# N1 – IUBMB 50th Anniversary Symposium: Protein Structure and Function

## N1-001
## Molecular biology of mammalian prions

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Transmissible spongiform encephalopathies (TSE) are fatal neurodegenerative diseases of humans and animals. The underlying infectious agent, the prion, accumulates not only in the central nervous system (CNS) but also in secondary lymphoid organs. I will revisit the role of the immune system in peripheral prion pathogenesis, while focusing on the mechanisms by which extraneural and extralymphatic prion infectivity develops. Interestingly, the same pro-inflammatory cytokines and homeostatic chemokines that are involved in lymphoid neogenesis and compartmentalization of immune cells appear to represent the crucial molecular switches responsible for the establishment of extraneural prion reservoirs.

## N1-002
## Regulation of the actin cytoskeleton by IRSp53 and MIM proteins

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The mechanism of actin cytoskeletal reorganization by Rho family small GTPases is complex and involves multiple protein interactions. We recently solved the crystal structure of the IRSp53-MIM actin bundling domain (IMD) of the human IRSp53. IRSp53 binds to the small GTPases Rac and Cdc42 and is involved in the assembly of both filopodia and lamellipodia in mammalian cells. It is thought to act as a scaffold, assembling complexes of actin regulatory proteins (such as Scar/WAVE, Mena/VASP) near the plasma membrane. We also found that it is an effector, inducing actin bundling directly through the IMD. The related protein, Missing in Metastasis (MIM) interacts with receptor tyrosine phosphatases and also organizes the actin cytoskeleton. Our studies aim to compare the activities of IRSp53 and MIM, using known structural information to create mutants and dissect the functions of these proteins in signalling to cell motility.
**Conclusions:** IRSp53 and MIM contain conserved actin bundling domains and are thus effectors as well as scaffold proteins. Both proteins bundle actin filaments by a conserved mechanism and this is important for their function in mammalian cells.

## N1-003
## Structural proteomics of transcription and translation proteins

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We have been studying structural and functional aspects of various transcription and translation proteins, such as RNA polymerases, transcriptional regulators, RNA helicases, RNA processing and modifications enzymes, aminoacyl-tRNA synthetases, and translation factors, mainly by X-ray crystallography, as a part of the national project of structural proteomics. The crystal structure of the bromodomain of human Brd2, as well as those of its complexes with different peptides corresponding to the acetylated histone H4 reveals many surprising features including the recognition of the site-specific acetylation pattern of the lysine residues (the ''histone code'') in epigenetic transcriptonal regulation. We have determined the crystal structure of the helicase fragment of Vasa bound with a single-stranded RNA and an ATP analogue. The observations illustrate the nucleic acid translocation, coupled to ATP hydrolysis, by the ''inchworm'' mechanism of the helicase superfamily II, to which the DEAD-box family belongs. Mechanisms of tRNA and amino acid recognition by aminoacyl-tRNA synthetases have been studied on the basis of their crystal structures and mutagenesis. For strict selection of amino acids, many synthetases have the ''editing

domain''. The crystal structures of a complex of archaeal leucyl-tRNA synthetase and a leucine tRNA demonstrate how the 3'-terminus of tRNA is relocated from the aminoacylation domain to the editing domain.

## N1-004
## Signaling via GTP-binding proteins of the Ras superfamily

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Guanine nucleotide binding proteins (GNBPs) cycle between a GDP-bound inactive and a GTP-bound active state. The switch-ON reaction involves the exchange of tightly bound GDP against GTP, while the switch-OFF mechanism involves the enzymatic cleavage of GTP to GDP. The first reaction is catalyzed by guanine nucleotide exchange factors GEFs, while the second is activated by GTPase-activating proteins GAPs. The inability of certain of these proteins to be down regulated leads to various forms of cancer. The biological function of the GTP-binding proteins relies on the ability to switch between two different conformations, only one of which has a high affinity to the downstream target. The common structural principles of the switch mechanism will be presented, and examples for how Ras and Rho proteins in their active GTP-bound form recognize downstream targets will be discussed, together with the biological consequences.

## N1-005
## 50 Years of IUB(MB)

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This will be an account of the history of the International Union of Biochemistry and Molecular Biology (IUBMB), which began life as the IUB, from its admission as a Union into the International Council of Scientific Unions (ICSU), in 1955, the occasion for this 50th anniversary celebration, but also mentioning the way in which idea to form a Union was conceived and implemented, beginning at the first International Congress of Biochemistry, held in Cambridge in 1949. The speaker was the first Secretary General of FEBS (1965–1967), the General Secretary of IUB (1973–1983) and President of IUBMB (1997–2000). He will offer prognostications for the next 50 years.

## N1-006P
## Isolation of a new toxin from *Tityus serrulatus* scorpion venom with action on the complement system

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Due to its diverse biological activities, the complement system (CS) provides key mediators of inflammation as natural response of the host tissue to any injury. The CS activation by animal venoms has been described and resulted in important discoveries. The aim of the present study was to isolate the toxin from *Tityus serrulatus* venom (TsV) with action on the CS and to evaluate its effects using *in vitro* assays.

**Methods:** The toxin was purified from TsV by ion exchange chromatography on CM-cellulose-52 followed by reverse phase HPLC (C-4) of lyophilized fraction I. Complement consumption by the toxin was evaluated using *in vitro* haemolytic assays, immunoelectrophoresis and two-dimension immunoelectrophoresis of complement components (factor B and C3).

**Results:** The isolated toxin is a single polypeptide chain showing a single band in SDS-PAGE, corresponding to an approximate Mr of 10 000. This protein induced a concentration-dependent reduction in haemolytic activity of the classical/lectin (CP) and alternative (AP) complement pathways, with an IC50 (sample concentration inhibiting 50% of the lytic activity) of 22.08 and 62.66 mg for CP and AP, respectively. Alterations in C3 and factor B electrophoretic mobility after incubation of normal human serum with toxin (45 mg), were identical to those obtained with zymosan (positive control).

**Conclusion:** Our results show that the toxin is able to activate the complement system leading to reduction of serum lytic activity and Factor B and C3 cleavage. Therefore, this toxin may play an important role in the inflammatory response observed upon scorpion envenomation.

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## N1-007P
## Interactions of HIV-1 integrase with modified analogs of viral DNA: implications for understanding integration mechanism

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Human immunodeficiency virus type 1 (HIV-1) is related to the class of retroviruses, which genome consists from RNA molecules. After RNA reverse transcription, a DNA copy of the viral RNA is integrated into genome of an infected cell. The viral DNA integration is one of the most important steps of the whole retroviral replication cycle, and it is effectuated by a viral enzyme, integrase (IN). It is known that IN recognizes specific sequences localized at viral DNA U3 and U5 LTRs, binds them and catalyzes two nucleotide removal reaction from 3'-ends of each strand. It is the first integration stage that is named 3'-end processing. Later IN mediates strand transfer reaction consisting in processed virus DNA embedding into the host DNA. In order to reveal what structural features of the viral DNA mostly determine the sequence-specificity of its recognition and processing by IN, in the current work we studied systematically how the double helix structure of viral DNA influences on the HIV-1 integrase activity in 3'-end processing reaction. For this purpose we synthesized a set of modified DNA duplexes, which sequence mimicked U5 LTR of the viral DNA. Nucleosides at different positions of processed and/or non-processed strands were consistently replaced by a non-nucleoside insertion, 1,3-propanediol residue, or nucleosides containing modified sugar residues: 2'-aminonucleosides and 2'-O-methylnucleosides. We concentrated our attention on the study of the integrase intetaction with viral DNA third adenosine situated close to the 3'-processing point. For this purpose we prepared a number of U5 substrate analogs containing unpaired nucleotides in the third position (instead of A/T pair) and 2,6-diaminopurine instead of adenine. We can conclude that IN is likely to recognize the viral DNA in the major groove forming hydrogen bond with N7-atom

and N6-amino group of the third adenine. In contrast, the complementary base, thymine, does not participate in any interactions with IN. Regarding other positions of the substrate DNA, we consider that IN recognizes a fine structure of the sugar-phosphate backbone rather than heterocyclic bases.

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## N1-008P
## Purification of fructose 1,6 bisphosphate aldolase from diabetic human placenta and inhibition effects of dihydroxyacetone-phosphate

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In diabetic complications there are changes in placental function, in particular with respect to the uptake, transfer and utilization of glucose and also in glycolysis and glycolitic enzymes. The placenta possibly plays a crucial role in protecting the fetus from adverse effects from the maternal diabetic conditions. Fructose-1,6-bisphosphate aldolase (FBP2) (EC. 4.1.2.13), a major glycolytic enzyme found in most cells, catalyzes the reversible cleavage of Fructose 1,6 bisphosphate into glyceraldehyde-3 phosphate and dihydroxyacetone phosphate. In mammalian tissues there are three isozymes of aldolase Type A, B and C. This enzyme, is a homotetramer with a molecular mass of 160 kDa, each subunit occurring in an alpha/beta barrel. In our study, we investigated the presence of aldolase in diabetic human placenta and then to purify and examine kinetic properties. The fresh placenta was obtained and the tissues were cooled, washed and perfused and weighed, then minced and suspended in three volumes (v/w) of buffer and homogenized. The homogenate was centrifuged and clear supernatant was obtained. Ammonium sulfate fractionation and phosphocellulose chromatography were applied to the supernatant. The quantitative estimation of aldolase activity present in the extracts were determined by measuring the rate of cleavage of fructose 1,6 bisphosphate spectrophotometrically. A unit of aldolase was defined as that amount of enzyme which catalyses the cleavage of 1 μmol of substrate per minute at 25 °C under conditions of assay. The specific activity was defined as the number of activity units per milligram of protein. With this procedure, aldolase was purified about 63-fold from diabetic placenta. Inhibition kinetics of FBP2 were studied using DHAP as allosteric inhibitor.

## N1-009P
## Structural and functional insights into *Histoplasma capsulatum*'s virulence factor CBP

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The dimorphic fungal pathogen *Histoplasma capsulatum* secretes a calcium-binding protein (CBP) that has been shown to be essential for virulence. However, the specific function of CBP and its role in pathogenesis remain a mystery. Recent collaborative work with *Histoplasma*-infected macrophages has provided a direct correlation between increased phagolysosomal pH and decreased calcium concentration, suggesting that the function of CBP may involve responding to or generating such an environment. The purpose of this study is to combine structural information on CBP with binding assays to link physical features with specific functions related to virulence and calcium binding. We have collected several NMR data sets in order to study the secondary and tertiary structure of CBP. Importantly, we will present the structure of CBP, representing the first 3D NMR structure of a fungal virulence factor. The preliminary structural studies reveal that CBP exists as a symmetric homodimer in solution and that the structure is unperturbed by calcium binding. Additionally equilibrium calcium-binding constants for CBP have been obtained by using both chromophoric chelator competition and mass spectrometry-based assays. We have demonstrated with our calcium binding assays that the $K_D$ for CBP is in the 100 μM range, which is relatively weak binding affinity considering that intraphagolysosomal calcium concentration is only 100 nM. These results, in conjunction with other surprising observations, suggest that the role of CBP is much more complex than simple calcium acquisition and emphasize the importance of future structural studies for understanding CBP's function.

## N1-010P
## Endothelium masking chemokine mutants as a novel principle in anti-inflammatory therapy

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The proinflammatory chemokine RANTES (regulated upon activation, normal T-cell expressed and secreted) is involved in the recruitment of a specific subset of immune cells leading to inflammation and its propagation by binding to two functionally important interaction partners. First, RANTES is immobilized via glycosaminoglycan chains of heparan sulphate proteoglycan coreceptors on the endothelium of inflamed tissues forming a chemotactic gradient. Secondly, it binds to and activates three chemokine receptors on leucocytes and other target cells. Here a new class of anti-inflammatory RANTES mutants is presented acting as coreceptor agonists and at the same time receptor antagonists. Several site-directed RANTES mutants were engineered that combine impaired receptor activation with enhanced coreceptor binding properties. Two RANTES variants additionally contain an aggregation impeding mutation as aggregation of RANTES was shown to be a prerequisite for some of its proinflammatory features. These chemokine variants should be able to mask the endothelium by competing with native RANTES and influence the inflammation at an early stage. Biophysical data on these RANTES mutants concerning their structural differences and their stability compared to wild type RANTES will be presented. The functionalities of the mutations were tested by *in vitro* glycosaminoglycan binding and chemotaxis assays. By combining these data two promising RANTES mutants stood out that will be assayed in an *in vivo* model of rheumatoid arthritis.

## N1-011P
## Gamma-glutamyl transpeptidase in Schistosomiasis

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Hepatic gama-glutamyl transpeptidase showed a significantly enhanced total activity in mouse schistosomiasis. The enzyme

was purified from the liver of normal mouse and compared to that from *Shistosoma*-infected one. The purified enzyme in both cases was separated into its molecular forms. The specific activities of these forms of the enzyme from normal and *Shistosoma*-infected mouse, respectively displayed some variations. A marked elevation was noticed for the isoforms III and V of the enzyme from *Shistosoma*-infected mouse compared to that from the normal one. These data suggest that structural changes of hepatic gama-glutamyl transpeptidase may occur in schistosomiasis.

## N1-012P
## Molecular modeling of human thromboxane synthase (CYP5A1): study of the effects of CYP5A1 gene mutations

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Thromboxane synthase (CYP5A1, EC 5.3.99.5) is the unique member of family 5 of the cytochrome P450 superfamily. CYP5A1 catalyzes the conversion of prostaglandin H2 (PGH2) to thromboxane A2 (TXA2), which is a potent mediator of platelet aggregation, vasoconstriction and bronchoconstriction, and plays an important role in major human diseases, including atherosclerosis, myocardial infarction, stroke, septic shock and asthma. Clinically, a deficiency in platelet CYP5A1 activity was shown to be associated with moderate to severe bleeding disorders, but the molecular mechanisms underlying this deficiency are not understood. Recently, the screening of the CYP5A1 genomic sequence in a population of 200 volunteers allowed us to identify eight missense mutations. In this study, we built a three-dimensional (3D) model of human CYP5A1 using the known tertiary structure of the human CYP3A4 solubilized structure, which shares 53% similarity (34% identity) with the CYP5A1 enzyme. The examined criteria indicated a reliable model structure. The model gave insight into the structural effects of naturally occurring mutations of the CYP5A1 allele. For each residue affected by a missense mutation, its location in the 3D structure and the putative changes in terms of biochemical properties brought about by the mutation were analyzed. Our 3D human CYP5A1 model provides a basic model for further studies of novels CYP5A1 mutations, and for identification of important residues involved in the specific activity of the enzyme. Moreover it would then represent a useful tool for studies of structural/functional relationships, reaction mechanism and potential drug interactions.

## N1-013P
## Steady-state kinetics of rat intestinal butyrylcholinesterase

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Butyrylcholinesterase (BChE; E.C. 3.1.1.8) was 260-fold purified from soluble fraction of rat intestine by Sephadex G-25 chromatography and procainamide-Sepharose 4B affinity chromatography. The enzyme was shown to be composed of tetrameric globular form by non-reducing electrophoresis. The hydrolysis of butyrylthiocholine iodide (BTCh) did not follow hyperbolic Michaelis–Menten kinetics. Hill plot displayed a biphasic character. At low substrate concentrations (0.05–0.5 mM), substrate activation (nH = 2.2) was observed whereas at high substrate concentrations (0.5–2.0 mM) substrate inhibition (nH = 0.375) was detected. Therefore steady-state kinetics was studied at the concentration range in which substrate activation was detected. Km, Kss, and kcat values were calculated as 0.060 ± 0.024 mM, 0.893 ± 0.392 mM and 2769 ± 280 per minute, respectively. The enzyme had higher catalytic efficiency towards BTCh than acetylthiocholine (ATCh) and propionylthiocholine (PTCh). kcat/Km. values were calculated to be 16210, 25650 and 46150 for ATCh, PTCh and BTCh respectively. Optimum pH value was determined as 7.2 after zero buffer extrapolation. Optimum temperature was examined 37 °C after zero time extrapolation. Energy of activation (Ea) and the temperature coefficient which is the factor by which the rate constant is raised by increasing the temperature 10 °C (Q10) were calculated as 4915 cal/mol and 1.30, respectively from the Arrhenius plot. Enthalpy of activation (ΔH) was found to be 3432 cal/mol from enthalpy graph.

## N1-014P
## Proteomic investigation of Taura syndrome virus regulated proteins in *Penaeus vannamei*

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Taura syndrome disease is generally a highly virulent disease in *P. vannamei*. TSV locates itself in the cytoplasm of infected epithelial cells of the shrimp's cuticle. We have produced and purified the viral particle from infected *P. vannamei* by Ficoll gradient ultracentrifugation. The purified TSV was characterized by RT-PCR detection and injected into specific pathogen-free shrimps. After 1 day of infection, TSV was detected in the haemolymph. Since the knowledge and understanding in the molecular level of TSV infected *P. vannamei* is far from adequate, two-dimensional polyacrylamide gel electrophoresis (2D–PAGE) was employed to analyze for proteomic changes upon TSV infection. The changes were observed as an increasing and decreasing expression of specific proteins. We have extracted protein from shrimp hemocytes and compare the expression profiles between infected and healthy shrimps at 72 h post-infection. There are 18 protein spots detected with significant modulation in their expression level. Eleven proteins are down-regulated while seven are up-regulated in comparison with the healthy shrimp. These proteins were subjected to be identified by matrix assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF MS) and MS/MS.

## N1-015P
## Proteins of oxygen evolving complexes of photosystem II studied by means of molecular modeling and vibrational spectroscopy

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A combination of homology and energetic modeling with vibrational spectroscopy [1] is used for the determination of the

structure of several proteins of the oxygen evolving complex (PsbQ, PsbP and PsbO) bound to photosystem II of higher plants, spinach and pea. Structural and sequence alignments of the individual proteins of the oxygen evolving complex were performed to get a basis for homology modeling. Three-dimensional models of oxygen evolving complex proteins are suggested. During the course of modeling methods of vibrational spectroscopy are used to get detailed information about the secondary structure content, the spatial arrangement and interactions of individual aromatic residues. These data serve as a feedback for the generation of three-dimensional models. For the purpose of vibrational spectroscopy proteins of the oxygen evolving complex are either prepared recombinantly or are isolated form the plant material. Thus gained models serve for the detection of interaction sites between PsbP and PsbQ by Raman and FTIR. Additionaly the models are used for fitting into images of the whole complex of photosystem II taken by electron microscopy. With this we are able to determine the spatial arrangement of individual proteins in the oxygen evolving complex.

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**Reference**

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## N1-016P
## Conformational and dynamics study of the human papillomavirus HPV-16 E2C complexed with its DNA target sequence

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Gene transcription and replication in Papillomavirus is tightly controlled by the E2 protein, which recognizes the consensus sequence ACCGNNNNCGGT. In the Human Papillomaviruses (HPV) E2 displays the ability to select between distinct binding sites that differ only in the central N4 spacer. Whether E2 activates or represses promoter activity depends on the relative affinity for each of its four binding sites localized on the viral genome as the concentration of the protein varies during the virus life cycle. The 80 residues dimeric Carboxy-terminal domain (E2C) is responsible for the specific DNA recognition. The E2C structures from the various viral strains can be subdivided into two families on the basis of the relative orientation of the two monomers and their recognition helices. High-resolution structural information is not available for the complexed form of HPV-16 E2C protein or other members of its family. We analyzed the structure and dynamics of the high risk strain HPV-16 DNA E2 binding domain bound to a 18mer DNA duplex containing the specific E2 recognition site by means of NMR methods. First, we have completely assigned the backbone resonances of the HPV16 E2C domain. Using Chemical Shift information, we were able to address several issues related to the conformational changes of this unique fold with respect to its unbound state. The DNA-free structure was refined against NMR data collected on the DNA-bound form, such as NOEs and Residual Dipolar Couplings (RDCs). One-bond RDCs analysis was performed to assess the conformational similarity with the DNA-free structure and the related members of the two E2C families. The results demonstrate that only modest protein changes accompany the DNA recognition. Careful backbone measurements of nuclear spin relaxation and exchange rates indicated that the DNA-free conformation, far away to be globally or locally disordered, nevertheless is characterized by diffused mobility and that the flexibility of the DNA recognition helix is particularly high. This plasticity provides potential regulatory conformational changes in regions far from the DNA recognition interface, an essential necessary role in these and other transcriptional regulators. In contrast, the dynamics analysis of the complexed form shows that mobility is considerably quenched and a large overall protection from solvent exchange is present when the DNA is bound, most notably in the DNA binding helices. Only the central β2-β3 loop in each monomer, which faces the DNA in the complex, appears disordered and flexible in both the bound and unbound protein, probably contributing to reduce the entropic costs of the recognition event.

## N1-017P
## Site-directed mutagenesis studies revealed the functional role of the conserved Lys 215 in the domain closure dependent phospho-transfer catalysed by human 3-phosphoglycerate kinase (hPGK)

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PGK is a typical two-domain hinge-bending enzyme. It is still unclear how the geometry of the active site is formed during domain closure and how the catalytic residues are brought into the optimal position for the reaction. Surveying the 3D structure of PGK in various open and closed conformations prompted us to assume that the residue Lys 215 moves in more than 10 Å distance during domain closure. This movement may allow direct participation of this side chain in the phospho-transfer process. To test this hypothesis two mutants of Lys 215 (K215A and K215R) were constructed from hPGK and comparative enzyme kinetic and substrate binding studies were performed. DSC and CD measurements could not reveal any detectable conformational change upon these replacements, while drastic decreases (2000- and 700-folds, respectively) were observed in the enzyme activities, approving the essential role of Lys 215 in the catalytic function. Among the kinetic constants of substrates only the *K*m value of MgATP is affected substantially (about 20-fold increase in case of K215A), while no change in the *K*m value of MgADP is detected upon mutation. The results suggest that in the functioning ternary complex Lys 215 strongly interacts with the γ-phosphate of MgATP. From the known crystal structure of MgATP binary complex, however, only a loose and possibly periodic interaction of its flexible phosphate chain can be assumed with this side chain. This initial loose interaction should be largely strengthened during domain closure and therefore Lys 215 moves together with the transferring γ-phosphate of MgATP, meanwhile this phosphate is positioned properly for catalysis.

## N1-018P
## Functional analysis of histidine residues 183, 199, 211, 298, 390, 437 and 661 of human muscle phosphofructokinase by site-directed mutagenesis

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Phosphofructokinase (PFK) activity is highly modulated by the binding of various allosteric effectors, being considered a key enzyme in the regulation of the glycolytic flux in most cells. The smallest active oligomeric form of mammalian PFKs is the tetramer, as found with the rabbit muscle isozyme, which was shown to isomerize to an inactive one upon protonation and then to dissociate slowly into dimers and monomers. Thus, pH plays a fundamental role on the regulatory properties of PFK activity, mediating the protonation of certain ionizable groups of the enzyme with a pK value of about 6.8 (most probably histidines). In order to identify these residues in human muscle PFK (HmPFK-M), histidines 183, 199, 211, 298, 390, 437 and 661, that are highly conserved in mammalian isozymes, have been mutated to alanine. In the absence of a crystal structure of eukaryotic PFK, sequence alignments based on the evolution of this enzyme by duplication/fusion of a prokaryotic gene, and the 3D structure of bacterial protein, suggest that H298 of HmPFK-M would be located in the active centre, whereas H661 (equivalent at the C-terminal domain) would correspond to the fructose bisphosphate allosteric site. The mutant cDNAs were expressed in a PFK-deficient yeast strain, and the recombinant enzymes were purified to homogeneity and characterized. Among all mutations, the pH-dissociating effect was only prevented in H661A with a pK value of 5.2 (as compare with the pK value of 6.8 for the wild type), without change in the pH sensitivity. The H298A mutant showed a fourfold decrease in fructose-6-P affinity, and H661A was not activated by fructose-1,6-$P_2$ and scarcely by fructose-2,6-$P_2$. These results are in agreement with the proposed evolutionary model of eukaryotic PFK.

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## N1-019P
## Characterization of MerR family member, PMTR (*Proteus mirabilis* transcription regulator)

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One class of heavy metal transcription regulator is the MerR family. Members of the MerR family bind DNA as homodimers and activate or repress the transcription of genes involved in efflux or detoxification in response to the specific ligands. Most of these transcription regulators bind cognate DNA in the presence or absence of ligand, typically repressing in the absence of ligand and activating when ligand has bound. The *Proteus mirabilis* transcription regulator, PMTR, is a member of the MerR family and confers resistance to normally toxic levels of zinc when introduced into *Escherichia coli* cells [1]. Here we determine a cognate DNA binding site from *P. mirabilis* and show *in vitro* binding of PMTR to this site. DNA footprinting results indicates that PMTR distorts its DNA binding site in a similar manner to those observed with TPP-BmrR [2], MtaN [3], and other MerR family members, thus suggesting the distortion mechanism for the transcription regulation. The isothermal titration calorimetry shows that PMTR binds copper cation with higher specificity than either zinc or cobalt cations. And the structural based amino acids sequence alignment [4] suggests that PMTR has significant homology to CueR and, to a lesser extent, ZntR. Thus, we propose that PMTR is the *P. mirabilis* homologue of the copper binding transcription regulator CueR.

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## N1-020P
## Purification and characterization of a novel ATP-dependent robust protein-unfoldase, Unfoldin

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We have isolated a novel ATP-dependent robust protein-unfolding activity from *S. cerevisiae* and designated Unfoldin. ATP, but not its hydrolysis, promoted binding of Unfoldin to substrates and unfolded their conformation. Protein sequencing revealed that Unfoldin was identical to YDL178w identified as an actin interacting protein 2 (AIP2), the function of which is poorly understood. Gel-filtration and low angle shadowing electron microscopy revealed that Unfoldin formed a homo-oligomeric complex consisting of 10–12 subunits arranged in an grapple-like structure with a ~2 nm central cavity. Removal of the C-terminal coiled-coil region of Unfoldin led to dissociation of the oligomer concomitant with the loss of both substrate binding and protein-unfolding activity. Unfoldin bound to all substrates so far examined *in vitro*, and modified their conformation as determined by the trypsin susceptibility assay. It is worth noting that the robust protein-unfolding activity of Unfoldin modulated the conformation of several pathogenic, highly aggregated proteins such as prion protein in β-sheet form associated with prion disease, amyloid β (1-42) peptide with Alzheimer's disease and α-synuclein with Parkinson's disease, in the presence of ATP. Protein-unfolding activity of Unfoldin depends on the growth stage of yeast and the most significant activity was observed at the log phase, suggesting the presence of a cofactor/s.

## N1-021P
## Structures of *Escherichia coli* NAD synthetase with substrates and products reveal mechanistic rearrangements

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The final step of the biosynthesis of the ubiquitous enzyme cofactor nicotinamide adenine dinucleotide (NAD) is the amidation of nicotinic acid adenine dinucleotide (NAAD). The latter reaction is catalyzed by the nicotinamide adenine dinucleotide synthetases

(NADS) using either ammonia or glutamine as amide donor. Here we report crystal structures of the strictly ammonia-dependent homodimeric NADS from Escherichia coli alone and co-crystallized with natural substrates and with the reaction product, NAD. The structures disclosed two NAAD/NAD binding sites at the dimer interface and an adenosine-triphosphate (ATP) binding site within each subunit. Comparison with the *Bacillus subtilis* NADS showed pronounced chemical differences in the NAAD/NAD binding sites and less prominent differences in the ATP binding pockets. The differences at the NAAD/NAD site between the two bacterial species are relevant for designing species specific antibiotics. In addition, the *Escherichia coli* NADS structures revealed unexpected mechanistic dynamic in the NAAD/NAD binding pocket upon NAAD-to-NAD conversion, which define a catalysis state and a substrate/product exchange state. The two states are adopted by concerted movement of the nicotinysyl moieties of NAAD and NAD, F170 and residues 224–228, which may be triggered by differential coordination of a magnesium ion to NAAD and NAD.

## N1-022P
## Identification of amino acid in unstructured region of ErmSF which has crucial role in methyl group transfer reaction

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ErmSF is one of the Erm proteins which dimetylates at canonical A2058 (*E. coli* numbering) in 23S rRNA to confer resistance to MLS (macrolide-lincosamide-streptogramin B) antibiotics on various microorganisms ranging from antibiotic producers to pathogens. Unlike other Erm proteins, ErmSF has long N-terminal end region (NTER) of which 25% is composed of arginine which is known to interact with RNA well. The structure of NTER was determined by NMR to be unstructured and was confirmed by various NTER truncated ErmSFs since the fact that these proteins showed reasonable but gradually decreased activity on truncation demonstrates that all the proteins retained structural integrity. However, *E. coli* producing mutant protein truncated up to R60 exhibited reduced resistance to erythromycin compared to *E. coli* expressing wild type ErmSF, but smaller inhibition zone than that of *E. coli* harboring empty vector. The purification of this protein yielded 2.4 mg of soluble protein per liter of culture that is enough amount of protein to confer the resistance to erythromycin but retained only 2% of methyl group transferring activity relative to wild type protein. Molecular modeling study suggested that this amino acid interact with RNA close to methylatable adenine to locate it at the active site.

## N1-023P
## Bacterial glutamine synthetase: two-cation-bearing active centres of the enzyme probed by $^{57}$Co emission Mössbauer spectroscopy

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Glutamine synthetase (GS; EC 6.3.1.2.) is a ubiquitous enzyme of nitrogen metabolism. GS from *Azospirillum brasilense*, having 12 subunits and 12 active centres per molecule (similar to other adenylylatable bacterial GSs activated by divalent cations, typically $Mg^{2+}$, $Mn^{2+}$ or $Co^{2+}$), yet has specific features as to its kinetic behaviour, metal specificity and secondary structure [1, 2]. Emission ($^{57}$Co) Mössbauer spectroscopy (EMS) was for the first time used for probing GS active centres, each having two metal-binding sites (n1 and n2) with different affinities, by comparing homobinuclear (with EMS-active $^{57}Co^{II}$ at both sites) and heterobinuclear (with $^{57}Co^{II}$ + natural $Mn^{II}$) active centres. Adding $^{57}Co^{II} + Mn^{II}$ to the metal-free enzyme, as compared to $^{57}Co^{II}$ alone, led to a partial redistribution of $^{57}Co^{II}$ between the sites, showing competitive binding of the cations. This is in line with their similar efficiency in supporting the activity of partly adenylylated GS [1]. Coordination symmetry of $^{57}Co^{II}$ at both sites was found to be altered by changing the GS adenylylation state. The results show the possibility for heterobinuclear catalysis by the GS, and EMS is promising for further studying enzyme–substrate interactions at the molecular level.

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## N1-024P
## Homology modeling and docking study of translationally controlled tumor proteins

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Translationally controlled tumor protein (TCTP) is a ubiquitous and highly conserved protein that is implicated in various functions. However its primary physiological functions as well as 3D structure still remain unclear. Antimalarial drug, artemisinin, has been reported to bind *Plasmodium falciparum* (Pf) TCTP in the presence of heme. That is, heme-catalyzed cleavage of the endoperoxide-bridge forms a free radical, which eventually alkylate Pf TCTP. Through homology modeling by MODELLER and docking studies using FlexiDock, the binding modes between the artemisinin , heme and Pf TCTP have been determined. The peroxide bridge of artemisinin was docked facing to Fe in heme in the distance of 2.6Å. Then, the activated artemisinin was docked into the Pf TCTP in 2.48Å from the sulfur of Cys14. Changes in the expression of TCTP have been reported to be associated with carcinogenesis. Human cellular retinol binding protein (CRBP) is also known to contribute to tumor growth and progression via retinoid-mediated signaling when its expression is modulated. The two proteins are found to have similar domains by domain search. Therefore, an attempt to establish the interactive relationship between the human TCTP and CRBP with retinol will be helpful in further understanding the cell signaling of TCTP. A possible binding site of retinol in TCTP was searched by multiple alignments of the sequences of TCTP and CRBP. Docking of retinol into the homology modeling deriven human TCTP structure was performed using a flexible docking program, QXP, and resulted in a stable TCTP-retinol complex structure with specific binding modes. These results may provide valuable information on the mechanisms of

the antimalarial activity of artemisinin and the cell signaling of TCTP.

## N1-025P
## Expression of *A. thaliana* G protein alpha subunit in *P. pastoris*

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Heterotrimeric G proteins play important roles in plant signal transduction pathways, including defense responses. An appropriate eukaryotic expression system was chosen for producing large quantities of high purity recombinant GPA1, Arabidopsis thaliana heterotrimeric G protein alpha-subunit. GPA1 was cloned into two expression vectors, pPICZC and pPICZαB; for intracellular and secreted expression respectively. Both plasmids harbor the AOX1 promoter, myc-epitope, his-tag and pPICZαB also contains the *S. cerevisiae* alpha-factor prepro signal sequence. The recombined plasmids were transformed into methylotropic yeast, *Pichia pastoris* strains GS115 and KM71H by LiCl transformation and inserts were verified by yeast colony PCR. Intracellular expression of GPA1 was achieved by induction of AOX1 promoter using methanol and was confirmed with Western blot analysis using anti-myc. The recombinant GPA1 is to be purified via $Ni^{+}$ chelating resin and purified protein will be biochemically characterized via GTP binding assays. This study describes the first report of expression of *A. thaliana* GPA1 gene in a eukaryotic system and points the direction for cloning and expression of the beta and gamma subunits of the heterotrimer. Availability of purified recombinant G protein alpha-subunit will enable comparison with its mammalian counterparts and facilitate experimental structural determination.

