxidation of PhIP and MeIQx have also been observed with human and rat liver microsomal samples *(222)*. However, the enzyme kinetic parameters for the *O*-demethylation of methoxyresorufin are similar for human and rat P450 1A2 *(222)*.

Important species differences also exist in the regioselectivity of P450 1A2-mediated oxidation of HAAs. Human P450 1A2 is regioselective for the *N*-oxidation (bioactivation) of HAAs, such as IQ, MeIQx, and PhIP, and this enzyme does not appreciably catalyze the ring-oxidation (detoxication) of the heteroaromatic ring systems. However, the P450 1A2 orthologues of experimental laboratory animals produce both *N*-oxidation and ring-oxidation products at comparable levels *(103,214,222,262)*. Human P450 1A2 also catalyzes the oxidation of the C^8-methyl group of MeIQx to form the alcohol, 2-amino-(8-hydroxymethyl)-3-methylimidazo[4,5-*f*]quinoxaline (8-CH_2OH-IQx), which undergoes further oxidation by P450 1A2 to form the the carboxylic acid, 2-amino-3-methylimidazo[4,5-*f*]quinoxaline-8-carboxylic acid (IQx-8-COOH) (Figure 5) *(262)*. IQx-8-COOH formation is the major pathway of metabolism and detoxication of MeIQx in humans *(103)*. The rat P450 1A2 orthologue catalyzes the detoxication of MeIQx through C-5

hydroxylation, but it does not catalyze IQx-8-COOH formation *(85,262-264)*. In the case of PhIP, human P450 1A2 is highly selective for *N*-oxidation, whereas rat P450 1A2 catalyzes both *N*-oxidation and 4′-hydroxylation of the phenyl ring of PhIP, to produce the detoxicated product, 2-amino-4′-hydroxy-1-methyl-6-phenylimdazo[4,5-*b*]pyridine *(220-222,265)*.

The metabolism of IQ, MeIQx, PhIP, and AαC (Figure 5) has been studied with rodent and human liver microsomes *(60,197,214-216,222,257,266-273)*, in experimental laboratory animals *(66,85,195,197,239,263,274,275,275-281)*, rodent hepatocytes *(195,265,282,283)*, human hepatocytes *(262,284)*, and HepG2 cells *(283)*. A number of metabolites of MeIQx and PhIP have also been identified in human urine *(98,101,103,176,264,285-293)*. P450-mediated ring-oxidation of MeIQx, IQ, and PhIP are major pathways of metabolism and detoxication in rodents *(85,214,222,239,274)* and in Cynomolgus monkeys *(196)*. P450 1A2 is not expressed in liver of Cynomolgus monkeys *(294)* and other P450s, including P450 3A4 and/or P450 2C9/10 appear to contribute to the ring and exocyclic *N*-oxidation of HAAs in this species *(196)*. These other P450s were reported to *N*-hydroxylate IQ to an appreciable extent, but did not catalyze the *N*-oxidation of MeIQx; IQ is a carcinogen in Cynomolgus monkeys, but MeIQx is not *(196)*. The P450-mediated *N*-demethylation of IQ and MeIQx is another important biotransformation pathway of IQ and MeIQx in rodents and nonhuman primates *(196,281)*. *N*-Demethylation of IQ is thought to be a detoxication pathway because the mutagenic potency of desmethyl-IQ is more than 60-fold weaker than IQ *(295)*. However, the P450-mediated *N*-demethylation of IQ or MeIQx is negligible with human liver microsomes *(215,222,233)*, human hepatocytes *(272)*, or in humans *(103,264)*. The microflora of the human colon catalyzes the oxidation of IQ and MeIQx at the C-7 atom of the heterocyclic ring *(296)*; these oxidation metabolites are not carcinogenic in rodents *(297)*.

Numerous studies have shown that P450 1A2 plays a major role in the metabolic activation of aromatic amines and HAAs and in the formation of DNA adducts in rodents *(63,298,299)* (and references cited therein). The pretreatment of human liver microsomes with various amounts of furafylline, a mechanism-based inhibitor of P450 1A2 *(300)*, led to a concentration-dependent inhibition of HONH-MeIQx, 8-CH_2OH-IQx, IQx-8-COOH, and HONH-PhIP formation by up to 95% *(215,216,220,222,261)*, indicating the important contribution of human P450 1A2 in the metabolism of these carcinogens. The formation of 8-CH_2OH-IQx and IQx-8-COOH, and the glucuronide conjugates of HONH-MeIQx and HONH-PhIP, were also inhibited to a similar degree in human hepatocytes pretreated with furafylline *(262,284)*. In humans, the contribution of P450 1A2 to the metabolism of MeIQx and PhIP was demonstrated in a pharmacokinetic study that used furafylline *(301)*. As much as 91% of the MeIQx and 70% of the PhIP consumed in grilled meat were estimated to undergo metabolism by P450 1A2 *(301)*. Thus, P450 1A2 significantly contributes to the metabolism of both MeIQx and PhIP in vivo in humans, but with marked differences in substrate specificity. Human P450 1A2 primarily catalyzes the detoxification of MeIQx by oxidation of the 8-methyl group, whereas it catalyzes the bioactivation of PhIP by oxidation of the exocyclic amine group (Figure 5) *(103,262)*. These metabolic studies support the notion that P450 1A2 is a major enzyme involved in the metabolism of MeIQx and PhIP in humans.

Conversely, the results from several studies employing transgenic rodents have led investigators to propose that alternative enzymes are involved in HAA- and arylamine-mediated toxicity and that P450 1A2 may even be protective against these carcinogens in animals *(211)*. The levels of DNA adducts of IQ and PhIP were found to be lower in some organs of P4501A2-knockout mice than in organs of wild-type mice; however, other P450s or enzyme pathways of activation also contributed to DNA adduct formation in specific organs *(302)*. In the neonatal mouse model, higher incidences of lymphoma and

hepatocellular adenoma occurred in female P4501A2-knockout mice than in wild-type mice exposed to high doses of PhIP (11 or 22 mg/kg) *(258)*, indicating PhIP-induced carcinogenesis is independent of P450 1A2 expression. Methemoglobin formation, a biomarker of exposure and toxicity to certain aromatic amines, was higher in P450 1A2-knockout mice than in wild-type mice exposed to 4-ABP *(303)*. Furthermore, P450 1A2 expression in wild-type mice was not associated with 4-ABP-induced hepatic oxidative stress or with 4-ABP–DNA adduct formation *(304)*. 4-ABP-induced hepatocarcinogenesis in P4501A2-knockout mice was also found to be independent of P450 1A2 *(259)*. These paradoxical effects may lead us to question the importance of P450 1A2 in HAA- and 4-ABP-mediated toxicity and malignancy *(211,305)*. We note that very high concentrations of HAAs and 4-ABP were employed in these transgenic rodent studies; the high doses may have triggered metabolic pathways that lead to formation of chemically reactive metabolites, by other P450s or Phase I enzymes, which may not arise under low-dose treatments. Indeed, liver microsomes from P450 1A2-knockout mice displayed significant *N*-oxidation activity of PhIP and 4-ABP *(258,259)*. The role of P450 1A2 in the activation as opposed to the detoxication of HAAs or AAs in the intact animal is likely to depend on the extent of Phase II metabolism, the degree of coupling of *N*-oxidation with Phase II enzymes, and cell type- and tissue-specific context, as well as the dose and pharmacokinetics of the compound under study *(211,305)*. Investigations in "humanized" mice containing the *P450 1A2* allele in place of the orthologous mouse gene *(280,306)* can be used to assess the role of human P450 1A2 in the DNA damage induced by HAAs and AAs, under realistic human exposure levels.

Peroxidases

Peroxidases, including prostaglandin H synthase (PHS), an arachidonic acid-dependent peroxidase, may play a significant role in the activation of aromatic amines and HAAs in extrahepatic target tissues of experimental animals, such as urinary bladder, colorectum, and mammary gland, where the P450 content is low *(307-318)*. Much of the data are consistent with a one-electron mechanism of arylamine or HAA oxidation by PHS, and the *N*-hydroxy intermediates do not appear to be involved in the metabolism by PHS *(310)*. However, a number of the PHS oxidized products of arylamines and AIAs generate a DNA adduct profile that is similar to those generated by P450s, suggesting a common DNA-reactive species, presumably an arylnitrenium ion, produced by different pathways in these cellular and enzyme model systems *(310,316,319-322)*.

N-Acetyltransferases

N-Acetyltransferases (NATs) are critical enzymes involved in the genotoxicity of aromatic amines and HAAs. There are two distinct *N*-acetyltransferase isoenzymes (designated NAT1 and NAT2, http://louisville.edu/medschool/pharmacology/consensus-human-arylamine-n-acetyltransferase-gene-nomenclature/). NAT2 is expressed primarily in the liver, whereas NAT1 appears to be more prominently expressed in extrahepatic tissues *(323,324)*. More than 25 genetic polymorphisms have been identified for both *NAT* genes that can affect the catalytic activity of NATs toward aromatic amines and HAAs *(323,325,326)*. NAT enzymes have a dual role in the metabolism of aromatic amines and HAAs: these enzymes can serve as mechanisms of bioactivation or detoxication. Some epidemiological studies suggest a role for NAT2 activity in human susceptibilities to various cancers from tobacco smoke and from consumption of well-done meats, where the exposures to aromatic amines and HAAs can be substantial *(327,328)*.

N-Acetylation is an important mechanism of detoxication of aromatic monoamines *(324)*: this biotransformation pathway is catalyzed by both NAT1 and NAT2 and serves as a competing pathway of *N*-oxidation *(203)*. The resulting acetamides are generally viewed as

poor substrates for P450-mediated *N*-oxidation (Figure 3) *(329)*. For many aromatic amines, the catalytic efficiency (k_{cat}/K_m) of *N*-acetylation by recombinant NAT1 is superior to that of recombinant NAT2, but the relative affinity (K_m) for each of the AA substrates investigated was higher for recombinant NAT2 *(203)*. Bz, an aromatic diamine, is an exception. The *N*-acetylation of one of the amine groups of Bz appears to facilitate P450-mediated *N*-oxidation of the non-acetylated amine group, to form the reactive *N*-4-hydroxyamino-*N*′-acetylbenzidine (HONH-*N*′-acetyl-Bz) metabolite *(318,330)*. Bz is preferentially *N*-acetylated by NAT1 *(331,332)*

N-Acetylation of the arylhydroxylamines also occurs, to form the arylhydroxamic acids, which can undergo bioactivation by *N*,*O*-acetyltransferase or sulfotransferases (SULTs) *(45)*. Direct activation of the *N*-hydroxy-AAs by *O*-acetylation also occurs and results in formation of the reactive *N*-acetoxy intermediates that readily bind to DNA *(60,194,333)*. NAT1 appears to function as an *O*-acetyltransferase (OAT) and as an *N*,*O*-acetyltransferase, when using acetyl coenzyme A or arylhydroxamic acids, respectively, as acetyl donors. NAT2 appears to act preferentially as an OAT and NAT (Figure 3). HAAs that contain the *N*-methyl-imidazo-2-yl-amine moiety (AIAs) are poor substrates for NATs and *N*-acetylation is not an important pathway of detoxication in rodents or humans. AαC and several other pyrolysate HAAs are substrates for rodent NATs. Nonetheless, the catalytic rates are ~1/1000 the level observed for the *N*-acetylation of AF *(60)*. In contrast to the parent HAAs, the HONH-AIA and HONH-HAA metabolites do undergo *O*-acetylation, primarily by NAT2, to form the reactive *N*-acetoxy species, which bind to DNA (Figure 2) *(63,334,335)*. *N*-Hydroxy-AαC is an exception and it undergoes *O*-acetylation by both NAT1 and NAT2 *(271)*.

A mouse model deficient in both NAT1 and NAT2, *Nat1/2(-/-)*, was employed to examine the pharmacokinetics of 4-ABP, AF and PhIP *(336)*. The metabolism of AF was severely affected and the plasma clearance was increased by 4-fold in *Nat1/2(-/-)* mice, whereas the clearance of 4-ABP was found to be less dependent on *N*-acetylation, and no difference in 4-ABP plasma clearance rates was observed between wild-type and knockout animals. PhIP did not undergo *N*-acetylation, nor was its clearance affected by NAT genotype *(336)*. In adult female rapid and slow acetylator rats congenic at the NAT2 locus, PhIP-DNA adduct formation was unaffected by NAT2 acetylator status in liver or any of the extrahepatic tissue examined, whereas MeIQx-DNA adducts, particularly in liver, were significantly lower in slow acetylators *(337)*. Similar findings were observed in congenic rapid and slow acetylator Syrian hamsters, PhIP-DNA adduct formation was independent of *N*-acetylator activity *(338)*. These data signify that PhIP genotoxicity in rodents is not influenced by NAT enzymes.

HONH-PhIP, like many other HONH-HAAs, undergoes activation by human NATs in subcelluar cytosolic assays *(339)*, and by recombinant NAT2 *(335)*, to form the reactive *N*-acetoxy-PhIP intermediate, which binds to DNA *(340,341)*. However, the level of PhIP-induced mutation and DNA adduct formation in Chinese hamster ovary cell lines cotransfected with *NAT2*4* (rapid acetylator) or *NAT2*5B* (slow acetylator) alleles with either P450 1A1 or P450 1A2 is comparable to cell lines only transfected with the P450s *(342,343)*. A similar result was demonstrated in *Salmonella typhimurium* bacterial strains expressing human NAT1 or NAT2 *(68,344,345)*, and PhIP appeared to be activated by other phase II enzymes, including SULTs *(68,345,346)*. A much more potent effect of NAT2 phenotype was demonstrated for the induction of mutagenicity and DNA adduct formation of MeIQx *(347)*, IQ *(342)*, and AαC *(348)*. The findings indicate that HONH-PhIP is a poor substrate for rodent and human NATs. Thus, metabolic data obtained with subcelluar fractions or isolated enzymes, particularly when high substrate concentrations are employed, may not be reflective of enzyme activity that occurs within cells. Therefore, the adverse

biological effects of NAT2 phenotype in the gene–environmental (cooked red meat) studies may reflect exposure to other HAAs such as MeIQx and AαC more so than PhIP. The identification of exposure to specific HAAs is very important in molecular epidemiological investigations that seek to assess the significance of HAAs and *NAT2* genetic polymorphism in cancer risk.

The role of NAT2 genetic polymorphism in cancer risk has been studied extensively, and the elevated risk of urinary bladder cancer in cigarette smokers who are slow *N*-acetylators is well documented *(50,77,78,349)*. This increased cancer risk has been attributed to the diminished capacity of slow *N*-acetylator individuals to detoxicate aromatic amines present in tobacco, some of these aromatic amines are bladder carcinogens *(1-3,34,326)* (Figure 3). However, the role of NAT2 phenotypes in cancer risk of HAAs is unclear *(204,323)*. NAT2 does not efficiently detoxicate most HAAs, but the *N*-hydroxylated HAA metabolites are substrates for *O*-acetylation by NAT2 and the resultant *N*-acetoxy intermediates readily bind to DNA *(60,63,334,335,339,350,351)*. As a result, the increased cancer risk may be markedly elevated in individuals who are both rapid P450 1A2 *N*-oxidizers and rapid *O*-acetylators *(327,328)*.

Sulfotransferases

The sulfotransferases (SULTs) are another Phase II enzyme involved in the metabolism of aromatic amines and HAAs. The SULTs belong to a super family of genes that are divided into two subfamilies: the phenol SULTs (SULT1), and the hydroxysteroid SULTs (SULT2) *(352-354)*. SULT1A1, 1A3, and 1B1 are expressed in all parts of the gastrointestinal tract, often exceeding the protein levels that are expressed in the liver *(355)*. In addition to the sulfating of phenolic xenobiotics, steroids, and estrogens, the SULT enzymes can serve to detoxicate or bioactivate HAAs or AAs *(68,356)*. Rat SULT1A1 catalyzes the formation of sulfamates of IQ and MeIQx *(357-359)* as detoxication products, but the sulfamation of PhIP does not occur in rats or other experimental laboratory animals *(195)*. The sulfamate of MeIQx is excreted in urine of humans *(264,285)*: its formation is presumably catalyzed by SULT1A1 *(359)*. Boyland *et al. (360)* demonstrated that rats dosed with aniline, 1-naphthylamine, or 2-NA excrete in the urine a very small amount of these aromatic amines as the sulfamate derivatives. The sulfamates of IQ and MeIQx are quite stable under the range of pH conditions that exist in urine *(285,357,358)*, whereas the sulfamates of many arylamines are labile *(360)*.

Human SULT1A1 and SULT1A2 catalyze the binding of the *N*-hydroxy metabolites of MOCA, AF, AAF, 4-ABP, PhIP, AαC, and MeAαC to DNA, although the *N*-hydroxy metabolites of MeIQx and IQ are poor substrates for both SULT isoforms *(68,202,205,345,356,361-363)*. The SULT-mediated metabolic activation of arylhydroxylamines and *N*-hydroxy-HAAs has been detected in human liver, colon, prostate, and female mammary gland cytosols, but not in pancreas, larynx, or urinary bladder epithelial cytosols *(361,364-366)*. SULT1E1, which is under hormonal regulation, catalyzes the binding of HONH-PhIP to DNA in cultured human mammary cells. Therefore, SULT1E1 was proposed to play a role in the bioactivation of PhIP in breast tissue *(367)*. However, a recent study failed to detect the SULT1E1 protein in breast tissue and factors in the cell culture media may have induced the expression of SULT1E1 protein in cultured human mammary cells *(365)*.

One common genetic polymorphism, an Arg213His polymorphism in the *SULT1A1* gene, has a strong influence on the level of enzyme protein and phenol sulfotransferase activity in platelets, which has been used for metabolic phenotyping *(205)*. The frequency of the variant *SULT1A1*2* allele exceeds 10% in Japanese *(368)*, African-Americans, and Caucasians *(369,370)*. The SULT1A1*2 protein has low enzyme activity and stability

compared to the wild-type SULT1A1*1 protein *(361)*. DNA binding studies using recombinant SULT1A1*1 and SULT1A1*2 have shown that SULT1A1*1 protein catalyses HONH-4-ABP and HONH-PhIP DNA adduct formation with much greater efficiency than the SULT1A1*2 variant *(205)*. Several molecular epidemiological studies have explored the roles of *SULT1A1*1* and *SULT1A1*2* genotypes and putative HAA exposure in breast *(371)*, colorectal *(370,372)*, and prostate cancer risk *(373)*. The expression of the variant allele *SULT1A1*2*, with diminished capacity for bioactivation of some HONH-HAAs, was associated with decreased risk of breast cancer for women who often ate well-done cooked meat *(371)*; however, this genotype was not associated with a decreased risk of colorectal *(370,372)* or prostate cancer *(373)*. The frequency of consumption of grilled meats and the extent of exposure to HAAs are uncertain in these subjects. In the absence of exposure to biologically relevant levels of HAAs, a genetic polymorphism would not be expected to be manifested as a risk factor *(374)*. Since SULTs are involved in both the metabolic activation and detoxication of HAAs and other dietary genotoxicants, as well as in maintaining hormonal homeostasis, it has been difficult to predict the impact of SULT enzymes in individual susceptibilities following exposure to cooked meat.