## N1-026P
## Functional properties of mammalian DNA polymerases lambda and DNA polymerase beta in reaction of DNA synthesis related to base excision DNA repair

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DNA polymerase lambda is a novel enzyme of the family X of DNA polymerases. The recent investigation demonstrated that Pol lambda having the properties in common with DNA polymerase beta. We have tested DNA substrates of different structures and found that activity of Pol lambda to incorporate dNTPs and their analogs was strictly dependent on DNA substrate. Pol lambda demonstrates a higher selectivity to gapped DNA in comparison to recessed or nicked DNA and can complete one-window gap DNA structures only with 5'-phosphate group. The influence of human replication protein A, apurinic/apyrimidinic endonuclease-1, flap endonuclease-1 and PCNA on the activity of Pol lambda was studied. The stimulation of strand-displacement synthesis catalyzed with Pol lambda was observed when FEN-1 was supplemented with PCNA. Based on our findings, Pol lambda has different enzymatic properties comparing to Pol beta. An important difference between these two enzymes is Pol lambda failure to catalyze strand-displacement DNA synthesis in the absence of FEN-1 and PCNA. However all properties of DNA polymerase lambda support a role of this enzyme in base excision repair.

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## N1-027P
## Modification of tryptophan residues with trihalocompounds and its application to protein chemistry

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Our research group is developing novel tryptophan chemistry tools which will enhance protein biochemistry. The initial discovery was of a photochemical reaction between chloroform and tryptophan, which produces a fluorescent product. Various halocompounds can be used to attach a desired group on to tryptophan and allow selective modification of accessible tryptophan residues in proteins. Using this tryptophan chemistry a rapid protein visualization method for polyacrylamide gel electrophoresis has been developed. In order for this technique to be compatible with proteomic studies we will demonstrate that this protein modification is compatible with protein identification by mass spectroscopy. Another application of this tryptophan photochemistry allows for selective modification of accessible tryptophan residues. This has been applied to the localization of proteins within cells. In this application *Escherichia coli* cells are reacted with halocompounds that are impermeable to the cell membrane. Thus proteins in the periplasm versus the cytoplasm can be identified. Halocompounds that are impermeable to the cell membrane versus those that are permeable can be used to determine the topology of membrane proteins. This will be shown by confirming the known topology of EmrE and SugE from *E. coli*. In addition tryptophan residues within proteins have been shown to have differential accessibility to halocompounds. The accessibility of tryptophan residues within globular proteins has been monitored using matrix assisted laser/desorption ionization mass spectrometry, to successfully identify surface exposed tryptophan residues.

## N1-028P
## Intein engineering for controllable protein splicing

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Inteins are protein intervening sequences found in various host proteins of many organisms. An intein catalyzes a protein splicing reaction to excise the intein itself and produce the mature host protein. Because inteins can function when inserted in non-native host proteins, controllable inteins may be used to control the maturation of any protein of interest through controllable protein splicing. However naturally occurring and characterized inteins are not controllable, because they function spontaneously without assistance of other molecules. In this study, a naturally occurring intein is converted into a controllable intein through protein engineering. A middle portion of the Ssp DnaB mini-intein, named IntM, was deleted from the intein sequence and replaced with an appropriate linker sequence. This produced a controllable intein that could catalyze the protein splicing reaction only when the IntM was supplied in trans. This synthetic

controllable intein, when inserted in a host protein of interest through gene transformation, allowed controllable maturation of the host protein, either *in vivo* by expressing the IntM from a controllable promoter or *in vitro* by adding a purified preparation of the IntM. Controllable inteins may be used as general switches for controlling protein functions (maturation through splicing). They may also allow cytotoxic recombinant proteins (e.g. proteases, endonucleases) to be first produced as non-toxic intein-containing proteins and then converted into mature proteins through protein splicing initiated *in vitro*.

## N1-029P
## Analysis of HIV-1 protease mutants to understand mechanisms of resistance

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The therapeutic efficacy of inhibitors of HIV-1 protease is limited due to the rapid selection of drug resistant mutants of the protease. Resistant mutants of HIV-1 protease with single amino acid substitutions (L24I, M46L, I50V, and G73S) have been examined using enzyme kinetics and crystallographic analysis in order to understand the molecular basis for resistance. These mutations alter residues located close to the active site, flap region and protease surface. Most mutants were observed to have decreased catalytic activity, inhibition or stability with a various level relative to the wild type enzyme. Crystal structures of mutant protease complexes with a clinical inhibitor and an analog of the p2-NC cleavage site were determined at resolutions of 1.35–1.05 Å in order to define the specific molecular changes associated with the altered activities. Each mutated residue showed altered interactions with neighboring residues that are consistent with the kinetic data.
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## N1-030P
## Detection of new molecular partners of hypoxia inducible factor 1α (HIF-1α)

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Transcription factor HIF-1 (Hypoxia Inducible Factor 1) is the major molecular effector of the hypoxia response. Its inducible subunit HIF-1α (in contrast to the constitutively expressed subunit HIF-1β) is upregulated by hypoxia but also by signals such as growth factors and chemical agents whose action is not yet well understood and is, in many cases, cell type specific. From the site of synthesis to the site of action on the promoters of its target genes, HIF-1α complexes with different proteins responsible for many of its properties. We have used the yeast two hybrid system in order to discover new interactions of HIF-1α, putative clues to the yet unraveled cellular functions of HIF-1. The N-terminal region (1-532 ) of the HIF-1α protein was used as bait to screen a mouse embryonic library. Among the selected clones, 30 were confirmed to be plasmid depended and are being further analyzed. The first clones characterized so far encode for novel HIF-1 partners. Interestingly, a subgroup of them suggests a molecular relationship of HIF-1α with cytoskeleton organizing molecules pointing to as yet unexplored functions of HIF-1.

## N1-031P
## NMR solution structure of hPrxVI, a 25 kDa 1-Cys human peroxiredoxin enzyme

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Peroxiredoxins (Prxs) are a family of thiol-specific antioxidant proteins and they are also known as thioredoxin peroxidases (TPx) or alkyl-hydroperoxide reductases. Prxs are divided into three classes, the 1-Cys, atypical 2-Cys, and 2-Cys Prxs, based on the number and position of cysteine residues directly involved in enzyme catalysis. Prxs contain a well conserved cysteine residue in the N-terminal region, which is aperoxidatic cysteine. Human Prx VI (hPrxVI) belongs to the distinct class of 1-Cys Prxs, which contains only one N-terminal conserved cysteine residue, and cannot use thioredoxin. Even though a physiological reducer for hPrxVI is still unknown, it has been shown that hPrxVI mediates the reduction of hydrogen peroxide with the use of electrons from a nonphysiological electron donor, dithiothreitol (DTT). The high resolution structure of the hPrxvi was determined using heteronuclear multidimensional NMR spectroscopy. NMR data shows that the secondary structure of hPrxVI in the reduced state consists of 10 β-strands and six α-helices. The secondary structure of the wild-type monomeric hPrxVI in solution is slightly different from that of the dimeric mutant form determined by X-ray crystallography. The topology of hPrxvi could be divided into two globular domains, which are a large N-terminal with thioredoxin fold and a small C-terminal domain. The N-terminal domain is comprised of three β-sheets and two α-helices, whereas the C-terminal domain is four β-sheets and one α-helix. Two domains are connected by a long flexible loop. This study will serves as a structural framework in understanding various biological functions of peroxiredoxins.

## N1-032P
## Identification and characterization of novel form of glutathione S-transferase in human kidney

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Recently, a novel isoform of GST without affinity for glutathione Sepharose (GSH-Sepharose) was isolated in normal human kidney. This isoenzyme might be clinically relevant, since in kidney tissue of patients with renal cell carcinoma (RCC) GST without affinity for GSH-Sepharose is either absent or accounts for significantly less total GST activity than in healthy subjects. The aim of this study was to characterize this form of GST. Renal GST without affinity for GSH-Sepharose has been isolated, identified with specific antibodies against classes Alpha, Mu and Pi by Western blot and further characterized with specific substrates. The results obtained have shown that GST without affinity for GSH-Sepharose is represented by one basic GST isoenzyme (pI 7.9) with subunit molecular weight of 25 000, which cross reacted with anti-GST Alpha antibodies. It exhibited high activity with

cumene hydroperoxide, specific class GSTA2 substrate. We conclude that renal GST without affinity for GSH-Sepharose (GST-pI 7.9) is Alpha member that possesses peroxidase activity typical for subclass GSTA2. Altered expression of this particular GST in the kidney of RCC patients might be responsible for their increased susceptibility to oxidative damage.

## N1-033P
## Structural studies with an acidic platelet aggregation inhibitor and hypotensive phospholipase A2 from *Bothrops jararacussu* venom: crystal structures of native and two complexed forms with pBPB and alfa-tocopherol inhibitors

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Phospholipases A2 are small stable calcium-dependent enzymes which belong to the superfamily of proteins which hydrolyzes the sn-2 ester bond of sn-3 membrane phospholipids to release arachidonic acid and lysophospholipids. These proteins are involved in several biochemical reactions and biological events being the study of chemical compounds that inhibit these molecules have a great relevance. This work presents the structural and functional analysis of the BthA-I, an acidic PLA2 isolated from the venom of the brazilian snake *Bothrops jararacussu.* Three high-resolution crystal structures were solved: the native BthA-I form and their complexes with pBPB (p-bromophenacyl bromide) and alfa-tocopherol (vitamin E), two known PLA2 inhibitors. The BthA-I is three to four times more active catalytically than BthTX-II and other basic Asp49 PLA2 from *Bothrops* venoms, however it is not myotoxic, cytotoxic or lethal. Although it showed no toxic activity, it was able to induce time-independent edema, with the activity being inhibited by EDTA. In addition, BthA-I-PLA2 caused a hypotensive response in rats and inhibited platelet aggregation. The structures were solved in the resolution range 1.45–1.90 Å. Native BthA-I structure presents an unusual dimeric conformation related to other class II PLA2s. The pBPB induced tertiary and quaternary changes in the BthA-I/pBPB crystal complex might be related to loss of certain pharmacological properties. The BthA-I/alfa- tocopherol complex whose crystals were grown in the same conditions of native protein presents a monomeric conformation with important structural changes.

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## N1-034P
## Amyloid oligomers: structure and function

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In amyloid diseases and *in vitro* soluble oligomers precede or assemble concomitantly to amyloid fibrils. They are highly heterogeneous and can serve as nuclei or building blocks for fibrillar growth. The oligomers rather than mature fibrils induce cytotoxicity. In our studies we address the following questions: which type of oligomers lead to amyloid formation, whether the partitioning between amyloidogenic pathways is governed by specific peptide sequences and whether cellular toxicity correlates with certain oligomer type. We showed that *de novo* carrier-protein albebetin assembles into two types of oligomeric intermediates: pivotal (comprised of 10–12 molecules) and amyloid-competent oligomers (26–30 molecules). Their stoichiometry was determined by atomic force microscopy. The former assemble into chains and rings with ''bead-on-string morphology'', while the latter give rise to fibrils and their appearance is concomitant with cross-beta-sheet formation. We suggest that transformation of the pivotal into the amyloid-prone oligomers is a limiting stage in albebetin fibrillation. Lysozyme from horse milk assembles into annular and linear protofillaments in a calcium-dependent manner. We showed that its oligomers, but not protofilaments, produce toxic effect on primary and tumour cells. The toxicity depends on the size of oligomers, 8-mer and 20-mers are more toxic than 4-mers. This suggests that oligomers can be considered as primary target for therapeutic intervention. We demonstrated the presence of significant autoimmune response to prefibrills of A-beta(25–35) peptide and human lysozyme in the sera of early Alzheimer's disease patients, which indicates that protein aggregation can be used as a diagnostic feature.

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## N1-035P
## Control of CD4 function by disulphide-bond switching

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CD4 is a co-receptor for binding of T cells to antigen presenting cells and is the primary receptor for the human immunodeficiency virus type 1 (HIV-1). Binding of HIV-1 to CD4 and a chemokine receptor leads to fusion of the viral and cell membranes and HIV-1 entry. The extracellular part of CD4 consists of four immunoglobulin-like domains. The disulphide-bond in the second domain is atypical in that it links adjacent strands in the same beta-sheet and is highly strained. We have shown that this disulphide-bond is cleaved on the cell surface by thioredoxin (*Nature Immunol* 2002; **3**: 727–732), a small redox active protein secreted by immune cells. Cleavage of the domain 2 bond leads to the formation of covalent dimers of CD4 on the cell surface linked intermolecularly through the domain 2 cysteines. Molecular modeling of this dimer implies that it has formed through swapping of the second domain (*Proteins* 2004; **57**: 205–212). The functional significance of dimer formation was tested by expressing wild-type CD4 or disulphide-bond mutant CD4 that does not form dimers (both domain 2 Cys were replaced with Ala) on the surface of 293T cells expressing the X4 or R5 chemokine receptor. These cells were examined for entry of seven patient-derived HIV-1 reporter viruses and fusion with 293T cells expressing seven different HIV-1 envelope proteins. Preventing covalent CD4 dimer formation increased entry of viruses 4 to 11-fold and increased cell–cell fusion up to 22-fold. The enhancement was independent of the chemokine receptor specificity of the HIV-1. These findings imply that HIV-1 enters susceptible cells through monomeric but not dimeric CD4.

## N1-036P
## Biochemical, structural and physiological analysis of the SPRY domain-containing SOCS box protein 2 (SSB-2)

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SSB-2 belongs to a family of four proteins (SSB-1 to -4) that have a C-terminal SOCS box motif, a central SPRY domain and a variable N-terminal region. The SPRY domain is common to many proteins with diverse functions and is involved in protein–protein interactions. The SOCS box has been shown to bind an elongin B/C complex to form an E3 ubiquitin ligase. These findings indicate that the SSBs may bind target proteins and function to mediate their ubiquitination and subsequent degradation. To determine the targets for SSB-2, we have sequenced proteins co-purified with SSB-2 over-expressed in cell lines, and also used SSB-2 affinity columns to purify interacting proteins from cell line and tissue lysates. With both these approaches, SSB-2 was found to interact with Prostate apoptosis response protein 4 (PAR-4). PAR-4 was initially characterized as being differentially expressed in prostate cells undergoing apoptosis and was soon after implicated in neuronal degeneration. PAR-4 has since been shown to act both in the nucleus as a transcriptional repressor and in the cytoplasm to inhibit atypical protein kinase C activity. To further dissect the molecular basis for the interaction of SSB-2 with PAR-4, the solution structure of SSB-2 lacking the SOCS box was determined by NMR spectroscopy. This is the first structure of a SPRY domain and it presents as a novel fold. We also sought to ascertain the biological function of SSB-2 via murine genetic deletion. These mice had a decreased rate of platelet production resulting in mild thrombocytopenia and a lowered blood urea nitrogen level. The mice appear healthy until approximately 6 months of age when they begin to exhibit stress-induced seizures which are associated with premature death. While we are still trying to reconcile our genetic and biochemical data, the interaction of SSB-2 with PAR-4 represents an interesting explanation for the age-dependant phenotype of SSB-2 deficient mice.

## N1-037P
## Biochemical and genetic characterization of phosphofructokinase from the dimorphic yeast *Yarrowia Lipolytica*

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*Yarrowia lipolytica* is a non-conventional yeast that has received increasing attention due to its capacity to grow in unusual carbon sources and to excrete organic acids, its potential as host for expression of heterologous proteins, and its ability to shift between yeast and hyphal form. However, biochemical studies on its central metabolic pathways are scarce. Regarding the regulatory steps of glycolysis, both hexokinase and pyruvate kinase have been shown to exhibit kinetic and regulatory properties significantly different from those of the enzymes from *Saccharomyces cerevisiae*. Thus, we have investigated the characteristics of the phosphofructokinase (Pfk) from *Y. lipolytica*. The enzyme was purified to homogeneity and its encoding gene isolated. YlPfk is a homooctamer of 869 kDa composed of 109 kDa subunits, and shows atypical kinetic properties. It did not exhibit cooperativity with respect to fructose 6-P ($h$ 1.1; $S_{0.5}$ 52 μM); was not very sensitive to ATP inhibition ($K_i$ 3.5 mM), while it was strongly inhibited by P-enolpyruvate ($K_i$ 61 μM). Fructose 2,6-$P_2$ did not activate the enzyme. *YlPFK1* gene codes for a protein of 953 amino acids, and its disruption abolished growth in glucose and Pfk activity. The disrupted strain grew in permissive substrates in the presence of glucose. The unusual properties of YlPfk and the intracellular concentrations of glycolytic intermediates during growth in glucose suggest an important role for this enzyme on the regulation of glycolysis in this organism, different from that played in *S. cerevisiae*.

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## N1-038P
## Inhibition of phenylethanolamine *N*-methyltransferase: a kinetic and mutagenic analysis

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Phenylethanolamine *N*-methyltransferase (PNMT) is an *S*-adenosyl-L-methionine (AdoMet)-dependent enzyme that catalyzes the terminal step in catecholamine biosynthesis, the conversion of noradrenaline to adrenaline. PNMT is found in high levels in the adrenal medulla where adrenaline serves as a hormone, and in the CNS where it is proposed to act as a neurotransmitter. There are currently a number of studies examining the possible role of CNS adrenaline in stress, and in particular, the regulation of cardiovascular function. Accordingly, PNMT has been the target for the design of inhibitors, especially to assist in examining the specific role of CNS epinephrine. Recently X-ray structures have been determined of human PNMT complexed with several potent inhibitors. These show that the enzyme has a similar overall fold to several other small molecule methyltransferases. However, it does have some significant differences from, for example, catechol-O-methyltransferase, in that it appears to have a cap over the active site. Thus the binding of substrates and/or inhibitors must be accompanied by a conformational change to make the active site accessible. We have used a combination of mutagenesis and kinetic analysis to examine the binding of substrates and inhibitors to PNMT. We have identified two residues that are particularly important for inhibitor binding. We also demonstrate that the inhibitors bind much more tightly in the presence of AdoMet, suggesting that the binding of the latter brings about a conformational change in the enzyme.

## N1-039P
## The structure and the interactions of the bifunctional Mason–Pfizer monkey virus nucleocapsid-dUTPase

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Betaretroviruses encode dUTPase, an essential factor in DNA metabolism and repair, in the pro open reading frame located

between gag and pol. Ribosomal frame-shifts during expression of retroviral proteins provide a unique possibility for covalent joining of nucleocapsid (NC) and dUTPase within Gag-Pro polyproteins. We show that recombinant betaretroviral NC-dUTPase and dUTPase have low catalytic activity as compared all other dUTPases. We determined the 1.55 angström resolution crystal structure of the enzyme. A comparison with this retroviral and other dUTPase structures reveals a significant difference in the orientation of the C-terminal arm that could be the reason of the low catalytic activity. We created a mutant dUTPase lacking the C-terminal arm to investigate how this different orientation could affect the catalytic activity. We demonstrated that the nucleocapsid-dUTPase is present in the infected cells. The role of dUTPase in faithful DNA replication and the nucleic acid binding role of NC suggests that NC-dUTPase might be present in the preintegration complex (PIC) of the retrovirus. Based on surface plasmon resonance experiments, we present the connections of NC-dUTPase to other proteins in the PIC (e.g. capsid and integrase). According to our current working hypothesis, the NC domain may help dUTPase function with adequate localization and/or the trimeric organization of dUTPase could enable the three NC domains with a concerted character. We determined the positions of the nucleocapsid domains in the organization of the NC-dUTPase with small angle X-ray scattering. Our limited tryptic digestion results also prove this highly exposed location of the NC domains as compared to the more compact dUTPase domain.

## N1-040P
## HmuR and HmuY – a novel mechanism of heme utilization in *Porphyromonas gingivalis* or an error in a rearrangement strategy?

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*Porphyromonas gingivalis* is a Gram-negative anaerobic bacterium associated with adult periodontal diseases. To acquire iron/heme it employs specific outer membrane receptors, gingipains and lipoproteins. We have identified and characterized *P. gingivalis* outer membrane heme receptor HmuR through insertional mutant construction, site-directed mutagenesis and analysis of the recombinant protein. The *hmuR* gene in *P. gingivalis* A7436 and W83 strains is cotranscribed with a *hmuY* gene, whereas in other strains the organization of this operon is different, possibly as a result of a rearrangement strategy. Physiological analysis revealed that *hmuR* and *hmuY* mutants were defective in growth in the presence of hemin and hemoglobin and exhibited reduced growth recovery when human serum was used as iron/heme source. The mutant cells demonstrated decreased ability to bind hemin and hemoglobin. The aim of this work was to examine an involvement of *hmuY-hmuR* operon in heme utilization in *P. gingivalis* A7436. For this purpose, a complementation analysis in *E. coli hemA aroB* mutant defective in the heme biosynthesis pathway and iron uptake was used. The *hmuY* and *hmuR* transcripts were further examined in *P. gingivalis* A7436 wild type and mutant strains grown under various iron/heme conditions. The recombinant HmuY protein was purified and characterized in regard to hemin and DNA binding. A theoretical model of the HmuY protein structure was constructed to support experimental results. In conclusion, our results show that HmuR functions to bind and transport heme across *P. gingivalis* A7436 outer membrane, whereas HmuY might play a regulatory role in heme utilization through HmuR receptor.

## N1-041P
## The interaction of an auxin, indole-3-acetic acid, with placental glutathione-S-transferase-Pi

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Glutathione-S-tranferases (GST) are a superfamily of enzymes, with an established role in detoxification. Two distinct supergene families encode proteins with GST activity; at least 16 genes encode proteins expressed in tissue cytosols and six genes are expressed in membranes. Cytosolic GSTs of mammals have been well characterized, and were classified into Alpha, Mu, Pi and Theta classes on the basis of a combination of criteria such as substrate/inhibitor specificity, primary and tertiary structure similarities and immunological identity. In this study, we investigated the interaction of a naturally occurring plant growth hormone of the auxin class, the indole-acetic acid (IAA) with human placental GST-Pi. Although of plant origin, in mammals, elevated IAA levels is found in cerebrospinal fluid, blood, lung, kidney, liver, brain and in such diseases like phenoylketonuria. The kinetic analysis of GST-Pi with IAA was done using glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates. The Ks values for GSH and CDNB were calculated as $1.947 \pm 0.171$ mM, and $2.751 \pm 0.534$ mM, respectively. The analysis of percent inhibition of IAA on GST-Pi at fixed (1 mM) GSH and CDNB concentrations displayed progressive inhibition with a nearly limit value of 70 % activity at 5 mM IAA. Further kinetic analysis of IAA with varying GSH (0.125–4 mM) and fixed CDNB (1 mM) revealed IAA to be a non-competitive inhibitor of GSH with a Ki value of $7.751 \pm 0.653$ mM. Lineweaver–Burk kinetics with IAA at fixed GSH (1 mM) and varying CDNB exhibited competitive inhibition, and the Ki value was calculated as $3.235 \pm 0.240$ mM.

## N1-042P
## Lister homologue of vaccinia virus complement control protein is two amino acids shorter, has putative glycosylation sites and other functional and structural differences

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Vaccinia virus complement control protein (VCP) is one of the proteins encoded by the vaccinia virus to modulate the host immune response. VCP down regulates the host inflammatory response by binding and inhibiting the activated third (C3b) and fourth (C4b) components of the complement system, and by binding to heparan sulfate on the surface of endothelial cells, thus preventing antibody binding. Because of its potentials as a therapeutic agent, and in regulating complement-mediated inflammation common in many disease conditions, there is a search for VCP that is most active. The extended structure of VCP, mobility between its sequential domains, charge distribution and type of residues at the binding regions are factors that have been identified to influence its ability to effectively bind and inhibit complement proteins. Here, we report that the Lister strain of vaccinia virus encodes a VCP homologue (Lis VCP) that is functional, has two amino acids less and has several differences compared to the well-characterized VCP from vaccinia

virus Western Reserve strain (WR VCP), and the human smallpox inhibitor of complement enzymes (SPICE) from variola virus. We also report that Lis VCP is the only orthopoxviral VCP homologue found to be glycosylated so far, and that glycosylation influences its pattern of complement inhibition. In addition, using site-directed mutagenesis, a number of modified VCP proteins have been generated, some of which may have greater ability to regulate the classical pathway of complement activation than the authentic WR VCP.

## N1-043P
## Functional expression and mutational analysis of two plant purple acid phosphatases

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Purple acid phosphatases (PAPs) contain binuclear metallic center with iron and zinc or manganese ions. Plant PAPs are divided into two groups differing in molecular weight and subunit structure. Several insect baculovirus expression systems to produce and purify two high molecular weight phosphatases from yellow lupin (*Lupinus luteus*) (LAP1) and red kidney bean (*Phaesolvus vulgaris*) (FAP1) were used. Both enzymes were previously isolated from plant material and characterized. They are members of two distinct subfamilies with two identical subunits connected by disulfidge bridge (FAP1) or non-covalently (LAP1). Several variants of proteins differing in fusion tags, baculovirus promoters and secretion signal peptides were designed. The maximal expression level (2.6 mg/l) was achieved when N-tagged phosphatases containing 6xHis and S-Tag fusion peptides were expressed under GP64 promoter and GP64 baculovirus signal sequence. Some of C-tagged phosphatase variants and proteins without fusion peptides were also enzymatically active. The proteins secreted into the medium were purified on TALON metal affinity resin. Mutants lacking the unique cysteine 119 in LAP1 and cysteine 372 forming a linkage between subunits in FAP1, were constructed. Seven variants, each with replaced one amino acid residue involved in metal binding were also designed. Substrate specificity, kinetic parameters and protein stability experiments were performed for all mutants. In conclusion, we show that unique cysteine residues in both enzymes may play role in a stability of proteins in solution. Preliminary experiments suggest that metal coordinating residues in LAP1 and FAP1 are crucial for enzyme activity.

## N1-044P
## Structure–function characterization of a new lectin from *Chromobacterium violaceum*

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*Pseudomonas aeruginosa*, an opportunistic pathogen responsible for numerous nosocomial infections in immunocopromised patients, produces a variety of carbohydrate-binding proteins that could be involved in host recognition and adhesion. One of them, PA-IIL, is a fucose binding lectin that is closely related to the virulence of the bacterium [1]. Searching in databases for proteins displaying sequence similarities to PA-IIL revealed new homologous proteins in other opportunistic pathogens like *Chromobacterium violaceum*. Human infection by *Ch. violaceum* is rare but when it occurs, it is associated with very high mortality rate [2]. In *Ch. violaceum* genome a hypothetical protein similar to PA-IIL and coded by the gene cv1741 was found. The gene was cloned and the recombinant protein CV-IIL, the product of cv1741, has been expressed in *E. coli* and purified to homogeneity. Binding affinities of the protein with different mono and oligosaccharides using enzyme linked lectin assay (ELLA) and isothermal titration microcalorimetry have been characterized and crystal structures of CV-IIL in complexes with a-D-methylmannoside and a-L-methylfucoside have been solved. The binding data together with structure analysis allowed comparison of CV-IIL and PA-IIL and brought more detailed view on fine specificity of both lectins. Structure basis of the protein/sugar complexes and the thermodynamics of their interactions could be helpful to design carbohydrate-based compounds that can be used as alternatives to antibiotics or new antiadhesive therapeutics.

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## N1-045P
## Fibroblast-derived factors modulate breast cancer cell proteomics

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Carcinomas arise from epithelial cells through the sequential accumulation of multiple genetic alterations that mainly affect cell proliferation, metabolism, cell–cell and cell–matrix interactions. Benign tumors usually remain encapsulated for undefined period of time and do not form metastasis. Malignant tumors acquire the ability to invade the underlying stroma, establishing new and dynamic interactions with both extracellular matrix molecules and neighboring fibroblasts. These new interactions generate signals that may influence the invasive behavior of tumor cells through the expression of undefined set of genes, difficult to predict "*a priori*" and to search individually. Thus the proteomic approach is of great utility to evaluate multiple responses of cells subjected to external influences. To investigate these interactions we have performed proteomic analyses of breast cancer cells (8701-BC) cocultured with normal fibroblasts. Coculture experiments were performed in which the two cell types were separated by a microporous membrane. The proteins were separated by 2D-IPG and detected by Nt-microsequencing, Maldi-Tof and Western Blot. The spots, with assigned identity, were grouped into functional categories, as previously described (Pucci-Minafra et al. *Annals NY Acad Sci* 2002; **963**: 122–139). The comparative analysis between control and cocultured cells revealed significant variations of the expression levels especially with regards to cytoskeletal proteins. In particular an increased expression level of vimentin and a decrease of cytokeratins were observed. These data might be related to a more marked epithelium–mesenchyme transition and/or an increase in the motility of 8701/BC cells when cocultured with fibroblasts.

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## N1-046P
## Influence of non-thermal coherent extremely high frequency electromagnetic radiation on some parameters of wheat germinating seedlings

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The study of biochemical parameters of germinating wheat seedlings accompanying by highly specific pattern of activity variation in a number of enzymatic systems can be convenient model for an estimation of processes occurring in the environment. The influence of low intensive non-thermal coherent extremely high frequency electromagnetic radiation (EMR) of mm – diapason on intensivity of wheat seedlings growth, general activity and isoenzimatic content of lactate dehydrogenase (LDG) and peroxidase (PO) have been investigated. The changes of LDG activity are different directed and depend on frequency of radiation and period of growth. The study of LDG isoenzimatic content of wheat germinating seedlings has revealed the increase of slow fraction. It may occur due to changes in correlation of genes responsible for LDG synthesis. The activity of peroxidase rises in all cases. The highest effect was observed at an exposition 20–30 min. The biologically effective frequencies in a narrow range 49–53 GHz with the expressed resonant frequencies close to 50 and 51.8 GHz are revealed. On the basis of the received results the assumption of presence of resonant interaction of low intensivity EMW of the mm-range with biological structures is made which results in increase of intensity of metabolic processes of developing plant organism.

## N1-047P
## Integrase HIV-1: inhibition of catalytic activity by modified oligonucleotides

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In context of searching for new effective medicines against AIDS, active investigations to inhibit integrase HIV-1 are carried out. Integrase accomplishes the integration of viral DNA into the host-cell genome, what is essential step of replicate cycle of HIV. As previously demonstrated (reference), conjugates of short oligonucleotides with aromatic intercalators are non-competitive inhibitors of integrase. They suppress catalytic activity of the enzyme by means of enzyme-substrate complex dissociation. However, applying of these conjugates as integration inhibitors *in vivo* is restricted by their low stability and high cytotoxicity of intercalators. In present work we have synthesized resistant to hydrolysis conjugates of oligonucleotides modified at sugar-phosphate backbone with molecules possessing low cytotoxicity, i.e. fluorescein, eosin, cholesterol etc. Besides, we have varied the length of the conjugates oligonucleotidic part in order to determine the optimal structure of the integrase inhibitor. The influence of such conjugates on the integrase catalytic activity has been investigated. Our results indicate that conjugates of the 11-mer 2'-O-methyl-oligonucleotide with fluorescein and eosin are the most perspective and potent inhibitors. It is shown that these conjugates suppress integration even at nanomolar concentrations. It should be underlined that activity of other DNA-binding enzymes is not affected by them. The mechanism of the conjugates action as integrase inhibitors is under study.

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## N1-048P
## Intramolecular cooperativity in a protein binding site assessed by combinatorial shotgun scanning mutagenesis

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Combinatorial shotgun alanine-scanning combined with phage-display was used to assess intramolecular cooperativity in the high affinity site (site 1) of human growth hormone (hGH) for binding to its receptor. A total of 19 side chains were analyzed and statistically significant data were obtained for 145 of the 171 side chain pairs. The analysis revealed that 90% of the side chain pairs exhibited no statistically significant pair interactions, and the remaining 10% of side chain pairs exhibited only small interactions corresponding to cooperative interaction energies with magnitudes $< 0.4$ kcal/mol. The statistical predictions were tested by double-mutation cycles measuring affinities for purified mutant proteins and were found to be accurate for five of six side chain pairs tested. The results reveal that hGH site 1 behaves in a highly additive manner and suggest that phage-display shotgun scanning could become a general approach to assess cooperative effects in other protein–protein interactions.

## N1-049P
## Domain organization of hordeivirus TGB1 movement protein

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Inter- and intracellular transport of many plant viral RNA genomes involves formation of ribonucleoprotein particles with a specific viral protein/proteins named movement proteins. In the case of poa semilatent virus (e.g. Hordeiviridae) TGB1 movement protein is responsible for this function. Secondary structure prediction methods suggested that TGBp1 consists of three distinct structural domains: unfolded N-terminal domain (NTD), containing alternate clusters of positively and negatively charged resides (aa 1-190), small beta-structural, presumably OB-fold domain (aa 191–290), and alpha/beta-structural domain including seven conservative helicase motifs (aa 201–576). Expression of a recombinant TGBp1 in *E. coli* was accompanied by formation of two main protein products in addition to full-length TGBp1. These additional proteins were formed by proteolysis exactly at predicted

interdomain TGBp1 regions. Histidine-tagged mutant proteins corresponding to the predicted domains were constructed, expressed in *E. coli* and purified with metal affinity chromatography. CD and FTIR-spectra measurements of mutant proteins confirmed secondary structure prediction. Gel-retardation assay showed that all tree domains possessed RNA-binding activity. HEL and OB-domains could bind RNA in cooperative manner and N-terminal domain in non-cooperative one. Biochemical studies including chemical cross-linking and sucrose gradient analysis showed that all domains had different oligomerization patterns. The proposed multimodular organization of TGBp1 is typical for many cellular proteins involved in mRNA metabolism.

## N1-050P
## Blocking the active site crevice of mono-ADP-ribosyltransferases with llama heavy-chain antibodies

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Some of the most potent bacterial toxins (e.g. Cholera-, Pertussis-toxin) are mono-ADP-ribosyltransferases (ARTs) which catalyse the transfer of the ADP-ribose moiety from NAD onto specific amino acid side chains of proteins. Members of the ART family also occur endogenously in mammals where they regulate important functions including lymphocyte activation and migration. Specific inhibitors of mono-ADP-ribosyl transferases would be useful tools for research on mammalian ARTs and the treatment of diseases mediated by bacterial ARTs. The unique features of the camelid heavy-chain antibodies provide a basis for designing specific ART-inhibitors. Produced by camelids (camels, dromedary, llamas) alongside convenional antibodies, these unusual antibodies are composed only of two heavy chains. Thus, the antigen binding site of camelid heavy-chain antibodies is formed solely by the heavy-chain variable domain (VHH). The third complementarity determing region (CDR 3) of these VHHs possesses the capacity to form long fingerlike extensions that can extend into cavities on antigens, e.g. the active site crevice of an ART. The recently elucidated 3D structure of ART2 revealed a Pac-man-like fold with a deep active site crevice for NAD-binding. In order to raise ART-specific camelid antibodies we immunized llamas using a cDNA prime/protein boost immunization strategy. Immuneserum and purified single chain antibodies indeed block the function of ART2. A phage-display library was generated from the immunized llamas in order to select and clone ART2-specific VHHs. We expect that these VHHs will open interesting perspectives for experimental and therapeutic interventions. Moreover, they will serve as molecular models for designing peptide mimetic and small molecular weight ART-inhibitors.

## N1-051P
## A protein engineering study of the role of glutamine 19 in the catalytic mechanism of actinidin

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Several amino acids in the active site of actinidin, a cystein protease from the kiwifruit (Actinidia chinensis), have been proposed for many years. However, the roles of the amino acids in catalytic mechanism were not investigated yet. The various suggested roles for the side chain of Gln19 in the active site of actinidin have been clarified by using site-directed mutagenesis. Wild-type actinidin and two variants (Gln19Ala and Gln19Ser) were produced in *Escherichia coli* expression system, and purified by immobilized-metal affinity chromatography. Firstly, the catalytic activity was compared in both wild-type and a Gln19Ala variant by using Z-Lys ONp (N-α-benzyloxycarbonyl-L-lysine p-nitrophenyl ester) and casein as substrates. This showed that mutation of Gln19 to Ala caused a decrease for hydrolysis of Z-Lys ONp and digestion of casein. In addition, the catalytic activity with a Gln19Ser variant was much lower than that with Gln19Ala. For two variants, the pH dependence of activity was similar to that for wild-type actinidin. Results of this study suggest that an essential role is played by the side chain of Gln 19 which forms a hydrogen-bond donor in the catalytic mechanism of actinidin.

## N1-052P
## Changes of glycoprotein patterns in rat placenta during early pregnancy caused by the demethylating agent 5-azacytidine

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DNA methylation is an important mechanism for regulation of gene expression during development. It can be changed by 5-azacytidine (5-azaC), a demethylating agent capable of incorporation into DNA instead of cytosine. During placentation the expression of different glycoproteins is crucial for normal development of placenta. DNA demethylation caused by 5-azaC might influence the normal expression of glycoproteins during placentation. The single dose of 5-azacytidine (5 mg/kg body weight) was administered to pregnant rats on day 8 of pregnancy. The animals were sacrificed on day 20 of pregnancy and placentas were measured and histologicaly analyzed. Glycoproteins were detected by Western-blot method using lectins: SNA, DBA, PHA-E and UEA-I. In treated animals significantly smaller placentas were found. Histological analysis demonstrated reduced labyrinthine layer in treated placentas, compared to controls. Comparison of glycosylation patterns of placental proteins between treated and control placentas have shown significant differences. Differences in glycosylation between cytosol and membrane proteins was found. Lectin UEA-I detected the cytosol glycoprotein GP 70, which was absent from control samples. PHA-E identified cytosol GP 70 only in the experimental sample. Comparing the samples of membrane proteins we found two glycoproteins GP 50 and GP 35 only in treated samples. These results support our hypothesis that proper methylation pattern of olygosaccharide genes is crucial for their regular expression and normal development of rat placenta. The demethylating agent 5-azacytidine disturbs this normal pattern, allowing in treated samples some new glycoproteins to appear in cytosol and membrane protein extracts.

## N1-053P
## Anti-catalytic antibodies for Angiotensin I-converting enzyme: inhibitory effect and fine epitope mapping

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Angiotensin I-converting enzyme (ACE) is a key regulatory enzyme in cardiovascular pathophysiology. ACE consists of

two homologous domains (N and C), each bearing Zn-dependent active site. 3D-structure of the C-domain is characterized by a central channel where the active site is located. Modeled 3D-structure of the N-domain possesses similar channel structure. Two anti-catalytic monoclonal antibodies (mAb) have been developed against human N-domain of ACE: 3A5 and i2H5. These mAbs inhibit the hydrolysis of tripeptide CbzFHL by N-domain with quite similar potency, however inhibition mechanisms are different. Both mAbs can bind to the free enzyme and E·S complex, forming E·mAb and E·S·mAb complexes, respectively. However, complex E·S·3A5 is productive (kcat is about 10 times lower than for E·S), but the complex E·S·i2H5 is non-productive (kcat is negligible). These mechanisms well correlate with predicted mAb epitope localization: i2H5 blocks the main entrance into the channel, whereas inhibitory effect of 3A5 is explained by strong conformational changes in the N-domain after 3A5 binding. Analysis of species specificity of mAbs binding and mutagenesis of the amino acid residues demonstrates that the epitope for i2H5 includes at least E403, K407, D412, R413, N416. Epitope for 3A5 is located at a distance from the channel entrances and includes at least K517, Y521, E522, K542, K557, D558, L562, Q568 and K572. Mutation of the amino acid residues (D558L and K557Q) increased significantly (threefold) apparent binding of 3A5 with the mutated N-domain in the plate precipitation assay, but abolished completely the inhibitory potency of this mAb. This result confirms that conformational changes in ACE upon binding of mAb 3A5 determine the anti-catalytic properties of this mAb.

## N1-054P
## How does a protein function?

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In order to perform their functions, proteins must store chemical energy and prevent its decay into heat for a sufficiently long time. Otherwise, the energy will be lost to the environment and won't be useful for work. McClare [1] suggested that an efficient way of keeping the energy in the protein is in the form of an excited state. This idea was developed by Davydov [2]. He assumed that the energy released in the hydrolysis of ATP could be stored as a vibrational excited state known as Amide-I. Davydov's model predicted an enhancement of the lifetime of the excitation, when compared with its lifetime in an isolated amino acid, in agreement with the need to prevent its loss into heat. Further work has shown that Amide-I excitations can be transferred in a very robust way from the active site of the protein to other regions in tens of ps [3]. Furthermore, lifetimes of tens of ps were measured for Amide-I in proteins and similar systems [4,5]. These values suggest that the energy stored as a vibrational excited state lasts long enough to reach any site in the protein (eventually the one where it is needed for work). Still missing is some understanding of how proteins can use the stored energy to undergo conformational changes. We are concerned here with a model that can describe this final conversion of the energy released in the hydrolysis of ATP into work.

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## N1-055P
## Oligopeptide repeats of Sup35 as the determinants of [PSI+] variability

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*Saccharomyces cerevisiae* [PSI+] prion appearance and replication arises from the ability of translation termination factor eRF3, also called Sup35, to switch to the self-propagating prion state. [PSI+] is a convenient model for studying the prion phenomenon, it reproduces all basic properties of mammalian prions, including the existence of heritable variants (strains). It is known, that diverse [PSI+] variants differ in mitotic stability and nonsense suppressor efficiency. However, the molecular basis of [PSI+] variability is unclear. Sup35p prion-forming domain (PrD) bears a region, composed of six copies of an imperfect oligopeptide repeat with the consensus sequence PQGGYQQ-YN. The deletion analysis of the repeat-containing Sup35 region has been performed for five 'strong' and five 'weak' [PSI+] variants in order to estimate how does the length of PrD influence maintenance of different [PSI+] variants. It has been shown that the minimal number of PrD repeats required to rescue [PSI+] depends on it's variant. [PSI+] transmission from wild-type Sup35 to Sup35 with the reduced number of PrD repeats weakened suppressor phenotype and reduced mitotic stability. The efficiency of [PSI+] transmission depended on [PSI+] variant and on the number of PrD repeats in Sup35p recipient protein. Sup35, bearing only four PrD repeats (Sup35R1-4), could adopt 'weak' [PSI+] variants twenty times more efficiently, than the 'strong' ones. When we have got SUP35wt genotype restored we also found out, that Sup35R1-4 had been unable to support 'strong' [PSI+]. Our data show, that Sup35R1-4 acquires conformation, incompatible with 'strong' [PSI+].

## N1-056P
## Characterization of DNA binding by HIV-1 integrase using Schiff-base formation

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One of the most important steps of HIV replication is insertion of the DNA copy of viral RNA into the host cell genome. In this context a viral enzyme forcing this process and named integrase is of particular interest. Integrase recognizes U3 or U5 sequences located at the both ends of the viral DNA (DNA-substrate). Each of two DNA 3'- ends undergo a cleavage of dinucleotide GT in a 3'- processing stage followed by their incorporation into the human genome, which acts as a target DNA. Thus for the normal function integrase should be

able to bind two DNAs (substrate and target) simultaneously. In order to understand whether the substrate and target binding sites are located at different regions of integrase and to identify amino acids lying in close contacts to DNA, we employed a methodology of covalent bond formation between DNA and protein. In the present study 21-base pair duplexes containing at different positions aldehyde groups linked to 2'-deoxyribose carbon atom were used. Those possessing a sequence of the U5 viral DNA domain were considered as integrase substrate analogs, and the duplexes with a random sequence were used as target DNA analogs. Under conditions when the enzyme was active, 2'-modified nucleotides formed a Schiff-base with lysine residues lying in proximity to CHO-group. The yield of integrase-DNA covalent complexes varied depending on the position of the aldehyde group and reached 50–60% in some cases. A high yield allowed preparing of sufficient amounts of complexes for trypsine-mediated hydrolysis. Resulting peptides were detected using mass-spectroscopy thus providing information concerning a place of DNA binding in the structure of integrase.

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## N1-057P
## Heterogeneity of a new aspartic proteinase from *Cynara cardunculus* L.

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Aspartic proteinases (AP) are a well-defined class of proteinases (EC 3.4.23) found in retrovirus, bacteria, fungi, animals and plants. These have high physiologic relevance since are involved in a number of normal and pathological processes. Veríssimo et al. and later Sarmento et al. purified two APs from flowers of *C. cardunculus* L. These enzymes, cardosins A and B, exhibit broad specificity for the residues acceptable in the P1 and P1' positions, although show the typical preference for residues with large and hydrophobic side-chains. A third proteolytic fraction, named cardosin $A_0$, was co-purified along with cardosins A and B. The goal of this investigation is the characterization of this proteolytic fraction. On SDS-PAGE it as cardosin A, even though non-reducing electrophoresis revealed migration differences. Further purification of cardosin $A_0$ by anionic change chromatography yielded four different enzymes whose activity was determined. It was found that these fractions are heterodimeric APs with different selectivity requisites. All these fractions are glycosilated at both sub-units. Additionally, the molecular weight of both sub-units of cardosin A and cardosin $A_0$ fractions were determined by ESI-MS. Furthermore, microheterogeneity was detected both by mass spectrometry and by 2D-electrophoresis.

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## N1-058P
## Investigating the mechanism of ligand binding to the maltose-binding protein

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The maltose-binding protein (MBP) binds maltose and higher oligosaccharides in a deep binding cleft between two independently folded domains. MBP, a 41 kDa protein, is normally in the open state in the absence of a ligand. Binding of the ligand triggers the domain closure during which the two domains rotate by about 30° respect to each other, burying the substrate. The ligand-bound closed form of MBP is recognized by the $MalFGK_2$ ABC transporter. Strong binding of MBP to the transporter activates substrate transport across the inner cell membrane. Additionally, the closed state of MBP has the correct conformation to bind to the chemotaxis receptor Tar and activates signaling. We have simulated the complete transition starting from the closed state of MBP to the open state upon the removal of the ligand as well as from the open state to the closed state by adding the ligand. The simulation endpoints are consistent with existing crystal structures and solution data. The pathway of the conformational transition is complex and includes a number of highly correlated conformational changes of local binding interactions. Importantly, we could identify a "door-stop" mechanism that assists the domain closure motion and secures the ligand in the binding cleft of the closed MBP. The latch consists of a salt bridge between Glu111 in the hinge region and Lys15 in the N-terminal domain. This salt bridge initially forms at the expense of breaking a hydrogen bond in the interdomain β-sheet, but it is restored upon completion of the domain closure. This door-stop mechanism could well be a general property of periplasmic binding proteins.

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## N1-059P
## The water channel aquaporin-2

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Located in collecting duct principal cells, aquaporin-2 (AQP2) is responsible for the regulated water reabsorbtion in the kidney and is indispensable for the maintenance of body water balance. Dysregulation or malfunctioning of AQP2 can lead to severe diseases such as nephrogenic diabetes insipidus, congestive heart failure, liver cirrhosis and pre-eclampsia. The structure of AQP2 is a prerequisite for understanding its function, its regulated shuttling to the apical membrane, and ultimately for developing therapeutics. Here we present the crystallization of recombinant human AQP2 into two-dimensional protein–lipid arrays and their characterization by atomic force microscopy and electron crystallography. These crystals are double-layered sheets that have a diameter of up to 30 μm and diffract to $(3\ Å)^{-1}$. The 3D density map, obtained by image processing and electron diffraction, shows the C-terminus, involved in the regulated shuttling, to be trapped in between the two layers in a fixed conformation. This gives a unique opportunity to study an *in vivo* flexible structure by electron crystallographic methods. This project serves as a test case for our novel methods development in electron crystallography, based on the open-source software package iplt (http://www.iplt.org).

## N1-060P
## The N-terminal domain of HypF aggregates under physiological-like conditions

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Denaturation is generally recognized as a crucial step that precedes the aggregation of a protein. It has been shown that the N-terminal domain of the bacterial protein HypF (HypF-N) undergoes aggregation at pH 5.5 in the presence of moderate concentrations of trifluoroethanol (TFE). Under these experimental conditions denatured protein molecules aggregate rapidly and the aggregated species develop into organized amyloid fibrils in 2–3 weeks. We have studied HypF-N under conditions close to physiological. HypF-N has been freshly prepared in 10 mM phosphate buffer at pH 7.4 and its conformational stability has been found to be unaltered compared to pH 5.5. Nevertheless, the protein undergoes aggregation without the addition of TFE. Binding of the dye thioflavine T, CD measurements and dynamic light scattering experiments indicate the formation of HypF-N aggregates in 7–10 days. Atomic force microscopy shows the presence of unbranched fibrils, morphologically similar to those observed in the presence of TFE. Under these conditions, however, the aggregates do not bind either Congo red nor ANS, suggesting a different, presumably less ordered, organization of the fibrillar species. The investigation of the aggregation process under physiological conditions is a fundamental passage that may contribute to the elucidation of the molecular mechanisms underlying the formation of amyloid aggregates in living organisms.

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## N1-061P
## SNW-induced complexes in the nuclei of *Dictyostelium discoideum* and *Schizosaccharomyces pombe*

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SNW proteins are nuclear coregulators that functionally cooperate with various transcription factors in the regulation of gene expression. Direct interactions were reported for SNW and both DNA binding factors, such as VDR, SMAD2, or CBF1, as well as various coregulators, such as NotchIC, SMRT, or pRb. The effects of SNW proteins may be due to their involvement in early-stage spliceosomes, which was documented in both lower and higher eukaryotes. We found SNW in complex with peptidyl-prolyl isomerases of the small cyclophilin family and suggested that SNW recruit the foldases to splicing and other complexes, increasing the rate of conformational changes and exchange of components. We show in *Dictyostelium discoideum* cells that the SNW homolog SnwA can dimerize and that it partitions in salt and nuclease resistant fractions upon the extraction of nuclear content. In wt-cells, SnwA is localized to large number of small domains that are distributed outside the regions of high DNA content, suggesting their location to interchromatin space. Both *Dictyostelium* and *S. pombe* cellular model systems demonstrate that nuclear accumulation of SnwA and Snw1p proteins, respectively, results in the formation of a detectable nuclear body. Similarly to the snRNP complexes induced by SMN protein, the SNW-induced compartment could be a useful tool. It can reflect temporal accumulation of the SNW functional partners or the clustering of SNW-containing domains. With the aim to characterize the protein composition of SNW-complexes by mass spectrometry, SnwA-bodies were isolated from *Dictyostelium* cells using immunomagnetic separation. TAP-tagged Snw1p expressed under autologous promoter was used to affinity purify the Snw1p complexes from *S. pombe* cells.

## N1-062P
## Changes of lamin B/PARP-1 transient interactions depend on PARP-1 activation and subsequent ADP-ribosylation

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Poly(ADP-ribose) polymerase-1 (PARP-1) and poly(ADP-ribosyl)ation are proposed to be important for the regulation of many cellular processes such as DNA repair, cell death, chromatin functions and genomic stability. Activation of PARP-1 is one of the early DNA damage responses. PARP-1 is responsible for post-translational modification of nuclear proteins in the response to numerous endogenous and environmental genotoxic agents. A functional interaction between PARP-1 and lamin B has recently been concluded from nuclear fractionation, *in vivo* crosslinking and immunoprecipitation experiments. Here we use confocal microscopy to verify and extend these findings. A nuclear colocalization of PARP-1 and lamin B is traced under physiological conditions where PARP-1 has a low basal activity, after DNA damage induced by gamma-irradiation when PARP-1 is activated, and in the early phase of apoptosis when it is partially inhibited. The results show that under physiological conditions lamin B is responsible for anchoring a major part of the total nuclear PARP-1 population within the nuclear matrix structure. Dramatic changes are induced upon DNA damage. The activation of the repair process and subsequent entry into apoptosis are accompanied by a gradual release of the PARP-1 from the lamin B. The results of immuno-blot analysis revealed that after PARP-1 activation, aside from its automodification, lamin B was a main target for poly (ADP-ribosyl)ation in the isolated nuclear matrix. These results are consistent with our hypothesis that poly(ADP-ribose) modification of lamin B and PARP-1 was responsible for the disruption of their interaction during DNA repair process and apoptosis.

## N1-063P
## Biochemical and biophysical characterization of the negative regulatory region of the Notch receptors

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Notch receptors are single-pass transmembrane proteins that regulate cell growth, differentiation, and apoptosis in multicellular organisms. During maturation the Notch precursor is processed by a furin-like protease yielding an extracellular (ECN) and a transmembrane (NTM) subunit that remain non-covalently associated as a heterodimer. Ligand binding to ECN induces signaling by initiating two successive proteolytic cleavages in

NTM, which enable the intracellular domain of Notch to gain access to the nucleus and induce transcription of target genes. The Notch proteins exhibit a highly conserved modular architecture in which different *in vivo* functions are attributed to distinct structural units. Adjacent to the ligand binding EGF repeats at the N-terminus, lies a Negative Regulatory Region (NRR) that maintains the integrity of resting Notch receptors. The NRR consists of an LNR domain, which contains the three cysteine-rich Lin12-Notch Repeats and protects the Notch polypeptide from ligand-independent cleavage by metalloproteases, and a heterodimerization (HD) domain that straddles the two subunits. Here we present our progress toward understanding the biochemical and biophysical features of these domains that are important for their negative regulatory function. We have used Nuclear Magnetic Resonance Spectroscopy and Circular Dichroism to probe the structure of individual modules and intermodular interactions within the NRR, and have identified the disulfide bond connectivity in this region using a combination of protease digestion and mass spectroscopy. This work represents the initial steps in elucidating the structural requirements that impose crucial restraints to prevent premature Notch receptor activation.

### N1-064P
### Epigenetic regulator proteins for phenotype alterations and proteinaceous transmission of conformational/prion diseases [CD] and delayed-type hypersensitivity [DTH]: structural relations in domains for binding redox- and metalloregulated RNA bioaptamers

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Antigen-specific [granulomatous, tuberculin-type] DTH and CD comprise several problems by heredity of protein mutations, aging and epigenetic [non-Mendelian phenotype] alterations. Some lack ''infectivity''. Others are transmissible (''infectious'') by *paucidisperse proteinaceous* prionic matter or by transfer factors of DTH [TF-DTH] resulting epigenetic alterations in recipients without involvement of antibody and exogenous foreign genome [virus]. From isolated epigenetic regulator and amyloidogenic proteins of TF-DTH and CD, novel relations were found [Wissler et al., *Protides Biol Fluids* 1986; **34**: 525–536; *Materialwiss Werkstofftech* 2001; **32**: 984–1008; *Ann NY Acad Sci* 2002; **961**: 292–297; 2003; **991**: 333–338; 2004; **1022**:163–184; *FASEB J* 2004; **18**: C62; 2005; **19**: 360.3 & 196.4; *Mol Biol Cell* Suppl. 2004; **15**: 479a–480a & L312; *Biophys J* 2005; **84**:2000]. Thus, prion, ure3-yeast, alzheimer precursor, huntingtin, parkin and fragile-X mental retardation proteins as well as some S100, IeF, ribosomal, chaperone, receptor, adaptor and serum proteins and several proteins associated with ataxia, amyotrophic lateral sclerosis, epilepsy, schizophrenia, deafness, cardiomyopathy, diabetes, cancer, muscular dystrophy, multiple sclerosis, ichthyosis, psoriasis, MHC, transport, gametogenesis, development, translation, transcription, growth factor and hormone precusors [FGF, VEGF, BMP, BDNF, leptin] contain newly found homologous domains as address for binding endogenous redox- and metalloregulated [copper ion-pre-structured] RNA bioaptamers [~2–200 bases], termed K/R3H [K/RxxxH], i.e. -t/s/xK/RxxxHx$_{7\text{-}9}$h/xx$_{7\text{-}9}$h/xx$_{5\text{-}20}$K/R/q/e/h/n/s/d- with accessory basic [R/K]n and SR/K/RS segments. Their sequence position may argue that only some CD are infectious [''*bioaptamer disease*''] and as to how copper-binding ATCUN motifs are formed upon protein maturation. The results suggest new aspects on proteinaceous transmission of epigenetic reprogramming and inheritance in CD, the ''infective folding'' still in search within the prion hypothesis and compliance with Crick's central dogma of molecular biology. On known resistance of infectious prionic matter to inactivation by usual sterilization procedures, the found TF-DTH/antigen/adjuvant relations suggest new alternatives in health care of transmissible CD.

### N1-065P
### Photophysics and chromophore cavity analysis of green fluorescent protein

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Green fluorescent protein (GFP) is a very commonly used marker in biology and medicine. The chromophore is an intrinsic part of the protein backbone, it is formed by an autocatalytic cyclization. The chromophore only fluoresces when it is located in the correctly folded GFP beta-barrel. The protein presumably prevents the excited state chromophore from twisting, which can lead to non-radiative relaxation by means of non-adiabatic crossing. Computational methods have been used to establish that (i) the chromophore cavity of wild-type GFP is not complementary with a planar chromophore, (ii) the tau one-bond-flip (OBF) in the chromophore model, 4-hydroxybenzylidene-2,3-dimethylimidazolidinone displaces a larger volume than the hula-twist (HT) or the phi OBF. However both the HT and phi OBF processes displace the same volume. A hula-twisting motion of the excited state chromophore is not necessarily a volume conserving motion, (iii) the protein matrix of GFP forms a cavity around the chromophore that is complementary to an excited state conformation in which the phenol and imidazolidinone rings are perpendicular to each other – a conformation that was obtained by a concerted positive 45° hula-twist of both chromophore dihedral angles, (iv) there is a significant variation in the dimensions of the chromophore cavity amongst all the GFP mutants and GFP-like proteins in the protein databank. Some of the cavities are not complementary with hula-twist motions.

# N2 – Media Relations Symposium

### N2-001
### The media - between science and fiction?

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Where does the public get its image of science and scientists? We are often told it comes from the media but the domain of fiction is often an overlooked area when it comes to science communication. Outlets from science fiction literature to Hollywood movies and TV series play a significant role in forming the public's view of science. Overall, it seems that the negative stereotypes have an overwhelming lead on the positive images of scientists. Importantly, the imagery of fiction spills over into the media. Reporting on modern scientific subjects draws on deeply seated archetypes and creates immediate identification and perhaps automatic judgement. We see this from the campaigns against ''Frankenfood'' to the deluge of newspaper articles on 'The brave new

world' of genetics. Such media coverage often serves as inspiration for new works of fiction and the image of modern science is caught in a vicious circle.

## N2-002
## The role of media in GMO debate in France

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The use of genetically modified organisms (GMO) for human consumption has not yet become a reality in most European countries although these new plant varieties are rapidly developing throughout the world. This raises the question of knowing if EU consumers are pioneers when refusing GMOs or if they behave as exceedingly conservative persons. Numerous debate and polls indicate that public opinion is poorly informed on the benefits and risks potentially generated by the use of GMOs. This raises the question on the role of media on the misinformation of consumers. This also reveals the poor capacity of the scientific community to explain its results and projects to citizens but also to politicians. Most of the scientists are also unable to adopt an appropriate language capable of counteracting the irrational arguments often used by GMO opponents. This leaves an almost entirely free space to opponents. The confrontation between scientists, media and public opinion about GMOs has a more general impact. It emphasizes the need of reassessing the role that should have science and its applications, particularly in biotechnology, in modern societies. A progress in this field is necessary and it is possible only if the three partners make an effort to allow a majority of citizens to have a sound appreciation of the new scientific and technical challenge. No satisfactory method has been found so far to bridge this gap. This presentation will report the experience of the author who participates in the GMO debate in France.

## N2-003
## Science and the media – a poor fit?

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Science Media Centre has developed to find the right scientist in the form and timeframe the journalist need, with other words to have it good and to have it now. Not because it is some kind of duty to the media; but because that is the way science can ensure a balanced public debate where the public have access to accurate, good, evidence-based science and the scientific approach to new developments. To those scientists who disagree with us and dismiss the idea that science should have to adapt to the media we answer that all players in this particular drama have too long a history of misunderstanding each other. Unless we can do better, we will weaken our ability to make wise judgements about science, undermining science and our ability as a society to make progress. Nothing less is at stake.

## N2-004
## Can genetics help us rethink communication? Public communication of science as a 'double helix'

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Public communication of science is still largely conceptualized within a "transfer" paradigm that describes it as a displacement of results and ideas from the specialists to the lay public, problematizing the public, the media, (sometimes) science, but very rarely the notion itself of communication. This paper is a preliminary attempt to see if the discourse about genes and the genome can help us to problematize the concept of communication in relation to science, rethink our models of public communication of science and more in general the metaphors we employ to describe communication. It is suggested that the relationship between science and the public could be better understood by viewing communication through metaphors drawn from contemporary biology, e.g. as "cross-talk" between the specialist and public discourse or as a "double helix" coupling the two dimensions under certain conditions.

## N2-005
## Who is manipulating whom?

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Genetic engineering made it possible to transfer different traits from one living organism into an other one. This technology led to produce transgenic plants with novel traits for agricultural purposes, such as herbicide, insect or virus-resistance. The global area of transgenic crops continued to grow from 1.6 million ha in 1996 to over 81 million ha in 2004. A large number of civil organizations, "greens" express concern about the potential long-term effect of spread of transgenes in nature and also some greens brand food developed through agri-biotech as unnatural and suggest as dangerous for human health, when such food is consumed. They are also opposing the introduction of this technology as they are against multinationals, who actually developed this technology. These extremists are well-trained media communicators, and they are getting media attraction by using pseudo-scientific information to influence public perceptions. They are well informed about the general low-level knowledge of the society about this new technology, and use several expressions, which could easily influence the public, such as "Frankenfood," "superweeds" etc. Their campaigns are well designed into different regions. In Europe they often argue about the overproduction, neglecting the fact that ag-food products are imported into Europe from other parts of the World more than 60% of the yearly needs. In Africa they usually influence the governments politically that US based multinationals just want to colonialize them, and they will not be able to sell agricultural products in the EU countries if they are growing GM crops. The societies are usually miss-informed by the different statistical analyses on public perceptions. Those surveys are in most cases badly designed and biased by the real facts. The media faces numerous challenges in attempting to inform the public about real hazards and dangers in this world without causing mass hysteria. They also face manipulation from a variety of sources, pressure groups, big businesses, political parties and so on.

## N2-006
## Challenge of covering science in the EU

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# N3 – Apoptosis and Signal Transduction

## N3-001
## The PIDDosome, a protein complex implicated in activation of caspase-2 in response to genotoxic stress

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Apoptosis is triggered by activation of initiator caspases upon complex-mediated clustering of the inactive zymogen, as occurs in the caspase-9-activating apoptosome complex. Likewise, caspase-2, which is involved in stress-induced apoptosis, is recruited into a large protein complex, the molecular composition of which remains elusive. We show that activation of caspase-2 occurs in a complex that contains the death domain-containing protein PIDD, whose expression is induced by p53, and the adaptor protein RAIDD. Increased PIDD expression resulted in spontaneous activation of caspase-2 and sensitization to apoptosis by genotoxic stimuli. Because PIDD functions in p53-mediated apoptosis, the complex assembled by PIDD and caspase-2 is likely to regulate apoptosis induced by genotoxins.

## N3-002
## Connecting signalling pathways regulated by exercise with cancer

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The LKB1 serine-threonine protein kinase was originally identified as a gene mutated in patients with an inherited cancer syndrome, termed Peutz Jeghers Syndrome. Mutations in LKB1 predispose subjects to developing multiple benign and malignant tumours. Since its discovery in 1998, research indicated that LKB1 functioned as a tumour suppressor, by inhibiting cell proliferation, but the mechanism by which LKB1 controlled the growth of cancer cells was not known. I will provide evidence that the cellular localization and activity of LKB1 is controlled in an unusual manner, through interaction with a ''pseudokinase'' termed STRAD and a scaffolding protein called MO25. I will also show that unexpectedly, the first physiological substrate of LKB1 that we identified, was the AMP-activated protein kinase (AMPK), an enzyme that is switched on during situations that deplete the level of cellular ATP and increase 5'-AMP, such as those that occur during stress and exercise. AMPK is believed to function as a sensor of cellular energy and to restore ATP levels by stimulating catabolic pathways, such as glucose uptake, as well as by inhibiting anabolic processes. I will present evidence that confirms the important role that LKB1 plays in regulating AMPK activity and cellular energy in exercising muscles. I will discuss how this work provides a glimpse of how signalling pathways originally believed to control cell growth in cancer cells, are actually linked with signalling networks activated by energy depleting processes such as stresses and exercise. I will finally speculate that exercise and the blood glucose lowering anti-diabetes drug metformin (glucophage), that exert their physiological effects by activating AMPK, might ''trick'' cancer cells into thinking that they do not have sufficient cellular energy to grow, and therefore be used to treat cancer.

## N3-003
## Interactions between small GTPase signalling pathways in tumour cell biology

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Small GTPases of the Ras, Rho and Ral families play important roles in tumour biology. Genetic alterations to small GTPases underscore their important role. Ras is mutated in some tumours while RhoA and RhoC are over-expressed particularly in more aggressive tumours. As well as their individual roles it is emerging that there are significant interactions between small GTPase signalling pathways. For example Rho signalling is required to suppress CDK inhibitory levels of p21Waf1 induced by Ras signalling. Interesting examples of interactions between small GTPase signalling pathways are emerging through studies on invasion/cell motility. We have delineated two modes of cell motility one dependent on Rho signalling through the ROCK family of Rho dependent kinases the other requires ROCK signalling to be down-regulated to permit Rac dependent lamelliopodium extension. In tumour cells with high levels of Rho-GTP, down-regulation of ROCK expression can be achieved via Ras dependent activation of ERK signalling. In other tumour cells that use Rac dependent lamellipodium extension, activation of Rho to the GTP bound state is suppressed by ERK-MAP kinase activation. This mechanism of suppressing Rho activation is a consequence of sustained ERK signalling inducing the transcription factor Fra-1 which then leads to inactivation of Beta-1 integrin signalling which would normally lead to activation of Rho. This mechanism may account for the long-standing observation that Ras signalling through the ERK-MAP kinase pathway leads to the inactivation of integrin signalling. Several lines of evidence indicate that the Ral GTPases RalA and RalB may play important roles in the malignant phenotype. In order to elucidate the roles of Ral proteins we have searched for new binding partners and generated knock-out mice for Ral-GDS, a Ras dependent activator of Ral. We have identified ZONAB, a protein that functions both as a transcription factor and as a component of tight junctions.