UDP-Glucuronosyltransferases

UDP-Glucuronosyltransferases (UGTs) catalyze the glucuronidation and elimination of numerous classes of xenobiotics, steroids, and endogenous compounds, as well as the detoxication of various carcinogens *(375-377)* (http://www.pharmacogenomics.pha.ulaval.ca/sgc/ugt_alleles/). The UGTs are present in the 1A, 2A, and 2B subfamilies and expressed in liver and extrahepatic tissues. Aromatic amines and HAAs undergo metabolism by UGTs. The UGT1A family contributes more to the metabolism of aromatic amines than does the UGT2B family *(378,379)*. 4-ABP, Bz, and *N*-acetyl-Bz were reported to be catalyzed most efficiently by UGT1A9, followed by UGT1A4, UGT2B7, and lastly by UGT1A1 *(379-382)*. Many of these isoforms are also involved in the *N*-glucuronidation of the respective arylhydroxylamines or the *O*-glucuronidation of *N*-arylhydroxamic acids *(383-386)*. The *N*,*O*-glucuronide conjugates of *N*-arylhydroxamic acids are fairly stable and are viewed as detoxication products *(40,383,387,388)*, whereas the *N*,*O*-sulfonates of *N*-arylhydroxamic acids are highly reactive species that bind to DNA and protein (Figure 3) *(389-391)*.

Depending upon the structure of the HAA and the UGT isoform, glucuronidation can occur at the exocyclic amine group or the endocyclic *N*-imidazole atom of the AIAs and the *N*-hydroxy-AIAs *(282,392-395)*. *O*-Glucuronide conjugates of ring-oxidized AIA metabolites are also prominent metabolites that are excreted in urine of rodents *(195,239)* and nonhuman primates *(196)*, but not in urine of humans *(98,103,176,264,293)*. The human UGT1A family of enzymes is principally involved in the *N*-glucuronidation of PhIP *(396-398)* and most likely MeIQx as well *(262)*. On the basis of studies with recombinant enzymes, the human UGT1A1 isoform, followed by UGT1A4, UGT1A8, and UGT1A9 are the most active enzymes involved in *N*-glucuronidation of PhIP and HONH-PhIP *(397)*; other studies reported that UGT1A9 *(399,400)* or UGT1A10 *(401)* were highly active isoforms in glucuronidation of HONH-PhIP. The N^2 atom of HONH-PhIP is the preferred site of conjugation for all of the recombinant UGTs studied, except for UGT1A9, where the *N*3 imidazole atom is the preferential site of conjugation *(401,402)*. The levels of formation of N^2-(ß-1-glucosiduronyl-2-(hydroxyamino)-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (HON-PhIP-N^2-Gl), the principal metabolite of PhIP excreted in human urine *(98,101,103,176)*, showed a high interindividual variability in formation, up to 28-fold, with human liver microsomes *(398)*. High and variable levels of UGT-catalyzed glucuronidation of HONH-PhIP were also detected with human colon microsomes, signifying that

extrahepatic UGTs, such as UGT1A10, may serve as an important enzyme of detoxication of HONH-PhIP in colon *(401,402)*.

The differential rates of UGT isoform activities reported for aromatic amines, HAAs, and their *N*-hydroxylated substrates should be viewed with caution. The discrepancy in enzyme activities observed among the different UGTs may be in part due to different systems used for screening enzyme activity: UGTs are membrane-bound and recombinant UGT-over-expressing baculosomes do not necessarily mimic activities that are observed for UGT-over-expressing cell lines *(397,401)*. Moreover, the complete activation of UGT activity in microsomal preparations requires the presence of detergents or the membrane-permeabilizing agent alamethicin *(394,402)* to overcome the latency associated with UGT-membrane bound enzymes; the assay conditions, buffers and cofactors were different in the studies cited above.

The *N*-glucuronidation of arylamines and arylhydroxylamines is viewed as a mechanism of transport of the carcinogenic intermediates, to the urinary bladder and colon (Figure 2 and Figure 6), and thought to contribute to the organotropism of aromatic amine carcinogenesis. The *N*-glucuronide conjugates of arylamines, HAAs, and their *N*-hydroxylated metabolites are eliminated in urine and bile of animal species and humans *(66,98,101,103,196,386,393,403,404)*. Arylamine and arylhydroxylamine *N*-glucuronide conjugates can undergo hydrolysis in the range of pH conditions that exist in urine *(383)*, whereas AIA and HONH-AIA *N*-glucuronide conjugates are stable *(103,275,358,393,394)*. The half-lives of the *N*-glucuronides of 4-ABP and HONH-4-ABP are 10.5 and 32 min, respectively, at pH 5.5; the half-lives of *N*-glucuronide conjugates of Bz and the *N′*-glucuronide of the HONH-*N′*-acetyl-Bz are 7.5 min and 3.5 h at pH 5.5 *(318,380,385)*. The regenerated arylamines can undergo bioactivation by P450s or peroxidases in the bladder epithelium *(238,318)*. The reactivity towards DNA of many arylhydroxylamines shows strong pH dependence: the level of DNA adduct formation at pH 5.0 is 10 to 50-fold higher than the level of adduct formed at pH 7.0 *(45)*. This enhanced reactivity at acidic pH is attributed to the formation of the nitrenium ion *(76,405)*. Thus, arylhdroxylamines that are eliminated in urine as the unconjugated metabolites, or produced by hydrolysis of the *N*-glucuronide conjugates, undergo protonation in the acidic bladder lumen, to produce reactive species that readily bind to DNA of the urothelium *(384,385)* (Figure 3).

The pH of urine has also been reported to have a strong influence on the levels of urinary Bz and its urothelial DNA adducts formed in humans: A high urine pH was inversely correlated with the proportions of free Bz, *N*-acetyl-Bz in urine of post-shift factory workers, and the average of each subject's urine pH was negatively associated with the urothelial adduct *N*-(deoxyguanosin-8-yl)-*N′*-acetylbenzidine *(332,406)*. When internal dose was controlled, subjects with a urine pH < 6 had 10-fold higher DNA adduct levels than subjects with a urine pH > 7 *(406)*. A more recent study has reported that urine pH is a risk factor for bladder cancer and a dose-response relationship in bladder cancer risk was observed with increasing urinary acidity among current smokers *(407)*. These findings are consistent with the biochemical properties of aryalmines and support a causal role of arylamines in bladder cancer. The glucuronide conjugates of HAAs, formed at either exocyclic or endocyclic nitrogen atoms of the AIA and HONH-AIA imidazole moieties are stable in weak acid *(103,275,358,393,394)*, and the reactivity of *N*-hydroxy-AIAs with DNA is not appreciably enhanced by weak acid *(339,408)*. These chemical properties may help to explain why AIAs are not bladder carcinogens in experimental laboratory animals and possibly in humans *(9)*.

The UGT metabolism of arylamines is also thought to contribute to the organotropism of aromatic amine-mediated large intestinal carcinogenesis. Studies on aromatic amines in rodents with surgically performed colostomies showed that tumors exclusively appeared

proximal to the colostomy, where the intestinal segments were in actual contact with the fecal stream *(403,409-411)*. These experiments provided strong evidence that the induction of tumors in the intestine was related to the transport of some form of the carcinogen via the bile into the intestines rather than by the blood stream. The *N*-glucuronide conjugates of arylhydroxylamines undergo hydrolysis by bacterial β-glucuronidases within the intestines, to release the arylhydroxylamine species *(66,412)*, which are bioactvated by NATs or SULTs expressed in the intestines, to form DNA adducts (Figure 6) *(22,66,339,361,413)*.

The N^2- and *N*3-glucuronide conjugates of HONH-PhIP are substrates for β-glucuronidases of *E. coli*. from the fecal flora of rodents and humans *(393)*. The liberated HONH-PhIP would be expected to form DNA adducts in colorectal tissue. However, the same level of PhIP-DNA adducts were reported to form in colon and other extrahepatic tissues of sham- and bile duct-ligated rats *(404)*, implying that the *N*-glucuronide conjugates of HONH-PhIP eliminated in bile or the blood stream are not involved in PhIP-DNA adduct formation in the colon or other extrahepatic tissues. Intestinal bacteria of rodents and humans have been reported to catalyze the reduction of HONH-PhIP back to PhIP *(393)*. Perhaps, this enzymatic reduction occurs before the HONH-PhIP (or other HONH-AIAs) in the fecal stream can reach the colonic crypt and damage DNA (Figure 6). In the rat, the bioactivated PhIP metabolites appear to be either transported from the liver through the blood circulation to extrahepatic tissues or the bioactivation of PhIP occurs directly within extrahepatic tissues *(404)*. The N^2-glucuronide conjugates of IQ, MeIQx and their *N*-hydroxylated metabolites are resistant towards the hydrolytic action of β-glucuronidases *(103,275,358,394)*. The *N*3-methyl group of these AIAs appears to sterically hinder the enzyme since the N^2-glucuronide conjugate of *N*-desmethyl-IQ is a substrate for bacterial β-glucuronidase *(275)*. Since significant interindividual variation in the *N*-glucuronidation of HAAs and HONH-HAA occurs in vitro *(398,402)* and in vivo *(98,101,103,176,264,288,289)*, it is of interest to further examine the interrelationship amongst genetic polymorphisms in UGT1A isoforms, HAA exposure, and cancer risk *(377,401,414-416)*.

Glutathione *S*-transferases and Glutathione Conjugates

The glutathione *S*-transferases (GSTs) are another important class of enzymes involved in the detoxication of many endogenous electrophiles and classes of xenobiotics, including aromatic amines and HAAs *(417)*. In humans, these enzymes are classified as Alpha, Mu, Omega, Pi, Sigma, Theta, and Zeta *(418)*. The enzymes occur as dimeric protein structures and named according to their subunit composition, for example GST A1–2 is the enzyme composed of subunits 1 and 2 in the Alpha class. The non-enzymatic reactions of GSH or other thiols also can occur with arylhydroxylamines, *N*-hydroxy-HAAs, their esterfied products, the oxidized nitroso derivatives, and in some cases, oxidized nitro-AIAs. The interaction of GSH or other thiols with arylnitroso compounds has been extensively examined. The reactions are complex and product formation is dependent on thiol concentration, pH, and substituent effects *(419-421)*. The initial product formed between the arylnitroso derivatives and GSH is a labile semimercaptal. However, the products formed by the reaction of 3-nitrosonitrobenzene and 4-nitrosonitrobenzene with GSH were sufficiently stable and characterized by NMR spectroscopy and mass spectrometry *(422)*. The short-lived semimercaptals can react in several ways as depicted in Figure 7A.

High exposures to 4-ABP result in depletion of glutathione (GSH) in liver of mice *(303)*. The depletion of GSH in primary hepatocytes, by L-buthione sulfoximine, resulted in a 15-fold increase in the formation of PhIP-DNA adducts *(423)*, and GSH depletion in vivo in rats resulted in a 5-fold increase in hepatic PhIP-DNA adducts *(404)*. An increase in the level of IQ bound to DNA also occurs in primary cultures of rat hepatocytes, following depletion of cellular GSH *(424)*. These findings show that GSH is protective against the genotoxicity of some AAs and HAAs.

The peroxidatic activity of met-Hb and H_2O_2 catalyzed the oxidation of *N*-acetyl-Bz, presumably to the reactive nitroso intermediate, which was trapped with GSH to form a stable sufinamide adduct. The GSH conjugate was characterized by electrospray ionization/mass spectrometry as *N*-(glutathion-*S*-yl)-*N*′-acetylbenzidine *S*-oxide *(425)*. The non-enzymatic reaction of GSH with the nitroso and *N*-hydroxy metabolites of AF produced the sulfinamide, *N*-(glutathione-*S*-yl)-2-aminofluorene S-oxide, and the sulfenamide, *N*-(glutathione-*S*-yl)-2-aminofluorene; analogous GSH conjugates were formed with the nitroso and *N*-hydroxy metabolites of 1-naphthylamine and 2-NA *(426)*. In rats treated with *N*-hydroxy-2-acetylaminoflourene, the two biliary conjugates were identified as 1- and 3-(glutathion-*S*-yl)-*N*-acetyl-2-aminofluorene: no S-N linked conjugates were reported *(427)*.

Sulfinamide and sulfonamide adducts were produced from the non-enzymatic in vitro reaction of the nitroso metabolite of Glu-P-1 with GSH *(428)*. Enzymatic reaction of GSTs from rat liver with the *N*-hydroxylated metabolite of Trp-P-2 produced three GSH conjugates *(429)*. One of the conjugates was found to be a more potent bacterial mutagen than HONH-Trp-P-2: the structure may have been the semimercaptal conjugate, on the basis of mass spectral data *(429)*. The structures of the two detoxicated products appear to be respectively, a sulfinamide adduct, and a stable S-C adduct that may have formed at the C-4 atom of Trp-P-2 (Figure 7B) *(429)*. GSH reaction products with the oxidized nitro derivatives of MeIQx and PhIP have been reported to form in rodent hepatocytes *(282,393)*. In these reactions, the thiol group of GSH displaced the nitro moieties, by direct nucleophilic substitution, to form 2-(glutathion-*S*-yl)-3,8-dimethylimidazo[4,5-*f*]quinoxaline *(282)* and 2-(glutathion-*S*-yl)-1-methyl-6-phenylimidazo[4,5-*b*]pyridine *(393)*; the GSH conjugate of NO_2-PhIP was also detected in rat bile and suggests that NO_2-PhIP formation occurs in vivo *(393)*. The S-C linked GSH reaction products with NO_2-MeIQx and NO_2-PhIP can form non-enzymatically (Figure 7C).

The effects of GSH and of purified human and rat GSTs on the covalent DNA binding of the reactive *N*-acetoxy derivatives of PhIP, IQ, and MeIQx, were studied in vitro. GSH alone slightly inhibited (10%) the binding of *N*-acetoxy-PhIP to DNA, but the binding was strongly inhibited in the presence of both GSH and GSTs. Among human GSTs, the isozyme A1-1 was most effective (90% inhibition), followed by A1-2 (40% inhibition), and P1-1 (30% inhibition); other GSTs studied appeared to have little to no activity towards *N*-acetoxy-PhIP *(430,431)*.

Analysis of the incubation mixture containing *N*-acetoxy-PhIP, GSH and GST A1-1 revealed the presence of oxidized GSH (GSSG) and reduced PhIP (Figure 7D), but no GSH adducts were detected, suggesting a redox mechanism is involved in the deactivation of *N*-acetoxy-PhIP. A short-lived GSH sulfenamide conjugate of PhIP may have formed and undergone an ensuing reaction with GSH to produce PhIP and GSSG *(430)*. GST P1-1 showed even higher substrate specificity for the inhibition of DNA binding of ATP-dependent metabolite(s) of HONH-PhIP than for *N*-acetoxy-PhIP *(432)*. The binding of *N*-acetoxy-IQ or *N*-acetoxy-MeIQx to DNA was unaffected by human or rat GSTs; however, GSH alone significantly inhibited (40%) their binding to DNA *(430)*.

The GST-dependent detoxication pathway may be an important determinant for the organ specificity of PhIP-carcinogenesis in rodents and possibly humans *(430,431)*. Human liver cytosol, which contains high levels of GST A1-1, catalyzes the GST-mediated detoxication of *N*-acetoxy-PhIP *(430)*, whereas the cytosol of colon, which contains about 100-fold lower levels of the GST A1-1 subunit than the liver *(433)*, does not display GST-mediated inhibition of *N*-acetoxy-PhIP binding to DNA *(430)*. The high levels of hepatic GST A1-1 activity may help to explain the lower levels of PhIP-DNA adduct formation in liver in comparison to pancreas or colorectal tissue of rats *(340,404)*. In humans, a polymorphism in

the 5′-regulatory region of *GSTA1* gene results in the diminished expression of the GSTA1 and GSTA2 subunits *(434)*. In two case control studies, individuals who possess the homozygous single nucleotide polymorphisms *hGSTA1*B* (*B/*B*) genotype and who would be predicted to have the lowest levels of GSTA1 expression in liver, were at a greater risk for developing colorectal cancer, especially among consumers of well-done cooked meat, than subjects with the homozygous *hGSTA1*A* (*A/*A) genotype and express high levels of GSTA1 *(431,435)*. Individuals who are homozygous *GSTA1*B*, could be at risk of developing colorectal cancer, possibly as a result of inefficient hepatic detoxication of *N*-oxidized derivatives of PhIP *(431)*. However, a third case control study failed to detect an elevated risk for colorectal cancer in subjects harboring the (*B/*B) genotype *(436)*. Urinary mercapturic acid conjugates of PhIP, if formed, could serve as biomarkers to assess the efficacy of detoxication of PhIP by GSTs. Thus far, mercapturic acid conjugates of PhIP or other HAAs have not been identified in urine of experimental laboratory animals or humans.

Biomonitoring Aromatic Amines, HAAs, and their Metabolites in Human Urine

There are only a few reports on the direct chemical analyses of carcinogenic arylamine metabolites in human urine *(406,437-440)*. On the basis of metabolism studies in experimental laboratory animals *(44,441)*, in vitro with human liver slices *(318)*, and in vivo in humans *(406,437-440)*, arylamine metabolites can be grouped according to: *(a)* a substitution on the amino group by acylation (acetylation or formylation) or by conjugation with sulfate or glucuronic acid, ***(b)*** *N*-oxidation, *(c)* ring oxidation, followed by sulfation or glucuronidation, and *(d)* in some instances, mercapturic acid formation. The analysis of carcinogenic aromatic amines in human urine has been done primarily by gas chromatography with electron capture detection or negative ion chemical ionization mass spectrometry (GC-NICI-MS), following chemical derivatization *(442-444)*. The procedures employed for the isolation of aromatic amines from urine generally include acid or base hydrolysis, followed by organic solvent extraction and/or solid phase extraction. Thus, the amount of aromatic amine measured represents the unmetabolized compound plus the Phase II conjugates. In one study, smokers were reported to have 1.5 to 2-fold higher levels of 2-NA, 4-ABP and *o*-toluidine in their urine than non-smokers: up to 204 ng *o*-toluidine, 21 ng of 2-NA and 15 ng of 4-ABP present in urine of smokers collected over 24 h *(445)*.