## N3-004
## Nur77 an orphan transcription factor is induced in several apoptotic pathways of T cells

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Nur77 was originally identified as an immediate early gene in response to NGF stimulation in PC12 pheochromocytoma cells. The role of Nur77 in TCR-mediated apoptosis has been demonstrated in T cell hybridomas. Nur77 was rapidly induced in T hybridoma cells undergoing TCR-mediated death. Expression of a dominant negative Nur77 protein blocked TCRmediated apoptosis in these cells. Furthermore thymocytes undergoing TCR-mediated death also express high levels of Nur77 and in transgenic mice expressing a dominant negative form of Nur77 antigen-induced apoptosis of thymocytes is blocked. In contrast, overexpression of full-length Nur77 in thymus resulted in massive

apoptosis of thymocytes. Here we show that retinoids modulate TCR-mediated apoptosis by regulating Nur77 expression and transcriptional activity leading to a change in the expression levels of the proapoptotic proteins Bim and FasL, in the thymus and T cell hybridomas respectively. We show that inhibition of negative selection by retinoids results in positive selection of thymocytes. We also show that retinoids are actively synthesized in the developing thymus. In addition we demonstrate that adenosine that is released from macrophages ingested apoptotic cells also induces apoptosis in thymocytes. Adenosine-induced death of mouse thymocytes also involved Nur77 and Bim. Our data demonstrate that Nur77 plays a role in several apoptosis pathways of T cells.

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## N3-005
## Expression of Secreted Frizzled Related Protein and associated Wnt signalling in breast cancer

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We examined the interplay between Wnt and Secreted Frizzled Related Protein-4 (sFRP4) in estradiol induced cell growth in breast cancer cells (MCF-7), and also determined the *in vivo* distribution of sFRP-4 in human breast cancer. MCF-7 Cells were treated with estradiol, sFRP-4 conditioned media and a combination of the two. Real-time RT-PCR and Western blot analysis were used to determine the expression of the sFRP-4 and its associated Wnt signalling molecules following treatment. Immunohistochemistry was performed to examine sFRP-4 expression patterns in human breast cancers. Estradiol treatment up-regulated the expression of the Wnt signalling genes Wnt-10b, beta-catenin and fz-4 ($P < 0.001$ for all genes). This up-regulation was not associated with an increase in the Wnt signalling pathway as measured by the levels of active beta-catenin. sFRP-4 conditioned media reduced MCF-7 cell proliferation, down regulated the Wnt signalling genes beta-catenin and fz-4 as well as down-regulating wnt signalling activity. sFRP-4 was able to reduce the proliferation of estradiol stimulated MCF-7 cells. Cytoplasmic sFRP-4 protein was expressed in all breast tumours examined, with intense staining evident in the lobular carcinoma *in situ* and the ductal carcinoma. These data demonstrate that sFRP-4 is a potent inhibitor of the Wnt signalling pathway in MCF-7 cells, acting not only to down-regulate the activity of the wnt signalling pathway, but also down-regulate the transcription of Wnt signalling genes. The results of these *in vitro* and immunohistochemical experiments warrant further investigation as to whether sFRP-4 expression can be indicative of prognosis in human breast cancer.

## N3-006
## Cysteine cathepsins as apoptosis mediators

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Apoptosis is the major way of eliminating potentially harmful and excessive cells. The pathway is severely impaired in cancer and cancer cells generally fail to die. A number of events in apoptosis is governed by proteolysis with caspases playing the major role. Recently, evidence has been provided that lysosomal proteases, the cathepsins, are linked with apoptosis in numerous pathways, including oxidative stress and TNF-α. The molecular mechanism(s) of cell death induction by the cathepsins are, however, less well understood. We were able to show that following major lysosomal damage cysteine cathepsins can activate caspases indirectly via proteolytic cleavage of the proapoptotic Bcl-2 homologue Bid *in vitro* and in various cellular models. In addition to Bid, several other cathepsin targets have been identified. Blocking cathepsins using 20 μM E-64d prevented mitochondrial destabilization and all other signs of apoptosis downstream of lysosomal permeabilization, whereas blocking caspases using 10–20 μM Z-VAD-fmk only blocked caspase-dependent signs of apoptosis downstream of mitochondrial rupture. Cathepsins thus lie upstream of mitochondria and caspases in the apoptotic cascade, although this may depend on the apoptotic stimulus and the model used, which will be further discussed.

## N3-007P
## Interferon gamma induces STAT 1 activation and SOCS 3 expression in spite of reduced STAT 1 RNA levels in human malignant melanoma cells

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**Purpose:** To correlate the induction of SOCS 3 (suppressors of cytokine signaling) by interferons (IFNs) on the mRNA and protein levels with the STAT 1 (signal transducers and transcription activators) phosphorylation at serine 727 (S727) and tyrosine 701 (Y701) in melanoma cell lines.

**Materials and methods:** In this study, we used a unique collection of 18 established malignant melanoma lines and five human non-malignant normal cells (two skin keratinocytes and three fibroblasts). STAT 1 expression and inducibility of its activated phosphoforms were examined by Western blots using immunoprecipitation and specific anti-STAT 1 antibodies. SOCS 3 protein levels were determined by immunoblots using polyclonal anti-SOCS 3 commercial antibody. STAT 1 and SOCS 3 mRNA levels were analyzed by Northern blot.

**Results:** In malignant melanoma lines, the SOCS 3 has been induced by IFN γ in 83% cases at both protein and RNA levels; induction by IFN α was observed in 17% at the protein level and in 0% at the mRNA level. IFN γ but not IFN α stimulated SOCS 3 expression in non-malignant cells (100%). IFN γ induced phosphorylation of STAT 1 at S727 (39% cases) and Y701 (89%); for IFN α the values were 11 and 78%, respectively. The STAT 1 transcripts expressed as the STAT 1/GAPDH ratio were reduced two- to three-fold in melanoma cell lines compared to normal cells.

**Conclusions:** Melanoma cell lines possessed significantly reduced levels of STAT 1 mRNA than non-malignant cells suggesting silencing of STAT 1 expression in tumor cells (I). IFN γ seems to be much more powerful inductor of SOCS 3 than IFN α, on both protein and mRNA levels (II). There does not seem to be simple correlation between SOCS 3 induction and STAT 1 protein phosphorylation (III).

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## N3-008P
## The role of glutathione depletion on apoptotic signal forming in HL-60 cell lines

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Apoptosis is a special form of cell death, which can be triggered by a variety of signals and pathophysiological conditions, including oxidative stress. The primary objective of this study is to determine reduced glutathione levels and caspase-3 activity at apoptosis in HL-60 with or without N-Acetyl cysteine. In this study, tert-butyl hydroperoxide used to decrease glutathione levels. Early apoptosis estimated using Annexin V on the flow cytometry. Glutathione concentration changes occurs between 0 and 3 min. At the present of N-acetyl cysteine, Glutathione values reach the control level in 2 min, but without N-acetyl cysteine the value of glutathione is half of the control level. At the present of N-acetyl cysteine 25% value of glutathione loses at 0 min but this value increases and reaches the control value at 1 min. On the other hand, without N-acetyl cysteine, glutathione value loses as 76% of its first value. At 2 min this value increases to 50% and conserves this state. Caspase- 3 activity is close the control value with or without N-acetyl cysteine. We concluded our results, the time range should be longer to determine caspase-3 activity. Cells estimated with Annexin V at the early apoptosis, there might be caspase-8 and / or caspase-9 enzyme cascade and then activation of caspase-3 occurs. Our studies have went on with caspase 8 and 9.

## N3-009P
## Expression of FasR and FasL in tumor cells and splenocytes at their simultaneous cultivation

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The very important role of the gene system Fas-receptor/Fas-ligand (FasR/FasL) is revealed at number of investigations of apoptosis in tumor cells and lymphocytes. As it was shown by us earlier interinduction of apoptosis can take place at simultaneous cultivation of tumor hepatocytes MH-22a and splenocytes. The study of different clonal lines of hepatocytes revealed some differences in ability of tumor cells as for induction of apoptosis by splenocytes, as at their ability for induction of apoptosis in splenocytes. The aim of present study was a revealing of FasR and FasL expression in mouse hepatoma cells MH-22a and histiocytic sarcoma J-774, and also in syngenic splenocytes in their combain cultivation. And for FasR and FasL expression revealing there was used the method of reverse transcription PCR (RT-PCR). The intensive expression of FasR and FasL were revealed at studying FasR and FasL expression in the hepatoma MH-22a cell population. The expression of FasR and FasL of two clonal lines of hepatoma was at the same level in difference in their level in the tumor hepatocytes population. Two studied clonal lines were different at intensity of their genes expression. The expression of FasR and FasL genes in tumor cells of histiocytic sarcoma J-774 was quite intensive. After combine cultivation of tumor cell population and splenocytes expression of FasR was decreased, but expression FasL was increased. Expression of FasR and FasL in splenocytes was at the same level as before as after experiments. The present examination has shown the perspectives of expression of FasR and FasL revealing at studying interinduction of apoptosis between tumor cells and lymphocytes. The knowledge of the gene system FasR/FasL functioning in tumor cells and lymphocytes could be used with the prognostic aims at tumor disease in human.

## N3-010P
## Caspase- and mitochondrial dysfunction-dependent mechanisms of lysosomal leakage and cathepsin B activation in DNA damage-induced apoptosis

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A lysosomal pathway, characterized by partial rupture of lysosomal membranes and cathepsin B activation, is activated during camptothecin (CPT)-induced apoptosis in U937 and Namalwa cancer cells. These lysosomal events occur simultaneously with mitochondrial permeabilization and caspase activation. In U937 cells, blocking mitochondrial permeability transition pore with cyclosporin A and bongkrekic acid reduces mitochondrial and lysosomal rupture, suggesting that lysosomal rupture may be dependent, in part, on mitochondrial disruption. Overexpressing bcl-xL, an anti-apoptotic protein known to preserve mitochondrial functions, also impedes lysosomal and mitochondrial disruption in both cell lines, indicating signaling between the two organelles. In addition, no evidence was obtained of bcl-2-like proteins targeting lysosomes. Caspase activities, including caspase-2L, are required for lysosomal and mitochondrial disruption, and lysosomal cathepsin B slightly participates in apoptosis propagation after CPT, although not essential for apoptosis activation. Our study provides evidence for the participation of a lysosomal pathway during DNA damage-induced cell death. Our data suggest that caspase activation and mitochondrial disruption represent cell context specific mechanisms by which DNA damage leads to lysosomal rupture, and that lysosomal cathepsins could slightly participate in apoptosis propagation after CPT.

## N3-011P
## Identification of the masking factor that physically interacts with γH2AX in apoptosis

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The C-terminus of histone H2AX becomes rapidly phosphorylated on serine-139, designated γH2AX, upon double-strand breaks induction, or DNA double-strand intermediates formed during cellular functions, including the execution phase of apoptosis. In the course of apoptosis, γH2AX is readily detected on histone gels and immunoblots. However, upon immunocytochemistry the γH2AX epitope is not accessible to specific antibodies in apoptotic cells. This inaccessibility occurs only in apoptosis; it is not due to any modification of the epitope, or to disassociation of H2AX from chromatin, indicating that the masking factor is a protein that physically interacts with γH2AX in the apoptotic environment. Considering the possible role of γH2AX in all different cell functions involving double-strand breaks, a striking difference stands out; only the apoptotic cells are destined to die. Therefore, it is possible that the masking factor in the apoptotic cell environment, either determines a distinct role for the apoptotic γH2AX, or indicates that DNA repair and apoptosis share common initial chromatin-related steps. We set up a nuclear import cell system to

determine the caspase that controls masking of γH2AX foci and we developed a protocol to reveal the γH2AX epitope in apoptosis. After revealing, the apoptotic γH2AX signal by immunocytochemistry appears very strong, colocalizes precisely with DNA, and is present in all apoptotic cells that exhibit all different stages of apoptotic chromatin condensation. Subsequently, we identified the masking factor by a strategy involving cell fragmentation, selective extraction, and affinity purification. Here, we provide evidence that the γH2AX masking factor plays a cardinal role in different cellular functions and in development.

## N3-012P
## The effect of interferon-α and farnesyl transferase inhibitor (R11577) on the anti-apoptotic pathways in human epidermoid cancer cells

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Interferon-α (IFNα) induces into human epidermoid cancer cells an EGF-mediated and ras/Erk dependent survival pathway. Recently, a new class of non-peptidomimetic Farnesyl Transferase Inhibitor (FTI) drugs, to which R115777 (Zarnestra) belongs, have been synthesized and tested. Therefore, we have evaluated the effects of the combination between R115777 and IFNα on the growth inhibition and apoptosis of KB and H1355 human epidermoid cancer cells. The combination induces a strong synergism on cell proliferation and apoptosis when cells are exposed for 48 h to both agents at a molar ratio of 1000:1, 250:1 (CI50 = 0.03–0.49 for KB and CI50 = 0.53–0.03 for H1355). Moreover, we have found that 500 IU/ml of IFNα alone induces an increase of Ras and Erk 1/2 activity measured with immunoblotting technique. R115777 used at low concentrations (0.07 μM) sligthly decreases the activity of Erk-1/2 and Akt, but the combination completely antagonized the effect of IFNα on the activity of the two enzymes. Moreover, we have demonstrated that IFNα induces an increase of the immunoconjugate formation and co-localization at confocal microscopy between Raf-1 and Bcl-2 and again R115777 is able to antagonize this effect. Using xenograft models of KB cells we have evaluated the antitumor activity of IFNα and R115777 combination also *in vivo*. Twice daily treatment of R115777 20 mg/kg given orally in combination with of IFNα $2 \times 10^6$ UI/kg given s.c. three times a week provided synergistic effect leading to enhanced inhibition of tumor growth without apparent toxicity. As a result, FTI seems to be capable to completely antagonize the Ras-mediated survival pathways induced by IFNα in human epidermoid cancer cells synergizing on anti-proliferative and apoptotic effects.

## N3-013P
## Retinoids (ATRA and 4HPR) induce caspase–independent DNA fragmentation and cell death in human B-lymphoma cells

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All trans retinoic acid (ATRA) and its synthetic analogue fenretinide (4HPR) are potent anticancer drugs. Only few reports are available about the effects of retinoids on B lymphoma cells. In our study non-Hodgkin B-lymphoma cells (HT58, BL41, BL41/95) were treated with ATRA and 4HPR. Both agents induced cell death time and dose dependently. Reactive oxygen species (ROS) production was elevated in 4HPR treated cells but not in ATRA treated cells. The depolarization of mitochondrial membrane, as an important step of apoptosis, occurred earlier after ATRA than 4HPR treatment in HT58 cells. ATRA induced the depolarization of mitochondrial membrane in most of the BL41 and BL41/95 cells but not 4HPR. Z-VAD-fmk, the general caspase inhibitor, decreased the DNA-fragmentation in ATRA treated cells but increased necrosis at the same time in HT58 cells. However, z-VAD-fmk did not influence the DNA-fragmentation in 4HPR treated cells. Endonuclease G was released from the mitochondria during 4HPR treatment, which could be an inducer for caspase-independent DNA-fragmentation. Our results suggest that natural (ATRA) and synthetic (4HPR) retinoids induce different apoptotic pathways in B lymphoma cells which can be an important information for their potential use in leukemia treatment.

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## N3-014P
## Role of beta-1 integrins in anoikis and invasiveness of multidrug resistant human breast carcinoma cells

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The aim of the study was to investigate the role of integrins in anchorage dependent apoptosis (anoikis) and *in vitro* invasion of human breast cancer cell line MCF-7 and its multidrug resistant subline MCF-7Dox. Acquisition of MDR was associated with markedly decreased expression of collagen specific alpha2/beta1 and alphav/beta3 integrins, laminin specific alpha3/beta1 and alpha6/beta1 receptors and significant up-regulation of fibronectin specific alpha5/beta1 integrin. The MDR subline were substantially more resistant to anoikis than their wild type counterparts. Furthermore, MCF-7Dox cells secreted MMP-9 collagenase and invaded Matrigel. We demonstrate for the first time that stimulation of b1 integrin signaling strongly sensitizes MDR cells to anoikis.

## N3-015P
## Role of Voltage Dependent Anion Channel (VDAC), Bax and Bid in cell death: an electrophysiological study

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Apoptosis is critical for normal nervous system development and is tightly regulated by an evolutionary conserved molecular program. There are several hypotheses regarding the mechanism of apoptosis. Some of these say that, Voltage Dependent Anion Channel (VDAC) is involved in apoptosis. VDAC is an abundant protein in the outer mitochondrial membrane, which forms large voltage gated pore (2.5–3 nm) in planar lipid bilayers, and act as the pathway for the movement of substances in and out of the mitochondria by passive diffusion. Also, there are many reports, which say that Bax and Bid proteins are the key molecules involved in cell death. Bax and Bid are the members of Bcl-2 family of proteins,

which are well-characterized regulator of apoptosis. The role of VDAC, Bax and tBid in the reported models is still very controversial. In order to resolve this controversy, we have explored the role of tBid and Bax in the gating of VDAC through electrophysiological experiments. In the present work we have shown that there is an increase in the channel conductance (VDAC) after addition of Bax and tBid through bilayer electrophysiological experiments. Based on our findings in the bilayer membrane experiments we hereby propose that tBid along with Bax interacts with VDAC and forms large pore. This will cause swelling in the mitochondria and finally rupture the outer mitochondrial membrane, thereby release cytochrome c and other apoptogenic molecules into the cytosol leading to brain cell death. Regulation of this tBid and Bax induced increase in pore size of VDAC will be an important therapeutic target for various neurological dysfunctions caused by brain cell death and needs to be verified in detail.

## N3-016P
## The study of apoptosis in different model systems

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Apoptosis, being important cell cycle regulatory element, plays critical roles not only in different physiological processes during fetal development and adult tissues but also in variety of pathological conditions. Therefore we conducted experiments, revealing the role of apoptosis in different organs of *Drosophila melanogaster* in norm and mutants, and in development of heart ischemic disease on stress induced myocardial infarction in rats. The aim of the first part of work was the finding out the features of course of apoptosis in different: organs of *Drosophila* in norm and at mutants of locus Lobe, connected with infringements of development. The time and sequence of approach of apoptotical changes in various organs of *Drosophila* were observed. We revealed a temporary sequence and organspecific apoptosis and the features of apoptosis at Lobe-mutants of *Drosophila* with infringements of development of eye-antennal imaginal disks. It is now believed that apoptosis can be the main factor of cardiomyocyte loss during the cardiovascular disease progression. The aim of this part of our work was to investigate the role of programmed cell death in stress induced myocardial infarction in rats, as the experimental model. Myocardial infarction was modeled by stress induction through everyday immobilization of rats. The rats were divided into four groups: (i) intacts; (ii) exposed to the stress; (iii) exposed to the stress plus everyday izoket preparation injections; (iv) exposed to the stress plus everyday progesteron injections. Experimental animals were stressed during 3, 7 and 11 days. Apoptosis in myocard were analyzed by agarose gel electrophoresis of DNA laddering and TUNEL analysis. Necrotic degradation occurs almost in all experimental groups. TUNEL showed no specific staining in intact animal group. As soon as in group subjected to stress during 3, 7 and 11 days TUNEL revealed widening of the apoptosis area and significant increase of number of apoptotically changed cells. In the third group decrease of amount of apoptotical cells, reduction of an infarction area and improvement of cardiomyocyte structure was revealed. The apoptosis inhibition directly depended from duration of the izoket injections. In the group, which was subjected to the stress with the simultaneous progesteron injection we observed suppression of apoptosis, reduction of infarction zone and the infringement miocard of structure.

## N3-017P
## Role of phospholipase D in somatostatin-mediated modulation of cell growth in human neuroblastoma SH-SY5Y cells

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Human neuroblastoma is the most frequent type of cancer in children under 1 year. In the last years, its treatment and survival rate have improved slightly, but it is necessary to develop an alternative treatment to improve its prognosis. SRIF negatively regulates cell growth and induces apoptosis, but the molecular mechanism implicated is not well known. Phospholipase D (PLD) also regulates cell growth and an antiapoptotic effect has been described for this enzyme. Several data support the hypothesis that PLD could be implicated in SRIF-mediated modulation of cell growth. Therefore, the purpose of our study was to examine the role of SRIF on cell growth and of PLD on these effects. Human neuroblastoma SH-SY5Y cells present PLD activity and express SRIF receptors. In the presence of 10% fetal calf serum, SRIF decreased cell proliferation, measured as [$^3$H]thymidine incorporation in a time- and dose-dependent manner whereas in serum-starved cells an increase was seen. In order to examine the role of PLD on these effects, phosphatidic acid (PA) was measured in [$^3$H]palmitate-labelled cells. In the presence of serum, higher [$^3$H]PA levels were found in comparison with serum-starved cells. After SRIF treatment, a decrease in the [$^3$H]PA formation was detected in the presence of serum whereas an increase was found in its absence. Since PA is the natural product of PLD activity, these results suggest that SRIF might regulate cell growth through the modulation of PLD and support a dual role of SRIF on cell proliferation. The knowledge of the mechanisms responsible for the control of cell growth will allow a better understanding of the mechanisms underlying the abnormal growth of tumor cells.

## N3-018P
## Apoptotic executive protein, caspase-3 is activated by a carbamate-derivative insecticide, carbofuran

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Carbofuran (CF), a carbamate derivative insecticide used in agriculture and household causes sterility, congenital anomalies and increases the risk of gastrointestinal, neurological and cardiac dysfunction as well as retinal degeneration in human and animals by contaminating air, water and food. In this study, we tested whether one mode of action of CF is via apoptotic pathway and if so, is it a caspase-dependent apoptotic mechanism or not? Vero cells were cultured in DMEM-Ham's F-12 medium with 10% FCS. Following the cellular confluency, cells were treated with 100, 250, 500, 1000 or 2000 μM CF for 12 h. Apoptosis was assessed by (i) TUNEL assay which depicts the apoptosis-induced fragmented DNA marker and (ii) fluorescently labeled anti-active caspase-3 antibody as a marker of caspase-dependent apoptotic pathway. Protein quantification was then performed by western blot analysis for the caspase-3 protein activation. The rate of apoptotic cells was evaluated by counting TUNEL-positive cells (apoptotic index) which was found to be 0.5% in untreated control group. CF increased the number of TUNEL-

positive cells in treatment groups in a dose dependent fashion (1.2–8%). CF-treated cells also displayed anti-active caspase-3 immunopositivity many of which coincided with the TUNEL-positive cells. Western blot analyses showed an significant increase in active caspase-3 protein which directly supported the immunofluorescent stainings. Conclusively, CF (i) induces a dose-dependent programmed cell death and increases the rate of apoptosis; (ii) the apoptotic cell death is directly linked to the caspase-3-dependent pathway.

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## N3-019P
## Homocysteine induces neuronal cell death with apoptotic features

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Elevated homocysteine (Hcy) level has been shown to cause neuronal cell death leading to a neurodegenerative condition, but the underlying mechanisms are unclear. The present study investigated the *in vivo* ability of Hcy stress induced by i.p. injection of methionine (Met) at concentrations of 0, 100, 300, 500 and 800 mg/kg Body Wt./day to affect the neuronal cell death of Wistar rats. Plasma Hcy levels and neuronal cell death (monitored by propidium iodide binding) were significantly elevated after Met injection for 2 weeks. Reactive oxygen species (ROS) levels and percentage of denatured DNA (evaluated by the metachromatic properties of acridine orange) were significantly increased in the neuronal mitochondria of Met-injected rats with elevated plasma Hcy. Mitochondrial membrane potential (MMP) was significantly decreased in these rats. Percentage of denatured DNA in the neuronal cells was increased in Met-injected rats. These findings suggest that neuronal cell death resulted from Met injection may attribute to elevated Hcy leading to the apoptosis.

## N3-020P
## Lack of cholesterol induces apoptosis through an ERK- and JNK-independent, and p38MAPK-dependent mechanism

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The mitogen activated protein kinases (MAPKs) ERK, JNK and p38MAPK regulate intracellular processes such as proliferation, differentiation and apoptosis. On the other hand, it is known that cholesterol is necessary for cell proliferation and cell survival. In the present study we analyze how changes in the cholesterol cell content or its biosynthesis modify the activity of the ERK, JNK and p38MAPK cascades. We also study how these changes affect the proliferation and apoptosis in NIH3T3 cells. In this work we use two different approaches: to decrease the cholesterol cell content we incubate the cells with lipoprotein deficient serum (LPDS) and to inhibit the cholesterol biosynthesis we use the non-functional analog 25-hydroxycholesterol (25-HC), which inhibit the enzyme HMG-CoA reductase. We show that both LPDS and 25-HC increase the levels of apoptosis in these cells and that this effect is higher in cells treated with LPDS + 25HC at the same time. This effect is reverted by addition of exogenous cholesterol. We also find that LPDS and 25-HC increase the activity of the three MAPKs cascades. Using different specific inhibitor for each MAPK, we have observed that only the SB203580, the specific inhibitor for p38MAPK is able to revert the apoptosis induced by LPDS and 25-HC, whereas neither the specific ERK inhibitor UO126 nor JNK inhibitor SP600125 has any effect on LPDS/25-HC-induced apoptosis. We also demonstrate that overexpression of the p38MAPK activator MKK6 without kinase activity decreases the levels of apoptosis induced by LPDS and 25-HC. Our data demonstrate that low levels of cholesterol induce apoptosis in NIH3T3 cells through a p38-dependent mechanism.

## N3-021P
## Etoposide-ionizing radiation combined treatment activates apoptotic JNK/ p53 pathway in K562 erythroleukemia cells

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Study of ability of chemotherapeutic agents and/or ionizing radiation to induce apoptosis in tumour cells is essential for setting up efficient therapies. Since drug and ionizing radiation resistance is an impediment to successful cancer therapy, we wanted to check if etoposide/ionizing radiation combined treatment could have synergic effect to improve apoptosis in K562, human erythroleukemia ionizing radiation resistant cells. To this aim we examined the role played by JNK/SAPK, p53 and mitochondrial pathways in apoptotic occurrence in such experimental model. Our results suggest that apoptosis induction, evident in 15 Gy, mainly in 15 Gy/etoposide exposed cells, may be mediated by JNK/SAPK nuclear translocation, linking extracellular stimuli triggered by IR/ etoposide combined treatment to the nucleus. Furthermore, JNK/SAPK pathway could be strictly linked both to p53, which discloses a significant expression increase in 15 and 15 Gy/etoposide, and to Bcl2, which declines in the same experimental conditions. p53 increase, paralleled by Bcl2 decline, could allow Bax homodimerization leading to potential membrane loss, cytochrome c mitochondrial release, and caspase-9 activation, which tightly binds to Apaf-1. Active caspase-9 activates in turn cell death effector caspase-3, which cleaves PARP, as demonstrated by 85 kDa fragment presence in nuclear extracts. These events suggest the activation of two different pathways which join in caspase-3 activation: JNK activates p53,which,in turn, regulates death effector Bax level, and, in parallel, modulates death suppressor Bcl2 decline. Thus, further investigations of such molecular mechanisms are useful to set up new therapeutical strategies, which, influencing apoptotic response. Overcome resistance mechanisms.

## N3-022P
## Tanshinone IIA elicits the cell death of human endothelial EAhy926 cells

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Tanshinone IIA, a major component extracted from a traditional herbal medicine *Salvia miltiorrhiza* BUNGE, is known to exhibit a potent cytotoxicity against various human carcinoma

cells *in vitro*. However, the mechanism by which tanshinone IIA has this anti-tumor effect, remains unknown. Since anti-neovascularization has been generally regarded as an effective strategy for the anti-cancer therapy, we decided to investigate the mechanism underlying tanshinone IIA-mediated the human endothelial cell death. In this study we demonstrate that tanshinone IIA elicits human endothelial cell death independent of oxidative stress. These events are partially calcium-dependent and actually dependent upon NAD(P)H: quinone oxidoreductase activity (NQO1). Tanshinone IIA induces an increase in intracellular calcium, which triggers cytochrome c release, thus causing a loss of mitochondrial membrane potential, resulting in the subsequent activation of caspases. Blocking the induction of $Ca^{2+}$ perturbation with BAPTA-AM, partially rescues cells from tanshinone IIA-induced cytotoxicity. Additionally, blocking NQO1 activity with dicoumoral or inhibiting caspase activities with the general caspase inhibitor, z-VAD-fmk, prevents the cell death induced by tanshinone IIA. Therefore, our results imply that tanshinone IIA-mediated cytotoxicity against human endothelial cells may be through the activation of NQO1, which induces calcium imbalance and mitochondrial dysfunction stimulating caspase activity.

## N3-023P
## Modulation of cell cycle proteins with a role on apoptosis of primary rat hepatocytes by ursodeoxycholic acid

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Ursodeoxycholic acid (UDCA) modulates cell death and cell cycle regulators through unclear mechanisms. The aims of this study were to characterize specific cell cycle control genes targeted by UDCA, and determine their role in apoptosis. Global gene expression of primary rat hepatocytes incubated with UDCA was determined using microarrays. Cell cycle proteins and gene expression were evaluated by immunoblotting and RT-PCR, respectively, after incubation with either UDCA, tauroursodeoxycholic (TUDCA), deoxycholic (DCA), or taurodeoxycholic (TDCA) acids. In addition, hepatocytes were infected with an adenovirus vector expressing a cyclin D1 gene or transfected with a cyclin D1 reporter plasmid. Apoptosis was assessed by Hoechst staining. Microarray data indicated that UDCA regulates several apoptosis- and cell cycle-related genes, including cyclin D1 and E-cadherin. E-cadherin was down-regulated by UDCA, while cyclin D1, almost undetectable in controls, increased its transcriptional activation and expression. However, when cyclin D1 was overexpressed, UDCA decreased cyclin D1 by approximately twofold and significantly reduced apoptosis. In addition, UDCA alone increased transcriptional activation of cyclin D1. Similar results were obtained after incubation of cells with TUDCA. In contrast, DCA and TDCA induced apoptosis but did not significantly change cyclin D1 and E-cadherin proteins, or cyclin D1 transcriptional activation. In conclusion, cyclin D1 and E-cadherin may play an important role in the modulation of apoptosis by bile acids. UDCA appears to function as a sensory molecule acting either directly or indirectly at the cyclin D1 level to promote cell survival.

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## N3-024P
## Calcium channels and male germ cell apoptosis

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The spermatogenesis is an elaborate process of germ cell proliferation and differentiation that leads to the production and release of spermatozoa from the testis. This complex process is dependent upon the hormonal stimulation as well as the dynamic interactions between the Sertoli cells, the somatic component of the seminiferous epithelium, and the germ cells. In fact, in the mammalian testis germ cells, differentiating from spermatogonia to mature spermatozoa, are in close contact with Sertoli cells which supply the nutrients and the hormonal signals essential for successful spermatogenesis. Spontaneous death of germ cells occurs normally during spermatogenesis by the process of apoptosis, leading to a loss of up to 75% of the potential number of spermatozoa, probably as a physiological mechanism limiting the clonal expansion of germ cells and the spermatozoa release. Such a process has stimulated a number of studies *in vivo* and *in vitro* by investigators working in the area of male reproductive endocrinology and toxicology, with the aim to define the cellular and molecular mechanisms of both spontaneous and toxicant-induced germ cell apoptosis. Among the most commonly toxicants inducing injuries of the male reproductive system, the 2-methoxyethanol glycol (2-ME), a major bioproduct of the paint industry, causes severe testicular lesions in many mammalian species, including men. By using the methoxyacetic acid (MAA), the proximate toxic metabolite of the 2-ME, germ cell death can be induced *in vitro* in seminiferous tubule cultures. In 18–21 days old rats, that have not yet completed the spermatogenetic process, the MAA-induced cell death concerns a large proportion of pachytene spermatocytes. We have recently shown that such MAA-induced apoptosis is significantly prevented by co-treatment with nifedipine and w-conotoxin (1), which block, respectively, L-type and N-type voltage-operated calcium channels (VOCC's). Ca++ channels in many different cell types activate upon membrane depolarization and mediate Ca++ influx in response to action potentials and sub-threshold depolarizing signals. Ca++ entering the cells through VOCC's serves as second messenger of electric signalling, initiating intracellular events such as contraction, secretion, synaptic transmission, and gene expression. L-type and N-type VOCC's are present on the Sertoli cell plasma membrane and mainly localized at the level of contact surface between Sertoli cells and pachytene spermatocytes in the adluminal compartment of the seminiferous epithelium. Such calcium channels are responsible for the substantial Ca++ influx in rat Sertoli cells and play a role in laminin-dependent [Ca2+]i raise in Sertoli cells and in Sertoli cell secretory process. In the previous study of MAA-induced germ cell death we have used the clusterin expression as the indicator of apoptosis. Clusterin is a ubiquitously expressed heterodimeric glycoprotein that is the major protein produced by cultured rat Sertoli cells. A common theme found in several tissues is the association of clusterin with tissue damage or injury. It has been shown that, in MAA-induced apoptosis, Sertoli cell-derived clusterin is very early accumulated in the cytoplasm of dying germ cells at a specific stage of differentiation, i.e. pachytene spermatocytes. In the present study, by using clusterin expression as a marker of apoptosis, we demonstrate that Sertoli cell P/Q-type VOCC's are also involved in the modulation of MAA-induced germ cell death. In neurones P/Q-type channels are primarily responsible for Ca++ entry that initiates release of fast neurotransmitters at synapses and they participate with N-type channels in mediating secretion of hormones and

neuropeptides. The interest for investigate a role of P/Q channels in germ cell apoptosis derives from the peculiar localization of such channels on Sertoli cell plasma membrane: in fact they have been identified in the zone of the seminiferous epithelium adjacent to the basal lamina, at the level of the blood–testis barrier.

## N3-025P
## Relation between liver polyamine metabolism and effect of L-Methionine in experimental cholestasis

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Cholestatic liver disease presents intrahepatic accumulation of toxic bile acids (BA), which increase cell-membrane fluidity and apoptosis of hepatocites and decrease polyamines contest. L-Methionine (L-Met), is required for the biosynthesis of polyamines (spermine and spermidine). Polyamines are essential for cell growth and differentiation, membrane stabilization and prevention of apoptosis. The aim of present study was to examine a possible relation between polyamine metabolism and L-Met effects in cholestatic liver injury. Wistar rats were divided into three groups: I – control (sham operated), II – bile duct ligated (BDL) rats, III – BDL rats treated with L-Met (150 mg/kg BW, *per os*). The animals were killed after 7 days treatment. Decreased activity of arginase (18.85 ± 1.65 vs. 25.08 ± 1.42 μmol/mg p ) and increased production of nitric oxide (NO) and citrullin (6.93 ± 1.09 vs. 2.72 ± 0.94 nmol/mg p and 3.14 ± 0.17 vs. 1.66 ± 0.07 μmol/mg p) was shown in liver of BDL rats compared with control ($P < 0.01$). Expanded level NO inhibits activity ornithine decarboxylase (via S-nitrosylation), and decreased concentration of polyamines in cholestatic liver, compared with control (spermine 520 ± 6.1 vs. 693 ± 6.3 nmol/g; spermidine 683 ± 8.3 vs. 885 ± 9.1 nmol/g; putrescine 95 ± 4.1 vs. 160 ± 4.7 nmol/g; $P < 0.01$). Oral administration of L-Met in BDL rats, prevents decreasing of polyamines concentration in liver. Decarboxylated S-adenosylmethinone, uses aminopropyl residues and increases biosynthesis of spermidine and spermine. Polyamine (PAO) and diamine oxidase (DAO) activity was significantly decreased (1.08 ± 0.06 and 1.1 ± 0.07 U/mg) in liver of BDL rats vs. controls (1.94 ± 0.16 and 1.8 ± 0.09 U/mg p), $P < 0.01$. Reduced polyamine catabolism in liver of BDL rats could point to an effort of maintaining high liver polyamine pool, taking into account their protective role. Administration of L-Met in BDL rats, results in normalization of DAO and PAO activity in liver. L-Methionine show hepato-protective role in cholestatic liver injury, prevention of decreased concentration polyamines.

## N3-026P
## Mechanisms of apoptosis induction by the vanillod capsaicin in prostate PC-3 cells

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Vanilloid receptor subtype-1 (TRV1), the founding member of the vanilloid receptor-like transient receptor potential channel family, is a non-selective cation channel that was originally described as a receptor for capsaicin, the pungent ingredient of hot chilli peppers, and its ultra potent analog from *Euphorbia resinifera*, resiniferatoxin (RTX), in primary sensory neurons where its activation elicits a sensation of burning pain. It has been also described that TRPV1 may be activated by protons, high temperatures, endogenous pro-inflammatory substances as well as anandamide, *N*-arachidonoyldopamine and some lipoxygenase products, which have been proposed as endovanilloids. This work was undertaken to study the effect of vanilloids on the proliferation of the androgen-resistant prostate cancer epithelial PC-3 cell line. We show here, by [$^3$H]-thymidine incorporation that capsaicin induced a dose-dependent prostate epithelial cell death that was not ameliorated by capsazepine, which in turn resulted to produce an additional cytotoxic effect, pointing to a receptor-independent mechanism. Capsaicin, as well as capsazepine induced apoptosis on PC-3 cells as inferred from DAPI staining of nuclei and flow cytometry analysis. The growth inhibitory effect of capsazepine was accompanied by ROS production and inner transmembrane potential ($\Delta\Psi_m$) perturbation, which are biochemical hallmarks of early apoptosis. To further confirm that the vanilloids-induced cell death was due to apoptosis we examined caspase 3 activation, an event that is commonly used as an apoptotic hallmark. Treatment of PC-3 cells with 20 mM capsaicin, 20 mM capsazepine or both, resulted in cleavage of pro-caspase 3 as evidenced by western blotting using antibodies that recognize full-length pro-caspase 3. Those results show that capsaicin may induce apoptosis in the androgen-resistant prostate tumor PC-3 cell line through mitochondria alteration and caspases-3 activation.

## N3-027P
## Paradoxical activation of pro-survival pathways in Jurkat T cells sensitive to the cytotoxic action of TNF-Related Apoptosis Inducing Ligand (TRAIL)/Apo2L

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Since a few years ago, the known biological activity of TNF-Related Apoptosis Inducing Ligand (TRAIL)/Apo2L was far limited to induce apoptosis in various cell lines, including some of hematopoietic origin. In more recent years, new regulatory, pro-survival and proliferation effects are being attributed to this cytokine and, what was more unexpected, this was not restricted to normal primary cells, but extended to neoplastic cell lines of leukemic and non-leukemic origin. In this panorama, we decided to investigate the possible recruitment of survival pathways in the response of Jurkat T leukemic cells exquisitely sensitive to the cytotoxic action of TRAIL. Jurkat T cells displayed the occurrence of apoptotic patterns within 3 h upon TRAIL administration, reaching, within 48 h, a dose-dependent increase in the percentage of dead cells (up to 85–90%). A parallel dose-dependent increase in the G0/G1 phase of the cell cycle was detected and reverted by the treatment with z-VAD-fmk, a broad inhibitor of caspases. Co-treatment of the cells with inhibitors of PI-3 kinase (LY294002) and nuclear factor kappa B (NF-kB) (SN50) pathways lead to an earlier significantly increased cytotoxicity, respectively in the form of apoptosis and necrosis. Consistently with the data obtained with the pharmacological inhibitors, the activation and nuclear translocation of both PI-3K and NF-kB were observed. Our results provide evidence that even in sensitive neoplastic cells TRAIL paradoxically activates pro-survival pathways which protect against TRAIL-mediated death since their inhibition leads to an earlier and increased cytotoxicity.

## N3-028P
## The role of c-Jun $NH_2$-terminal kinase in aberrant neurofilament phosphorylation

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The complex I inhibitors MPTP and Rotenone cause the degeneration of dopaminergic neurones in which the c-Jun $NH_2$-terminal kinase (JNK) signalling cascade has been implicated. We have employed a differentiated mouse neuroblastoma N2a cell model to investigate the involvement of JNK in MPTP-induced collapse of the neurofilament network. Treatment with cytotoxic concentrations of MPTP (5 mM) or Rotenone (100 μM) caused rapid and sustained JNK phosphorylation and ERK dephosphorylation accompanied by cell death. In contrast, exposure of cells to sub-cytotoxic concentrations of either compound resulted in lower, transient JNK activation in the presence of sustained ERK activity. However, in the presence a specific mixed lineage kinase inhibitor (CEP-11004) MPTP- and rotenone-induced cell death was significantly attenuated. Previous work in our laboratory has established that exposure of N2a cells to sub-cytotoxic MPTP levels causes an aberrant increase in neurofilament heavy chain (NF-H) phosphorylation, perikaryal accumulation of NF-H and inhibition of axonal outgrowth, prior to cell death. In this study we show that, whilst normal NF-H phosphorylation can be mediated by ERK, selective inhibition of JNK using CEP-11004 can significantly attenuate MPTP-mediated aberrant NF-H phosphorylation and perikaryal NF-H accumulation. In doing so axon-like processes and viability are maintained. These data suggest that JNK is the predominant kinase involved in aberrant NF phosphorylation in this PD model, and may have implications in Lewy body formation. This study provides further evidence that modulation of JNK activity could have a role in Parkinson's disease therapy.

## N3-029P
## Efficient TRAIL-R1/death receptor 4-mediated killing of melanoma cells by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)

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Malignant melanoma is characterized by unbroken high mortality, increasing incidence and marked therapy resistance. The death ligand TRAIL bears high potential as an new anticancer agent, as after binding to the death receptors TRAIL-R1/DR4 or TRAIL-R2/DR5, it triggers apoptosis in most cancer cells, whereas normal cells are spared. For melanoma, however, only weak responsiveness of primary cultures was reported, and in particular a minor role for DR4 was supposed. For assessing susceptibility of melanoma, we studied the functionality of DR4 and DR5 in melanoma cells as well as their expression *in vivo*. In seven melanoma cell lines, investigated, DR5 was consistently expressed whereas, significant expression of DR4 was found in only two. However in clear contrast to previous considerations, high sensitivity to TRAIL-induced apoptosis was characteristic for DR4-positive melanoma cells, whereas DR4-negative cells showed less and delayed response or were resistant. Employment of selective DR4/DR5 blocking antibodies unequivocally proved the prevalent role of DR4 in those melanoma cells, where it was expressed. Full activation of apoptosis-related signalling cascades (caspases -8, -10, -9, -3 and -7, BID, XIAP and DFF45) was seen in sensitive cells, and activation of the mitochondrial pathway became clearly evident due to caspase-9 and Bid cleavage as well as due to complete suppression of TRAIL sensitivity after Bcl-2 overexpression. As shown here for the first time, DR5 as well as DR4 were also significantly expressed in the majority of melanomas, examined by immunohistochemistry. Thus, DR4 expression *in vivo* and high efficiency of DR4-mediated apoptosis may strongly suggest to reassess the suitability of TRAIL and especially of DR4-based strategies for melanoma treatment.

## N3-030P
## Mechanism underlying increased susceptibility to apoptosis in Lck-deficient T lymphocytes

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We have previously demonstrated increased susceptibility to apoptosis in non-transformed T lymphocytes deficient in the Src-protein tyrosin kinases (PTK), Lck. In this study, we sought to characterize the molecular mechanism responsible for this phenomenon. Our data indicate that, compared to normal T lymphocytes, Lck-deficient T cells exhibit heightened susceptibility to apoptosis upon withdrawal of growth factors, including IL-2 and IL-4. Induction of apoptosis takes place via the intrinsic cell death pathway and is associated with the release of cytochrome c from the mitochondria and activation of caspases 3 and 9. Subsequently, this leads to DNA damage and the activation and stabilization of the tumor suppressor p53. Lck-deficient T cells expressed significantly reduced levels of Bcl-2, but surprisingly higher levels of pro-caspase 9 protein, suggesting a link between Lck and expression of pro- vs anti-apoptotic mediators. Finally, increased apoptosis sensitivity in Lck-deficient cells was observed in the absence of any increase in the level of Bax, a key pro-apoptotic mediator. Taken together, our findings demonstrate that Lck-deficient cells have reduced levels of Bcl-2, a critical anti-apoptotic protein, which has a detrimental impact on mitochondrial integrity in a Bax-independent manner.

## N3-031P
## Organ-specificity of HIF-1α level and DNA fragmentation in rats exposed to chronic hypoxia

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Although hypoxia-inducible factor-1α (HIF-1α) is the main transducer of hypoxia, its capacity to accumulate *in vivo* during chronic hypoxia is yet to be addressed. HIF-1α persistence in hypoxic tissues is relevant when different organs are exposed to the same decrease in arterial blood $PO_2$. We test the hypothesis that hypoxemia causes different HIF-1α responses in various organs. To assess how HIF-1α affects downstream genes regulation, we selected apoptosis as a phenotype linked to cell viability. We measured HIF-1α (immunoperoxidase staining, quantitative immunofluorescence and western blot) and DNA fragmentation

(TUNEL) in heart, liver, kidney, gastrocnemius and brain of rats exposed to 10 or 21% $O_2$ for 2 weeks. HIF-1α accumulation in hypoxic tissues has the capacity to become a sustained phenomenon during hypoxia, but each organ responded differently, despite the same arterial $PO_2$. While marked in brain, muscle and kidney cortex, HIF-1α increase was undetectable in heart and liver. In kidney medulla, HIF-1α was high in both normoxia and hypoxia. By contrast, the apoptotic response to hypoxia was marked in heart, slight in kidney medulla and undetectable in the other organs, indicating that the apoptotic pathway may be triggered by HIF-1α-independent mechanisms. These data emphasize that the array of responses elicited by inadequate $O_2$ supply with respect to needs is very specific for each tissue and cell.

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## N3-032P
## A central role for MAO-A activity in apoptosis in SH-SY5Y cells

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Recent work has shown that the MAO gene is a target of MAPK signalling pathways associated with the stress response (DeZutter and Davis, *P. N. A. S.* 2001; **98**, 6168–6173). In turn hydrogen peroxide is a product of MAO catalysed dopamine deamination which can damage cells. In this study apoptotic cell death was induced in SH-SY5Y human neuroblastoma cells by exposure to 1 μM staurosporine (STS). Caspase 3 activation peaked after 3 h and was linked with caspase 9 (but not caspase 8) activation, signifying the importance of the mitochondrial cell death pathway. MAO-A activity was also significantly increased by threefold, peaking after 1 h treatment, and linked with increased MAO-A protein levels. Clorgyline, a specific and irreversible inhibitor of MAO-A, protected cells from apoptosis by 50%, suggesting an involvement of MAO in early apoptotic events. The MAPK enzymes JNK and p38 were activated within 20 min of STS treatment, whilst ERK phosphorylation diminished immediately. In the presence of clorgyline, activation of pJNK was delayed; and phosphorylation of ERK and p38 remained at control levels. Levels of survival proteins Bcl-2 and pAkt (PKB) were reduced due to STS induced apoptosis, whereas clorgyline reversed these effects. In this work we have shown that MAO-A may play an important role in the early events of STS-induced neuronal cell death.

## N3-033P
## Role of p53 in Newcastle disease virus induced cytotoxicity in tumor cell lines

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Newcastle disease virus (NDV) is an infectious agent causes serious infections in birds, but it is apparently non-pathogenic in mammalian species including humans. Previous observations and small-scale clinical trials indicated that NDV exerts oncolytic effect. Isolates of NDV were found to have selective affinity to transformed cells. We previously showed that the NDV isolate MTH-68/H causes apoptotic cell death *in vitro* in PC12 rat phaeochromocytoma cells. The aim of the present study was to extend MTH-68/H cytotoxicity testing in human tumor cell lines and to analyze certain biochemical aspects of its oncolytic effect. MTH-68/H was found to be able to kill a wide range of transformed cells by apoptosis independently of the presence of functional p53. Apoptosis was accompained by virus replication in two tumor cell lines tested. Proliferation of non-transformed mouse and rat fibroblast cell lines, and human primary fibroblasts was not affected by MTH-68/H treatment. A human glioblastoma cell line with repressible expression of the p53 protein did not show any difference in MTH-68/H sensitivity in its p53-expressing and p53-depleted state. Since progression of human tumors often leads to the loss of p53 function, novel therapeutic approaches that do not rely on a functional p53 protein may widen the scope of anticancer treatment. The selective, p53-independent oncolytic action of MTH-68/H observed in the present cell culture studies makes it a promising alternative therapeutic option against advanced human cancers.

## N3-034P
## Induction of apoptosis by transduced p27

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Plasma membranes of cells are generally impermeable to proteins and peptides. The potential for intracellular therapeutic use of proteins, peptides and oligonucleotides has been limited by the impermeable nature of the cell membrane to these compounds. To achieve an efficient intracellular drug and DNA delivery, attempts were made to target microparticulate drug carriers into cytoplasm bypassing the endocytotic pathway. TAT peptides derived from the HIV-1 TAT protein facilitate intracellular delivery of proteins and small colloidal particles. TAT protein enters into the cells when added to the surrounding media. Protein transduction has been widely used to analyze biochemical processes in living cells. The present study analyzed the effects of cell cycle on the uptake of proteins responsible for regulation of cell cycle. The proteins (p27, p23, Mp27) were transdused into different cell lines (NALM, MOLT, Raji, SuDHL, and K562) and their effects on proliferation of the cells were measured. A transduced p27 did not remarkable influence on proliferation of examined cell lines. Mutated p27 inhibited the proliferation of examined cell lines up to 30%. On the other hand, a transduction of p23 protein, truncated form of p27, inhibited the proliferation all of examined cell lines 30–60%. Also the effects on expression of host p27 protein were examined, as well as an influence on induction of apoptosis.

## N3-035P
## Analysis of p53 and p73 binding sites by ChIP-on-chip technology

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p73 is, together with p63, a member of the p53-family, which is able to bind to p53 DNA binding sites, transactivate p53-responsive genes and induce apoptosis when exogenously expressed in cells. The different p53-family members seem to cross-talk and cross-regulate between each other. p73 is expressed in the cells as several different C-terminus variants with different transactivational activities whose tumorigenic and/or oncogenic properties have not been completely unravelled. p73 plays also a crucial role in neurogenesis, thus indicating that this p53-family member can transactivate distinct target genes whose functions are completely unrelated to p53 pathways. In our work, we used the *in vivo* ChIP-on-chip technology on newly established stable cell lines

inducible for p53, p73 gamma and p73 epsilon to investigate specific or common targets among the family members. We found that, together with the common ones, not only p53 and p73, but also p73 gamma and epsilon have distinct targets. The transactivational activity of p53 and the p73 isoforms was then analysed on some of the common and unique targets and the cross-talk between p53 and its family members was also investigated.

## N3-036P
## Structure and expression of p53 gene and altered phosphorylation of p53 protein in human vestibular schwannomas

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Human vestibular schwannomas (VS) arise from the vestibular branch of the 8th cranial nerve. Majority of these tumors are sporadic and some occur as bilateral VS associated with neurofibromatosis type 2 (NF2) which is an autosomal dominant disorder. We have analyzed the structure and expression of the p53 gene in human VS for tumor specific alterations, if any, at this gene locus. We found loss of heterozygosity (LOH) at the p53 gene locus in approximately 50% cases. We found the p53 mRNA in all the tumor samples analysed. However, there was an increase in the level of p53 mRNA in the VS samples compared to that of the normal control. Analysis of the p53 protein showed a variable level of p53 protein in the tumor samples compared to the normal control. We also observed variable levels of phosphorylation of the p53 protein in the tumors and it correlated to that of the patients age. The LOH at the p53 gene locus is suggestive of a possible genetic instability in these patients. Increased level of p53 mRNA in the tumors suggests a possible deregulation of p53 gene in these tumors. Increased level of p53 protein is indicative of an anti-apoptotic function of this protein. The altered phosphorylation of the p53 protein indicates that the p53 protein could be involved in the age-related rate of proliferation of these tumors. These results indicate that the p53 gene may have an important role in these tumors.

## N3-037P
## Apoptosis and expression of cyclin A in human leukemia cell lines K-562 and HL-60

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Using light and electron microscopy we studied the distribution pattern of cyclin A and tried to define the relationship between expression of cyclin A and cytotoxicity of doxorubicin throughout the apoptosis. Cyclin A at the light microscope level was detected by the streptavidin-biotin-peroxidase technique and at the ultrastructural level by streptavidin-gold method. Flow cytometry analysis was used to estimate percentage of cells in phases of cell cycle. Studied cells were treated with doxorubicin in the range 0.5–10 μM. Changes in morphology of the cells and expression of cyclin A were dependent on concentration of doxorubicin. Doxorubicin inhibited cell growth of both lines in dose dependent manner. The cells treated with 0.5 μM of doxorubicin were smaller compared to cells treated with 5 especially 10 μM. In our experiment the number of apoptotic cells and positive cyclin A labelling was growing with dose of doxorubicin. Treatment of cells with doxorubicin involved decrease of G1/G0 phase and growth of cells at G2/M phase compared to control. At the ultrastructural level cyclin A was seen in the nucleus and cytoplasm but in cells treated with higher doses of doxorubicin intense gold labelling in cytoplasm was observed. These data suggest that increase of apoptotic cells might be caused by overexpression of cyclin A. Future studies are required to clarify whether cyclin A may have pro-apoptotic role.

## N3-038P
## Pyrimethamine induces apoptosis of freshly isolated human T lymphocytes bypassing CD95/Fas molecule but involving its intrinsic pathway

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Pyrimethamine (pyr), a folic acid antagonist, may exert, in addition to antiprotozoan effects, immunomodulating activities including induction of peripheral blood lymphocyte apoptosis. However, the molecular mechanisms underlying this proapoptotic activity remain to be elucidated. Here we show that pyr, used at pharmacologically relevant concentration, induced *per se* apoptosis of activated lymphocytes via the activation of the caspase 8- and caspase 10-dependent cascade and subsequent mitochondrial depolarization. Importantly, this seems to occur independently from CD95/Fas engagement. The proapoptotic activity of pyr was further confirmed in a patient with autoimmune lymphoproliferative syndrome (ALPS), an immune disorder associated with a defect of Fas-induced apoptosis. In this patient, pyr treatment resulted in a "normalization" of lymphocyte apoptosis with a significant amelioration of laboratory parameters. Altogether these results suggest a mechanism for pyr-mediated apoptosis that seems to bypass CD95/Fas engagement but fully overlaps CD95/Fas-induced subcellular pathway. On these bases, a reappraisal of the use of pyr in immune lymphoproliferative disorders characterized by defects in CD95/Fas-mediated apoptosis should be taken into account.

## N3-039P
## Involvement of caspase-1 in microglial cell death *in vitro*

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Purified microglial cells can survive in mCSF-containing medium, and these cells undergo apoptosis when transferred to the normal

medium. In this study we examined the molecular mechanism underlying this death. Mixed glial cell cultures were prepared from rat embryo and grown for 10–14 days in DMEM containing 10% FCS. The microglia was taken out by shaking the culture, and purified by panning. The isolated microglia were maintained in serum free DMEM containing mCSF (1 ng/ml). Cell death was induced by replacing the mCSF containing medium to normal DMEM. During the death, the expression of phosphorylated Akt and MAP kinase was decreased. This death was inhibited by non-specific caspase inhibitor zD or by the combination of casapse-1 inhibitor YVAD-fmk (1000 nM) and caspase-3 inhibitor DEVD-fmk (1000 nM). PI3-kinase inhibitor wortmannin (500 nM) and LY294002 (100 nM) induced apoptosis in the microglial cells maintained by mCSF. This death was inhibited by DEVD, and YVAD did not show any effect. In the early onset, these cells exhibited mitochondrial dysfunction, such as reduced potential and release of cytochrome C into cytosol. On the other hand, these changes were observed in the late stage of the apoptosis induced by MEK inhibitor PD98059 (0.1 mM) and U0126 (0.01 mM), and this death was prevented by YVAD and partially by DEVD. IL-1 receptor antagonist did not inhibit apoptosis induced by MEK-inhibitors. These data indicate that mCSF maintains phosphorylation of Akt and MAP kinase, and that inhibitors for Akt kinase induce death via mitochondria-caspase-3 dependent pathway whereas inhibitors for MAP kinase induce caspase-1 dependent pathway.

## N3-040P
## Role of TNF alpha in apoptosis induction and cell proliferation

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TNF-alpha is a pleiotropic cytokine which can induce apoptosis is sensitive cells, but also regulated cell proliferation, cellular activation and differentiation. To be better estimated role of TNF on PC cell line, originally developed from patients with myelodisplastic syndrome at Institute of Oncology Sremska Kamenice, Novi Sad, we monitored the kinetics of changes after *in vitro* treatment with or without TNF-alpha in presence anti-CD 45 and CD 95 MoAb, IL-4 and GM-CSF. We monitored the cell viability, by cell enumeration, intracellular metabolic activity by determination of total LDH activity, cell proliferation, cell membrane molecule expression as well as apoptosis and necrosis using flow cytometry (Becton Dickinson) after 2, 6, 8 and 24 h under some experimental conditions. Our results showed that in comparison with untreated cells, TNF-alpha induced significantly increase in apoptosis and necrosis, in PC cells, which expressed high level of CD95 and TNF alpha receptors. Pretreatment of PC cell with anti-CD45 and anti CD95 monoclonal antibodies modulated cell death induced by TNF. In addition, presence of TNF in cell culture medium induced significantly decrease in cell proliferation, stimulated by IL-4, or GM-CSF. However, no changes in CD13 and CD33 antigen expression following cell proliferation, determined after 4 days stimulation in comparison to percentage expression before treatment. No changes in intracellular LDH activity before and after cell proliferation induced with different cytokines. We conclude that sensitivity to apoptosis limited cell proliferation estimated on this cell line.

## N3-041P
## Involvement of ER stress responses in cyclosporin A-induced apoptosis in PC12 cells

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Cyclosporin A (CsA), an immunosuppressive agent and a calcineurin inhibitor, is widely used to treat allograft rejection and various autoimmune disorders. CsA also has been known to induce apoptosis by the mechanisms still not fully understood. In this report, we investigated the role of endoplasmic reticulum (ER) stress response in CsA-induced cell death mechanism. When PC12 cells, a rat pheochromocytoma cell lines, were treated with 40 μM CsA, LDH release from the cells was time-dependently increased 24 h, showing fourfold higher level than that of the control at 48 h after the treatment. CsA increased the expression of ER stress genes such as BiP/GRP78, CHOP/GADD153, and XBP-1 in dose- and time-dependent manners at both mRNA and protein levels. CsA (40 μM) also induced the activation of ER-specific initiator caspase-12 as well as the final common executor caspase-3. Another calcineurin inhibitor, FK506, also showed the similar effect on ER stress gene expression in PC12 cells, suggesting the involvement of calcineurin in the processes of ER stress response or ER stress-induced apoptosis. These results strongly indicate the involvement of ER stress-mediated apoptosis in the mechanism of CsA-induced apoptosis.

## N3-042P
## Expression and mitochondrial localization of human cell induced death effector – a (CIDEa) in yeast

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Mitochondria are gatekeepers of the programmed cell death, "decision makers" in apoptosis. CIDEa, CIDEb proteins are related to both N terminals of the heterodimeric DNA fragmentation factor DFF, consisting of the 40-kDa caspase-3-activated nuclease (DFF40 or CAD), & its 45-kDa inhibitor (DFF45 or ICAD) [1]. The DFF45&DFF40 complex is cleaved by caspase-3 and released nuclease then causes apoptotic DNA fragmentation. CIDE-induced apoptosis is not sensitive to caspase inhibitors but is inhibited by DFF45. The N-domain of CIDEa binds to the homologous domain on DFF45 opposing its inhibitory effect on DFF40. However, mitochondrial localization and CIDEb(a) dimerization is likely required for induction of apoptosis [2]. In this work we have confirmed the ability of human CIDEa to be imported into mitochondria of yeast *S. cerevisiae*, where CIDEa/CIDE-like homolog has not yet been identified. The human CIDEa clone (Invitrogen ORF, No IOH22361) has been transposed using the clonase reaction into the yeast Gateway expression vector pYES2-DEST52 (Invitrogen), which was then introduced into yeast strains W303 and JB516. CIDEa expression was induced by galactose. The CIDEa import into the inner membrane was proven by immunodetection of fractionated mitochondria and its identity was verified by Western blotting and by MALDI-TOF-assisted peptide mapping of the trypsinized samples of isolated yeast mitochondria. Thus we have demonstrated that even yeast mitochondrial protein import apparatus is able to direct ectopically-expressed human CIDEa into mitochondria.

**References**

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## N3-043P
## Adenosine metabolizing enzymes in apoptotic mouse liver induced by anti-mouse Fas monoclonal antibody

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Apoptosis, also known as programmed cell death, is a ubiquitous mode of cell death known to play an important role during embryogenesis, development and adult cellular homeostasis. The FAS-FAS ligand system (cell surface death receptor) is recognized as a major pathway for the induction of apoptosis in cells and tissues. It is also involved in the mechanism responsible for tissue disruption in many autoimmune diseases and in fulminant hepatitis. On the other hand, adenosine is an endogenous nucleoside that is considered a key regulator of neuro-endocrine-immune functions being implicated as a physiological signal in the receptor-mediated and receptor-independent mechanism of apoptosis. Having in mind all the facts, the aim of this study was to investigate possible implications of adenosine in antiFAS induced apoptosis in mouse liver. Considered to that we have been measured the activity of 5'-nucleotidase (an adenosine producing enzyme) and adenosine deaminase (involved in adenosine degradation). The experiment was performed on mice allocated to following groups: I – control; II group treated intraperitoneally with anti-mouse FAS monoclonal antibody (40 μg/mouse weighing 50 g); III treated with IGF (6 ng/g BW) and IV treated simultaneously with antiFAS and IGF. The mice were sacrificed 48 h after. The activities of enzymes were measured in 10% liver homogenates according to the reference spectrophotometric methods. During antiFAS administration the activity of 5'-nucleotidase decreased significantly ($P < 0.001$) compared to the control value. Administration of IGF simultaneously with antiFAS showed significant recovering of 5'-NT activity. The activity of adenosine deaminase slightly increased ($P < 0.05$) in liver of antiFAS treated mice but did not change in IV group. The obtained results showed that adenosine is not involved in mechanism of FAS ligand induced fulminant hepatitis since the activity of adenosine producing enzyme is diminished. The elevation of adenosine deaminase activity seems to be compensatory effect because of cell death and nucleic acid degradation. We have also proofed protective effects of IGF.

## N3-044P
## T lymphocytes isolated from peripheral blood of patients undergoing coronary-artery bypass graft operation reveal increased expression of bax protein

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**Introduction:** In many studies profound but transient immunosuppression was described after various types of operations. Decline in the absolute count of peripheral blood lymphocytes as well as disturbances in their function seem to be one of the most important factors that contribute to such condition. In was shown that apoptosis of lymphocytes is mainly responsible for postoperative impairment of the immune system. Bcl-2 family proteins play an important role in the control of this process. Bax protein role is pro-apoptotic and its involvement in the process of apoptosis during coronary artery bypass graft operation (CABG) was the subject of our study.

**Material and methods:** We examined the expression of bax protein in T lymphocytes (CD3+ cells) isolated from peripheral blood of patients undergoing CABG operation. The studied group consisted of 12 patients aged from 56 to 70 years treated on stable angina pectoris and scheduled for non-urgent CABG operation with extracorporeal circulation (ECC). Peripheral blood samples were taken seven times: 1, just before anesthesia; 2, 2 h after the beginning of surgery; 3, immediately after surgery; 4, 6 h after surgery; 5, 18 h after surgery; 6, 30 h after surgery; 7, 48 h after surgery. Detection of bax protein in T lymphocytes was performed by flow cytometric methods.

**Results:** We observed dynamic changes in the mean fluorescence intensity (MFI) of bax+ T lymphocytes from first to following measurements. It increased in second, third and got down in the following checkpoints of measurement. The most significant changes were noted between stages: first vs. third and third vs. sixth ($P = 0.01$), first vs. second and third vs. seventh ($P = 0.02$), second vs. seventh ($P = 0.03$) and third vs. fifth ($P = 0.04$).

**Conclusion:** Our results may indicate on increased apoptosis in the second and third checkpoints of experiment i.e. during and just after the operation. It is possible that apoptosis of lymphocytes during CABG operation is mediated by differences of bax protein expression and depends mainly on mechanical damages of lymphocytes in ECC. Further researches are required for better understanding of this phenomenon.

## N3-045P
## Role of nuclear factor-kappaB in ischemia and reperfusion in isolated rat cardiomyocytes

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NF-kappaB is a pivotal transcription factor implicated in the regulation of physiological processes, and it is activated during cellular responses to stress, hypoxia and ischemia-reperfusion (I/R). However, the importance of NF-kappaB inhibition for therapeutic purposes is still open especially taking into account evidence of the protective role of NF-kappaB activation in ischemic preconditioning. To test whether the role of NF-kappaB as a promoter or antagonist of apoptosis depends on the stress stimulus, we determined the influence of NF-kappaB activity on cell damage elicited by a variety of inducers within adult rat cardiomyocyte primary culture. Cardiomyocyte damage was induced by I/R subjecting cells to anoxia and reoxygenation, by exogenously added 100 μM hydrogen peroxide or the inflammation stimulator bacterial lipopolysacharide (100 ng/ml). Since compound ME10092 is a potent inhibitor of NF-kappaB activity, we used it

as a regulator of NF-kappaB activation. We found, that stress factor induced over-activation of NF-kappaB in isolated cardiomyocytes caused cell apoptosis and death. In contrary, the inhibition of NF-kappaB translocation by ME10092 significantly increased number of viable and non-apoptotic cells. On the other hand, the higher concentrations of ME10092 ($>1$ μM) completely reduced the stimulated NF-kappaB activation (till non-stimulated cell control level), which in turn decreased the number of viable cells and increased the number of apoptotic cells. This finding suggests that while some activation of NF-kappaB translocation could play a cardioprotective role, the stress factor induced over-activation of NF-kappaB is detrimental for cell survival.