There is one report on the detection of of *N*-acetyl-4-ABP and the *N*-glucuronide of 4-ABP in urine of smokers, by liquid chromatography-electrospray ionization/tandem mass spectrometry (LC-ESI/MS/MS) methods *(440)*. In that study, the geometric mean (95% CI) of the total 4-ABP concentration was 1.64 pg/mg creatinine (1.30–2.07) in nonsmokers (N = 41), and significantly greater, at 8.69 pg/mg creatinine (7.43–10.16) in smokers (N = 89) ($p < 0.001$). Other studies reported no major differences in the excreted levels of 2-ABP and 4-ABP in urine between smokers, passive smokers and nonsmokers *(444)*, or in the levels of aniline and *o*-toluidine in smokers as opposed to non-smokers urine *(442)*. Significantly higher concentrations of aniline, *o*-toluidine, *m*-toluidine, 2-NA, and 4-methyl-1,3-phenylenediamine were detected in the urine of factory workers who smoked than in urine of nonsmoking factory workers, and there was a significant increase in the renal excretion of unaltered 4-chloroaniline and *m*-toluidine in slow *N*-acetylators as opposed to rapid *N*-acetylators among the smoking workers, indicating NAT enzymes are involved in the detoxication of these chemicals *(446)*. Aniline, *p*-toluidine, 2-NA, and 4-chloro-*o*-toluidine were also detected in the urine of nonsmoking subjects who were not occupationally exposed to aromatic amines *(446)*. Another study reported up to 50-fold higher levels or *o*-toluidine in occupationally exposed individuals than in non-occupationally exposed subjects *(111)*. Certain aromatic amines have been observed to undergo decomposition in urine within a few hours and may explain why some arylamines have been difficult to detect in

urine *(444)*. In contrast to some arylamines, MeIQx, PhIP, and AαC are stable in the urine matrix *(447)*.

Various analytical approaches have been devised to isolate HAAs from human urine: such techniques have included solvent extraction *(24,301)*, solid-phase enrichment (SPE) *(447)*, treatment of urinary HAAs with blue cotton and ion exchange chromatography *(448)*, the use of molecularly imprinted polymers *(292)*, and immunoaffinity methods *(286)*, followed by quantification by GC-NICI-MS *(24,301,449)*, or LC-ESI/MS/MS *(292,447)*, or alternatively, followed by HPLC with UV or fluorescence detection *(291,448)*. [^{14}C]-MeIQx, [^{14}C]-PhIP, and their [^{14}C]-radiolabeled metabolites have also been measured in human urine by accelerator mass spectrometry (AMS) *(176,264,287)*. Urinary metabolites have also been detected by LC-ESI/MS/MS *(98,101,103,293)*, or indirectly, after chemical reduction or acid hydrolysis of HONH-PhIP conjugates, with detection by LC-ESI/MS/MS or GC-NICI-MS *(289,450)*.

Most of the studies that have examined HAA biomarkers were conducted with subjects on a controlled diet, eating well-done cooked meat *(252,285,291,451-454)*. However, there are reports on the identification of HAAs, including MeIQx, PhIP, APNH, AαC, in urine of subjects on a free-choice diet *(448,453,455-457)*. In the case of AαC, urinary levels of this carcinogen were associated with tobacco usage and not meat consumption *(456)*. Many of the early biomonitoring studies focused on MeIQx and PhIP because they are the two most mass-abundant HAAs formed in cooked meat *(10)*. The metabolism pathways of pyrolytic HAAs in humans are unknown *(458)*. The plasma half-life of MeIQx was estimated at 3.4 h and the plasma half-life of PhIP was estimated at 4.6 h in humans *(301)*. These short half-lives are consistent with the rapid elimination of MeIQx and PhIP in urine after consumption of cooked meat *(285,286,291,301,452,454,459)*. The metabolism of both HAAs is extensive. The amounts of non-metabolized MeIQx ranges from about 1 – 6% of the dose, whereas the amount of unaltered PhIP in 0 – 24 h post-meal urine ranges from about 0.5 to 2% of the dose *(93,285,291,452,454,459)*. In one study in Japan, MeIQx, PhIP, and the tryptophan pyrolysate mutagens Trp-P-1 and Trp-P-2 were detected in the urine of healthy volunteers on a normal diet, but they were not found in urine of hospitalized patients receiving parenteral alimentation *(448)*. This finding shows that the exposure to HAAs occurs from food and that these compounds are not formed endogenously. However, APNH, the reaction product formed from norharman and aniline in the presence of P450 3A4 or 1A2 *(460)*, was detected in 24-h urine samples at levels ranging from 21 to 594 pg in subjects on a non-restricted diet; similar levels were measured in urine from inpatients receiving parenteral alimentation *(457)*. These results suggest that APNH is a novel endogenous mutagen/carcinogen; the biological significance of this rodent carcinogen for human cancer development requires further study.

Widely ranging concentrations of HAAs have been detected in urine of individuals on unrestricted diets evaluated world-wide; such differences are probably attributable to variability in the concentrations of HAAs in the diet *(448,453,455,456)*. Some biomonitoring studies have examined the amount of MeIQx and PhIP recovered in urine following acid hydrolysis. The hydrolysis of urine provides an estimate of the contribution of phase II conjugation to the metabolism of these AIAs *(103,285,455,461)*. Acid treatment (1 N HCl, 80 °C for 8 h) increased the levels of MeIQx in urine by as much as 18-fold *(103,285,453,454)*, while the amounts of PhIP generally increased by only several fold or less *(103,285)*. The increase in MeIQx content is attributed to the acid-labile MeIQx-N^2-SO_3H and MeIQx-N^2-Gl conjugates present in urine *(285)*, whereas the acid-labile N^2- and *N*3-glucuronide conjugates of PhIP make up only a very minor percentage of the urinary metabolites of PhIP and explains the modest increase in the amounts of PhIP in urine, following acid treatment *(98,103,176)*.

Interindividual differences in the urinary excretion of MeIQx and PhIP have been reported in subjects on controlled diets *(452)*. Metabolic phenotypes may be expected to influence the levels of HAAs excreted in urine. For example, higher P450 1A2 activity was associated with significantly lower levels of unmetabolized MeIQx in the urine of omnivores (P = 0.008), when adjusted for the amount of meat eaten *(459)*. However, the levels of PhIP in urine were not associated with P450 1A2 activity *(286)*, a finding that is surprising since the contribution of P450 1A2 to the clearance of PhIP was estimated to account for 70% of the elimination of PhIP in a pharmacokinetic study *(301)*. The contribution of P450 1A2 to the metabolism of PhIP may have been obscured since GSTs reduce *N*-oxidized metabolites of PhIP back to the parent amine *(430,434)*.

N-Oxidation is an important biotransformation pathway of MeIQx and PhIP in humans. The levels of the urinary N^2-glucuronide conjugate of HONH-MeIQx were reported to range from 2 – 17% of the ingested dose of MeIQx *(288)*, but the major urinary metabolite of MeIQx, the carboxylic acid, IQ-8-COOH, which is also produced by P450 1A2 *(261,262)*, ranged from 32 to 65% of the ingested dose *(103)*. The N^2- and *N3*-glucuronide metabolites of HONH-PhIP account for up to ~24 – 54% of the ingested dose of PhIP in urine within 24 h *(176,287,289)*. The large variation in the urinary levels of IQx-8-COOH and HONH-MeIQx and HONH-PhIP *N*-glucuronide conjugates is likely due to the wide range of P450 1A2 content expressed in liver *(222,242)*, combined with varying levels of UGT activity, and other competing pathways of metabolism *(264,287-289,402)*.

The urinary level of the N^2-glucuronide conjugate of HONH-MeIQx did not correlate to P450 1A2 activity (N = 66 subjects), whereas the level of the N^2-glucuronide conjugate of HONH-PhIP did correlate to P450 1A2 acitivity, when caffeine was employed as the metabolic probe for P450 1A2 phenotyping *(288,289)*. The pathway of IQx-8-COOH formation *(261,262,264)*, which was discovered after these metabolism studies were completed *(288,289)*, is a competing reaction pathway of MeIQx-*N*-oxidation and may have obscured the relationship between HON-MeIQx-N^2-Gl and P450 1A2 activity. The interindividual variability in enzymatic reduction of the *N*-hydroxy-HAAs *(430,462,463)* is likely to contribute to the variability of urinary excretion of *N*-glucuronide conjugates of HONH-MeIQx and HOHN-PhIP and weaken the association between these HAA urinary biomarkers and P450 1A2 activity. There was no evidence for an inverse association between NAT2 phenotype activity and the amounts of HON-MeIQx-N^2-Gl or HON-PhIP-N^2-Gl excreted in urine *(288,289)*; a finding that is consistent with the poor rates of *N*-acetylation of MeIQx and PhIP by NAT2 *(335,339)*. In a pilot study, individuals with a rapid P450 1A2 phenotype and who excreted high levels of HONH-PhIP-N^2-Gl urine had the lowest level of colon PhIP-DNA adducts *(176)*. Thes data indicate that *N*-glucuronidation plays an important role in the detoxication of HONH-PhIP *(176,400,402,464)*. Definitive conclusions from a small data set (N = 10 subjects) are tenuous and further investigations using a much larger study group should be pursued to confirm the protective role of UGT enzymes and the usefulness of the HONH-PhIP-N^2-Gl urinary biomarker in HAA carcinogenesis studies.

The concurrent analysis of MeIQx and PhIP is important since the urinary excretion levels of either HAA by themselves can serve only as an approximate measure for the other, in assessing exposures in humans consuming unrestricted diets *(455)*. The interrelationship between the oxidative metabolism of MeIQx and that of PhIP in urine samples from 10 volunteers on a controlled diet was examined by calculation of the urinary metabolic ratio (MR) (% dose of urinary metabolite/% dose of unmetabolized urinary HAA) values for several of their P450 1A2-catalyzed oxidation products *(103)*. The employment of MR values to assess enzyme metabolizing activity must be done with caution because MR values can be influenced by changes in urine flow rate *(465)*. The extent of MeIQx and PhIP

metabolism and the MR for IQx-8-COOH and HON-PhIP-N^2-Gl and HON-PhIP-*N*3-Gl, the major P450 1A2 oxidative urinary metabolites of MeIQx and PhIP, were significantly correlated for a given subject (Figure 8). The MR values were independent of urine flow rate and support the notion that P450 1A2 is an important enzyme in the metabolism of both procarcinogens in vivo *(103)*. A study on a larger number of subjects will be required to firmly establish the MR values and the inter-relationship between P450 1A2-mediated metabolism of MeIQx and that of PhIP.

Aromatic Amine and HAA DNA Adducts

Some of the early structural characterization of arylhydroxylamine DNA adducts were reported by Kriek *(466)* and by King and Phillips *(467)*, who proposed that covalent linkage should occur between C8 atom of dG and the arylamine nitrogen of *N*-hydroxyaminofluorene. The structural assignments were later confirmed through the use of nuclear magnetic resonance spectroscopic techniques *(468)*. The characterization of many other arylamine DNA adducts followed these studies: both acetylated and non-acetylated adducts were identified in vitro and in vivo *(37,76,469,470)*.

Synthesis and Characterization of DNA Adducts

The structures of a number of the arylamine DNA adducts were originally obtained by reacting the synthetic arylhydroxylamine derivatives with DNA, followed by enzymatic digestion and spectroscopic characterizations *(37,76,469,470)*. The reactivity of many arylhydroxylamines towards DNA is enhanced under slightly acidic pH as opposed to neutral pH; this increase in reactivity has been ascribed to the formation of the aryl nitrenium ion *(76,405)*. In contrast to arylhydroxylamines, the chemical reactivity of a number of *N*-hydroxy-HAAs with DNA is only modestly enhanced under acidic pH conditions and alternative reaction conditions were employed to produce the presumed nitrenium ion *(339,408,471)*. Many arylhydroxylamines and *N*-hydroxy-HAAs primarily bind to dG at the C8 atom of guanine. However, this site is only weakly nucleophilic, and the dG-C8 adducts (and presumably dA-C8 adducts) have been proposed to be rearrangement products that are preceded by electrophilic substitution at the nucleophilic *N*7 atom of dG. This scheme has been postulated to be a general reaction for activated aromatic amines and HAAs *(472,473)*. Minor reaction products of arylhydroxylamines and *N*-hydroxy-HAAs are also formed at the N^2 atom of dG and the C8 and N^6 atoms of dA *(37,474-476)*. The structures of prominent arylamine and HAA adducts are shown in Figure 9.

DNA adducts of HAAs have been synthesized by biomimetic reactions of the *N*-hydroxy-HAA intermediateswith deoxynucleosides or DNA, in the presence of ketene gas, or acetic anhydride, so as to produce the reactive *N*-acetoxy intermediate and facilitate the formation of the nitrenium ion *(63,339-341,408,471,474,475,477,478)*. *N*-Acetoxy-IQ and *N*-acetoxy-MeIQx *(339,408)* have lifetimes of seconds or less, but *N*-acetoxy-PhIP has been isolated and characterized by mass spectrometry *(341)*. The imidazo moiety of AIAs may facilitate the formation of the oxime tautomer and influence the chemical reactivity of *N*-hydroxy-AIAs with DNA. The oxime structure favors *O*-acetylation of the *N*-hydroxy-AIAs by acetic anhydride, to produce the *N*-acetoxy intermediates, instead of *N*-acetylation to form the hydroxamic acids. The dG-C8 adducts of IQ *(408,474,479)*, MeIQ *(170)*, MeIQx *(474)*, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx) *(477)*, PhIP *(340,341,480)*, AαC and 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC) *(478,481)*, and the glutamic acid and tryptophan pyrolysate mutagens *(471,482)* are formed by biomimetic reactions; these DNA adducts also occur in tissues of experimental laboratory animals (see above citations, and *(63,100)*, and references therein) (Figure 9).

Isomeric dG-N^2-adducts of IQ and MeIQx are also formed in vitro and in vivo; adduct formation occurs at the C-5 atoms of the heteronuclei of these HAAs *(474,483-485)*. Recently, an hydrazine-linked *N*7-dG adduct with IQ, and a dA adduct of IQ formed in vitro by reaction of dG or dA with *N*-acetoxy-IQ was reported *(475)*, and a dA adduct of MeIQx was also detected in liver of rats *(476)*. Bond formation within these dA adducts is believed to occur between the N^6 atom of adenine and the C-5 atom of the IQ or MeIQx heteronucleus to form 5-(deoxyadenosin-N^6-yl)-IQ (dA-N^6-IQ) or 5-(deoxyadenosin-N^6-yl)-MeIQx (dA-N^6-MeIQx). The formation of dG-N^2 and dA-N^6 adducts of IQ and MeIQx, indicate that both nitrenium and carbenium ion resonance forms exist for these activated HAAs *(474,475)*.

The overall yield of DNA adducts formation with deoxynucleosides or DNA with arylhdroxylamines or *N*-hydroxy-HAAs are generally several percent or lower. More recently, non-biomimetic approaches have been developed to synthesize aromatic amine- and HAA-DNA adducts in high yields, using the Buchwald–Hartwig reaction of cross-coupling of primary and secondary amines with aryl halides. High-yield synthesis of dG and dA adducts of arylamines *(486)*, dG-C8 and dG-N^2 adducts of IQ, and the dG-C8 adduct of PhIP (dG-C8-PhIP) *(487-490)* have been achieved with this chemistry. The phosphoramidites of these adducts have been site-specifically incorporated into oligonucleotides to explore the effect of adducts in perturbations of DNA structure, and the fidelity of polymerases during translesional synthesis *(42,491-500)*. The results demonstrated that each arylamine-DNA or HAA-DNA adduct structure and the location of the adduct within the sequence context of the oligonucleotide affected the solution structure of the DNA and the fidelity and the catalytic efficiency of the polymerases in a unique manner.

Aromatic amine and HAA DNA Adduct Formation in vitro and in Experimental Animal Models

The early investigations on the measurements of arylamine-DNA adducts in experimental animals employed tritium-labeled carcinogens. Adduct identification was achieved by HPLC with radiometric detection and by co-chromatography with unlabeled DNA adducts, which served as UV standards *(37,76,469)*. More recent methods to detect and quantitate arylamine- and HAA-DNA adducts include: ^{32}P-postlabeling *(501,502)*; immunohistochemistry (IHC) *(503,504)*; GC-NICI-MS of alkaline-treated DNA, a technique that cleaves the bond between the guanyl C8 atom and the amino group of aromatic amines or HAAs *(169,505)*; accelerator mass spectrometry (AMS) for the detection of tritiated or ^{14}C-labelled adducts *(89,166,506)*, and LC-ESI-MS/MS methods *(100,480,507-513)*.

Five DNA adducts of 4-ABP are formed, when HONH-ABP is reacted with calf thymus DNA at pH 5.0 *(76)* (Figure 9). *N*-(Deoxyguanosin-8-yl)-4-ABP (dG-C8-4-ABP) is the principal adduct and accounts for 80% of the total adducts formed, followed by dA-C8-ABP (15% of total adducts) and then *N*-(deoxyguanosin-N^2-yl)-4-ABP (dG-N^2-N^4-4-ABP) (~5% of the total adducts). Two other minor dG adducts have been identified: 3-(deoxyguanosin-N^2-yl)-4ABP (dG-N^2-4-ABP) and *N*-(deoxyguanosin-N^2-yl)-4-azobiphenyl *(514,515)*. In urothelial cells of male Beagle dogs *(37)*, dG-C8-4-ABP accounted for 76% of the total binding, followed by dG-N^2-N^4-4-ABP (15%), and then followed by dA-C8-4-ABP (9%), 2 days after the oral administration of [^{3}H]-4-ABP *(37)*. DNA adducts of 4-ABP were quantified, by ^{32}P-postlabeling and immunohistochemistry (IHC), in liver and bladder of male and female BALB/c mice, following treatment with 4-ABP at a range of concentrations (from 0, 7 up to 220 ppm) in the drinking water for 28 days *(516)*. The principal adduct in both tissues, for both sexes, was dG-C8-4-ABP. The level of adduct formation increased as a function of dose and correlated with the incidence of liver tumors, in female mice. However, the relationship between adducts and tumorigenesis was distinctly

nonlinear in the bladders of male mice, and tumor incidence rose rapidly at doses above the 50 ppm dose 4-ABP. Toxicity and cell proliferation may have increased the tumor incidence in the bladder.