## N3-046P
## Alpha-fetoprotein protects hepatocellular carcinoma cells escaping from the immune surveillance of lymphocytes

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**Objective:** Alpha-fetoprotein (AFP) could sustain the growth of hepetocellular cancinoma cells (HCC) *in vivo*, the present investigation to explore the functionary mechanism of AFP in HCC escaping from the host immune surveillance.
**Methods:** Human lymphocytes, Jurkat cells line were co-cultured with the human HCC line, Bel7402 cells, utilized trypan blue staining exclusion and calculated the number of the viability cells; Western blot was used to detect the express of Fas ligand (FasL), caspase-3 and Survivin of the two types cells. Confocal microscope was utilized to analyze Fas or FasL express in the membrane of Bel 7402 cells.
**Results:** It showed that AFP (10–80 mg/l) could induce Jurkat cells apoptosis obviously; when co-cultured Jurkat cells with Bel7402 cells, it demonstrated that Jurkat cells had an ability to stimulate Bel7402 cells to death, but when administrated with AFP (20 mg/l) in the co-cultured cells, it indicated that AFP could increase the viability of Bel7402 cells and inhibit the growth of Jurkat cells. Western blot detection discovered that AFP could promote Jurkat cells to express caspase-3 and restrain the expression of FasL; AFP was able to repress the expression of Fas and caspase-3 of Bel7402 cells; It also indicated that AFP had less effect on the expression of Survivin in the two kinds cells; Confocal microscope measured further confirmed that AFP had an ability of enhancing the expression of FasL and suppressing the expression of Fas in the membrane of Bel 7402 cells.
**Conclusion:** AFP could counteract the apoptosis inducing of HCC mediated by Jurkat cells; Survivin had not play an important role in HCC escaping form immune monitor; AFP through down regulate Fas and caspase-3 express in HCC that led to cancer cells escape from the host's immune surveillance.

## N3-047P
## Identification of methyl-a-D-glucopyranoside as the active compound from *Tulbaghia violacea* in the induction of apoptosis

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*Tulbaghia violacea* Harv is a medicinal plant indigenous to South Africa. It has traditionally been used to treat a variety of disease conditions including cancer of the esophagus, fever, colds, asthma, tuberculosis, and stomach problems. However the mechanisms of action against these diseases, and the active constituents of the plant are unknown. We have evaluated the pro-apoptotic activity of *T. violacea* in order to understand its medicinal properties. We demonstrate the occurrence of morphological and biochemical changes, typical of apoptosis, in Chinese hamster ovary (CHO) cells treated with the aqueous extract from *T. violacea*. The shrinkage of the cells and the subsequent detachment from each other was observed. Depolarization of mitochondrial membrane potential was detected by using TMRE and FACS, and the activation of caspase-3 was detected. Phosphatidylserine (PS) translocation from the inner to the outer surface of the cell membrane was detected by using both APOPercentageTM apoptosis assay and Annexin-V-PE binding assay on FACS. Agarose gel electrophoresis revealed the fragmentation of chromosomal DNA. Overall these convincingly characterize the induction of apoptosis with the *T. violacea* extract. We have purified three pro-apoptotic fractions from the *T. violacea* extract by using bioactivity-guided fractionation. Characterization of these fractions by X-ray crystallography, NMR, MS, IR and microanalysis techniques showed the presence of methyl-á-D-glucopyranoside as the major component. Methyl-á-D-glucopyranoside was confirmed to be active in the induction of apoptosis, using commercially sourced material, and we propose a mechanism of action involving hexokinase in the activation of the mitochondrial transition pore and generation of reactive oxygen species in inducing apoptosis. The identification of this novel apoptosis inducing secondary metabolite presents opportunities for novel anti-cancer therapies to be developed.

## N3-048P
## The estimation of nitric oxide synthases and lipid peroxidation in A549 and MCF-7 cells after photodynamic treatment

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Photodynamic therapy (PDT) is a rather novel method for the treatment of cancer, involving the administration of photosensitizer drugs, preferentially taken up by tumour cells and activa-

ted in the presence of light resulting in tissue destruction. Photodynamic therapy, induces a strong oxidative stress and triggers the vascular-mediated response with massive neutrofil recruitment and these events are prone to be highly sensitive to NO mediation. NO play an important and dualistic role in malignant cells after PDT. In tumours producing high levels of NO, the PDT-induced reduction in tumour blood flow, vascular occlusion and consequent ischaemia may be diminished, while the inflammatory reaction triggered by PDT may be suppressed. On the other hand, elevated NO levels may maintain vessel dilation during PDT light treatment, which can diminish the decrease in tumour oxygenation and sustain in this way the oxygen-dependent generation of phototoxic damage. Interaction of reactive oxygen species with cellular signal transduction pathways may result in upregulation of genes encoding NOS. Oxidative stress causes damage to cellular macromolecules such as lipids, nucleic acids and proteins. Among these targets, peroxidation of lipids is particularly more damaging because the formation of lipid peroxidation products leads to a facile propagation of free radicals. Nitric oxide can both promote and inhibit lipid peroxidation. The aim of our study was to monitor the level of expression of nitric oxide synthases and the level of lipid peroxidation after photodynamic treatment using immunocytochemical assay. Photosensitizing drug – Photofrin was administered to cancer cells deriving from two lines: MCF7 – breast adenocarcinoma cells and A549 – lung carcinoma cells. In our study we observed differential expression of three different isoforms of nitric oxide synthase. The level of lipid peroxidation was significantly higher for cells after PDT comparing to that in the control cells.

## N3-049P
## Transactivation activity of Nur77 discriminates between calcium and cAMP signals

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Calcium and cAMP are the two second messengers that play a role in neuronal signaling. Transcription of the immediate early gene Nur77, encoding transcription factor which control the expression of other genes, can be enhanced in response to calcium influx or cAMP synthesis. However, function of the Nur77 proteins is regulated by phosphorylations that were shown, by other authors, to inhibit the transactivation activity of Nur77 proteins and to promote the export of Nur77 proteins from the nuclei to the cytoplasm. We demonstrate that cAMP analog induced strong increase, while calcium ionophore induced weak increase in the transactivation activity of Nur77 proteins, despite an increased transcription of the Nur77 gene in PC12 cells treated with calcium ionophore. Further, we found that Nur77 proteins were expressed in the nuclei of PC12 cells following stimulation with cAMP analog but not after stimulation with calcium ionophore. However, expression of Nur77 proteins was increased in the cytoplasm of cells treated with calcium ionophore. In conclusion, our results suggest that cAMP-induced and calcium-induced processes may differentially regulate activity of Nur77 at the level of translocation of the Nur77 proteins from the cytoplasm into the nuclei and that the process of translocation is promoted by cAMP signal but not by calcium influx.

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## N3-050P
## Apoptosis-inducing factor-mediated and caspase-independent apoptosis induced by evening primlose extract in Ehrlich ascites tumor cells

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**Introduction:** *Oenothere biennis* L., one species of evening primrose, is a herbal plant. We previously demonstrated that evening primrose extract (EPE) induced apoptosis in Ehrlich ascites tumor cells (EATC), while mouse embryo fibroblast cells (NIH3T3) used as a normal cell model, showed no effect of cell viability by treatment of EPE. Furthermore, our results demonstrated the rapid increase in intracellular peroxides levels, loss of mitochondrial membrane potential and the release of cytochrome c to cytosol, suggesting that the rapid increase in intracellular peroxides levels after addition of EPE triggers off induction of apoptosis. The aim of this study was to identify the role of caspase on EPE-induced apoptosis in EATC. We therefore examined the effect of EPE on translocation of Bax to mitochondria, caspase activation and translocation of apoptosis-inducing factor (AIF) to nuclei.

**Results:** We identified that EPE elicited the translocation of Bax to mitochondria and AIF to nuclei, but no activation of caspase-3-like protease. We also demonstrated that the rapid EPE-induced increase in hydrogen peroxide levels caused the translocation of Bax to mitochondria, and then mitochondrial cytochrome c was released. One of the main consequences of mitochondrial cytochrome c release is the activation of caspase-3. However, no caspase-3 activation was observed. On the other hand, AIF was translocated from mitochondria to nuclei. The EPE-induced translocation of AIF was suppressed with the addition of catalase.

**Conclusion:** There results suggest that the rapid intracellular peroxide levels after addition of EPE triggers off induction of apoptosis, which is AIF-mediated and caspase-independent.

## N3-051P
## 5-Fluorouracil differentially modulates apoptosis of colorectal cancer cells

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5-Fluorouracil (5-FU), the most important drug used for colorectal cancer (CRC) chemotherapy, is a potent antitumour agent that affects pyrimidine synthesis by inhibiting thymidylate synthetase. Treatment of cancer cells with 5-FU is thought to induce apoptosis. However, several CRC patients are resistant to 5-FU treatment. We studied two human colorectal cancer cell lines: HCT116, with wild-type p53 expression and microsatellite instability; and SW480, with mutant-type p53 expression and microsatellite stable. Supplementation with 8 μM 5-FU was more cytotoxic in HCT116 than in SW480 cells ($P < 0.001$).

Nuclear fragmentation and caspase-3, -8 and -9 activities were markedly increased in HCT116 cells after 5-FU exposure ($P < 0.01$), providing further evidence of apoptotic cell death. In addition, wild-type p53 expression in 5-FU-treated HCT116 cells was almost 25-fold increased compared with controls ($P < 0.001$), whereas mutant p53 expression was not altered in SW480 cells. Pro-apoptotic Bax remained unchanged during 5-FU treatment. Anti-apoptotic Bcl-2 protein, almost undetectable in SW480 cells, was not modulated in HCT116 cells after 5-FU exposure. In conclusion, HCT116 cells are more sensitive to 5-FU than SW480 cells. Up-regulation of wild-type p53, perhaps associated with a deficient DNA-mismatch repair system that allows incorporation of FdUTP, provides a mechanistic explanation for increased death of HCT116 cells after 5-FU treatment. Further, 5-FU induces a p53-dependent apoptotic pathway, which in turn is independent of Bax and Bcl-2 protein expression.

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## N3-052P
## PH-PxxP domain of RalGPS2 is a dominant negative for the RalA activation in PC12 cells

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RalGPS2 is a murine Guanine nucleotide Exchange Factor (GEF) for the small GTPase Ral; it codes for a 590 aa polypeptide that contains a Ras-GEF domain, a PxxP motif and a PH domain. PH domains may bind to phosphatidylinositol lipids and could serve to anchor RalGPS2 to the membrane. In particular, with protein-lipid overlay assay we have demonstrated that RalGPS2 binds to PIP2. Using GFP-fusions in HEK293 cells we demonstrated that the PH-PxxP domain localized heavily in membranes and caused ruffling and presence of vesicles (*Ann NY Acad Sci* 2002; **973**: 135). The expression of RalGPS2 in PC12 cells, stimulated to differentiate with NGF, induced a marked inhibition of differentiation and this was associated to an increase of RalA-GTP, in agreement with the observation that Ral pathway antagonized the Ras induced differentiation (Rameh LE et al. *J Biol Chem* 1999; **274**: 8347). Construct with the GEF domain alone resulted in cytoplasmic localization of the protein, and in an increase of Ral A-GTP, while the PH-PxxP domain caused a marked inhibition of RalA activation. The expression of PH-PxxP domains fused to GFP was observed only in highly differentiated cells, where it localized in the citoplasm and membranes; it also induced an increased production of neurites. The PH domain alone localized mainly in membranes and produces microspikes. These results are in agreement with the proposed role of Ral in PC12 cells as an antagonist of NGF-induced differentiation and suggest that PH-PxxP domains could function as a dominant negative for Ral pathway. Fos-luciferase assay on NIH 3T3 cells showed that the GEF domain of RalGPS2 could promote the transcription of genes involved in cellular growth; the whole RalGPS2 also promoted transcription but to a lower extent. PH domain alone did not produce any effect. This result is consistent with the results obtained in PC12 cells and indicate that the PH-PxxP domains of RalGPS2 could regulate the GEF activity.

## N3-053P
## Study of cannabinoid and vanilloid signal transduction pathways in prostate cell lines

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The endocannabinoid system is emerging as a new neuromodulatory system with multiple physiological functions which are being elucidated in the recent years. One of the most interesting action of cannabinoids is the inhibition of different tumoral cell growth. Recently, a role for cannabinoids in the apoptotic prostate cell death has been shown. These findings open the attractive possibility of the endocannabinoid system as a putative target for the management of tumoral diseases, becoming a focus of intense and growing research. The aim of this work is to investigate the mechanisms underlying the antitumoral properties of cannabinoids and related molecules, i.e. vanilloids on prostate cell lines, in an attempt to provide the bases for new therapeutic approaches for prostate cancer. One of the strategies is the analysis of different kinase cascades activated after treatment of cells with several agonists and antagonists. Phosphorylation or activation of proteins involved in different mitogenic or apoptotic cascades (ERK, JNK, p38MAPK, PI-3K, …) are analysed by classical techniques of Western blot. Once, we are testing involvement of sphingomyelin hydrolysis and ceramide generation upon cannabinoid and vanilloid stimulation, using classical biochemical techniques. Recent findings suggest that ceramide production is an important step in the apoptosis induction by cannabinoids. Therefore, it is essential to study the role of the ceramide pathway in prostate cancer cells.

## N3-054P
## A role for cathepsin D in the liberation of Bax from the inhibitor 14-3-3θ

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The intrinsic pathway of the apoptotic process is activated by release of cytochrome c from mitochondria and subsequent activation of proteases of the caspase family. The pro-apoptotic protein Bax is under normal conditions mostly localized to the cytosol where it is kept inactive through sequestration by proteins such as Ku70, some isoforms of the ubiquitously expressed 14-3-3 proteins and the peptide humanin. In response to apoptosis, Bax is released from inhibitory interactions by e.g. degradation or cleavage of the inhibitory proteins, and translocates to mitochondria, resulting in release of cytochrome c. The lysosomal protease, cathepsin D, has been shown to redistribute to the cytosol during staurosporine-induced apoptosis in human fibroblasts and is essential for translocation of Bax to mitochondria. We show, by co-immunoprecipitation, that Bax interacts with both Ku70 and 14-3-3θ in control fibroblasts. During apoptosis total and cytosolic amount of Ku70 is unchanged as analysed by western blot, but the total amount of 14-3-3θ decreases with time of exposure. Inhibition of cathepsin D with pepstatin A prevents the 14-3-3θ decrease, indicating that cathepsin D participates in the degradation of 14-3-3θ. Preliminary results from *in vitro* cleavage experiments show that 14-3-3θ is cleaved by cathepsin D. Further studies will answer if the interaction between Bax and 14-3-3θ, is disrupted during staurosporine-induced apoptosis and if cathepsin D is indispensable in the process.

## N3-055P
## The activation mechanism of the pro-apopoptic Bcl2-associated X protein (Bax): an insight through molecular dynamics

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Bax, a pro-apopoptic member of Bcl-2 family proteins, has a central role in the mitochondria-dependent apoptosis. Usually, it resides in the cytosol as a quiescent protein and traslocates into mitochondria after apopoptic stimuli. Bax protein has seven amphipatic helices clustered around two central, mostly hydrophobic helices. It belongs to the Bcl2-subfamily characterized by four homology domains (BH 1-4). BH3 domain (helix 2) seems to be a functionally important region for the interaction with other Bcl-2 family members or other Bax proteins after activation. The activated Bax molecule travels to the external mitochondria membrane where the aggregation process occurs; this event causes the cyctocrome c molecules to come out from mitocohondria such triggering the apoptosis via caspase 9. An apopoptic stimulus activates Bax by a supposed conformational change, which however remains rather unclear. To better draw a possible scenario which describes the confomational change, we have performed a 10 ns molecular dynamics simulation of human Bax protein. By using essential dynamics it is possible to distinguish between functional internal movements and unrelevant thermal fluctuations. The essential dynamics analysis of Bax trajectory supports an activation mechanism by which helices 2 and 9 move away from helices 4 and 5 such increasing the accessibility of the protein structural core to the solvent. Moreover, such helices movements involve its own rotations that widens the exposed surface of the BH3 domain, which is made up by protein segments of higher hydrophobicity. These hydrophobic surfaces could drive the aggregation process of Bax molecules and their anchoring to mitochondria external membrane.

## N3-056P
## Arrested apoptosis in lens fiber cells: a possible role of α-crystallin

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A role for αB-crystallin in inhibition of caspase-3 activity was suggested from *in vitro* and tissue culture experiments. However, no animal model study has yet been used to probe the significance of α-crystallin as an anti-apoptotic agent. We demonstrated that morphological abnormalities in lens secondary fiber cells of αA-/αB-crystallin gene double knockout (DKO) mice are consistent with, and likely result from, elevated DEVDase and VEIDase activities, corresponding to caspase-3 and caspase-6 respectively. Immunofluorescence microscopy revealed increased levels of caspase-6, and the active form of caspase-3, in specific regions of the DKO lens, coincident with the site of cell disintegration. TUNEL assay illustrated a higher level of DNA fragmentation in lens secondary fiber cells from DKO mice, compared to wild type mice. Almost every nucleated fiber cell in lenses from DKO mice, regardless of the location in the lens, was TUNEL-positive. Regions of morphological change, or cell loss, in lenses from DKO mice coincide with intense TUNEL signal, suggesting an apoptotic character of cell disintegration. These studies suggest that α-crystallin plays a role in suppressing caspase activity in normal lenses, resulting in retention of lens fiber cell integrity following degradation of nuclei, mitochondria, and other organelles, which occurs during the apoptosis-like pathway of lens cell terminal differentiation.

## N3-057P
## Cathepsins cleave Bid in a pH-dependent way during apoptosis

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Bid, a pro-apoptotic Bcl-2 family member, is cleaved by caspases during apoptosis and the fragment (tBid) acts on mitochondria in aiding cytochrome c release. The formation of tBid is, however, observed also when caspases are inhibited, raising the possibility that other proteases may cleave Bid. Lysosomal proteases, cathepsins, translocates to the cytosol during apoptosis and may exert their pro-apoptotic function by cleaving Bid. The proteolytic activity of cathepsins is optimal at acidic pH and to establish if cathepsins have preoteolytic function when released to the cytosol, the cytosolic pH of apoptotic cells was examined. When apoptosis was induced by TNF-α, the cytosolic pH was decreased from 7.2 to 5.7, thus indicating that the proteolytic activity of cathepsins may be maintained in the cytosol during apoptosis. *In vitro* cleavage experiments show that both cathepsin B and D can cleave recombinant Bid. Cathepsin B cleaves Bid into a ~15 kDa fragment both at pH 5.7 and 6.5. Cathepsin D cleaved Bid into a ~15 kDa fragment at pH values between 5.7 and 6.8, but most effectively at lower pH. In addition, a larger fragment was also formed (18–19 kDa) at pH 5.7. Sequence analysis confirms both cleavage sites to be cathepsin D specific. These results indicate that both cathepsin B and D may act in proteolytic processing of Bid in the cytosol and that the cytosolic acidification observed during apoptosis facilitates proteolytic activity of cathepsins released from lysosomes during the apoptosis process.

## N3-058P
## Diazo-signaling: apoptosis triggering histone H1.2 exists partly in the tyrosine quinone diazide form

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Apoptosis, an intrinsic cell death program, is a strictly controlled process, deregulation of which can lead to various diseases and pathological conditions, including neurodegeneration, autoimmunity, and cancer. Mitochondria are crucial determinants in the life and death of cells, a central step being the release of cytochrome c (Cc) across the outer mitochondrial membrane into the cytoplasm, where it initiates the activation of caspases. Histones belong to one of the protein classes most nitrated under nitrative stress. Linker histone H1 can be embedded into lipid membranes. In macrophage-like RAW 264.7 cells, histone H1.2 nitrated at the only tyrosine residue (Tyr71) is a preferential substrate for "denitrase", a putative enzyme whose presence leads to a rapid decrease of the nitrotyrosine content. The same H1.2 form of histone H1, but not the other forms, plays the role of a signal molecule in the triggering of the Cc release. Here we show that in RAW 264.7 cells histone H1.2 is converted into a highly reactive tyrosine quinone diazide (Tyr-QD) form. At physiological pH values, Tyr-QD can react with some amino acids and bases of nucleic acids, guanine being found the most reactive.

## N3-059P
## Cellular effects of ECP in mammalian cell lines

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Eosinophil Cationic Protein (ECP) is one of the major components of eosinophilic secondary granules with a molecular mass ranging from 16 to 21.4 KDa depending on the glycosilation degree. Is has been shown that ECP is bactericidal, helminthotoxic, elicits the Gordon phenomenon when injected and is cytotoxic to tracheal epithelium. In addition, it has been described that ECP exerts a cytostatic but not cytotoxic effect on mammalian cell lines. Carreras et al. have tested ECP variants finding out that the Trp35/Arg36 and the Trp 10 regions are necessary for the bactericidal activity on gram positive and gram negative bacteria (*Mol Cell Biochem*, in press). The mechanism of its cytotoxicity is not understood, however it is suggested to be pore-forming activity which destabilizes lipid membranes. Previous studies have been performed with Onconase (ONC), another ribonuclease belonging to the pancreatic RNase superfamily which also shows a cytostatic and cytotoxic activity to numerous tumor lines. Cells treated with ONC displayed apoptotic features involving the mitochondrial apoptotic machinery. The aim of this work is to identify such determinants of the citotoxicity induced by ECP on the HL-60 cells. Apoptosis induced on ECP-treated cells was analyzed by specific apoptosis features such as loss of cell viability, chromatin condensation, cell blebbing and DNA fragmentation. Furthermore, apoptosis in the HL-60 cells was accompanied by the activation of the specific proteolytic cleavage of poly (ADP-ribose) polymerase (PARP) and the activation of caspases.

## N3-060P
## Phytosphingosine induced protein phosphatase mediated cell cycle arrest and apoptosis

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Spingomyelin and its metabolites, sphingolipids, are known to be involved in diverse types of signal transduction, including cell proliferation, differentiation, and apoptosis. Sphingosine, sphinganine, and phytosphingosine are structural analogs of sphingolipids. Sphingosine and sphinganine are known to play important roles in apoptosis. However, the study of phytosphingosine has recently begun. In the present study, we examined the phytosphingosine induced apoptosis and cell cycle arrest in human T-cell lymphoma Jurkat cells. Sphingosine induced apoptosis from an early stage. On the other hand, phytosphingosine induced DNA fragmentation, as well as G2/M cell cycle arrest to reduce cell viability. DNA fragmentation was inhibited by Z-VAD-FMK, a pan-caspases inhibitor, suggesting that phytosphingosine-induced apoptosis is caspase dependent. Phytosphingosine perturbed mitochondria for induction of apoptosis by releasing of cytochrome c and reducing mitochondrial membrane potential. Western blot assays revealed that phytosphingosine dephosphorylated survival signal kinase Akt, but no effect to the pro-apoptosis kinase JNK. Okadaic acid (OA) inhibited phytosphingosine induced dephosphorylation of Akt and OA attenuated phytosphingosine induced apoptotic stimuli. Furthermore, overexpression of Bcl-2 prevented phytosphingosine induced apoptosis, but did not prevent cell cycle arrest. These results suggest that phytosphingosine activated protein phosphatase 2A or protein phosphatase 2A-like phosphatase, and dephosphorylated Akt pathway factors resulting in the cell cycle arrest and induction of mitochondria-involved apoptosis independently.

## N3-061P
## Integrin signal replacement by transglutaminase 2 in anoikis

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Not ligated integrin receptors exert dramatic effects on cytoskeletal architecture and may kill the cells via anoikis. Before dying, the expression of transglutaminase 2 (TG2) is induced in MCF-7 mammary and Hep2G hepatic carcinoma cell lines when placed on non-adhesive substratum, whereas Hep3D hepatoma cells die without expressing detectable levels of TG2 protein under similar conditions. Here we show that, (i) TG2, if present, elicits a temporary formation of filopodial processes in the early phases of anoikis and delays cytoskeletal desintegration by several days. (ii) The effects of TG2 on cytoskeletal organization are independent from its transglutaminase activity, as evidenced by the effects of overexpression of catalytically inactive TG2 in Hep3D cells. (iii) TG2 activates filopode formation by a pathway different from Rho activated kinase and, phosphorylates myosin light chain kinase at the same Thr18 and Ser19 residues, which are also targeted by the Integrin-linked kinase. Our findings propose a transient modulatory role for TG2 in anoikis and offer explanation for the occurrence and role of TG2 in distinct cells under normal and pathological conditions.

## N3-062P
## Synergistic induction of prostate cancer cell death by γ-tocotrienol and lovastatin is mediated by direct geranylgeranyl pyrophosphate inhibition through a caspase-independent pathway

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γ-Tocotrienol, a vitamin E acyclic isoprenoid, downregulates the mevalonate pathway through post-transcriptional suppression and increased degradation of 3-Hydroxy-3-Methylglutaryl Coenzyme A reductase. Here in we explore molecular events underlying mevalonate deprivation imposed by γ-tocotrienol, the non-discriminant inhibitor of HMG CoA reductase lovastatin and their combination in prostate cancer cells. γ-Tocotrienol moderately inhibited growth of LNCaP androgen responsive and DU-145 androgen unresponsive cells. Morphologic features of apoptosis and caspase-3 activation were observed only in LNCaP cells without modulation by wide spectrum caspase inhibitors. Survival decrease was disproportional to caspase activity. Cleaved poly(ADP-ribose) polymerase (PARP) was modestly elevated at higher γ-tocotrienol

concentrations. Geranylgeraniol (GGO), a mevalonate pathway intermediate with proapoptotic effect through c-Jun N-terminal Kinase and lovastatin had a significant synergistic effect in γ-tocotrienol-induced cell death and caspase-3 activation. Proteolytic cleavage of PARP was moderately increased by GGO and prenylation of RAP1A small G-protein was modestly inhibited by lovastatin. Interestingly, in DU-145 cells lovastatin augmented γ-tocotrienol-induced cell death concominantly with inhibition of RAP1A and significant increase in caspase-3 activity. These data are suggesting that unprenylation is not due to decreased levels of geranylgeranyl pyrophospate but rather due to γ-tocotrienol induced inhibition of geranylgeranylation, leading to GGO accumulation and to secondary caspase-3 activation. Whether inhibition of N-linked glycosylation of EGFR underlying the androgen-unresponsive phenotype is also responsible for the effect in DU-145 cells remains to be elucidated.

## N3-063P
## Identification and characterization of p53-interacting proteins and its target genes in *Drosophila melanogaster*

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Mutation of the tumor suppressor gene *p53* are frequently found in different types of cancer. The p53 protein plays a vital role in safeguarding the integrity of the genome by inhibiting the cell cycle, or bringing about apoptosis in case of different cellular stresses. Identification of the *Drosophila melanogaster* homologue of p53 (Dmp53) opens up possibilities to apply a combination of biochemical and genetical tools for studying p53 functions in flies. To find genes activated by Dmp53 upon genotoxic effects we brought about DNA damage by UV-C irradiation in wild-type and *Dmp53* mutant *Drosophila* larvae and analyzed the gene expression profile by oligo microarray and real time PCR. We have found several interacting partners of the Dmp53 using the yeast two-hybrid system. Some of the interacting factors such as Uba2, a small ubiquitin-related modifier (SUMO)-activating enzyme, Ubc9, the only known SUMO-conjugating enzyme and PIAS, a SUMO-ligase are involved in the post-translational modification called sumoylation. We are working on understanding of the sumoylation process on Dmp53. Another Dmp53-interacting partner is the Bip2 protein, a TBP (TATA-binding protein)-associated factor, which also interacts with hp73, a member of the p53 family. Bip2 has a negative regulatory effect on the transactivation of p53 and p73. Daxx-like protein (DLP) is also a Dmp53-interacting protein whose human homologue is an apoptosis-related factor, but its role in *Drosophila* is not known.

## N3-064P
## Sulindac activates nuclear translocation of AIF, DFF40 and endonuclease G but not induces oligonucleosomal DNA fragmentation in HT-29 cells

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Sulindac is one of the most widely studied non-steroidal anti-inflammatory drugs in the prevention of colon cancer. Thus, from the viewpoint of colon cancer chemotherapy it is important to reveal the mechanism of sulindac-induced cell death. This study was undertaken to dissect the molecular mechanism underlying sulindac-induced apoptosis in human colon cancer cell line HT-29 (mutant p53), focusing on nuclear translocation of AIF, DFF and endonuclease G. On induction of apoptosis by sulindac, it was associated with decreased mitochondrial membrane potential, nuclear expression of active caspase-3, cleavage of poly(ADP-ribose) polymerase, translocation of mitochondrial proteins to the nucleus, and morphological evidence of nuclear condensation. However, sulindac led to only disintegration of nuclear DNA into high molecular weight DNA fragments of about 100–300 kbp as determined by a pulse-field gel electrophoresis, suggesting a predominantly AIF-mediated cell death process. In summary, our findings indicate that sulindac induces large-scale DNA fragmentation without oligonucleosomal DNA fragmentation. This result suggests that nuclear translocation of DFF and endonuclease G are not sufficient for the induction of oligonucleosomal DNA fragmentation in HT-29 cells.

## N3-065P
## Complexity of interactions between TNF-R1 signalosome proteins in "immune escape" of COLO 205 cells

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To determine the possible cause of resistance of COLO 205 cell line to the TNF-α-induced apoptosis in colorectal cancer cells we addressed the question of the impact of cellular proteins in antiapoptosis. Translation inhibitor cycloheximide (5 μg/ml) and selected metabolic inhibitors were used. Individual treatment with TNF-α (10 ng/ml) or CHX did not affect cell viability, whereas coincubation of TNF-α along with CHX caused time-dependent cell death (6, 12, 24 h of study). Apparently, based on the morphological criteria evaluated by electron microscopy cells died by apoptosis. From these observations we concluded that CHX sensitizes COLO 205 cell to TNF-α-induced apoptosis by decreasing the level of short-lived proteins which inhibited the TNF-α death signal. Since at the level of TNF-R1 signalosome the antiapoptotic FLIP protein inhibits the TNF-α-induced cell death whereas at the same time STAT-1 kinase was shown to accelerate apoptosis we decided to examine them by proteomic approach. The protein array screening, immunoprecipitation, Western-blot and confocal microscopy were used to identify the presence and interactions of FLIP and STAT-1 kinase with the TNF-R1 signalosome proteins FADD and TRADD, respectively. The CHX-induced sensitization that enabled TNF-α to trigger apoptosis was associated with the decreased level of FLIP protein. In contrast, STAT-1 neither contributed to anti- nor to apoptosis induced by combined treatment with CHX and TNF-α. Moreover, CHX-mediated TNF-α-induced apoptosis was modulated by calcium homeostasis and cPKC. It appears that "immune escape" from TNF-α-induced cytotoxicity in great part is determined by both TNF-R1 ligation of FLIP, and the additional calcium-dependent inactivation of apoptotic proteins in COLO 205 cancer cells.

## N3-066P
## Estrogens modulate VGF-derived peptides induced contraction of rat isolated gastric fundus

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The VGF protein (non-achronymic name) is localized in subpopulations of neurons from both central and peripheral nervous system, as well as in endocrine tissues. This protein is a precursor of various peptides and their biological activity has been investigated. TLQP30 and TLQP21 peptides, both derived from the C-terminal region of VGF precursor, are able to induce a dose-dependent contraction of rat isolated gastric fundus, with TLQP21 more potent than TLQP30. Previous results demonstrated that TLQP21 activity, due to interaction with specific VGF receptors on the gastric mucosa, is mediated by prostaglandin production (PGF2a and PGE2). Pretreatment of gastric fundus with the non-selective COX inhibitors indomethacin ($10^{-5}$ M) and naproxen ($10^{-6}$ M) significantly inhibited TLQP21 contractile response. Since marked sexual differences were observed, we studied the role of sexual hormones on gastric contraction *in vivo* and *in vitro*. Purpose of the present study was to investigate the modulatory effect of estrogens on TLQP21 activity. Following 24 h subcutaneous treatment with diethylstilbestrol (DES), TLQP21-induced contraction of gastric musculature from female rats was significantly reduced. Moreover, such inhibitory effect was observed also *in vitro* when fundus strips were pre-incubated with estrogen receptor agonists (DES or bEstradiol, 5–10 mM), as well as with the antiestrogen tamoxifen (10–20 mM). This inhibitory effect was rapid (15–20 min) dose-dependent and reversible (60 min). In the same experimental conditions, the response elicited by acetylcholine or KCl was unaffected by estrogen. We hypothesize that the rapid effect involves a non-genomic mechanism and we are investigating the processes activated by estrogen modulation.