The reaction of *N*-hydroxy-2-aminonaphthalene (HONH-2-NA) with DNA in vitro, at pH 5.0, results in formation of three DNA adducts (Figure 9) *(76)*. The major adduct is an imidazole ring-opened derivative of *N*-(deoxyguanosin-8-yl)-2-NA (dG-C8-NA, 50% of the total adducts), followed by lower levels of 1-(deoxyguanosin-N^2-yl)-2-NA (dG-N^2-NA, 30% of total adducts), and 1-(deoxyadenosin-N^6-yl)-2-NA (dA-N^6-NA, 15% of total adducts). These same three DNA adducts were formed in target (urothelium) and nontarget (liver) tissues of dogs 2 days after oral administration of [^{3}H]-2-NA *(37)*. A 4-fold higher binding level of 2-NA was found in the urothelial DNA than formed in liver DNA. The major adduct in both tissues was the ring-opened dG-C8-NA, followed by lower levels of dA-N^6-NA and dG-N^2-NA. The dG-N^2 adduct persisted in the liver, and this adduct and the ring-opened dG-C8-NA adduct persisted in the bladder. The differential loss of adducts indicates that active repair processes are present in both tissues. The relative persistence of the ring-opened dG-C8-NA adduct in the target but not in the nontarget tissue suggests that this adduct is a critical lesion for the initiation of urinary bladder cancer.

Peroxidative enzymes, such as PHS, catalyze both the *N*-oxidation and ring-oxidation of 2-NA; 2-amino-1-naphthol is a major ring-oxidation product *(319)*. PHS catalyze the binding of 2-NA to DNA and produced the same three adducts arising from *N*-hydroxy-2-NA (*vide supra*). Three other adducts were also formed from 2-imino-1-naphthoquinone, the oxidative product of 2-amino-1-naphthol. The major adduct is N^4-(deoxyguanosin-N^2-yl)-2-amino-1,4-naphthoquinoneimine (dG-N^2-NAQI) (Figure 9) *(37,319)*. This adduct and two other minor adducts, accounted for approximately 60% of the total DNA binding that was obtained by incubation of 2-NA with PHS in vitro. The DNA adducts derived from 2-imino-1-naphthoquinone were reported to account for approximately 20% of the 2-NA bound to urothelial DNA in dogs, but these peroxidative DNA adducts were not detected in liver DNA *(319)*. The remaining adduction products in urothelium were derived from HONH-2-NA. PHS expressed in the bladder could play a significant role in bioactivation of 2-NA directly in the bladder, and could contribute to carcinogenesis of 2-NA and other arylamines that serve as substrates of PHS.

The reaction of calf thymus DNA with HONH-*N*′-acetylBz at pH 5 gives rise to *N*-(deoxyguanosin-8-yl)-*N*′-acetylbenzidine (Figure 9) *(517,518)*. The structural isomer, *N*-(deoxyguanosin-8-yl)-*N*-acetylbenzidine and the nonacetylated derivative, *N*-(deoxyguanosin-8-yl)-benzidine, have not been identified in rat or mouse liver DNA *(518)*. However, benzidine diimine, a reactive intermediate formed during the enzymatic peroxidation of Bz, can undergo deprotonation of the cationic diimine to form its nitrenium ion, which reacts with dG to form *N*-(deoxyguanosin-8-yl)-benzidine. This adduct was detected in vivo in the dog urothelium *(519)*.

The reaction of HONH-MOCA in vitro, at pH 5 or 7, with DNA produces *N*-(deoxadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol and *N*-(deoxadenosin-8-yl)-4-amino-3-chlorotoluene as the major adducts (Figure 9); these lesions are also formed in rat liver *(520)* and in dog urinary bladder epithelium *(521)*, following treatment with MOCA. The preferred reactivity of MOCA with dA is atypical of most AAs and HAAs, which primarily react with dG *(37,63,522)*. The chemistry of MOCA-DNA adduct formation is also unusual: the adducts contain only a single ring derived from MOCA. The incipient DNA adduct formed appears to undergo fission at the methylene bridge of MOCA to form *N*-(deoxadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol or *N*-(deoxadenosin-8-yl)-4-amino-3-chlorotoluene *(520)* (Figure 9).

dG-C8 adducts of monocyclic alkylanilines as well as 2- and 4-chloroaniline are also formed by reaction of the corresponding *N*-(acyloxy)arylamines with dG and DNA *(523,524)*. A study with 3,5-DMA revealed that this arylamine forms a C8 adduct with dG but also forms adducts at the N^6 and C8 atoms of dA, and a unique adduct with dC *(525,526)*. The formation of these DNA adducts are consistent with the involvement of nitrenium ion chemistry. It is noteworthy that several monocyclic arylamines have been reported to form sulfinamide adducts with Hb in rodents, but dG-C8 or other DNA adducts attributed to the nitrenium ion were not detected in liver or extrahepatic tissues *(524,526)*. Therefore, biologically available *N*-hydroxy-AAs that form Hb adducts do not necessarily produce DNA adducts *(524)*. The quinone imine or quinone methide electrophiles may contribute to the DNA damage and adduct formation of monocyclic alkylanilines *(123)*.

The doses of HAAs employed in many experimental laboratory animal studies exceeded daily human exposures by more than a million-fold; many of these studies are reviewed in *(63,100)*. In early studies, several variants of the ^{32}P-postlabeling method *(501,527)*, followed by DNA adduct separation by 2-dimensional thin layer chromatography or HPLC *(63,479,528,529)*, were used to discern HAA-DNA adducts. A myriad of lesions were detected in a number of these DNA binding studies; many of the adduction products were subsequently shown to be incompletely digested oligomers of the dG-C8-HAA adducts *(167,479,530)*. For many HAAs, DNA adduct formation is greatest in the liver of rodents, a result perhaps attributable to the high levels of P450 1A2 expression *(63)*. However, DNA adducts are formed in all tissues investigated, even in tissues that do not develop cancer, indicating other factors, such as tumor promotion, are involved in tumorigenesis *(9,159)*.

DNA adduct formation of MeIQx *(166)* and IQ *(167)* was found to occur in a near linear dose-response relationship in liver of rodents over a wide range of doses. DNA adducts were formed at dose levels approaching human exposures for both of these AIAs, as well as for PhIP *(531)*. In contrast to many other HAAs, PhIP shows levels of adduct formation in rodents that are lower in liver than in extrahepatic tissues; adduct levels are particularly elevated in colon and pancreas *(340,404)*, and in prostate of male rats *(155)*, and mammary glands of female rats *(532)*. Both GSTs and UGTs, which are expressed at high levels in the liver, catalyze the detoxication of reactive PhIP metabolites *(397,402,430,464)*, thus accounting for the relatively lower level of PhIP–DNA adduct formation in the liver.

The isomeric dG-N^2 adducts of IQ and MeIQx are minor adducts formed in vitro with the *N*-acetoxy-IQ and *N*-acetoxy-MeIQx (<10% of total adducts) *(474)*, but their contribution to the total amount of DNA adducts formed in rodents is greater *(479,483,485,533)*. The dG-N^2-IQ adduct became the prominent lesion in slowly dividing tissues of non-human primates that underwent chronic treatment with IQ *(483)*, suggesting preferential repair of the dG-C8-IQ adduct. The contribution of dG-N^2-MeIQx to the total adducts in rats was significantly more important than that observed in vitro when calf thymus DNA was reacted with *N*-acetoxy-MeIQx *(485)*. dG-N^2-MeIQx was the major adduct detected in liver of rats 24 h after gavage with a 0.5 mg/kg dose. Thus, isomeric dG-N^2-AIA adducts are prominent lesions formed in slowly dividing tissues rodents and non-human primates, particularly during chronic exposure.

DNA Adduct Formation of Aromatic Amines and HAAs in Human Tissues

The analyses of DNA adducts from human tissues have often been conducted on biopsy samples of patients that were obtained during clinical diagnosis of cancer *(27,534)*. The DNA adducts formed are likely attributed to recent exposures; however, the most relevant time to measure DNA adduct formation is when tumor initiation is in progress and not many years later when the cancer has been diagnosed *(27,32)*. Hence, the assumption made is that current adduct levels are reflective of the levels that existed during the time of cancer

initiation. This assumption may be valid for inviduals subjected to long-term habitual exposure to genotoxic agents, such as those exposures that occur through smoking or by frequently consuming well-done cooked meats; however, only few studies have investigated the variations in DNA adduct levels in individuals over time *(27,535)*.

dG-C8-4-ABP was first detected by ^{32}P-postlabeling in human urinary bladder tissue biopsy samples and exfoliated uorothelial cells *(28,536)*. Subsequently, the adduct was detected by GC-NICI-MS methods in lung and urinary bladder mucosa; dG-C8-4-ABP was found at levels ranging from <0.32 to 49.5 adducts per 10^8 nucleotides in the lung, and from <0.32 to 3.94 adducts per 10^8 nucleotides in bladder samples *(505)*. dG-C8-4-ABP has also been detected, by IHC, ^{32}P-postlabeling or GC-NICI-MS methods in bladder and lung tissues from smokers and ex-smokers *(502)*, and by IHC in the liver of Taiwanese subjects with hepatocelluar carcinoma *(537)*. In pancreas tissue, a major adduct was observed that was chromatographically identical to dG-C8-4-ABP in eight of 29 organ donors, at levels ranging from 0.2 to 1.1 adducts per 10^8 nucleotides. Pancreas tissue displays low enzyme activities for P450-mediated *N*-oxidation and prostaglandin hydroperoxidation of 4-ABP, but high levels of 4-nitrobiphenyl reductase and NAT1-mediated *O*-acetyltransferase activity are present *(538)*. The dG-C8-4-ABP adduct levels in pancreas did not correlate with the number of cigarettes smoked per day or the length of smoking history; other sources of environmental exposure to 4-ABP or the exposure to 4-nitrobiphenyl or other nitroarenes, produced during combustion *(122)*, may contribute to arylamine-DNA adduct formation in pancreas.

The DNA present from the induced sputum of smokers, representing DNA of the lower respiratory tract, was shown to possess significantly higher levels of 4-ABP-DNA adducts than were found in the sputum of non-smokers, when assessed by IHC *(539)*. 4-ABP-DNA adducts were also detected in female breast tissue biopsy samples, when visualized by IHC *(504)*; the levels of 4-ABP-DNA in tumor-adjacent normal tissues, but not in tumorigenic tissue, were correlated to the frequency of women's smoking. 4-ABP-DNA adducts were also detected in laryngeal biopsies by IHC *(540)*, and adduct levels were significantly higher in smokers than were the levels measured in tissue from non-smokers.

The putative dG-C8 adduct of 4-ABP, measured as 4-ABP after acid treatment of DNA, was detected in biopsy samples from 37 out of 75 bladder cancer patients, corresponding to 86 ± 22 adducts per 10^8 nucleotides (Mean ± SE). The amount of 4-ABP-DNA adducts in the bladder of current smokers was elevated in subjects with more aggressive grade levels of bladder tumors *(541)*. In another study, the putative dG-C8 adducts of 4-ABP and *o*-toluidine were measured as the parent amines, after acid treatment of DNA from epithelial and submucosal bladder tissue of bladder cancer patients: 4 and 11 of 12 tumour samples contained adducts of 4-ABP (1.9 ± 4.1 adducts per 10^8 nucleotides) and *o*-toluidine (2.9 ± 1.5 adducts per 10^6), respectively *(542)*. The levels of the putative dG-C8 adduct *o*-toluidine, but not 4-ABP, were significantly higher in epithelium of smokers than in non-smokers. The detection of high levels *o*-toluidine-releasing DNA adducts is suggestive of a causal role of *o*-toluidine in the human bladder cancer.

LC-ESI-MS/MS methods have been employed to directly quantitate dG-C8-4-ABP in human tissues. dG-C8-4-ABP was detected in urinary bladder epithelium in 12 out of 27 subjects in DNA extracted from tumor tissue or non-tumor surrounding tissue *(543)*. The levels of adducts ranged from 5 to 80 adducts per 10^9 bases, but a correlation was not observed between tobacco smoking and adduct levels *(543)*. In another pilot study, dG-4-C8-ABP was identified, by LC-ESI-MS/MS, in six of 12 human pancreas samples *(512)*. The levels ranged anywhere from 1 to 60 adduct per 10^8 nucleotides; again, there was no correlation observed between the level of adducts, and smoking preference, age, or gender.

The prediction of the relationship between 4-ABP exposure from tobacco smoke and adduct levels is not straightforward, being confounded by environmental exposure to 4-nitrobiphenyl and a variable persistence of dG-C8-4-ABP in the tissues. Because the activation or detoxification processes of 4-ABP metabolism, as well as DNA repair mechanisms, can be tissue-specific, a correlation between tobacco usage and DNA adducts in different tissues may not exist.

Another environmental source of exposure to 4-ABP is hair dyes *(120,121)*. The relationship between 4-ABP-DNA adduct levels and hair-dye usage has only been examined in one study, which determined the levels of the putative 4-ABP-DNA adduct, by ^{32}P-postlabeling, in exfoliated breast epithelial cells in milk from lactating mothers *(544)*. The adduct levels were associated with the use of hair coloring products (odds ratio 11.2), but not with tobacco usage, in a statistically significant manner. Some commercial permanent hair dyes are known to contain 4-ABP *(120,121)*.

The presumed *N*-(deoxyguanosin-8-yl)-*N*′-acetylbenzidine adduct was identified by ^{32}P-postlabeling of DNA from exfoliated urothelial cells of workers in factories manufacturing Bz in India *(332)*. This finding supports the hypothesis that *N*-acetylation and the ensuing formation of HONH-*N*′-acetyl-Bz is an important bioactivation pathway for at least one Bz-related adduct in humans *(318)*; this bioactivation pathway is analogous to the pathway proposed for Bz activation in rodents *(518)*. Moreover, the same Bz adduct was identified in white blood cells of exposed workers, and there was a significant correlation between WBC and exfoliated urothelial cell Bz adduct levels *(545)*. This was the first study in humans to show a relationship for a specific carcinogen adduct in a surrogate tissue and in urothelial cells, the target for urinary bladder cancer of Bz.

The major DNA adduct isolated from exfoliated urothelial cells collected from urine of a subject after an accidental acute exposure to MOCA, was detected as *N*-(deoxadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol, by the ^{32}P-postlabeling method *(546)*. The adduct was found in cell samples obtained between 4 and 98 h after initial exposure but not in samples collected at later times. The level of DNA adducts 4 h after exposure was determined to be 516 adducts/10^8 nucleotides.

The putative dG-C8-MeIQx adduct was detected in the colon and kidney of some individuals at levels of several adducts per 10^9 DNA bases, when assayed by ^{32}P-postlabeling *(547)*. A GC/MS assay, following hydrolysis of putative dG-C8-HAA adducts, was employed to measure the levels of HAA adducts in DNA of the colorectal mucosa *(169)* and lymphocytes from colorectal cancer subjects *(175)*; the levels of the putative dG-C8-PhIP adduct were found to be in the range of several adducts per 10^8 DNA bases. In the latter study, the adduct was found in lymphocytes of about 30% of the subjects, and the adduct levels varied by a factor of 10-fold between the lowest and the highest level. The level of lymphocyte PhIP DNA adducts was not significantly higher in smokers or high meat consumers than individuals who ate meat less frequently *(175)*. A subset of younger individuals carrying two mutated *GSTA1* alleles had higher adduct levels than homozygous wild-type and heterozygous subjects. This finding is consistent with the reported activity of the GSTA1 protein in the detoxication of *N*-oxidized metabolites of PhIP *(430,548)*. In another study, a PhIP-related DNA adduct (the presumed dG-C8-PhIP adduct) was detected by the ^{32}P-postlabeling method in 106 of the 150 colorectal tissues analyzed: similar levels of adducts were detected in tissues from controls, polyp patients, or cancer patients *(549)*.

Adducts attributed to dG-C8-PhIP were frequently detected, by the ^{32}P-postlabeling method, in exfoliated breast epithelial cells in milk of lactating mothers *(30)*. Thirty samples from the 64 subjects contained the presumed PhIP-DNA adduct, and the mean level of adduct

formation was 4.7 adducts/10^7 nucleotides. In an ensuing study, PhIP-DNA adducts were detected, by IHC, in mammary tissue of 82% of the women with breast cancer (N = 106) and also found in 71% of the tissue samples of the healthy control patients (N= 49) *(173)*. An interactive effect was observed among well-done meat consumption and NAT2 genotype and the level of PhIP-DNA adducts. This observed interactive effect is surprising since HONH-PhIP does not appear to be bioactivated by human NAT2 at appreciable levels *(342,343)*. A very high percentage of pancreas and prostate tissue biospecimens were also positive for PhIP-DNA adducts, when assayed by IHC *(174,177)*. The mean level of the PhIP-DNA adducts was ≥ 2.7 adducts/10^7 nucleotides, by IHC, in both patients and the healthy control populations *(173,174,177)*. The levels of PhIP-DNA adducts reported in breast, pancreas and prostate tissues of humans are comparable to the adduct levels observed in these corresponding tissues of rodents treated with either a single acute dose or chronic carcinogenic doses of PhIP (10 - 50 mg /kg bw) *(155,404,550)*. These findings imply that PhIP-DNA adduct formation occurs with far greater efficiency in humans exposed to ppb levels of dietary PhIP than the adducts which occur in the rodents given high, carcinogenic doses of PhIP.

PhIP-DNA adducts were also measured, by AMS, in breast tissue of female cancer patients who had received a dose of [^{14}C]PhIP (20 μg PhIP/70 kg body weight) via oral administration prior to surgery *(172)*. The estimates of PhIP-DNA adducts obtained by AMS ranged from 26 to 480 adducts/10^{12} nucleotides *(172)*, or nearly 1,000 to 10,000-fold lower than the levels of adducts reported by the IHC or ^{32}P-postlabeling techniques cited above. The large discrepancy in estimates of PhIP adducts in breast tissue obtained by IHC *(173)* and ^{32}P-postlabeling *(30)*, as opposed to the precise AMS method *(172)*, suggests that these biochemical assays are detecting a variety of lesions in addition to or other than dG-C8-PhIP. Moreover, the dG-C8-PhIP adduct is underestimated, when determined by ^{32}P-postlabeling in comparison to LC/MS methods *(551,552)*; dG-C8-PhIP adduct measurements should be conducted by quantitative LC/MS techniques.

DNA Adducts of Aromatic Amines and HAAs in the Oral Cavity

The oral cavity is the portal of entry for carcinogens that are ingested in the diet or inhaled through smoking. The bioactivation of aromatic amines and HAAs can occur directly by P450s 1A1, 1A2 or 1B1 that are expressed in buccal cells of the oral cavity *(553,554)*, or by P450 1A2 expressed in salivary glands *(555)*. The *N*-hydroxy metabolites also can form by the action of P450 1A2 in the liver *(233,339)* and reach the oral cavity through systemic circulation *(404)*, followed by phase II activation in cells of the oral cavity. Peroxidases in saliva *(556)* may also catalyze the bioactivation of HAAs and arylamines *(308,312,314,316,322,519)*. The potential of the oral microflora to contribute to metabolism and DNA adduct formation of HAAs or arylamines has not been investigated.

Both ^{32}P-postlabeling *(557-560)* and IHC techniques *(561-563)* were employed to screen for DNA adducts in cells of the oral cavity. Several of the studies reported differences in total DNA adduct levels between smokers and non-smokers; however, the complexity of the adduct profiles and the inability to identify specific DNA adducts precluded any interpretation on the principal DNA damaging agents and their significance in the risk of development of oral cancer or cancers of other organs *(557-563)*. Some of the lesions detected were believed to be derived from polycyclic aromatic hydrocarbons or aromatic amines *(557,561-563)*. As an extension of those studies, a selective LC/MS method was recently employed to detect DNA adducts in saliva derived from carcinogens formed in tobacco smoke and cooked meats. The dG-C8 adducts of 4-ABP, PhIP, AαC, and MeIQx were identified in saliva samples from volunteers on unrestricted diets, by LC-ESI/MS/MS^n, at the MS^3 scan stage mode with a linear quadrupole ion trap MS *(178)*. DNA adducts of PhIP were found most frequently: dG-C8-PhIP was identified in saliva samples from 13 of

29 ever-smokers and in saliva samples from 2 of 8 never-smokers. dG-C8-AαC and dG-C8-MeIQx were identified solely in saliva samples of 3 current smokers, and dG-C8-4-ABP was detected in saliva from 2 current-smokers. The levels of these different adducts ranged from 1 to 9 adducts per 10^8 nucleotides. Moreover, the employment of the linear quadrupole ion trap MS permitted the acquisition of product ion spectra of the aglycone adducts $[BH_2]^+$, at the MS^3 scan stage, for unambiguous identification of the carcinogen-DNA adducts (Figure 10) *(476,552,564)*. Some HAAs induce oral cancer in rodents during long-term feeding studies *(9)*. Moreover, an appreciable level of metabolic activation of Trp-P-2 was observed in rat tongue *(565)*, and elevated levels of salivary DNA adducts were reported in rats fed with MeAαC, resulting in severe atrophy of the salivary glands *(566)*. Thus, saliva may be a promising non-invasive fluid to monitor exposure and DNA damage of some HAAs and aromatic amines.

Exfoliated epithelial buccal cells and leukocytes are the principal mammalian cells present in saliva *(567,568)*. The time frame from new cell production to exfoliation of the buccal cell from the mucosal surface is estimated to be between 5 and 12 days *(569)*, and the leukocytes, which originate mainly from the gingival crevice, and then migrate into the oral cavity, are predominantly short-lived neutrophils and other granulocytes *(568,570)*. Given the short life spans of both buccal and leukocyte cell types, the DNA adducts present in saliva are likely to occur from recent exposures to carcinogens. It is not known whether adducts are formed in both cell-types and whether they preferentially form in one type. Studies that examine kinetics of PhIP-DNA adduct formation in cells of the oral cavity of humans exposed to defined amounts of PhIP, combined with studies that can unravel the myriad of plausible enzymes that contribute to PhIP adduct formation in oral cells are required. Moreover, the level of adduct formation in the oral cavity must be shown to correlate to target tissues of cancer, for the validation of this biomarker.

Aromatic Amine and HAA Protein Adducts

Carcinogen blood protein adducts have been used as an alternative to DNA adducts for human biomarkers of several different classes of carcinogens, including aromatic amines, polycyclic aromatic hydrocarbons, and aflatoxin B_1 (AFB_1) *(31,49,571-574)*. The research on protein adducts originates from the pioneering studies of the Millers and is based upon the paradigm of chemical carcinogenesis in which electrophilic species or electrophilic metabolites of carcinogenic compounds react with nucleophilic centers on proteins as well as DNA *(35,36,575,576)*. The use of Hb as a dosimeter for alkylation agents was introduced by Ehrenberg and his collaborators *(577,578)*. The biomonitoring of carcinogen adducts with serum albumin (SA) also has been examined for several different classes of carcinogens *(31,49)*. The biomonitoring blood protein carcinogen adducts is advantageous, because up to several hundred mg of Hb or SA, as opposed to ~100 μg DNA, can be obtained from a 10 mL blood sample. Moreover, stable carcinogen protein adducts are expected to accumulate and follow the kinetics of the lifetime of Hb or half-life of SA, during chronic exposure. In humans, the lifetime of Hb is 120 days and the half-life of SA is between 20-25 days *(31)*. Thus, the steady state levels of Hb and SA carcinogen adducts would be, respectively, about 60 and 29-fold higher after chronic exposure than after a single dose *(578,579)*. Carcinogen protein adducts of aromatic amines and HAAs are formed through their *N*-oxidized metabolites and represent a measure of the biologically effective dose *(31,38)*. However, there are caveats in the application of blood protein adducts for human risk assessment. The adduction of carcinogens to blood proteins does not represent genetic damage, and adduct formation with Hb occurs in the erythrocyte, which may not reflect the genetic damage that occurs in the target organ. In the case of SA adducts, the adduct can form in the hepatocyte, the cell where SA is biosynthesized *(580)* and where metabolic activation of arylamines and HAAs occurs *(60)*.

Hemoglobin Adducts

A number of aromatic amines form adducts with Hb, via a sulfinamide linkage (Figure 2), in experimental laboratory animals *(38,80,524)*. The existence of AA-Hb sulfinamide adducts for many arylamines demonstrates that the *N*-oxidation is a common metabolic pathway for this class of genotoxicants. The percent of the arylamine dose bound to Hb as a sulfinamide linkage ranges over 100-fold in rodents, depending upon the structure of the chemical *(38,48,524)*. The highest levels of arylamine-Hb adducts were reported for 4-ABP, where over 5% of the dose is bound to Hb in the form of the sulfinamide adduct *(80)*. In a hallmark study, Hb sulfinamide adducts of 15 aromatic amines were determined in nonsmokers and smokers, and significant differences between smokers and nonsmokers were observed for Hb adducts of 4-ABP, 3-aminobiphenyl, 2-NA, *o*- and *p*-toluidine, 2,4-dimethylaniline, and 2-ethylaniline; some of these arylamines are human bladder carcinogens *(581)*. In a study among factory workers to exposed to Bz and Bz-based dyes, the Hb-adduct levels of acetylBz, Bz and 4-ABP correlated strongly with each other *(111)*. The levels of *N*-acetyl-Bz adducts were 20-fold higher than the levels of Bz-sulfinamide adducts and the results are consistent with P450 activation of *N*-acetyl-Bz to form HONH-*N*′-acetylBz as a major reactive metabolite in vivo *(318,582)*.

The validation of a protein carcinogen adduct as a biomarker requires that the biomarker correlates to exposure, DNA damage, and cancer risk. The Hb-sulfinamide adduct of 4-ABP fulfills these requirements. The levels of 4-ABP-Hb sulfinamide adduct formation were shown to correlate with the number of cigarettes smoked per day *(581,583,584)*. Subjects with rapid *N*-acetylator phenotypes have decreased levels of the 4-ABP-Hb adduct in comparison to slow *N*-acetylator phenotypes *(583,584)*. Thus, *N*-acetylation, a detoxication pathway and a competing metabolic fate to *N*-hydroxylation, results in a decreased level of the biologically effective dose of 4-ABP in rapid *N*-acetylator phenotypes. The levels of 4-ABP-Hb adducts were also shown to correlate with the amount of dG-C8-4-ABP adduct present in exfoliated urothelial cells *(536)*. Elevated levels of 4-ABP-Hb and other arylamine-Hb sulfinamide adduct levels are associated with increased bladder cancer risk, both in smokers as well as non-smokers *(50,77,78,584,585)*.

HAAs also react with Hb and SA. However, the levels of IQ, MeIQx, and PhIP bound to Hb are low in experimental laboratory animals (~0.01% of the dose), and the levels of HAA-SA adducts are only several fold higher *(49,88,90,263,586-588)*. The low level of HAA-Hb sulfinamide formation does not seem to be attributed to poor reactivity of the *N*-hydroxy-HAA metabolites with Hb. In the case of IQ, a metabolic study conducted in vitro showed that the *N*-hydroxy-IQ metabolite penetrates the human erythrocyte and induces methemoglobinemia, and that a portion of the IQ bound to Hb (~10%) was released by acid and recovered as IQ *(586)*. Thus, *N*-hydroxy-IQ does appear to form a sulfinamide adduct with Hb. The *N*-hydroxy and nitroso derivatives of Glu-P-1 were also shown to modify the thiol groups of Hb *(589)*. The low levels of HAA-Hb sulfinamide formation with HAAs in rodents suggest that there is little free *N*-hydroxy-HAA present in the blood that is available to react with Hb. The inefficient binding of HAAs to Hb will probably preclude the development of HAA-Hb adducts as biomarkers in humans *(88,90)*.

Serum Albumin Adducts

Aromatic amines also react with SA. Human SA is of 585 amino acids in length and is the most abundant protein in plasma (~45 mg/mL). Its roles include maintenance of osmotic pressure and transport of endogenous (i.e., fatty acids, bilirubin, steroids) and exogenous (drugs) chemicals *(590)*. The single tryptophan residue at position 214 of rat SA is a selective site of binding for several activated arylamines *(391)*. This amino acid reacts with *N*-sulfonyloxy ester of *N*-acetyl-4-aminobiphenyl, to form an adduct with a stable 4-ABP-

tryptophan-linkage (Figure 11) *(390)*. The same adduction product was shown to form by reaction of synthetic sulfate ester of *N*-hydroxy-*N*-acetyl-4-aminobiphenyl, and the sulfate esters of *N*-hydroxy-*N*-acetyl-2-aminofluorene and HONH-*N*,*N*′-diacetylbenzidine with human SA in vitro (F. F. Kadlubar, personal communication) *(391)*. SULT enzymes play a critical role in the formation of these adducts in hepatocytes *(391)*. To our knowledge, an analytical method has not been established to biomonitor this arylamine-tryptophan SA adduct in humans.

Some of the adduct(s) of IQ, MeIQx and PhIP formed with SA in rodents or produced in vitro with rat or human SA are acid-labile *(88,586,587,591,592)*. An adduct formed between IQ and rat SA adduct was characterized by MS, ^{1}H NMR, and amino acid analysis and shown to contain a sulfinamide linkage formed through the SA-Cys^{34}: this adduct accounted for about 10% of the total SA adducts formed in rats (Figure 11) *(586)*. The Cys^{34} is one of 35 conserved cysteine residues in SA across species *(590)*. Thirty-four of these cysteines are involved in 17 disulfide bonds. The single unpaired Cys^{34} is present either as a free thiol or in an oxidized form: this residue is present partially as disulfide linkages with low molecular-weight thiols *(593)*. The SA-Cys^{34} is thought to be responsible for many of the antioxidant properties of SA and accounts for ~80% of the net free thiols in plasma *(594,595)*. The scavenging properties of this Cys^{34} residue to reactive carcinogenic and toxic electrophiles are well documented *(596)*. Adducts at the Cys^{34} have been identified with reactive metabolites of various toxicants in rodent or human SA, including: MeIQx *(587)*, PhIP *(592,597)*, acrylamide *(598)*, sulfur mustard *(599)*, benzene *(600)*, and acetaminophen *(601)*, in addition to IQ *(586)*. Acid-labile adducts of MeIQx and PhIP have been reported to form with SA; these adducts may be sulfinamide linkages at the Cys^{34} residue *(587,591)*. A plausible scheme for HAA-SA sulfinamide adduct formation, based upon studies with IQ *(586)*, is depicted in Figure 11. The *N*-hydroxy-HAA metabolites can undergo further oxidation by P450 1A2 or by transition metals, to form the nitroso-HAA intermediates *(602)*, which can react with the SA-Cys^{34}, to form HAA-SA-Cys^{34} sulfinamide adducts. There is one report in the literature on the measurement of putative acid-labile MeIQx-SA-Cys^{34} sulfinamide adducts in a pilot human study. The level of this adduct, if present, was reported to be below the LOD of the GC/MS assay (29 attomol MeIQx/mg SA) *(587)*. It is unlikely that the sulfinamide adduct of MeIQx with human SA can be used as a dosimeter for human AIA exposure.

The binding of ^{14}C-PhIP to SA was shown to be much greater than the binding of ^{14}C-MeIQx to SA in humans, by AMS measurements *(92,588)*. Moreover, PhIP-SA adduct formation was up to 40-fold greater in humans than in rats, given comparable doses of chemical *(92)*. The levels of PhIP adducts formed with human SA *(90)* may be sufficient to establish MS methods of biomonitoring towards this adduct(s). An acid-labile PhIP-SA adducts(s) was (were) detected in human subjects on a non-controlled diet; levels were 10-fold higher in meat-eaters than vegetarians (6.7 ± 1.6 vs. 0.7 ± 0.3 fmol PhIP/mg protein; mean ± SE) *(591)*. The structure(s) of the adduct attributed to the acid-labile lesion has not been determined. It seems likely that some portion of the acid-labile PhIP adduction products were formed at the Cys^{34} residue in human SA *(592,597)*. The chemical stability of the adduct(s) is (are) unknown. The same study revealed the presence of acid-labile PhIP-Hb adducts in human subjects; the adduct levels were about 2-fold lower than the levels of the acid-labile PhIP-SA adducts *(591)*. These findings suggest that HONH-PhIP forms sulfinamide adducts with SA and Hb in humans. The results contradict the data reported on PhIP blood protein adduct formation in humans by AMS measurements, where the levels of PhIP-Hb adducts were about 40 to 50-fold lower than PhIP-SA adducts *(90)*. However, the PhIP blood protein adduct data was obtained following a single dose, in the AMS study, whereas the adduct levels in the population-based study represent an integral value of

chronic exposure over the lifespan of the blood proteins *(591)*. Further investigations on the implementation of PhIP blood protein adducts in human population studies are warranted

Biomonitoring of HAAs in Hair

Human hair and animal fur have served as matrices for biomonitoring of chemicals such as nicotine, other drugs and narcotics, and hormones *(23,25,603)*. Studies with experimental laboratory animals have shown that ^{3}H-labeled PhIP accumulates in melanin-rich tissues, including including fur *(24)*. The radioactivity cleared from the body within several days, but stayed in the hair, and was present in the cortex of the distal hair shafts 4 weeks after the exposure. Following digestion of the hair matrix, chemical analyses showed that the radioactivity represented unmetabolized PhIP *(24)*. However, the exposures in the animal studies occurred at levels exceeding the levels of PhIP or other HAAs in the human diet by at least 4 orders of magnitude. Nevertheless, Alexander and co-workers established a method to quantitate PhIP in mouse fur and then applied the technique to measure PhIP in human hair by GC/NCI/MS *(24,604)*. Thereafter, Kobayashi and collaborators established a method to quantitate PhIP in human hair by LC-ESI/MS, employing the selected ion monitoring mode *(605,606)*. Both methods require up to several hundred mg of hair and entail lengthy extraction procedures for chemical analysis. The pre-rinsing of the hair shaft prior to digestion of the hair matrix is required in order to remove HAAs that may have been deposited on the external surface of the cuticle by exposures from the fumes of cooking oils or airborne particulates generated by the frying or grilling of meats *(13,14)*. The pre-rinsing procedure is essential to distinguish dietary intake of PhIP from airborne exposure.

A more recent study reported a simplified method for the extraction and analysis of HAAs in hair, by employing base hydrolysis to digest hair, followed by tandem solvent/solid phase extraction for a clean-up method *(607)*. The quantification of PhIP was performed by LC-ESI/MS/MS in the selected reaction monitoring mode: the LOQ value was 50 pg PhIP/g of hair, when 50 mg of hair was assayed *(607)*. The analytical method was employed to measure HAA adducts in a pilot study of 12 human volunteers. PhIP was detected in the hair of all six omnivores (non-hair dye users) at levels ranging from 290 to 890 pg/g hair, whereas PhIP was detected in the hair from one out of six vegetarians, and at a level just above the LOQ (65 pg/g hair) *(607)*. These findings demonstrate that PhIP exposure occurs primarily through meat consumption (Figure 12). MeIQx and AαC were below the LOQ (50 pg/g hair) in hair samples from all of the omnivores as well as the six vegetarians.

The levels of PhIP measured in hair of subjects in the United States (290 to 890 pg/g) *(607)* are within the same range of the levels of PhIP detected in hair of subjects in Norway (60-7500 pg/g hair) *(608)* and in subjects of Japan (180-3600 pg/g hair) on unrestricted diets *(606)*. The levels of PhIP in hair samples from two omnivores in the United States were found to vary by less than 24% over a 6 month interval *(607)*. A study on twenty Japanese volunteers reported a reasonably good correlation after adjustment for hair melanin content between intakes of PhIP, MeIQx, Trp-P-1 estimated with a FFQ and the mean PhIP content of hair samples collected 1 - 3 months apart *(609)*. These findings signify that the exposure to PhIP and its accumulation in hair are relatively constant over time.