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## N3-067P
## ApoJ-Ku70-Bax interaction regulates Bax-dependent apoptosis

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Bax is a pro-apoptotic member of Bcl-2 family proteins and is central to mitochondria-dependent apoptosis. It resides in the cytosol as a quiescent protein and translocates into mitochondria after apoptotic stimuli. Ku70 is a 70 kDa subunit of the Ku complex, which has an important role in DNA double-strand break (DSB) repair and apoptosis induction in the nucleus. The Ku heterodimer binds the ends of various types of DNA discontinuity, and is involved in the repair of DNA breaks caused by an incorrect DNA replication , V(D)J recombination, isotype switching , physiological oxidations, ionizing irradiation and some chemotherapeutic drug effects. In the present abstract we report that Ku70 interacts with pro-apoptotic protein Bax in the cytosol and regulates its mitochondrial translocation, suggesting that Ku70 regulates Bax-mediated apoptosis. On the basis of the previous published finding that showed an interaction between Ku70 and Bax we demonstrated by immunoprecipitation and immunofluorescence their interaction with ApoJ. Furthermore we demonstrated the interaction among ApoJ- Ku70-Bax after damage induction and a cytostatic treatment in a human colon carcinoma cell line. Moreover we provide that the proapototic activation of Ku86 is mediated by serin phosphorylation, and that its interaction with Ku70 in the cytoplasm forming the Ku heterodimer is ApoJ and Bax independent. The releasing of Bax from Ku70 is induced by somatostatin treatment, laser irradiation and anticancer drugs. These data may provide valuable information in the development of therapies that control apoptosis-related diseases.

## N3-068P
## Crosstalk between mitochondria and cytosol in abiotic and biotic stresses and in apoptosis in plant and mammalian systems

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In recent years the role of mitochondria in biotic and abiotic stresses and in apoptosis is receiving increasing importance since these organelles play a major role in the cell response to abiotic and biotic stresses and since ATP is required for apoptosis to occur. At present, there are no exhaustive answers to the following questions: – what is the role of mitochondria in the cell response to abiotic and biotic stresses? – what is the role of mitochondria in apoptosis? In particular, is there involvement of the mitochondrial permeability in these processes? We address these points by investigating in isolated mitochondria and/or in cell homogenates from plant and mammalian systems the role of some mitochondrial protein carriers which mediate traffic of some metabolites participating in these processes. We show: 1. The occurrence of two novel proline carriers, i.e. the proline its own carrier and the Proline/Glutamate antiporter, in mitochondria isolated from seedlings of durum wheat chosen as a plant model system because durum wheat crops are frequently grown under abiotic stresses. 2. The involvement of the ADP/ATP translocator (ANT) in providing ATP outside mitochondria and in participating to the mitochondrial permeability transition in apoptosis with a gradual ANT modification to become a component of the mitochondrial permeability transition pore.

## N3-069P
## Modulation of neurotoxicity associated with Alzheimer's disease-linked amyloid precursor protein mutations by tauroursodeoxycholic acid

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Amyloid-beta peptide (Abeta) is derived from the amyloid precursor protein (APP). The early onset familial forms of Alzheimer's disease (FAD) are linked to autossomal dominant mutations in APP and presenilins 1 and 2 (PS1, PS2) genes, which increase the production of total Abeta and promote AD neuropathology. In addition, we have shown that tauroursodeoxycholic acid (TUDCA) modulates exogenous Abeta-induced apoptosis. In this study, we used mouse neuroblastoma cell lines that express either wild-type APP (APPwt), APP with the Swedish mutation (APPswe), or double-mutated human APP and PS1 (APPswe/deltaE9), all exhibiting endogenous Abeta aggregation. We sought to characterize neurotoxicity associated with

AD-linked APP and PS mutations as well as the potential protective role of TUDCA. The results showed that cell viability was 10 and 30% decreased in APPswe and APPswe/deltaE9, compared with APPwt cells ($P < 0.05$ and $P < 0.01$, respectively). Nuclear fragmentation and caspase-2 and -6 activation were readily detected in APPswe and APPswe/deltaE9 cells ($P < 0.05$). In addition, FAD mutations resulted in significant TNF-R1, Fas, p53 and Bax up-regulation, whereas Bcl-2 was strongly down-regulated. TUDCA inhibited cell death, p53 and Bcl-2 family protein changes ($P < 0.05$), but not death receptor expression, suggesting modulation of apoptosis through mitochondrial pathways. Moreover, TUDCA reduced p53-dependent nuclear fragmentation and caspase-6 activation induced by wild-type p53 overexpression ($P < 0.05$). In conclusion, our results demonstrate that FAD mutation-induced neurotoxicity proceeds through a p53-dependent apoptotic pathway, which in turn can be specifically modulated by TUDCA.

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## N3-070P
## Sequential induction of heme oxygenase-1 and manganese superoxide dismutase in brain astrocytes under nitrosative stress

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Nitric oxide (NO) is a widely recognized mediator of physiological and pathophysiological signal transmission. In an attempt to better understand the molecular actions of NO in astrocytes, a pattern of protein expression in response to NO donor, sodium nitroprusside, was characterized by two-dimensional gel electrophoresis and MALDI-TOF mass spectrometry. Among several proteins of altered expression levels, heme oxygenase-1 (HO-1) has been identified as an inducer of manganese superoxide dismutase (MnSOD), playing a cytoprotective role under the condition of nitrosative stress. We present evidence that the sequential induction of HO-1 and MnSOD protects astrocytes from NO toxicity: (i) both HO-1 and MnSOD expression were induced by NO; (ii) NO-mediated increase in MnSOD activity was partly abolished by HO-1 inhibitor Zn(II) protoporphyrin IX (ZnPP); (iii) pre-treatment of astrocytes with a non-toxic dose of NO protected the cells against the later treatment with a toxic dose of NO; (iv) inhibition of HO-1 by ZnPP sensitized astrocytes to the nontoxic dose of NO, resulting in a marked cytotoxicity; and (v) adenovirus-mediated overexpression of MnSOD protected astrocytes from the NO toxicity. The molecular action of NO in astrocytes appears to be dose-dependent. While a high dose of NO exerts cytotoxicity leading to the tissue damage in central nervous system, a low dose of NO may act as an important signaling molecule in astrocytes with concurrent induction of cytoprotective proteins such as HO-1 and MnSOD.

## N3-071P
## Mammalian cell differentiation and apoptosis mediated by forkhead transcription factor FOXO subfamily

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The forkhead transcription factors class O (FOXO) subfamily is widely accepted as a regulatory factor of cell cycle arrest, apoptosis, cell survival, and differentiation by regulating expression of a numerous number of target genes under the control of phosphorylation by Akt. We previously analyzed the expression and transcription activities of FOXO subfamily that identified as a potent regulator of $H_2O_2$-induced luteal cell apoptosis during the process of bovine estrous cycle. To further analyze the effect of oxidative stress for FOXO subfamily, $H_2O_2$ and Glucose oxidase, a glucose-dependent generator of $H_2O_2$, were added into luteal cells, HepG2, HeLa and others, resulting in the elevation of FOXO1a expression and subsequent increase of cell death in dose- and time-dependent manners. In response to $H_2O_2$ stimuli, nuclear translocation of FOXO1a and its transcription activity were synergistically induced, yielded to increase of expression of pro-apoptotic genes, Bim and BCL6. To ensure the inducible effect of apoptosis by FOXO1 subfamily, HepG2 and HeLa cells stably expressing dominant negative FOXO3a were established and incubated with $H_2O_2$, resulting in the loss of induction of cell death. Further protein analysis revealed that exogenously introduced p53 could associate with FOXO3a protein under the oxidative stress, and thereby, suppressed transcription activity of FOXO3a, indicating that molecular interaction of FOXO protein with other factors is critical for modulation of molecular roles of FOXO. Because the expression of FOXO4 was strictly regulated in the adipogenic differentiation process of mouse 3T3-L1 cells, FOXO subfamily potentially involves in a critical role in adipocyte development, as well as in cell death.

## N3-072P
## The metilotrophic yeast *Hansenula polymorpha* as the model for screening for genes involved in apoptosis

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Programmed cell death is one of the key events in the development of multicellular organisms. In recent years it has been convincingly shown that unicellular organisms possess active cell death program(s) too. Yeast *Saccharomyces cerevisiae* has been successfully used as a model to study the mechanisms of apoptotic regulation. The metilotrophic yeast *Hansenula polymorpha* is one of the most well studied non-*Saccharomyces* yeast. It has a number of significant differences from *S. cerevisiae* one of them is that *H. polymorpha* is more dependent on mitochondrial respiration and apparently has mitochondrial respiratory complex I (like mammals), which is absent in *S. cerevisiae*. Mitochondrial respiration is one of the key elements of apoptosis, therefore one might expect that *H. polymorpha* has a more advanced apoptotic mechanism compared to *S. cerevisiae*. During functional screening of *H. polymorpha* we found a number of mutants with hypersensitivity to several harsh treatments. Some of these show features typical for apoptosis: mitochondrial thread-grain transition, DNA fragmentation, cell shrinkage and high percent of dead cells. To learn more about the cell death cascade of *H. polymorpha* we have characterized the dynamics of mitochondria hyperpolarization and the generation of reactive oxygen species in the wild type and selected "apoptotic" mutants. Our results suggest the use of *H. polymorpha* for screening for genes involved in apoptosis could be a promising way to fill gaps in our understanding of the active cell death mechanism.

## N3-073P
## Modulation of lead nitrate action by cAMP in alveolar macrophages

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Lead is one of the heavy metals that its poisoning is responsible for a variety of pathological states. Macrophages are of the most important cells in the immune system that play immunoregulatory role against poisoning. In this study the mechanism of lead nitrate action on alveolar macrophages was investigated using anion superoxide production, DNA fragmentation and flow cytometry. The results show that addition of cAMP (30 μM) to the alveolar macrophages culture pre-treated with lead nitrate increased the survival rate and decreased the anion superoxide production and DNA fragmentation of the cells after 3 and 6 h of culture. Cytological analysis by fluorescent staining confirmed the results. Also flow cytometery analysis indicated that cAMP suppresses apoptosis in alveolar macrophage pre-treated with lead nitrate. It is suggested that lead nitrate induces apoptosis in macrophages and cAMP is able to modulate the effect.

## N3-074P
## Antitumoral properties of vanilloids, capsaicin and capsazepine, in xenograft prostate tumors in mice

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Advanced prostate cancer frequently involves the acquisition of an androgen-independent phenotype which demonstrates resistance to many chemotherapeutic agents. Here, we investigated the role of the vanilloids, capsaicin and capsazepine in the growth regulation of the human cancer androgen-resistant cell line PC-3. To study the *in vivo* effect of casaicinoids in prostate tumor growth, PC-3 cells were grown as xenografts in nude mice. Subcutaneous injection of either capsaicin or capsazepine (5 mg/kg b.w.) in nude mice was started 4 weeks after tumor cell implantation. A significant reduction of tumor growth was observed in mice treated during 14 days either with capsaicin or capsazepine. However, capsazepine had a greater effect on tumor suppression and was better tolerated than capsaicin. To identify the mode of cell growth inhibition induced by capsaicinoids in the xenograft tumors, we labeled the dissected tumors with the terminal deoxynucleotidyl transferase (TdT) dUTP nich-end labeling (TUNEL) reaction technique. The data showed a increase number of positive labeling cells in capsaicin- or capsazepine-treated tumors, indicating that capsacicin and capsazepine were capable of inducing apoptotic cell death *in vivo*. Our data suggest a role for capsaicinoids against androgen-independent prostate cancer cells *in vivo* and suggest that capsazepine is a promising anti-tumor agent in hormone-refractory prostate cancer.

## N3-075P
## Upregulation of MMP-9 and tuning of intracellular signal elements in 4-AP induced rat epilepsy model

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In the CNS, significant activation of matrix metalloproteinases (MMP-2, -9) was observed after epileptic seizures. As MMPs can degrade extracellular matrix proteins and adhesion molecules (like laminin, integrins), epilepsy induced enhancement of MMP-9 activity might result in changes of the adhesive contact between the cells and the extracellular matrix (ECM) thus, may affect the intracellular signaling pathway. It is known that degradation of cell–ECM interaction can lead to the cell death. Here we used a 4-aminopyridine (4-AP) induced focal epilepsy model, in which no expanded cell loss can be observed, to investigate the spatial distribution of MMP-9 and to follow changes of some components of signal system after seizures. During Halothan anesthesia, 4-AP crystal (0.15–0.3 mg) was placed on the right occipital cortex of adult SPRD rats for 40 min. Prefrontal, frontal and parietal EEG were registered to monitor epileptic activity for 6 h in anesthesia. Samples were collected from different areas of the brain 6, 12, 24 h after drug administration. MMP-9 and -2 level was analyzed by zymography. The regulation of MAPK pathway (activation/phosphorylation of p42/p44 ERK), the 14-3-3 chaperon, and the proapoptotic BAD proteins were detected using Western-blot analysis. We applied immunprecipitation to investigate BAD and 14-3-3 chaperon colocalization. Six hours after 4-AP treatment, extensive induction of MMP-9 was observed in the epileptic focus and a less robust activation in the mirror focus. We found a small activity of MMP-9 in the ipsi- and contralateral prefontal cortical and hippocampal areas. In the thalamus, striatum, brain stem no significant MMP-9 could be detected. Although, EEG registration demonstrated that the epileptic seizures spread to all studied areas of the brain. Within the ipsi- and contralateral occipital cortical and hippocampal areas, maximal phosphorylation of ERK1/2 was detected after 6 h 4-AP application compared to control samples. We observed a decrease of degree of 14-3-3 and BAD colocalization in the cortical and hippocampal areas. Summarizing our findings, we demonstrated that MMP-9 is upregulated in the 4-AP induced focal epilepsy model 6 h after drug administration. In parallel with the MMP-9 induction, we observed maximal phosphorilation of p42/44ERK at 6 h, which refers to the activation of the MAPK pathway. We experienced decrease of colocalization of BAD and 14-3-3 that can refer to the inicial phase of degenerative processes. Thus, this model could be suitable to study the interaction between extracellular proteinases and intracellular signal components in connection with determination of cell survival.

## N3-076P
## Retinoids promote glucocorticoid-mediated apoptosis of mouse thymocytes

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Previous work in our department has shown that the vitamin A derivative all-trans and 9-cis-retinoic acid (RA) induce apoptosis

in mouse thymocytes. Previous work has also shown that mouse thymocytes express Retinoic acid receptor (RAR) alpha and gamma, but not RARbeta. In our experiments we investigated whether retinoids are able to influence the apoptosis induced by glucocorticoids, and if yes which receptors are involved. In addition to dexamethasone RAR and RXR agonists and antagonists were administered, and the degree of apoptosis was detected by cell cycle analysis. All RARalpha agonists promoted the death by dexamethasone, while RARbeta, gamma and RXR agonists were ineffective. This suggests that RARalpha plays a crucial role in the effect. At the same time an RARalpha antagonist was also effective in promoting glucocorticoid mediated apoptosis implying that RARalpha does not regulate apoptosis in a direct transcriptional way. It has been known that nuclear receptors can also affect transcription by interacting with other transcription factors in a ligand dependent manner. Using immunprecipitation we have proven that GR and RARalpha interact in the presence of both ligands and this interaction leads to enhanced transcription activity of GR resulting in an enhanced expression of GILZ, one of the target genes of GR. Our data suggest that retinoids might promote glucocorticoid-induced death of T cells, and this might have pharmaceutical implications.

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## N3-077P
## C-Raf protects against ROS-induced apoptosis

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The role of the cytoplasmic (Ras-Raf-MEK-ERK) cascade in cell survival is well established. However, the molecular mechanisms through which Raf controls apoptotic cell death are largely unknown. Previous experiments have pointed to the suppression of mitochondrial events which precede the release of apoptogenic factors. In our studies we used the IL-3 dependent promyeloid cell line 32D to analyze the role of Raf and Raf-dependent signaling pathways in the protection against the proapoptotic effects of ROS. The data obtained so far in our study using DCF (2,7-dichlorodihydrofluorescein diacetate) loaded cells demonstrate that growth factor withdrawal results in a burst in ROS production within 4–8 h after removal of IL-3 (almost twofold elevation in ROS production). Consistent with this observation, presence of Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a cell-permeable, water-soluble derivative of vitamin E with potent antioxidant properties significantly delayed apoptotic cell death. On the contrary, use of a direct-acting oxidative stress-inducing agent tert butyl hydroperoxide (t-BHP) lead to a dose dependent increase in cell death, which could be reversed by exogenous growth factor or expression of a constitutively active Raf kinase (v-Raf). 32D cells protected from cell death by v-raf have elevated ROS generation, which further increases following IL-3 removal. Presence of Trolox enhances the protective effect of Raf. Preliminary date suggest that mitochondrial $Ca^{2+}$ levels are significantly increased upon growth factor removal in 32D but not in 32D v-Raf cells. Taken together these data suggest a critical role for C-Raf in the protection against ROS-induced cell death, which may involve effects on mitochondrial $Ca^{2+}$ levels.

## N3-078P
## Somatostatin downregulates dntm1 gene expression in a pituitary tumor cell model

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Methylation-mediated silencing of tumor suppressor gene promoters is a common finding in human cancers. DNA methylation is maintained and established by the DNA methyltransferases DNMT1, DNMT3a and DNMT3b. The pituitary tumor cell line GH3 expresses high dnmt1 levels, and is characterized by low expression of tumor suppressor genes that are regulated by promoter hypermethylation, such as, p16/Ink4 and Zac1. One of the most common therapies for pituitary tumors involve the use of somatostatin analogues, which inhibit hormone secretion and cell proliferation. The aim of the present study was to analyze the effect of somatostatin on dnmt1 gene expression in GH3 cells. Treatment of GH3 cells with somatostatin resulted in decreased dnmt1 mRNA levels after 1 h of treatment, which was followed by an increase in p16 and Zac1 expression after 5 h. The effect was pertussis toxin sensitive and abolished by the phosphotyrosine phosphatase (PTP) inhibitor orthovanadate, indicating the involvement of Gi and a PTP. Dnmt1 promoter contains a p53 consensus binding site and its expression can be repressed by p53. Treating GH3 cells transfected with a reporter vector containing a p53 binding site with somatostatin resulted in increased p53 relative luciferase activity. These data demonstrate that somatostatin inhibits dnmt1 gene expression, probably by activating p53. DNMTs with their ability to affect global promoter methylation have a central role in cell transformation and tumorigenesis. Elucidation of the pathways controlling their expression may provide additional pharmacological targets for controlling the aberrant growth of tumors.

## N3-079P
## Critical role of PTEN in aging, increased susceptibility to injury and impaired healing

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Clinical and experimental data indicate that in individuals $>70$ years of age, the stomach has increased susceptibility to injury and delayed healing, but the precise mechanisms are not fully defined.

**Experimental design**: Fisher F344 rats, 3 months of age (young) and 24 months old (aging) were used. Rats were treated with either placebo or indomethacin (anti-arthritis drug) for 2–24 h. In addition, we used aged- and neonatal-human microvascular endothelial cells (HMVEC). Studies *in vivo*: (i) extent of gastric injury; (ii) *in vivo* angiogenesis (new blood vessel formation); (iii) expression of PTEN and survivin mRNAs and proteins by RT/PCR Western blot and immunostaining; (iv) PI3K/Akt activation; (v) expression of 112 genes involved in apoptosis using oligo GEN apoptosis array. *In vitro*: in aged- and neo-HMVEC we examined PTEN expression, cell viability and angiogenesis *in vitro*.

**Results**: Gastric mucosa of aging rats exhibits: (i) increased PTEN ($320 \pm 20\%$) and $>40\%$ reduced survivin expression (both $P < 0.01$ vs. young); (ii) 14-fold increased injury by indomethacin ($P < 0.001$ vs. young); (iii) increased activation of Bax. casapases -2, -3 and -8 and p53 and decreased Bak-1, Bid 3 and survivin. Aged HMVEC had dramatically increased PTEN, reduced viability completely blocked *in vitro* angiogenesis. PTEN downregulation in aged HMVEC with siRNA completely restored angiogenesis.

**Conclusions:** (i) PTEN is strongly overexpressed in aging stomach and aged endothelial cells. (ii) Overexpressed PTEN inhibits PI3K/Akt, predisposes gastric mucosa to increased injury and impairs angiogenesis. (iii) PTEN plays a critical role in aging.

## N3-080P
## Selective effect of unductors of apoptosis on the phosphorylation state and the endoribonuclease activity of 26S proteasomes from different cellular compartments of K562 cells

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The 26S proteasome, composed of a 20S catalytic core and two associated 19S regulatory complexes, is a large multicatalytic protease which catalyzes the non-lysosomal proteolytic pathway in eukaryotic cells. The research of the structure and functions of 26S proteasomes is one of intensively developing sections of cell molecular biology. Proteasomes isolated from nuclei, cytoplasm and culture medium differ in the subunit composition. For the first time it has been shown that these subpopulations vary considerably in phosphorylation on tyrosine and threonine residues. We have shown that inductors of apoptosis induce changes in the tyrosine and threonine phosphorylation of proteasomes from different compartments in K562 human chronic myelogenous leukemia (CML) cells. Population of proteasomes was found to be heterogeneous in their RNase activity. The characteristics of the RNase activity of different subpopulations of proteasomes differ. We show that endoribonuclease activity of 26S proteasomes is changed under the action of inductors of programmed cell death. Treatment of K562 cells with inductors of apoptosis – doxorubicin (adriamycin) and diethylmaleate – lead to the reduction of different subpopulations of proteasome RNase activity. Inductors of apoptosis of K562 cells stimulate RNase activity associated with 26S proteasomes isolated from culture medium. However, RNase activity associated with both cytoplasmic and nuclear 26S proteasomes is inhibited by inductors of apoptosis of K562 cells. The enzymatic activity under study has been shown to be specifically and selectively dependent on phosphorylation of subunits of 26S proteasomes. The specificity of the subpopulation of proteasomes exported from the cell has been demonstrated. Proteasome involvement in the coordinated control of stability of various specific messenger RNA molecules is suggested, and one of the mechanisms of this control might be the export of specific subpopulation of proteasomes from the cell.

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## N3-081P
## Analysis of post-translational N-myristoylation of caspase-cleavage product of human gelsolin

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Recently, it was demonstrated that protein N-myristoylation could occur post-translationally, as in the case of the pro-apoptotic protein Bid. Our previous study showed that cytoskeletal actin was post-translationally N-myristoylated during apoptosis and targeted to mitochondria. In this study, to detect the post-translational N-myristoylation of other caspase substrates, the susceptibility of the newly exposed N-terminus of several caspase substrates to protein N-myristoylation was evaluated by *in vivo* metabolic labeling. It was found that the newly exposed N-terminus of the caspase-cleavage product of human gelsolin, an actin regulatory protein, efficiently directs the protein N-myristoylation. *In vivo* metabolic labeling of COS-1 cells transiently transfected with full-length truncated gelsolin (tGelsolin) revealed the efficient incorporation of [$^3$H] myristic acid into this molecule. When COS-1 cells transiently transfected with cDNA coding for epitope-tagged full length gelsolin were treated with staurosporine or etoposide, the apoptosis inducing agents, an N-myristoylated 44 kDa C-terminal fragment of gelsolin was generated as demonstrated by *in vivo* metabolic labeling. Immunofluorescence staining coupled with MitoTracker revealed that exogenously expressed tGelsolin localized to intracellular membrane compartments, but not to mitochondria. To study the role of this modification on the anti-apoptotic activity of tGelsolin, we constructed the bicistronic expression plasmid tGelsolin-IRES-EGFP capable of overexpressing tGelsolin concomitant with EGFP. Overexpression of N-myristoylated tGelsolin in COS-1 cells using plasmid tGelsolin-IRES-EGFP inhibited etoposide-induced apoptosis, whereas cells expressing non-myristoylated tGelsolinG2A mutant did not exhibit resistance to apoptosis. These results indicated that post-translational N-myristoylation of tGelsolin did not direct mitochondrial targeting, but this modification might be involved in the anti-apoptotic activity of tGelsolin.

## N3-082P
## Neuroglial interactions and intercellular signaling during photodynamic injury of isolated crayfish stretch receptor

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Photodynamic (PD) therapy is used for treatment of cancer including brain tumors. However, PD injury of normal glial cells and neuroglial relationships occurring under PD impact are not explored. We used a simple model system, isolated crayfish mechanoreceptor, consisting of two sensory neurons enveloped by satellite glial cells. Neuron functional state was monitored electrophysiologically. Double fluorochroming of this preparation with Hoechst 33342 and propidium iodide allowed visualization of alive, necrotic and apoptotic cells. PD effect of Photosens (AlPcSn) inhibited and then irreversibly abolished neuron activity. In the next 8 h percent of necrotic glial cells progressively increased. Apoptosis of glial cells became significant at 6–8 h after the treatment. Neuron nuclei progressively shrank, but their apoptotic fragmentation did not occur. PDT-induced death of some glial cells was accompanied by increase in their total number. Preliminary neuron inactivation by a laser beam focused to its body increased percent of apoptotic but not necrotic glial cells. Therefore, the neuron supports survival of surrounding glial cells. Inhibition of adenylate cyclase by MDL-12330A or tyrosine phosphatase by sodium orthovanadate protected glial cells from PD-induced necrosis but not apoptosis. In contrast, adenylate cyclase activation by forskolin enhanced PD-induced apoptosis of glial cells. Inhibition of tyrosine kinase by genistein did not affect necrosis or apoptosis of glial cells. These data show involvement of adenylate cyclase and tyrosine phosphatase signaling pathways in PD-induced death of glial cells.

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## N3-083P
## Signal transduction survival pathways in the interaction between the bone marrow microenvironment and acute myeloid leukemia

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Relapse is common in acute myeloid leukemia (AML) patients due to emergence and outgrowth of minimal residual disease (MRD). New strategies to effectively eradicate these MRD cells are currently needed to improve survival of patients. Our research focuses on how aberrant signal transduction, e.g. constitutive phospho-AKT (pAKT) expression, and interaction(s) between leukemic cells and the bone marrow microenvironment (BM-ME) contribute to the emergence, persistence and outgrowth of MRD and prognosis of the patient. MRD cells are detected with flow cytometry (FACS) using expression of leukemia-associated phenotypes (Feller et al. *Leukemia* 2004). We want to study pAKT expression in AML blasts at diagnosis, in MRD and at relapse and investigate whether changes occur during and after therapy. Evidence suggests that leukemic cells can be protected from apoptosis when attached to the BM-ME. By co-culture of AML blasts with the BM-ME we want to elucidate whether AKT signaling contributes to this described protective effect. A FACS assay was developed for the quantification of phosphorylated protein expression in AML/MRD cells. It was validated using cell lines which constitutively express pAKT. Without LY294002, a specific inhibitor of PI3K-dependent AKT phosphorylation, pAKT expression (as mean ratio MFI Ab/isotype ± SD, $n = 5$) was: 2.2 ± 0.4 (U937) and 2.6 ± 0.7 (Jurkat). With the inhibitor, pAKT expression was absent in U937 (1.0 ± 0.2) and strongly reduced in Jurkat (1.5 ± 0.7) cells. Preliminary experiments suggest a good correlation between results obtained with FACS and Western Blot. Experiments with primary patient samples are ongoing. We anticipate that the results of this study will contribute to a more effective treatment using specific targeting of signaling proteins, to eradicate MRD.

## N3-084P
## FasL interfering protein – cloning, expression and characterization

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Fas ligand is a cytokine that induces apoptosis by binding to Fas which is a cell-surface protein mediating apoptosis. The Fas/FasL system is implicated in the pathogenesis of several human diseases ranging from AIDS to autoimmunity and lymphoproliferation, transplant rejection, hepatitis multiple sclerosis and neurodegeneration. It is conceivable that modulating the activity of the Fas/FasL pathway would have clinical applications for the treatment of these patients. The cDNA corresponding to extracellular domain of mature rat Fas (spanning 21-176aa) was amplified by RT-PCR from rat brain mRNA and was cloned into pET28a (+) expression vector. FasL Interfering Protein (FIP) was expressed in *E. coli* Rosetta strain. FIP was purified under denaturing conditions using nickel resin and subsequently, the protein was renatured by dialysis. The fluorimetric assay was performed to ensure whether renaturation process was successful. Purified protein preparation was subjected to ESI-Q-TOF analysis to determine its homogeneity. Functional interactions between FIP and FasL were confirmed by co-immunoprecipitation of FIP-FasL complexes using anti-FasL polyclonal antibody. Those complexes were visualized by immunoblotting with anti-HisTag monoclonal antibody. To determine FIP activity in biological system, apoptiosis was induced in astrocytes using immunosuppresive drug – FK506.

## N3-085P
## JNK phosphorylation of 14-3-3 regulates nuclear targeting of c-Abl in the apoptotic response to DNA damage

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The ubiquitously expressed c-Abl tyrosine kinase localizes to the cytoplasm and nucleus. Nuclear c-Abl is activated by diverse genotoxic agents and induces apoptosis; however, the mechanisms responsible for nuclear targeting of c-Abl remain unclear. Here we show that cytoplasmic c-Abl is targeted to the nucleus in the DNA damage response. The results demonstrate that c-Abl is sequestered in the cytoplasm by binding to 14-3-3 proteins. Phosphorylation of c-Abl on Thr-735 functions as a site for direct binding to 14-3-3. We also show that, in response to DNA damage, activation of the c-Jun N-terminal kinase (JNK) induces phosphorylation of 14-3-3 and release of c-Abl from 14-3-3. In concert with these results, expression of an unphosphorylated 14-3-3 mutant attenuates DNA damage-induced nuclear import of c-Abl and apoptosis. These findings indicate that 14-3-3 is a pivotal regulator of intracellular c-Abl localization and the apoptotic response to genotoxic stress.

## N3-086P
## Cytotoxic and cytostatic effects of 3-hydrogenkwadaphnin, in human normal and leukemia cell lines

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3-Hydrogenkwadaphnin is a recently characterized daphnane-type compound isolated from *Dendrostellera lessertii* with high anti-tumor activity in animal model. Herein, we report time- and dose-dependent effects of this compound on growth, IMPDH inhibition and apoptosis in a panel of human cancer cell lines (K562, HL-60 and Molt4). The drug decreased growth in $<24$ h of treatment. However, longer exposure times, or higher concentrations, were required to promote cell apoptosis. Cell cycle analyses revealed the accumulation of cells in their G1 phase as early as 12 h after drug exposure but sub-G1 population was recorded after 24 h. Occurrence of apoptosis was constantly accompanied by morphological and biochemical variations among the drug treated cells. Despite these observations, none-activated normal human PBL were insensitive to the drug action. In addition, treatment of K562 and Molt4 cells with a single dose of the drug for 24 h leads to inhibition of IMPDH activity by almost 38 and 44%, respectively. Restoration of the depleted GTP concentration by exogenous addition of gunosine (25–50 μM) reverses the drug-effects on cell growth, DNA fragmentation and apoptosis. However, the drug effects were potentiated by exogenous addition of hypoxanthine to the drug-treated cells. Furthermore,

reduction of the drug potency on the non-proliferative (retinoic acid treated) HL-60 cells by almost 40%, compared to the proliferative cells, clearly indicates type II IMPDH as one of the main targets of the drug.

### N3-087P
### Neutralization of human MRP8/14 cytotoxicity by Vitamin E

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Migration inhibitory factor-related protein (MRP8) and MRP14 are two small anionic proteins, with zinc and calcium binding capacity. They belong to the S-100 protein family and abundantly found in cytosolic fraction of neutrophils. They form a heterodimeric complex in a calcium dependent manner that called MRP8/14 or calprotectin. MRP8/14 is a multifunctional protein with broad spectrum of antimicrobial and antitumoral activity that significantly elevates in serum and body fluids during inflammatory conditions. Since MRP8/14 cytotoxicity may be mediated via generation of reactive oxygen species (ROS) in target cells, we purified (>98%) human MRP8/14 from neutrophils, then examined its cell death inducing activity against MOLT4 and K562 at the presence of vitamin E (alpha-tocopherol). Treated cells were seeded into 96-well plates and relative cell number was measured using MTT assay. Our results demonstrate that human MRP8/14 suppress growth of MOLT4 and K562 in time- and dose-dependent manner and vitamin E (50–200 μM) significantly inhibits MRP8/14 cytotoxicity in both cell lines, suggesting possibility of ROS involvement in cell death inducing pathway of MRP8/14. Since vitamin C makes vitamin E a more effective antioxidant by freeing it from alpha-tocopheryl oxidative free radical, we used a combination of vitamin E (200 μM) and C (30 μM), but did not find a significant synergistic enhancement of vitamin E effect. In conclusion we suggest that in some inflammatory states, when plasma/serum concentration of MRP8/14 is relatively high, natural antioxidants such as vitamin E may be important in neutralization of cytotoxic effect of this protein, especially against normal cells.