The hair biomarker represents an integrated exposure to PhIP over a time period of weeks to months and may be a superior method to assess exposure to PhIP than the Food Frequency Questionnaires (FFQ), which is often used in molecular epidemiology studies *(19)*. PhIP levels in hair appear to be a good biomarker of long-term exposure to HAAs; however, this hair biomarker is not a predictor of DNA damage *(607)*. Moreover, levels of PhIP accumulated in hair of individuals are highly variable. This large variation in the levels of PhIP in hair reflect in part the different concentrations of PhIP in the diet *(10)*. The pharmacokinetics and metabolism of PhIP are also likely to influence the levels of PhIP that

accumulate in hair. Because of the large interindividual differences in the hepatic P450 1A2 protein content *(222,242)*, the amount of unmetabolized PhIP in the bloodstream that reaches the hair follicle, following first-pass metabolism, is expected to widely range and may affect the levels of PhIP accrued in hair. The pigmentation of hair also may affect the amount of PhIP incorporated into hair. Eumelanin *(610)*, a pigment that is more predominant in black hair than in lighter-colored hair *(611)* has a high affinity for PhIP. Thus, dark-haired individuals may sequester larger amounts of PhIP than light-haired individuals, on the basis of findings from a study that reported mice with dark pigmented fur accrued more PhIP in their fur than mice with light pigmented fur *(610)*. Approximately 25% of the male population and 42% of the female population have been reported to use hair dyes in the United States, Europe, and Japan *(612)* with permanent hair dye being the most commonly used hair dye product. The oxidizing conditions used to develop the desired hair dye colors are likely to produce oxidation products of PhIP *(120,612,613)* during the dye development process and would escape detection by current analytical methods. A robust analytical method to measure PhIP in users of hair dyes still requires development and validation.

Epidemiology of Cooked Meats, Potential Role of HAAs in Human Cancer

Many epidemiological investigations have examined the interrelationships among consumption of cooked red meat, its effect on human cancer risk of the digestive tract, prostate gland, the mammary gland, and the potential causal role of HAAs in the etiology of these cancers *(19,70,124)*. The 2007 WCRF/AICR report on nutrition and cancer concluded that red meat and processed meat are “convincing causes” of colorectal cancer and that there is “limited evidence” that they also cause esophagus, stomach, pancreas, lung, endometrial and prostate cancers *(614)*. Although several classes of carcinogens are present in red meat and processed meat and multiple mechanisms of carcinogenesis are likely to be at play *(615-617)*, the exposure and causal role of HAAs in cancer development through eating meat cooked well-done has been an area of great research interest. The majority of epidemiologic studies that investigated dietary consumption of well-done meat in relation to various tumor sites reported a positive association between cancer risk and well-done meat consumption (see reviews *(124,618)* and references therein). However, some studies have shown no associations between well-done meat and cancer risk *(124,618)*. Fewer studies have attempted to estimate intake of specific HAAs. A number of them have shown associations with cancer or colorectal adenoma risk *(615,619-621)*, whereas others have not *(622)* (reviewed in *(618)*). Thus, overall, the dietary data have been suggestive but inconsistent.

A similarly large number of studies have explored the associations of polymorphisms in genes or pathways involved in the metabolism of HAAs (e.g., NAT2, P450 1A2, P450 1B1) with cancer risk and their results have been quite inconsistent *(324,623-625)*. However, whole-genome association studies have recently demonstrated that common genetic variants only have small effects on risk. In particular, it can be assumed that an effect of a genetic polymorphism in a xenobiotic metabolism enzyme involved in carcinogen bioactivation (or detoxication) would be unlikely to be manifested when there is a low, biologically insufficient level of exposure to the carcinogen. Thus, it is probably important to consider both the exposure and the genetic variants to be able to detect an association with disease risk.

A smaller number of studies have examined the combined effects of dose (e.g., well-done meat or HAA intake, smoking) and metabolic genotypes or phenotypes, and these studies were mainly investigating colorectal cancer or adenoma. Interactions were suggested between intake of red meat, well-done meat or HAA and variants in *NAT2 (626-630)*; *NAT1 (631)*, *AHR (632)*, *CYP1B1 (632,633)* and *SULT1A1 (633)*, as well as in a combination of metabolic genes (*CYP1A2*, *CYP2E1*, *CYP1B1*, *CYP2C9*) *(634)*, in relation to colorectal

cancer or adenoma risk. However, other studies failed to replicate these associations *(627,635-637)*. Similar interactions were found with NAT2 and meat intake for bladder cancer *(638)*. Again, these data are suggestive but they do not show a high level of consistency across studies.

Because of its high interindividual variation, P450 1A2 activity may be relevant for cancers associated with exposure to HAAs. This enzyme, which is prominently expressed in liver *(224)* and other enzymes catalyzing the activation and/or detoxification of HAAs (and aromatic amines) that are inducible by lifestyle factors or modulated by genetic polymorphisms may account for interindividual differences in susceptibility to these carcinogens *(256)*. Two case-control studies support the concept that rapid P450 1A2 activity in combination with rapid NAT2 activity is a risk factor for colorectal cancer in individuals eating well-done cooked meat, which is a rich source of HAAs *(327,328,639)*. In one of the two studies, this association was limited to smokers (Figure 13) *(328)*, which makes biological sense since smoking induces P450 1A2. These findings, if confirmed, would support the hypothesis that individuals with high metabolic phenotypes in P450 1A2 and NAT2 activities will have elevated levels of some types of HAA-DNA adducts, which may lead to the development of cancer. However, a third study failed to find any modifying effect of NAT2 or P450 1A2 activity, also measured by urinary caffeine metabolites, or an association of HAA with adenoma *(631)*.

A critical limiting factor in most epidemiological studies is the uncertainty in quantitative estimates of chronic exposure to HAAs (or other carcinogens). For most molecular epidemiology studies, the extent of HAA exposure is difficult to assess and, thus, the association of HAAs formed in cooked meat and cancer risk has been difficult to establish. The extent of exposure to HAAs from meat in molecular epidemiology studies is often inferred by FFQ often combined with pictures of meat cooked at different levels of doneness *(19,134,190)*. Intake estimates for meats cooked at a specified level of doneness and with various methods of high-temperature cooking (pan-frying, broiling, and barbecuing/grilling) are based on usual frequency and portion size. HAA intake estimates are then derived using corresponding HAA meat content values *(615)*. There are clear difficulties in quantifying cooking doneness by such methods, the day-to-day variation in diet can be large, and the conventional FFQ *(640)*, can be especially problematic when exposure to the compound of interest spreads over a range of food items at varying levels of concentrations. Moreover, the accuracy of the FFQ is particularly challenging in the assessment of levels of HAA formation, because the levels of HAAs formed are highly dependent on the type of meat cooked and, especially, the method, temperature, and duration of cooking. These variable parameters can lead to differences of HAA concentrations by more than 100-fold *(10,125,126,134,189,641)*. Furthermore, a number of cooked meat samples assayed for HAAs across all levels of doneness categories were reported to have no detectable HAAs of any kind *(641)*. Clearly, the uncertainties in HAA concentrations in daily staples can result in poor estimation of chronic exposure to these compounds. The limitations of the questionnaire-based exposure assessment methods are likely to be a major reason for the inconsistency in the epidemiologic data. Thus, the required data are not currently available to fully characterize the relationship between HAAs and human cancer risk. Stable, long-lived biomarkers of HAAs are required for any reliable assessment of HAA exposure for use in population studies.

Conclusion

The demonstration of exposure and chemical-specific adducts in target tissues, combined with the correlation of specific DNA adducts with mutation spectra in tumor related genes provide a mechanistic understanding of the causal role for a chemical in the development of

cancer *(20,26,642)*. The laboratory research conducted on AFB_1, a fungal toxicant and a potent animal carcinogen that is found as a contaminant in various crops *(15)*, is the prime example of where biomarkers of carcinogen exposure have been employed to identify and refine cancer risk estimates *(642,643)*. The positive associations observed between dietary AFB_1 exposure and the incidence of hepatocellular carcinoma in Asia and Africa were greatly strengthened by the application of validated biomarkers, which included DNA and SA adducts, and a characteristic mutation spectrum in the *p53* tumor suppressor gene that is linked to a DNA adduct of AFB_1 *(571,572,642,644,645)*. There is also promising biomarker data that support a role of aristolochic acid, a carcinogen present in the plant species of the genus *Aristolochia*, as a causal agent of urothelial cancer in subjects of the Balkans *(564,646)* and Taiwan *(647)*.

With regard to aromatic amines, the causal role of some these compounds in human bladder cancer was revealed through epidemiological studies conducted world-wide on factory workers occupationally exposed to high levels of the procarcinogens *(1-3,105,106)*. Later, laboratory studies elucidated the biochemical mechanisms of aromatic amine metabolism and adduction products with protein and DNA *(45,76)*, which set the stage for the employment of arylamine-Hb and DNA adducts as biomarkers of exposure. The implementation of these biomarkers in human epidemiology studies has strengthened the association of arylamines with cancer risk and has also implicated aromatic amines in tobacco-associated bladder cancer *(28,31,50,52,536,581,583)*.

Such extensive biomarker data have yet to be established in molecular epidemiology studies examining the potential cancer causal role of HAAs or other genotoxicants that are formed at the ppb concentrations in cooked foods. The daily exposure to numerous genotoxicants in cooked foods makes dietary hazard assessment a challenging task, particularly when carcinogenesis involves numerous steps *(71)*, and when dietary, environmental, and genetic factors can impact the biological potency of the procarcinogens *(9,22,157-159,413)*. Ultimately, the incorporation of biomarkers in molecular epidemiology studies may help to disentangle the uncertainty about the relative contributions of various dietary genotoxicants to cancer risk *(24,185)*. Although a causal link with cancer has not been established for HAAs, many epidemiology studies have associated frequent consumption of well-done cooked meat products to colorectal cancer, and less consistently, prostate and breast cancer *(124,618)*. Our current knowledge about the biochemistry of HAAs indicates that the underlying biochemical mechanisms of HAA metabolism, DNA adduct formation, DNA repair, and mutations are comparable to those involved in aromatic amine carcinogenicity.

Even though aromatic amines and HAAs are structurally related classes of chemicals and share some common pathways of metabolism, the strategies employed for human bimonitoring of these procarcinogens are different. The assessment of chronic exposure to many arylamines has been successfully done through measurement of Hb sulfinamide adducts formed by reaction of the arylnitroso intermediates with the Hb-$Cys^{93\beta}$ residue. This adductome approach has been employed to measure 15 aromatic amine Hb sulfinamide adducts *(581)*. HAAs also undergo extensive *N*-oxidation in humans, as demonstrated by the urinary metabolite profiles of MeIQx and PhIP *(98,101,103,176,222,288,289,292)*, but the HAA-Hb sulfinamide adducts appear to be formed at insufficient levels to exploit this protein adduct for human biomonitoring. An alternative potential adductome approach that can be applied to measure reactive carcinogenic and toxic electrophiles is through their adduction products formed at the Cys^{34} residue of SA *(596)*. Human SA-Cys^{34} may be screened for the sulfenamide or sulfinamide adducts of PhIP *(90,591,592)*, or adducts formed with *N*-oxidized intermediates of other HAAs; however, the structures of these human SA-Cys^{34} adducts have not yet been fully characterized by spectroscopic techniques and the chemical stability of the adduct(s) is unknown. Further studies on the

characterization of PhIP-SA adducts are required to validate this biomarker prior to its application in population-based studies.

The *N*-glucuronide conjugates of many arylamines and arylhydroxylamines undergo facile hydrolysis in the urinary bladder and as a result are not readily measured *(76)*, whereas the *N*-glucuronide conjugates of AIAs and HONH-AIAs are stable in the mildly acidic pH conditions of urine *(103,293)*. Therefore, the direct monitoring of urinary *N*-glucuronide metabolites of MeIQx and PhIP and their *N*-hydroxylated metabolites may be used to examine metabolic phenotypes such as P450 1A2 or UGTs *(176,459)*; however, the short half-life of these metabolites in urine reflects recent dietary intake only, making it unsuitable for assessment of chronic but intermittent exposures.

The biomonitoring of HAA levels in hair or macromolecular HAA adducts has the potential to assess long-term exposure to these carcinogens. On the basis of the current state of knowledge of HAA exposure and the literature on HAA biomarkers, several biomarkers of PhIP seem to be most promising for employment in molecular epidemiology studies. A very high percentage of humans in different parts of the world contain PhIP in their hair *(604,607,609)*. The implementation of this hair biomarker in epidemiology studies can confirm chronic exposure to PhIP, but it does not represent DNA damage or necessarily represent cancer risk *(607)*.

Putative PhIP-DNA adducts have been detected with very high frequency in human pancreas, prostate and female mammary gland, or in exfoliated epithelial cells from human milk samples of women in the United States, by IHC or ^{32}P-postlabeling methods *(30,173,174,177)*. Surprisingly, the levels of PhIP-DNA adducts formed in these tissues are comparable to the adduct levels reported in the corresponding tissues of rodents given a single acute dose or chronic carcinogenic doses of PhIP, which exceeded human dietary levels by a million-fold or more *(155,404,550)*. The frequent detection of PhIP-DNA adducts at such high levels in human tissues is alarming. However, the proof of identity of PhIP-DNA adducts by IHC and ^{32}P-postlabeling detection methods is equivocal. If the high levels of PhIP-DNA adducts in human tissues are confirmed by selective and quantitative mass spectrometry methods, PhIP would be recognized as a major dietary DNA-damaging agent. Furthermore, the interpretation of the rodent biochemical and carcinogenicity data *(9,155,191)* extrapolated across species to assess the human health risk of PhIP and possibly other HAAs would require re-examination *(96,164,179,181,183,184)*. Currently available mass spectrometry instruments have the requisite sensitivity to measure PhIP-DNA adducts in human tissues and biological fluids *(178,480)*. Thus, there is an urgent need to corroborate the DNA adduct binding data obtained by ^{32}P-postlabeling and IHC methods with quantitative mass spectrometry techniques.

In conclusion, rapid through-put methods are still needed to be developed for analyses of HAA biomarkers by mass spectrometry methods in large scale human epidemiology studies. The employment of novel analytical approaches and mass spectrometry techniques to measure HAA biomarkers in large prospective studies with appropriate biospecimens presents the most potential to characterize better the health risks of dietary and tobacco-associated HAAs in human cancers.

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Abbreviations

AMS	accelerator mass spectrometry
AIAs	aminoimidazoarenes
AAs	aromatic amines
FFQ	Food Frequency Questionnaire
GC-NICI-MS	gas chromatography with negative ion chemical ionization-mass spectrometry
GST	glutathione *S*-transferase
HAAs	heterocyclic aromatic amines
Hb	hemoglobin
IHC	immunohistochemistry
LC-ESI/MS/MS	liquid chromatography-electrospray ionization/tandem mass spectrometry
MOE	margin of exposure
MR	metabolic ratio
NATs	*N*-acetyltransferases
OAT	*O*-acetyltransferase
PBPH/PD	physiologically based pharmacokinetic/pharmacodynamic
PHS	prostaglandin H synthase
SA	serum albumin
SULTs	sulfotransferases
UGTs	Uridine diphosphate-Glucuronosyltransferases
4-ABP	4-aminobiphenyl
HONH-4-ABP	*N*-hydroxy-4-aminobiphenyl
IFP	2-amino-1,6-dimethylfuro[3,2-*e*]imidazo[4,5-*b*]pyridine
AAF	*N*-acetyl-2-aminofluorene
AF	2-aminofluorene
HONH-AF	*N*-hydroxy-2-aminofluorene
Trp-P-1	2-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole
Trp-P-2	2-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole
Glu-P-2	2-aminodipirido[1,2-*a*:3′,2′-*d*]imidazole

Glu-P-1	2-amino-6-methyldipirido[1,2-*a*:3′,2′-*d*]imidazole
APNH	9-(4′-aminophenyl)-9*H*-pyrido[3,4-*b*]indole
AMPNH	9-(4′-amino-3-methylphenyl)-9*H*-pyrido[3,4-*b*]indole
IQx	2-amino-3-methylimidazo[4,5-*f*]quinoxaline
I*g*Qx	2-amino-3-methylimidazo[4,5-*g*]quinoxaline
7-MeI*g*Qx	2-amino-3,7-dimethylimidazo[4,5-*g*]quinoxaline
7,9-DiMeI*g*Qx	2-amino-3,7,9-trimethylimidazo[4,5-*g*]quinoxaline
MeIQx	2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
HONH-MeIQx	*N*-hydroxy-2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
IQx-8-COOH	2-amino-3-methylimidazo[4,5-*f*]quinoxaline-8-carboxylic acid
8-CH_2OH-IQx	2-amino-8-(hydroxymethyl)-3-methylimidazo[4,5-*f*]quinoxaline
MeIQx-N^2-Gl	N^2-(ß-1-glucosiduronyl)-2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
HON-MeIQx-N^2-Gl	N^2-(ß-1-glucosiduronyl)-2-(hydroxyamino)-3,8-dimethylimidazo[4,5-*f*]quinoxaline
MeIQx-N^2-SO_3H	N^2-(3,8-dimethylimidazo[4,5-*f*]quinoxalin-2-yl-sulfamic acid
DMIP	2-amino-1,7-dimethylimidazo[4,5-*b*]pyridine
TMIP	2-amino-1,5,6-trimethylimidazo[4,5-*b*]pyridine
4,8-DiMeIQx	2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline
7,8-DiMeIQx	2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline
MeIQ	2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline
IQ	2-amino-3-methylimidazo[4,5-*f*]quinoline
HONH-IQ	*N*-hydroxy-2-amino-3-methylimidazo[4,5-*f*]quinoline
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
HONH-PhIP	*N*-hydroxy-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
HONH-PhIP	HON-PhIP-N^2-Gl, N^2-(ß-1-glucosiduronyl-2-(hydroxyamino)-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
HON-PhIP-*N*3-Gl	*N*3-(ß-1-glucosiduronyl-2-(hydroxyamino)-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
PhIP-N^2-Gl	N^2-(ß-1-glucosiduronyl-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
PhIP-*N*3-Gl	*N*3-(ß-1-glucosiduronyl-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
MeA*α*C	2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole
A*α*C	2-amino-9*H*-pyrido[2,3-*b*]indole
HONH-A*α*C	2-hydroxyamino-9*H*-pyrido[2,3-*b*]indole

Bz	benzidine
***N*-acetylBz**	*N*-acetylbenzidine
HONH-*N′*-acetylBz	*N*-4-hydroxyamino-*N′*-acetylbenzidine
2-CA	2-chloroaniline
4-CA	4-chloroaniline
3-EA	3-ethylaniline
2-NA	2-naphthylamine
HONH-2-NA	*N*-hydroxy-2-aminonaphthalene
3,5-DMA	3,5-dimethyaniline
dG-C8-ABP	*N*-(deoxyguanosin-8-yl)-ABP
dG-N^2-N^4-4-ABP	*N*-(deoxyguanosin-N^2-yl)-ABP
dG-N^2-ABP	3-(deoxyguanosin-N^2-yl)-4-ABP
dA-C8-4-ABP	*N*-(deoxyadenosin-8-yl)-4-ABP
***N′*-acetyl-dG-C8-Bz**	*N*-(deoxyguanosin-8-yl)-*N′*-acetylbenzidine
dG-N^2-2-NA	1-(deoxyguanosin-N^2-yl)-2-NA
dG-C8-2-NA	*N*-(deoxyguanosin-8-yl)-2-NA
ring-opened-dG-C8-NA	ring-opened-*N*-(deoxyguanosin-8-yl)-2-NA
dA-N^6-2-NA	1-(deoxyadenosin-N^6-yl)-2-NA
dG-N^2-NAQI	N^4-(deoxyguanosin-N^2-yl)-2-amino-1,4-naphthoquinoneimine
dG-C8-MeIQx	*N*-(deoxyguanosin-8-yl)-MeIQx
dG-C8-4,8-DiMeIQx	*N*-(deoxyguanosin-8-yl)-4,8-MeIQx
dG-N^2-MeIQx	5-(deoxyguanosin-N^2-yl)-MeIQx
dG-C8-IQ	*N*-(deoxyguanosin-8-yl)-IQ
dG-C8-MeIQ	*N*-(deoxyguanosin-8-yl)-MeIQ
dA-N^6-IQ	5-(deoxyadenosin-N^6-yl)-IQ
dG-N^2-IQ	5-(deoxyguanosin-N^2-yl)-IQ
dG-C8-AαC	*N*-(deoxyguanosin-8-yl)-AαC
dG-C8-MeAαC	*N*-(deoxyguanosin-8-yl)-MeAαC
dG-C8-PhIP	*N*-(deoxyguanosin-8-yl)-PhIP

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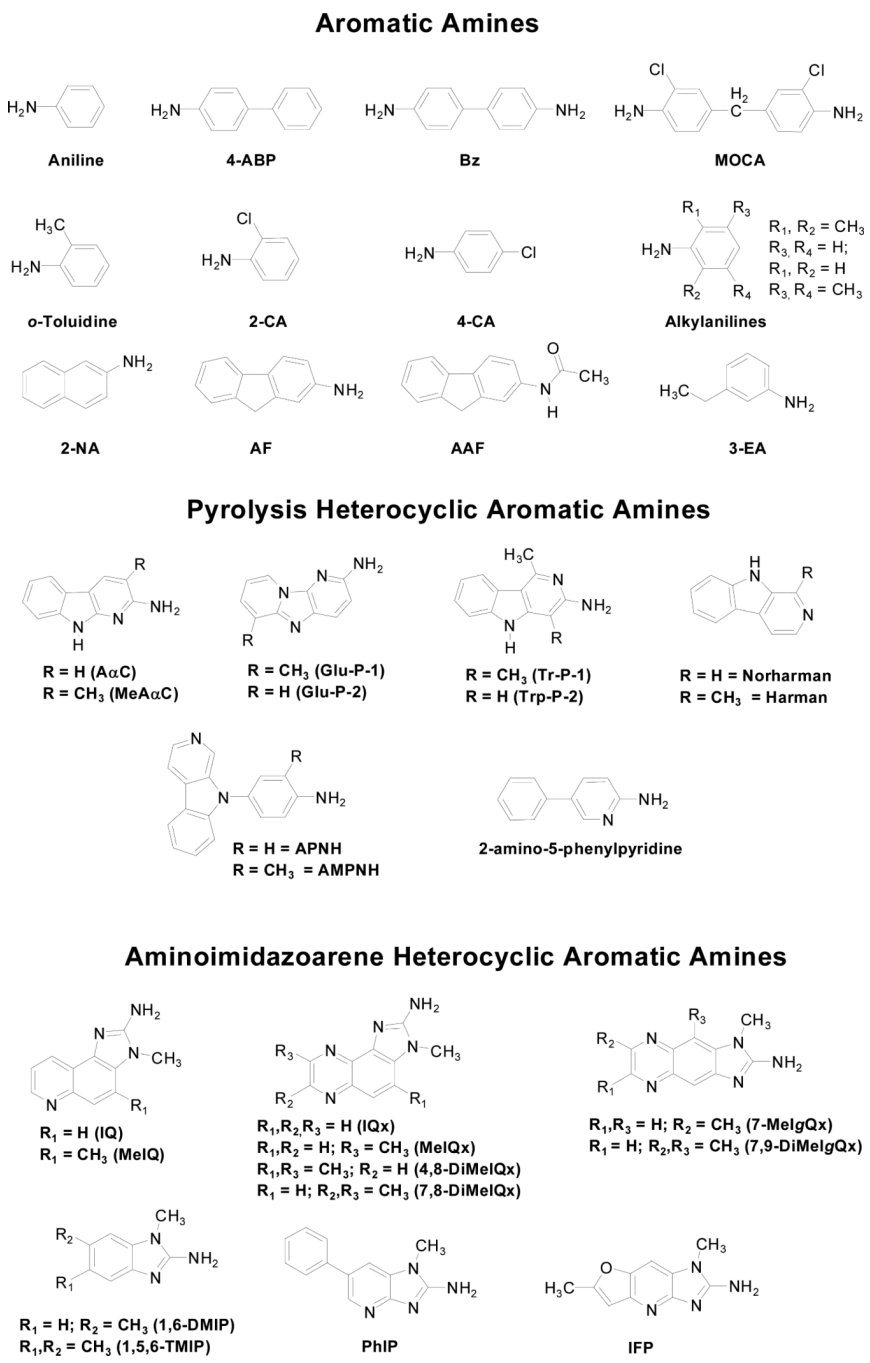

Figure 1.
Chemical structures of prevalent aromatic amines and HAAs

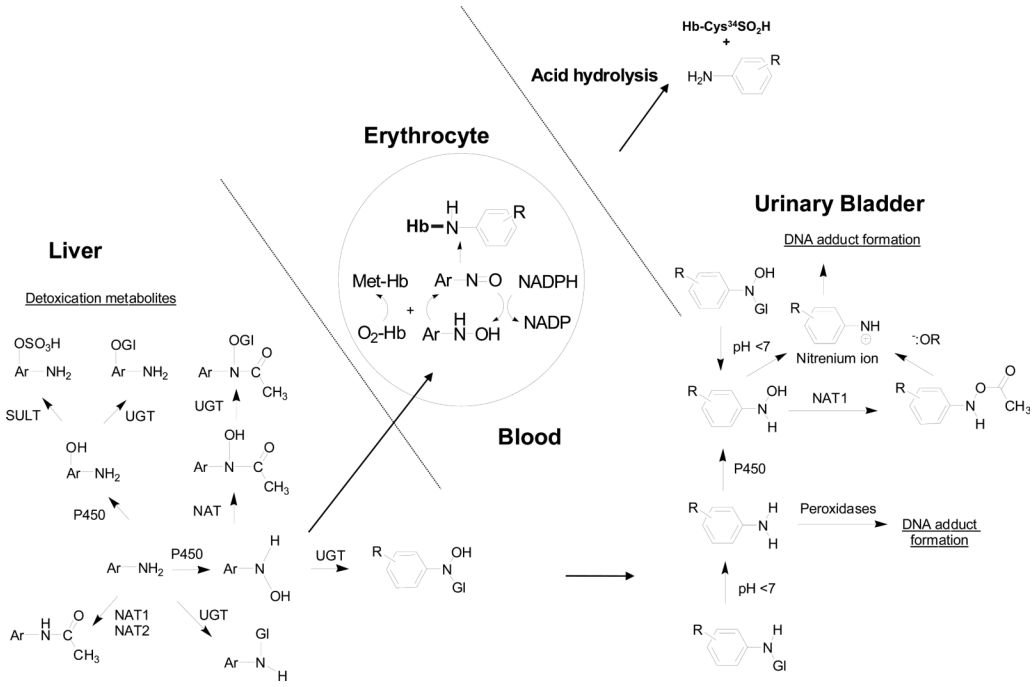

Figure 2.

Mechanisms of arylamine-induced methemoglobinemia, arylamine-Hb sulfinamide adduct formation, and arylamine-DNA adduct formation in the urinary bladder. The arylhydroxylamine metabolite can undergo oxidation to the arylnitroso intermediate within the erythrocyte and react with the Hb-Cys$^{93\beta}$ to form an arylamine-Hb sulfinamide adduct. A portion of the arylhydroxylamine is excreted in urine in the unconjugated form or as an *N*-glucuronide conjugate. Hydrolysis of the *N*-glucuronide conjugate by the mildly acidic pH conditions of urine, regenerates the arylhydroxylamine, which undergoes protonation to form the corresponding arylnitrenium ion and reacts with DNA in the urothelium.

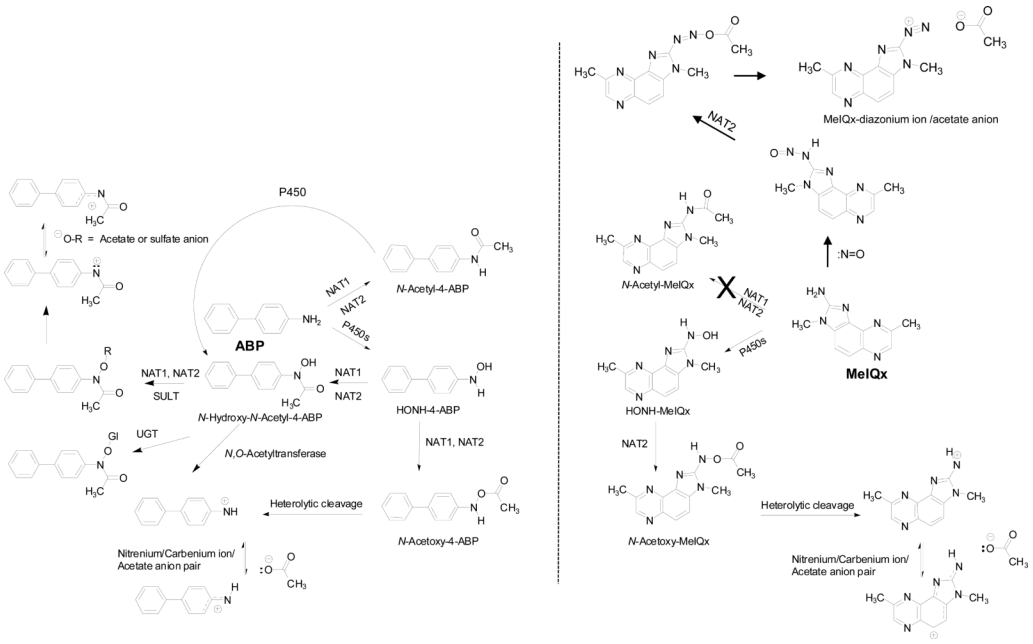

Figure 3.

The metabolism of 4-ABP and MeIQx as prototypes of aromatic amines and HAAs. NAT enzymes effectively detoxicate arylamines, by *N*-acetylation; however, many HAAs are poor substrates for NATs. NATs also catalyze the formation of *N*-arylhydroxamic acids, which can undergo bioactivation by NAT1 and NAT2, or SULTs, or undergo detoxication by UGTs. NAT1 and NAT2 also serve as an *N*,*O*-acetyltransferase or *O*-acetyltransferase and produce reactive *N*-acetoxy esters of the arylhyroxyalmines and *N*-hydroxy-HAAs, which are formed by P450s. *N*-Nitroso-MeIQx formation can occur by reaction with nitric oxide under inflammatory conditions. The *N*-nitroso-MeIQx intermediate has been proposed to undergo metabolic activation by NAT2 to produce a reactive diazonium ion of MeIQx that may damage DNA *(209)*.

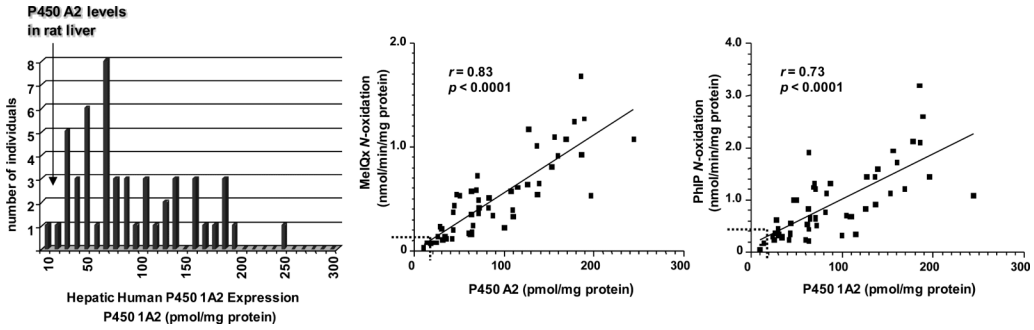

Figure 4.

Levels of expression of P450 1A2 in human liver microsomes and correlation between P450 1A2 expression and rates of *N*-oxidation of MeIQx and PhIP *(222)*. The checkered lines depicted in the correlation regression curves show the upper levels of P450 1A2 expression and rates of *N*-oxidation of MeIQx and PhIP in rat liver microsomes.

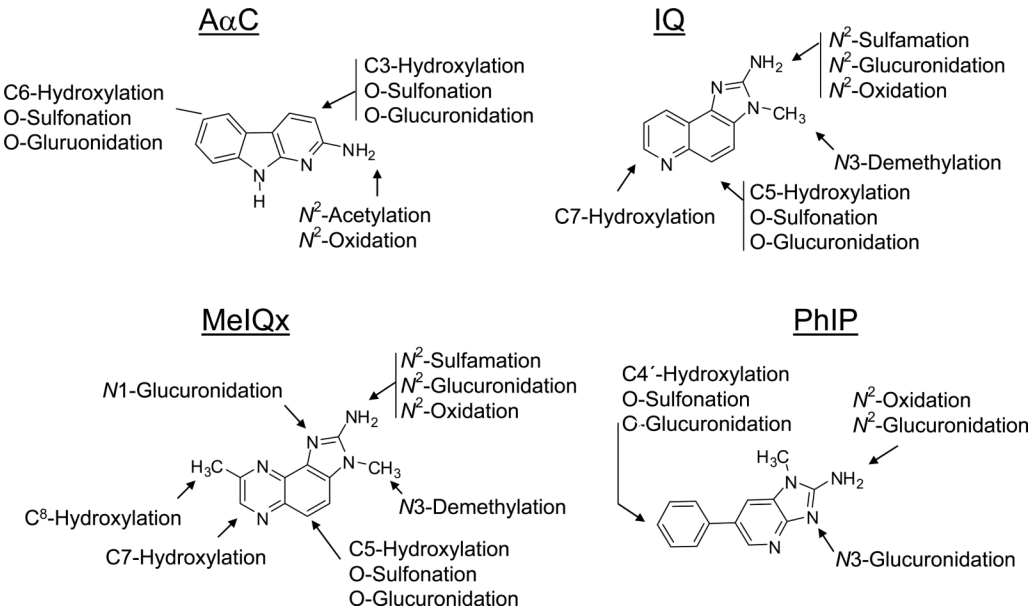

Figure 5.

Major pathways of metabolism of AαC, IQ, MeIQx and PhIP in experimental laboratory animals and humans.

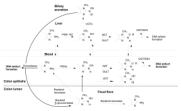

Figure 6.
Metabolism of aromatic amines and HAAs by UGTs, and the role of UGTs in transport of the genotoxic arylhydroxylamine and *N*-hydroxy-HAA metabolites to the colon, to form DNA adducts.

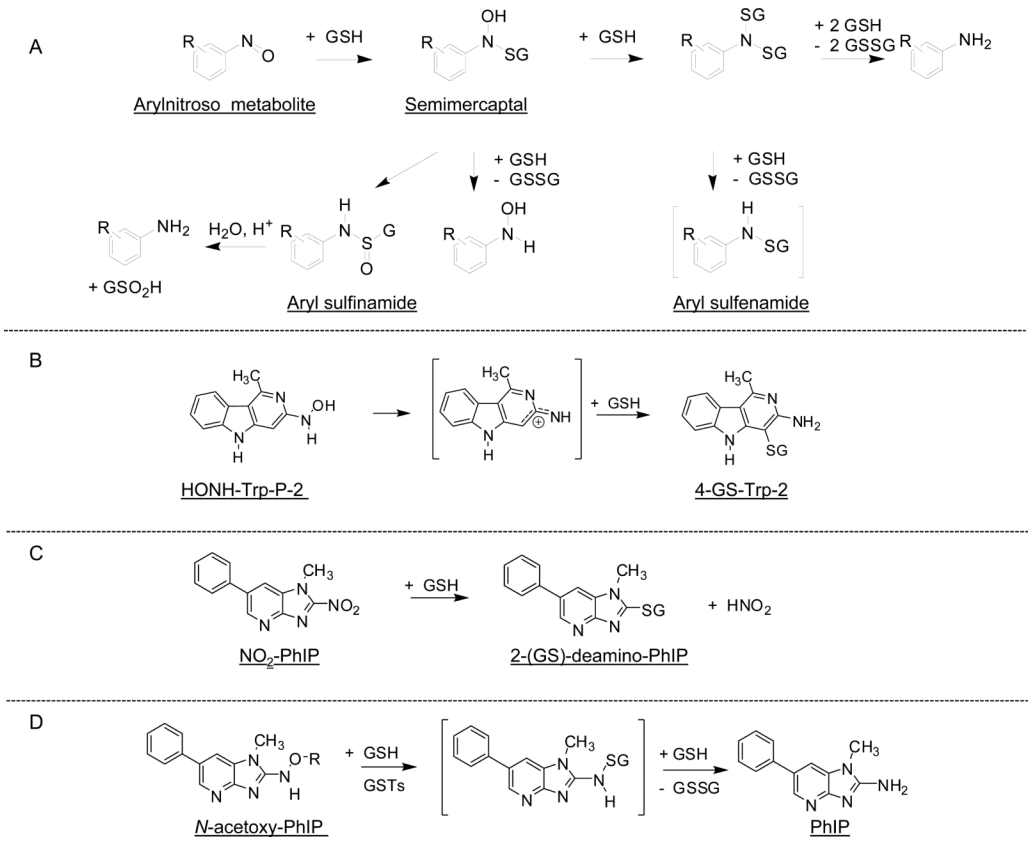

Figure 7.
Reaction pathways of nitrosoarenes, nitroso-HAAs, or nitro-HAA intermediates with GSH and GSTs *(393,419,420,429,430)*.

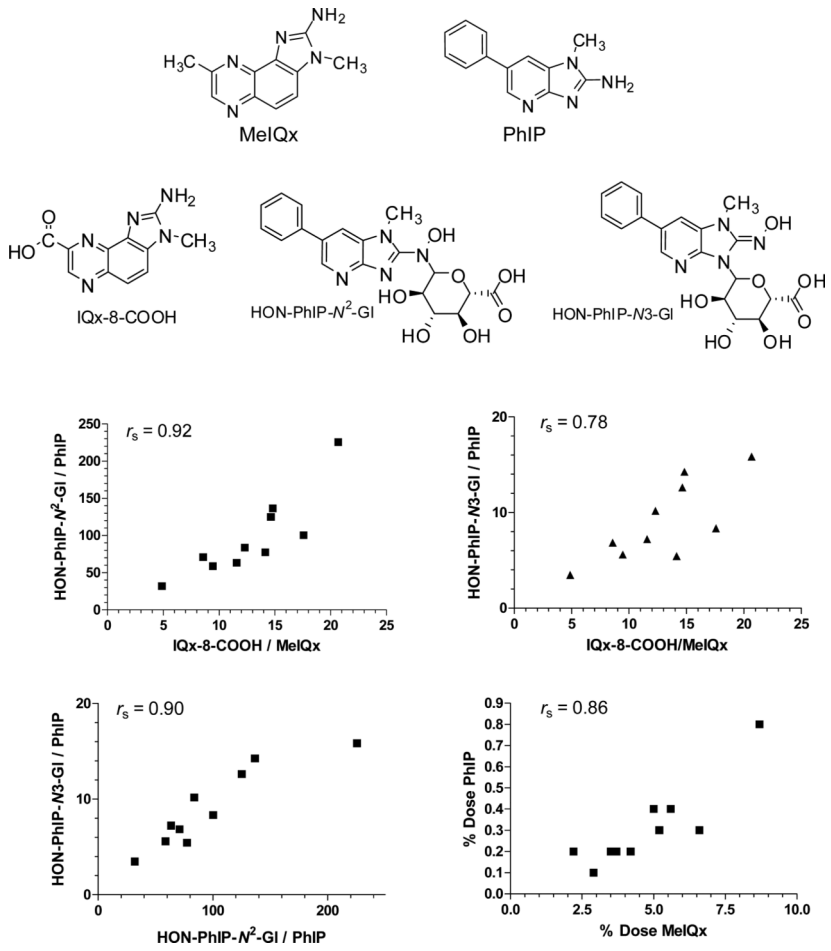

Figure 8.

Scatter plots relating the percentage of the unmetabolized dose of MeIQx and PhIP, and relating the MR of P450 1A2-oxidized MeIQx and PhIP metabolites (% of dose of metabolite/% of dose of unmetabolized MeIQx or PhIP) eliminated in urine collected for 10 h after meat consumption *(103)*.

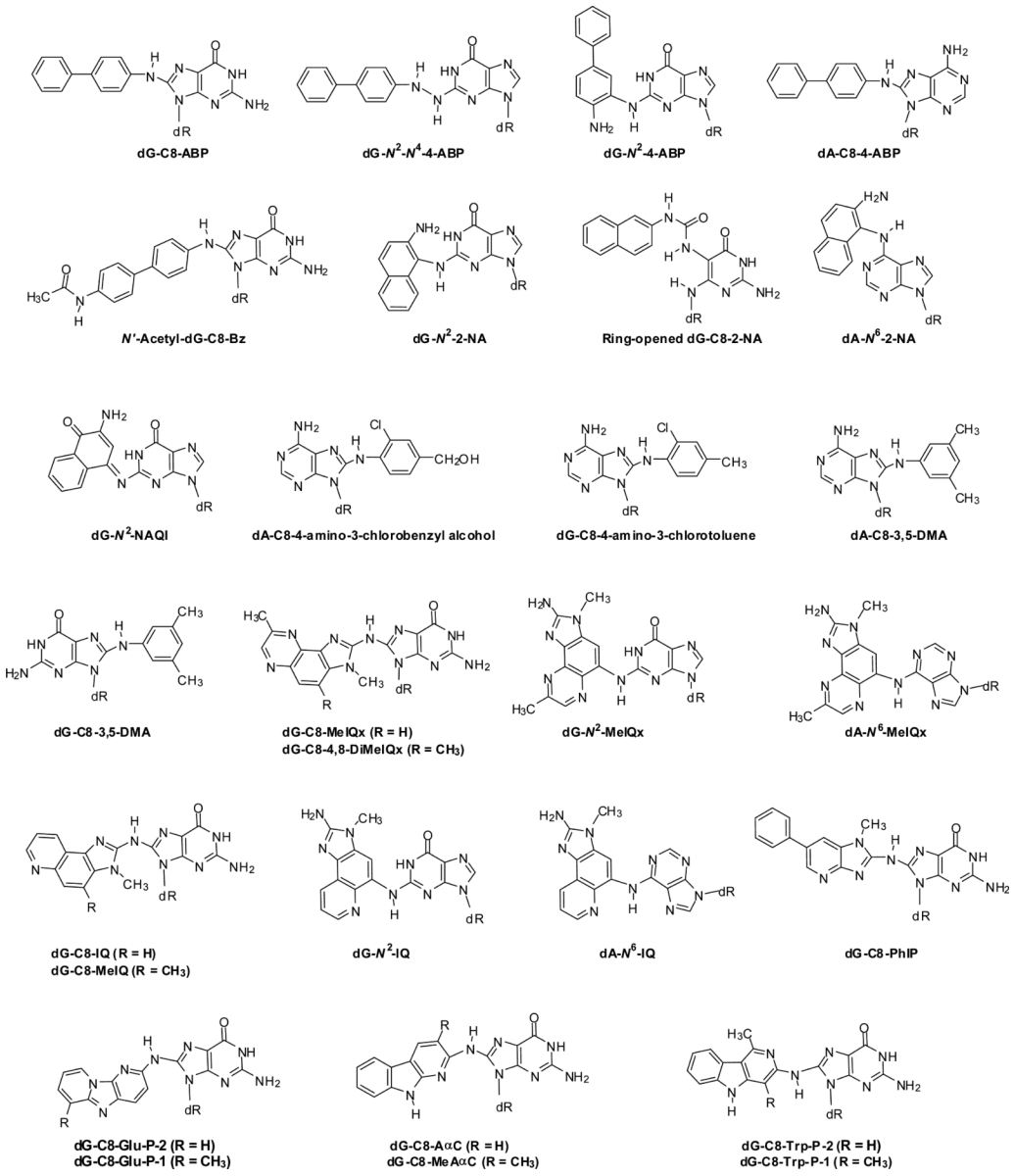

Figure 9.
Structures of DNA adducts of aromatic amines and HAAs.

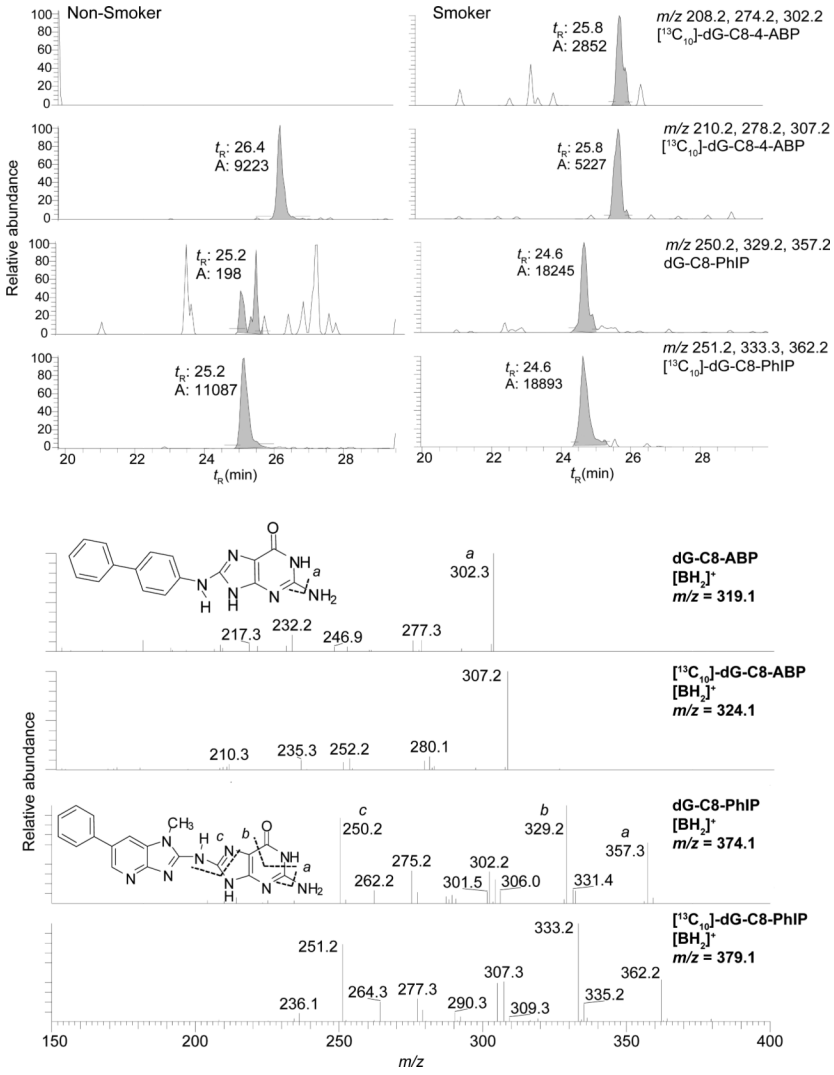

Figure 10.

LC/ESI/MS/MS^3 traces of dG-C8-4-ABP and dG-C8-PhIP adducts in saliva DNA, acquired with a linear ion trap MS *(178)*: (A) non-smoker and (B) current smoker. The MS^3 product ion spectra of the aglycone $[BH_2]^+$ and the $[^{13}C_{10}]$-dG-labeled internal standards, added to DNA at a level of 1 adduct per 10^7 DNA bases, are presented in the lower panel (adapted from *(178)*).

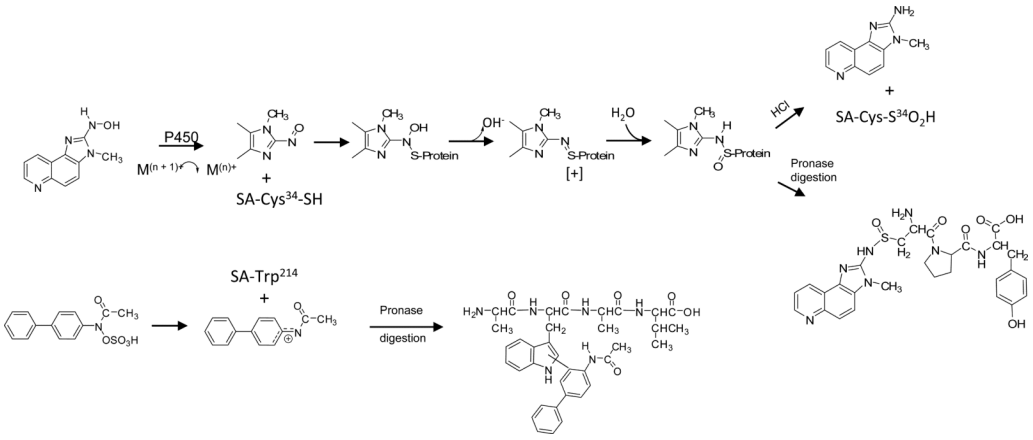

Figure 11.

Mechanism of formation of protein adducts of 4-ABP and IQ with rat SA. The of *N*-sulfonyloxy ester of *N*-acetyl-4-aminobiphenyl reacts with the sole tryptophan residue of rat SA *(390)*. In the case of IQ, the HONH-IQ metabolite undergoes further oxidation, by either transition metals or P450 1A2, to form the nitroso metabolite *(602)*. Nitroso-IQ reacts with SA-Cys^{34} residue to form the semimercaptal, which undergoes rearrangement to the sulfinamide structure. A tetrapeptide containing *N*-acetyl-4-ABP adducted to ala-trp-alaval and a tripeptide containing the IQ adduct at cys-pro-tyr are recovered upon digestion of SA with pronase. In analogy to arylamine-Hb sulfinamide adduct chemistry (see Figure 2), acid treatment of the IQ-SA sulfinamide adduct results in hydrolysis and the generation of IQ and the SA-Cys^{34} sulfinic acid *(587)*.

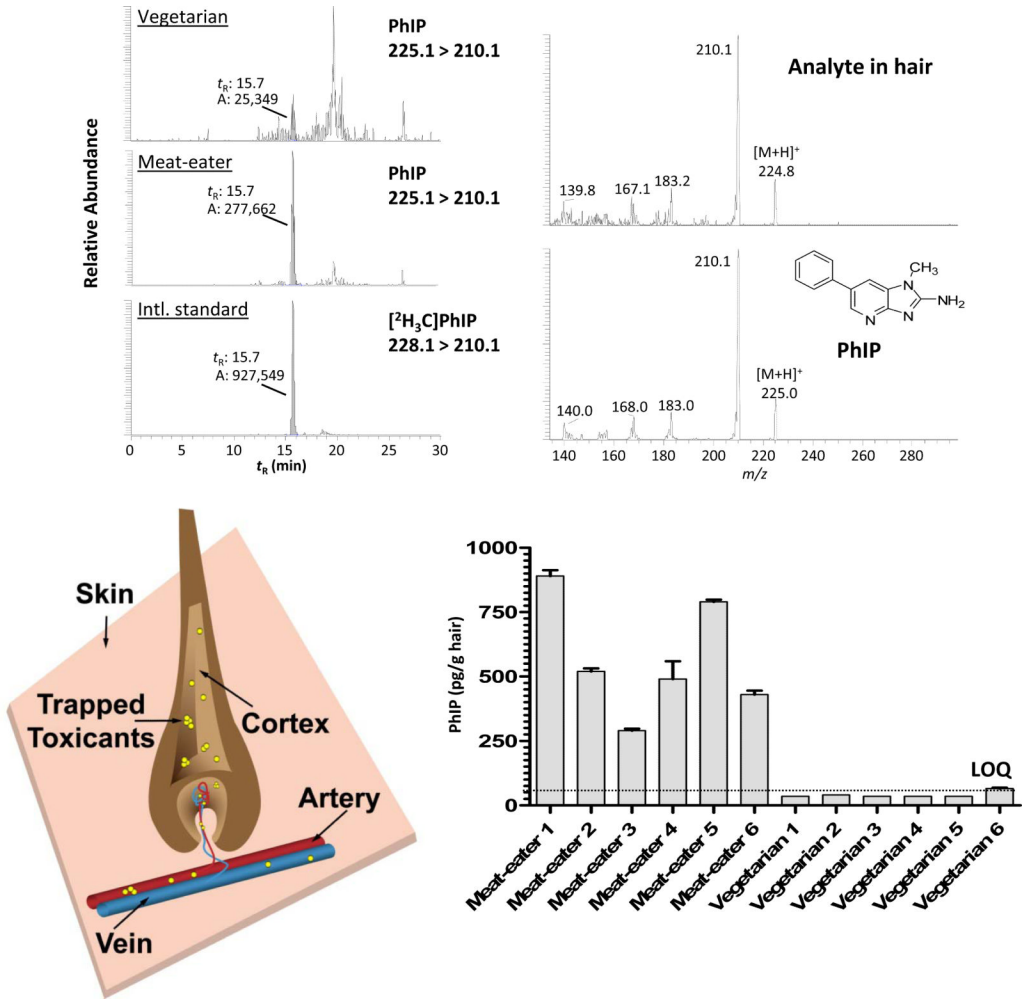

Figure 12.

Bioaccumulation of PhIP in hair of omnivores and vegetarians. The melanin in the hair follicle has high affinity for PhIP, and sequesters the carcinogen from the blood stream, following first-pass metabolism *(24)*. The LC-ESI/MS/MS trace shows the presence of PhIP in hair of an omnivore but not in the hair sample of a vegetarian. The identity of PhIP was confirmed by its product ion spectrum *(607)*.

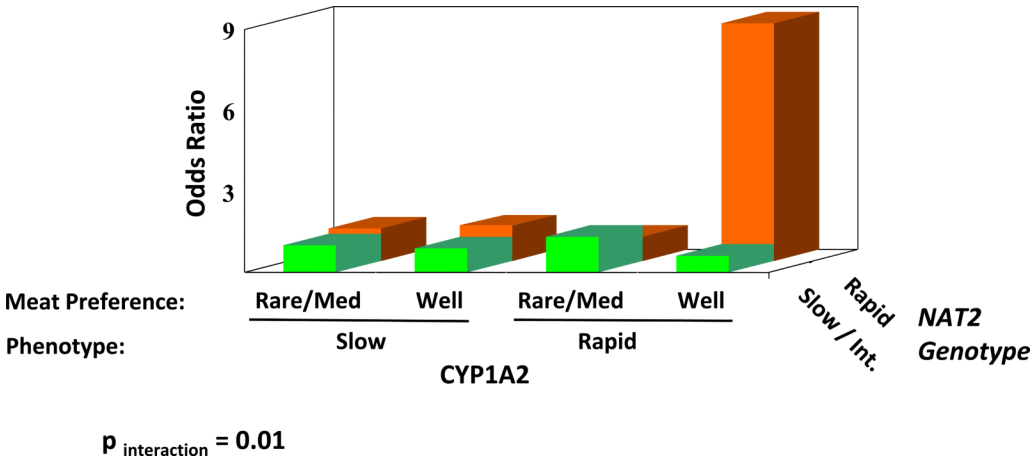

Figure 13.
Three-way interaction of red meat preference, *NAT2* genotype and P450 1A2 phenotype on the risk of colorectal cancer among ever-smokers (149 cases and 216 controls), p interaction = 0.01 *(639)*.