### N3-088P
### Regulation of $p21^{CIP1/WAF1}$ mRNA and protein in cardiac myocytes

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Terminally-differentiated cardiomyocytes, the contractile cells in the heart, are susceptible to oxidative stress that induces apoptosis. We studied the regulation of $p21^{CIP1/WAF1}$ in cardiomyocytes exposed to $H_2O_2$ (an example of oxidative stress). RT-PCR indicated that $p21^{CIP1/WAF1}$ mRNA was upregulated from 2 h in response to 0.1 mM $H_2O_2$. $p21^{CIP1/WAF1}$ protein (assessed by immunoblotting) was similarly upregulated despite ~95% inhibition of global protein synthesis, suggesting that $p21^{CIP1/WAF1}$ is regulated translationally and constitutes a significant aspect of the apoptotic response. Phosphorylation of $p21^{CIP1/WAF1}$ protein by PKB/Akt increases its stability, but inhibition of the pathway with LY294002 indicated that, in cardiomyocytes exposed to $H_2O_2$, this was not a significant factor. Transfection experiments with luciferase reporter genes indicated that $H_2O_2$ suppressed the expression of luciferase whether a constitutive promoter or the promoter for $p21^{CIP1/WAF1}$ was used, providing additional evidence for translational regulation of full-length $p21^{CIP1/WAF1}$ mRNA. Further studies focused on the $p21^{CIP1/WAF1}$ gene. We identified a region of high homology within intron 1 of rat, mouse and human $p21^{CIP1/WAF1}$ genes, which overlapped with a published EST sequence. This novel sequence is expressed in the $p21^{CIP1/WAF1}$ mRNA in cardiomyocytes and, although cardiomyocytes express both the novel and classical $p21^{CIP1/WAF1}$ transcripts, the two mRNAs are differentially regulated in response to $H_2O_2$. We hypothesize that the novel sequence may promote translation of $p21^{CIP1/WAF1}$ in the context of inhibition of global protein synthesis. Further investigations focus on the precise role of the classical and novel $p21^{CIP1/WAF1}$ transcripts in cardiomyocyte apoptosis.

# N4 – ISN Symposium on System Neurochemistry: From Genes to Drugs

### N4-001
### SK3 channels as potential pharmaceutical targets to counteract age-dependent memory decline

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In the study, young (4–6 months) and aged (22–24 months) mice were trained in a trace fear-conditioning paradigm. Trace conditioning is a hippocampus-dependent form of associative learning in which the conditioned stimulus (tone) and the unconditioned stimulus (foot-shock) are separated by a defined time interval. Young mice had a better memory of the tone footshock association than aged mice when the tone was presented again the next day. In accordance, long-term potentiation (LTP) of the Schaffer collateral-CA1 pathway was lower in hippocampal brain tissue of aged mice when compared to LTP in hippocampus of young mice. Semi-quantitative RT-PCR and western blot analysis showed highly elevated levels of the small-conductance Ca2+-activated K+ channel SK3 transcript and protein in the hippocampus of aged, but not young mice. Most strikingly, antisense-mediated downregulation of SK3 channels in the hippocampus of aged mice restored the learning and memory ability as well as LTP. In summary, the data suggest that increased SK3 channel expression in the hippocampus of aged mice represents a mechanism, which contributes to the age-dependent decline in cognition and synaptic plasticity. An intervention that selectively reduces the function of SK3 channels may therefore be a novel mechanistic approach for pharmacological treatments that might ameliorate or even prevent the memory deficits associated with aging.

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## N4-002
## Genetic manipulations of hormonal signaling in the hippocampus

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Glucocorticoids (GCs), the adrenal steroids released during stress, compromise the ability of neurons to survive neurological injury. In contrast, estrogen protects neurons against such injuries. We designed three genetic interventions to manipulate GCs actions, which reduced their deleterious effects in rat both *in vitro* and *in vivo*. The most effective was a chimeric receptor combining the ligand-binding domain of the glucocorticoid receptor (GR) and DNA-binding domain of the estrogen receptor. Expression of this receptor reduced hippocampal lesion size after neurological damage by 63%, and reversed the outcome of the stress response by rendering GCs protective rather than destructive. Our findings elucidate three principal steps in the neuronal stress response pathway, all of which are amenable to therapeutic intervention. GCs are also implicated in reducing adult hippocampal neurogenesis. There has been little evidence for the presence of type 1 GR or type 2 (mineralcorticoid) receptors in neuronal precursor cells (NPC), and therefore suggested that GCs must indirectly inhibit NPC proliferation, though the mechanism has remained obscure. We demonstrate that GR mRNA is transcribed and yields a cytoplasm-localized receptor in isolated NPC from the adult hippocampus. Treatment of NPC grown *in vitro* with GCs induces decreased proliferation index, and a down-regulation of Nestin, a protein marker that is down regulated as NPC stop dividing and differentiate. This response is blocked using the GR-specific antagonist indicating that the GCs response is mediated by the glucocorticoid receptor. The apparent responsiveness of NPC to GCs suggests that neurogenesis may be directly modulated via GR signaling pathways.

## N4-003
## SC35 promotes prolonged stress-induced 3'alternative splicing of acetylcholinesterase

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Long-lasting 3' alternative splicing of neuronal acetylcholinesterase (AChE) pre-mRNA occurs following stress, replacing the synaptic transcript, AChE-S with the 'read-through' variant, AChE-R. At the 5' end of AChE, novel alternative first exons were identified. Some of the newly confirmed transcripts displayed stress and/or glucocorticoid-dependent regulation, predicting combinatorial complexity with the known 3' alternative AChE mRNA transcripts. Searching for the corresponding molecular events, we found elevated levels of the splicing factor SC35 in stressed as compared with naïve mice. To test the physiological significance of this finding, we co-transfected an ACHE minigene with several splicing factors. SC35 facilitated a shift from AChE-S to AChE-R mRNA, whereas ASF/SF2 caused the reverse affect. Transfections with different chimeric constructs comprised of SC35 and ASF/SF2 RRM / RS domains identified the SC35 RRM as responsible for AChE's 3' alternative splicing. In post-stress PFC neurons, increased cytosolic SC35 mRNA and nuclear SC35 protein levels coincided with selective increases in AChE-R mRNA. In the developing ventricular zone, cortical progenitor cells displayed transient SC35 elevation concomitant with dominance of AChE-R over AChE-S mRNA. Together, these findings highlight the complexity of AChE in both health and disease and point to an interactive relationship of SC35 with cholinergic signals in the long-lasting consequences of stress on nervous system plasticity and development.

## N4-004
## Stress, drugs and transcription. Specific inactivation of the GR in the brain, new insights on drug addiction

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Several evidences suggest that glucocorticoid hormones are involved in determining the propensity of an individual to develop cocaine abuse. These hormones activate two related transcription factors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor. We previously showed using the Cre/loxP system that the selective inactivation of the GR gene in mice brains (GRNesCre) leads to a profoundly flattened dose-response function for cocaine intravenous self-administration and a suppression of sensitization, two experimental procedures considered relevant models of addiction. The absence of GR does not influence the behavioral responses to an acute cocaine injection. We showed that these behavioral effects are associated with a change in the impulse activity of midbrain DA neurons which is considered one of the biological substrates of motivation and reward. When compared to control animals, GRNesCre mice showed both lower firing rate and lower bursting activity of midbrain DA neurons demonstrating the essential role of GR on dopaminergic transmission. To determine in which cell type the function of GR is required to modulate cocaine abuse, we generated animal models in which GR is selectively inactivated in either presynaptic dopamine neurons (GRDATCre) or post-synaptic cells (GRD1Cre). For this latter model, we used a previously developed transgenic mouse line that expresses the recombinase under the control of the dopamine receptor 1A gene (YAC-D1Cre; T. Lemberger and G. Schutz, Heidelberg). To target the expression of the Cre recombinase specifically in the dopaminergic neurons, we generated a mouse transgenic line that expresses the transgene under the control of the Dopamine Transporter gene (DAT). For this purpose and to ensure a correct expression of the transgene we used a Bacterial Artificial Chromosome. Behavioral and electrophysiological studies of these animal models will be presented, implications for the molecular mechanisms that underlie drug addiction and reward will be discussed.

## N4-005P
## Neuregulin-1 isoform alpha is remarkably localized in human brain and strongly reduced in schizophrenia

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Numerous studies have reported linkage between the neuregulin 1(NRG-1) gene and schizophrenia. In the central nervous system (CNS) NRG-1 proteins function in neuronal migration, differentiation and survival of oligodendrocytes. The NRG-1 gene codes for at least 15 different isoforms, which may be classified on the basis of their molecular structure. All isoforms contain either an alpha or a beta epidermal growth factor-like domain, which interacts with the estrogene receptor B (ErbB receptor)-tyrosine kinases to activate them. While brain-associated NRG1 beta forms have extensively been investigated, much less is known about the occurrence and distribution of NRG1-alpha forms in the CNS. This holds especially true for the human brain. We show by immunocytochemistry, Western blot analysis and Polymerase Chain Reaction that NRG1-alpha is widely distributed in neurons and radial glial cells of developing human brain (earliest appearance at 16th gestational week). In the adult human brain this NRG1 isoform is restricted to a few cortical neurons and some white matter interstitial neurons. Certain hypothalamic neurons receive a NRG1-1alpha immunoreactive innervation. When stereologically analyzing the number of NRG1-alpha expressing neurons in postmortem brains of schizophrenics ($n = 10$) and matched controls ($N = 10$) it was found that there is a statistically significant reduction in cell number in schizophrenics. Moreover, in schizophrencis these neurons show an altered position. We conclude that NRG-1 alpha plays a role in human brain development and is involved in the pathophysiology of schizophrenia.

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## N4-006P
## Inhibition of L-[14C]glutamate uptake and release in nerve terminals by DL-threo-beta - benzyloxyaspartate under centrifuge-induced hypergravity

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Glutamate acts within the mammalian central nervous system as the predominant excitatory neurotransmitter and as a potent neurotoxin. The balance between these physiological and pathological actions of L-glutamate is thought to be kept in check by the rapid removal of the neurotransmitter from the synaptic cleft. The majority of uptake is mediated by the high-affinity, $Na^+$-dependent excitatory amino acid transporters. The present study assesses a transporter inhibitor ability to affect L-[14C]glutamate uptake by synaptosomes under the normal and altered gravity conditions (rats were rotated in a long-arm centrifuge at 10G during 1-h period). DL-threo-beta-benzyloxyaspartate (DL-TBOA) is a competitive non-transported inhibitor of the glutamate transporters. We found that DL-TBOA inhibited in dose-dependent manner the L-[14C]glutamate uptake (100 μM glutamate, 30 s incubation period) as in control as in hypergravity. IC50 values calculated on the basis of curves of non-linear regression kinetic analysis was 18 ± 2 μM and 11 ± 2 μM ($P < 0.05$) before and after exposure to artificial gravity, respectively. Inhibition caused by 10 μM DL-TBOA was significantly increased from 38.0 ± 3.8 to 51.0 ± 4.1% in control and tested group, respectively ($P < 0.05$). Glutamate transport is of electrogenic nature and thus depends on the membrane potential. Depolarization leads to stimulation of glutamate efflux mediated by reversal of the carriers. We examined the effect of DL-TBOA on the high KCl-stimulated L-[14C]glutamate release in $Ca^{2+}$-free medium to elucidate reversed transport via the glutamate transporters. We have found that DL-TBOA inhibited L-[14C]glutamate release in dose-dependent manner as in control as in hypergravity. Application of 10 μM DL-TBOA caused a decrease of 15.2 ± 2.2% of L-[14C]glutamate efflux in control and 26.2 ± 3.9% after loading. Thus, DL-TBOA had complex effect on glutamatergic transmission, inhibited uptake and release of L-glutamate, and perhaps, became more potent under centrifuge-induced hypergravity.

## N4-007P
## Neuronal death and astrocytes survival during hypoxia/reoxygenation are affected by opposite modulation of Iron Regulatory Proteins and ferritin biosynthesis

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Iron is the most abundant metal in the brain, but when in excess can catalyze the production of ROS, which can cause brain injury, such as during the ischemia/reperfusion. Ferritin exerts a cytoprotective effect against the iron-catalyzed production of ROS, but its role in brain injury caused by hypoxia/reoxygenation is unclear. Ferritin expression is regulated mainly at post-transcriptional level by iron regulatory proteins 1 and 2 that bind specific RNA sequences (IREs) in the 5'UTR region of ferritin mRNA. Here we show that hypoxia decreases IRP1 binding activity in type-1 astrocytes and enhances it in cortical neurons. These effects were reversed by reoxygenation in both cell types. In astrocytes there was an early increase of ferritin synthesis during hypoxia and reoxygenation. Conversely, in cortical neurons ferritin synthesis increased only during the late phase of reoxygenation. Analysis of ferritin mRNA levels suggested that ferritin synthesis is regulated transcriptionally in neurons and both at transcriptional and post-transcriptional level in astrocytes. The different regulation of ferritin expression may account for the death of neurons and for the survival of astrocytes exposed to the injury elicited by hypoxia/reoxygenation. In fact, exposure of neurons to hypoxia/reoxygenation determined impairment of mitochondrial activity, marked lipid peroxidation and apoptosis. Under the same experimental conditions, astrocytes did not show any appreciable reduction in cell viability, lipid peroxidation or apoptosis. Moreover, the greater vulnerabililty of cortical neurons to hypoxia/reoxygenation was strongly attenuated by the exogenous administration of ferritin during hypoxia/reoxygenation, suggesting a cytoprotective role exerted by this iron-segregating protein.

## N4-008P
## Hypotonic swelling inhibits glutamate and GABA uptake in rat brain synaptosomes

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Hyponatremia leads to hyperexcitability of neurons, seizures and coma. It is well established that uptake of neurotransmitters is a sodium-dependent process. Therefore we suggest that inhibition of neurotransmitter uptake can lead to the clinical manifestations of hyponatremia. Decreasing of sodium concentration down to 92 mM in incubation medium, which corresponds to lowering the osmolarity down to 230 mOsm/l, leads to a 45% decrease in glutamate uptake and a 46% decrease in GABA uptake. However, this effect was mediated by the non-specific lowering of osmolarity rather than by decreasing sodium concentration. Hypotonic shock was able to reduce glutamate uptake in the presence of protein kinase inhibitors staurosporine and genistein, the phosphatase inhibitor okadaic acid, the phosphatidylinositol 3-kinase inhibitor wortmannin, and cytoskeleton modulators colchicine and cytochalasin B. Therefore we suggest that intracellular signalling is not mediating the effect of osmolarity reduction on neurotransmitter uptake.

## N4-009P
## Neurosteroids directly stimulate neuroprotective catecholamine synthesis and secretion

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Catecholamines protect and promote recovery from neural damage, while their deficiency in the brain plays a critical role in the progression of neurodegenerative disorders. Brain neurosteroids dehydroepiandrosterone (DHEA), its sulfate ester DHEAS and allopregnanolone (Allo) exert neuroprotective effects in various experimental models. We hypothesized that these neuroactive steroids may exert a direct tonic paracrine effect on neuroprotective catecholamine production. Our data are as follows: DHEA, DHEAS and Allo increased rapidly (within 10–30 min) and dose-dependently (EC50 at the nM range) the secretion of norepinephrine and dopamine from PC12 cells, an established neuron-like cell model to study catecholamine turnover. Their effect was exerted by increasing actin depolymerization and filament disassembly, the fast-response cellular system regulating trafficking of catecholamine vesicles. Actin filament stabilizer phallacidin, completely prevented steroid-induced catecholamine secretion. DHEAS and Allo, but not DHEA, stimulated also catecholamine production by increasing mRNA and protein levels of tyrosine hydroxylase, the rate-limiting enzyme of catecholamine biosynthesis. Our findings show that DHEAS and Allo exert a direct tonic effect on catecholamine synthesis and secretion, suggesting that part of their neuroprotective effects may be exerted by increasing levels of neuroprotective catecholamines in the brain.

## N4-010P
## TPPP/p25: the role of a new intrinsically unstructured protein in neurodegeneration

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TPPP/p25, the first representative of a new protein family, identified as a brain-specific unfolded protein induces aberrant microtubule assemblies *in vitro* [1], suppresses mitosis in *Drosophila* embryo [2] and is accumulated in inclusion bodies of human pathological brain tissues [3]. Our prediction and experimental data validate that TPPP/p25 is a new member of the ''intrinsically unstructured'' protein family. The comparison of these characteristics with that of a-synuclein and tau, involved also in neurodegenerative diseases, suggests that there are similarities in their well-defined unstructured segments interrupted by ''stabilization centers'', phosphorylation and tubulin binding motives. SK-N-MC neuroblastoma cells were transfected with pEGFP-TPPP/p25 construct and a stable clone denoted K4 was selected and used to establish the effect of this unstructured protein on the energy state/metabolism of the cells. Analysis of the mitochondrial membrane polarization revealed that the high-energy phosphate production in K4 clone is more extensive than in the control cells. In accordance, the biochemical analysis demonstrated 1.5-fold higher ATP level in the TPPP/p25 expressing cells. At pathological conditions TPPP/p25 is localized in inclusion bodies in multiple system athrophy, it tightly co-localizes with alpha-synuclein, partially with tubulin and not with vimentin. Immunohistochemistry of pathological human brain tissues rendered it possible to classify among pathological inclusions on the basis of immunolabelling of TPPP/p25, and suggest this protein to be a potential linkage between Parkinson's and Alzheimer's diseases.

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## N4-011P
## Differential regulation of steroid 5α-reductase isozymes expression by androgens in the adult rat brain

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The enzyme 5α-reductase (5α-R) is present in many mammalian tissues, including the brain. The physiological importance of 5α-R in the brain derives from its capability to convert testosterone (T) to a more potent androgen, dihydrotestosterone (DHT), and to convert progesterone and deoxycorticosterone (DOC) to their respective 5α-reduced derivatives, precursors of allopregnanolone and tetrahydroDOC, potent allosteric modulators of the gamma-aminobutyric acid receptor (GABA(A)-R). 5α-R occurs as two isoforms, 5α-R type 1 (5α-R1) and 5α-R type 2 (5α-R2). We studied the effects of T and DHT on the mRNA levels of both

5α-R isozymes in the prefrontal cortex of the adult rat, using an accurate and precise method that combines the high specificity of one-step quantitative RT-PCR with the sensitivity of capillary electrophoresis. Our results demonstrate that both isozymes of 5α-R are expressed in the cerebral cortex of adult rats. The gene expression of 5α-R type 2 is under the positive control of T and DHT. The gene that codes for 5α-R type 1 is not constitutive, because its expression is negatively regulated by T and DHT. These results open up a new research line that may lead to a better understanding of the role of 5α-R isozymes in the physiology of the central nervous system.

### N4-012P
### Transcriptome analysis and comprehensive expression profiling in human tissues and animal models for identification and characterization of relevant drug targets in chronic pain

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Target selection and prioritization are strategic decision points in the drug discovery process. Axxam has developed a platform based on gene expression profiling which include high density oligonucleotide microarrays, real time quantitative PCR and *in situ* hybridization, in addition to extensive collections of human tissues and animal models. Gene expression information obtained with these technologies have been used to identify genes encoding for promising targets in chronic pain. We have focused our attention on genes encoding druggable targets like proteases and GPCRs. Whole transciptome analysis using HG-U133 GeneChip (Affymetrix®) has been performed through a panel of 22 human tissues including DRG, spinal cord and samples form the brain. DRG specific markers have been examined. Statistical and pathway analysis have been applied to microarray data in order to identify genes specific to DRG, dorsal Horn of the spinal cord, and neuronal tissues vs. other peripheral non-neuronal tissues. In house generated and curated databases for "druggable" targets has been used for additional filtering of the generated gene lists. Quantitative real time PCR has been applied to validate microarray data and to generate extensive expression profiling of selected targets in a panel of more than 50 human non-pathological tissues through Axxam proprietary GeneTrawler®. Furthermore, to gain higher confidence in the selected targets and in their involvement in mechanisms of chronic pain, we have studied their expression regulation in tissues form multiple rodent pain models (i.e.: CCI, axotomy, STZ diabetic rats). Some relevant examples will be shown in more detail.

# N5–Mitochondrial Dysfunction in Disease

### N5-001
### Mitochondria in HIV-1-induced cell death

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The envelope glycoprotein complex (Env) of human immunodeficiency virus-1 (HIV-1) can induce apoptosis by a cornucopia of distinct mechanisms. A soluble Env derivative, gp120, can kill cells through signals that are transmitted by chemokine receptors such as CXCR4. Cell surface-bound Env (gp120/gp41), as present on the plasma membrane of HIV-1 infected cells, can kill uninfected bystander cells expressing CD4 and CXCR4 (or similar chemokine receptors, depending on the Env variant) by at least three different mechanisms. First, a transient interaction involving the exchange of lipids between the two interacting cells ('the kiss of death') may lead to the selective death of single CD4-expressing target cells. Second, fusion of the interacting cells may lead to the formation of syncytia which then succumb to apoptosis in a complex pathway involving the activation of several kinases (cyclin-dependent kinase-1, Cdk1; checkpoint kinase-2, Chk2; mammalian target of rapamycin, mTOR; p38 mitogen-activated protein kinase, p38 MAPK; inhibitor of NF-kB kinase, IKK), as well as the activation of several transcription factors (NF-kB, p53), finally resulting into the activation of the mitochondrial pathway of apoptosis. Third, if the Env-expressing cell is at an early stage of imminent apoptosis, its fusion with a CD4-expressing target cell can precipitate the death of both cells, through a process that may be considered as contagious apoptosis and which does not involve Cdk1, mTOR, p38 nor p53, yet does involve mitochondria. Activation of some of the before mentioned lethal signal transducers have been detected in patients' tissues, suggesting that HIV-1 may indeed trigger apoptosis through molecules whose implication in Env-induced killing has initially been discovered *in vitro*. In particular, it appears that the mitochondrial pathway plays an important role in HIV-1-induced cell killing.

### N5-002
### Mitochondrial regulation of caspase activation

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It is now well established that the mitochondria play an important role in the regulation of apoptotic cell death by mechanisms, which have been conserved during evolution. Thus, it seems that a host of lethal agents target the mitochondria and stimulate their release of cytochrome c and other proteins, which can trigger caspase activation and apoptosis. This release is governed by the Bcl-2 family of proteins and occurs by a two-step process, which is initiated by dissociation of the hemoprotein from cardiolipin, the phospholipid that anchors it to the outer surface of the inner mitochondrial membrane. Mounting evidence suggests that a decrease in the level of cardiolipin affects cytochrome c binding to the inner membrane, thus leading to higher levels of soluble cytochrome c in the mitochondrial intermembrane space. Among factors known to affect the cardiolipin level are saturated free fatty acids (16:0, 18:0), calcium dysregulation and reactive oxygen species (ROS). These factors, particularly calcium and ROS, have long been recognized as triggers of cell death and, more recently, as modulators of mitochondrially-mediated apoptosis. In this presentation, I will discuss the significance of the disruption of the cardiolipin-cytochrome c interaction for cytochrome c release and apoptosis and for mitochondrial dysfunction in disease.

## N5-003
## Mitofusins: from mitochondrial architecture to oxidative metabolism

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Mitochondria play a central role in many cellular functions, which include apoptosis, cell differentiation, bioenergetics and metabolism. Respiration and oxidative phosphorylation are coupled activities and oxygen consumption is strongly dependent on the availability of ADP. This explains why cells match rates of substrate oxidation and ATP production to rates of ATP utilization. In spite of this, several evidences suggest the existence of additional levels of control of mitochondrial energy metabolism that are superimposed to the chemiosmotic model of operation. Mitochondria frequently form tubular structures or networks. The mitochondrial network is a highly dynamic structure that is regulated through changes in the rates of mitochondrial fission and fusion. Mitochondrial fusion in yeast and in *Drosophila* depends on the integrity of the mitochondrial transmembrane GTPase, Fzo protein and mammalian cells express two homologs named mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2). We have analyzed the impact of up-regulation and knock-down of Mfn2 protein in energy metabolism in muscle cells and the nature of the mechanisms involved. Mfn2 deficiency reduces fuel oxidation and mitochondrial membrane potential and represses nuclear-encoded subunits of OXPHOS complexes. An inverse pattern of changes was detected in cells in response to Mfn2 up-regulation. In addition, the effect of Mfn2 overexpression on mitochondrial metabolism was mimicked by a truncated Mfn2 mutant inactive as a mitochondrial fusion protein. In all, we conclude that Mfn2 stimulates the rate of fuel oxidation in cells by regulating OXPHOS expression through signals that are independent of its role as a mitochondrial fusion protein.

## N5-004
## The cAMP cascade regulates mitochondrial respiration in mammalian cells. The role of complex I

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Conditions leading to arrest of cell replication are associated with decline of the activity of complex I of the respiratory chain and increased level of ROS in a variety of cell cultures *in vivo*. Short term (60 min) activation of the cAMP cascade by cholera toxin results in marked enhancement of the rotenone sensitive NADH-ubiquinone oxidoreductase activity of complex I, a rate limiting step of the respiratory chain and disappearance of ROS from the cells. Experiments on fibroblast cultures from patients with pathological mutations of nuclear genes of complex I were also carried out. The results showed that mutations in the NDUFS4 gene (18 kDa subunit), causing complete suppression of complex I NADHubiquinone oxidoreductase activity, prevented ROS formation in the fibroblast cultures. Mutation in the NDUFS1 gene (75 kDa, FeS protein) causing severe depression of the NADH-ubiquinone oxidoreductase activity of complex I, was associated with production of ROS, reversed by activation of the cAMP cascade.

## N5-005
## The iron-sulphur protein Rli1p and mitochondria play an essential role in the biogenesis of cytosolic ribosomes

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Mitochondria perform a central function in the biogenesis both of mitochondrial and extra-mitochondrial iron-sulfur (Fe/S) proteins. It is unknown to date why this biosynthetic pathway is indispensable for life, the more so as no essential mitochondrial Fe/S proteins are known. Here, we show that the soluble ATP binding cassette (ABC) protein Rli1p carries N-terminal Fe/S clusters. Mutations in critical cysteine residues of Rli1p abolish association with Fe/S clusters and lead to loss of cell viability. Hence, the essential character of Fe/S clusters in Rli1p for the first time explains the indispensable character of mitochondria in all eukaryotes. Further, we provide evidence that Rli1p performs a crucial role in the biogenesis of ribosomes. We report the tight association of Rli1p with ribosomes by using co-precipitation and co-sedimentation methods. Rli1p also associates *in vivo* with Hcr1p, a protein involved in rRNA processing and translation initiation. Depletion of Rli1p resulted in a strong defect in the nuclear export of the small and large ribosomal subunits to the cytosol and in an impairment of late steps of rRNA processing and subsequently a translational arrest. These data demonstrate a surprising functional connection between two central cellular processes, namely Fe/S protein maturation involving mitochondria and the biogenesis of cytosolic ribosomes.

## N5-006
## Brain mitochondrial injury induced by oxidative stress-related events is protected by tamoxifen

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This study evaluated the effect of the synthetic, nonsteroidal antiestrogen drug tamoxifen on brain mitochondria function. We observed that tamoxifen concentrations above 30 nmol/mg protein induced a slight decrease on RCR and ADP/O ratio. However, only higher concentrations of tamoxifen (* 70 nmol/mg protein) affected the phosphorylative capacity of mitochondria. Those effects were characterized by a decrease on mitochondrial transmembrane potential and repolarization level and an increase on repolarization lag phase with a decrease in ATP levels. Moreover, our results also show that tamoxifen presented a potent capacity to inhibit hydrogen peroxide for-

mation and reduced the extent of lipid peroxidation induced by the pro-oxidant pair ADP/$Fe^{2+}$. Tamoxifen also exerted some protection against mitochondrial permeability transition pore (MPTP) opening, although in a less extension than that promoted by cyclosporine A, the specific inhibitor of the MPTP. However, in the presence of tamoxifen plus cyclosporine A, the protection observed was significantly higher when compared with that induced by both agents alone. Furthermore, tamoxifen avoided the oxidation of thiol groups and GSH depletion promoted by $Ca^{2+}$. These results show that tamoxifen can afford protection against brain mitochondrial injury promoted by several oxidative stress-related events such as hydrogen peroxide production, lipid peroxidation and the induction of the MPT. Since numerous neurodegenerative diseases are intimately related with mitochondrial dysfunction, future therapeutical strategies could be designed taking in account this protective role of tamoxifen.

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## N5-007P
## The crosstalk between the endoplasmic reticulum and mitochondria in Alzheimer's disease

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Accumulating data suggest a central role for mitochondria in neurodegenerative disorders, namely Alzheimer's disease (AD). One of the possible mechanisms leading to neurodegeneration in AD is due to amyloid β-peptide (Aβ) induced mitochondria dysfunction. Moreover endoplasmic reticulum (ER) receives growing attention since this cellular compartment is an important site for Aβ 1-40/42 generation and accumulation. The aim of our study is to investigate the interrelation between mitochondria and ER in Aβ-induced cytotoxicity. We therefore exposed NT2 cells (ρ+), a clonal human teratocarcinoma cell line capable of differentiation into terminal neurons, to Aβ1-42 and evaluated cell viability. In contrast, to NT2 parental cells, NT2 cells that rendered incapable of oxidative phosphorylation via depletion of their mitochondrial DNA (ρ0 cells) were unaffected by exposure to Aβ1-42. These data suggested that a functional mitochondrial respiratory chain is required for Aβ toxicity. Since, it was described the crosstalk between ER and mitochondria during apoptotic cell death process, we further explored the role of an ER stressor, thapsigargin, in NT2 ρ0 cells death. To access the correlation between both cellular compartments we additional analyzed the effects of Aβ and thapsigargin on NT2 cells expressing endogenous mitochondrial DNA (mtDNA), mtDNA from AD subjects (AD cybrids), and mtDNA from age-matched control subjects (control cybrids). When exposed to Aβ, events associated with programmed cell death are activated in AD NT2 cybrids to a greater extent than they are in control cybrids or the native NT2 cell line, suggesting a role for mtDNA-derived mitochondrial dysfunction in AD degeneration. Moreover, inhibitors of ER $Ca^{2+}$-release prevented Aβ induced increase of cytosolic $Ca^{2+}$. Taken together, these results support a mechanistic link between Aβ accumulation, ER and mitochondrial dysfunction in neuronal demise in AD.

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## N5-008P
## Is there a link between dNTP pools, mitochondrial function and mutagenesis?

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Nucleic acids are central macromolecules of all cells and a balanced supply of their building blocks – the nucleotides – is essential for genomic stability. Imbalances in the precursor pools are connected with development of health problems. Two biosynthetic pathways, the *de novo* and the salvage pathway, produce dNTP's. The enzymes responsible for thymidine salvage are the thymidine kinases, TK1 and TK2. So far, mitochondria seem unable to obtain dNTP's by the *de novo* pathway. Therefore, the mitochondrial nucleotide pool is maintained by either import of cytosolic dNTP's or by salvaging dNTP's within the mitochondria. A role for TK2 in human disease was documented by the discovery of mutations in the TK2 gene of individuals diagnosed with Mitochondrial DNA Depletion Syndrome (MDS). These findings highlight the importance of mitochondrial dNTP pools in disease. We showed that mitochondrial activity plays a role in maintaining the stability of the nuclear genome. Together, these results suggest a link between nucleotide metabolism and DNA repair. Therefore, it is conceivable that mitochondrial dysfunction causes impairment of nucleotide biosynthesis that ultimately contributes to mutagenesis. Since mitochondria are involved in pyrimidine biosynthesis, we hypothesize that cells with deficient TK2 activity or mitochondrial dysfunction may create misbalance in the dNTP pools resulting in increased mutation frequency. To test our hypothesis and gain better understanding of the role of dNTP pools in mitochondrial-mediated mutagenesis, we measured nucleotide pools in cells with decreased expression of TK2 and dysfunctional mitochondria. We found that mitochondrial dysfunction affects dNTP pools.

## N5-009P
## CREB, but not AMPK, mediates upregulation of nuclear genes encoding mitochondrial proteins during mitochondrial proliferation in skeletal muscle

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A symptom of several mitochondrial disorders, e.g. MERRF syndrome, is excessive proliferation of malfunctioning mitochondria (ragged red fibers). In order to understand this process, we studied mitochondrial proliferation during skeletal muscle differentiation, i.e. formation of multinucleated myotubes in muscle cell lines. Myogenesis is accompanied by a five- to sixfold stimulation of total ATP turnover, which is covered by a threefold increase of mitochondrial activity. Levels of mRNAs and proteins for nuclear encoded Cytochrome c, Cytochrome c oxidase subunit IV and mitochondrial transcription factor A increased together with total cellular RNA and protein, respectively. We used the Cytochrome c promoter as a model in luciferase reporter assays and focused on candidate regulatory factors, which may have important effects on its activation. As levels of AMP increase, while ATP, ADP and phosphocreatine remain constant during myotube differentiation and since its stimulator AICAR activates Cytochrome c promoter activity, we hypothesized the involve-

ment of AMP-activated kinase (AMPK) in Cytochrome c promoter activation. However, C2C12 lines overexpressing a constitutively active form of AMPK failed to show an increased Cytochrome c promoter activity. On the other hand, mutagenesis studies revealed a critical role for cAMP responsive element binding proteins for promoter upregulation. The phosphorylation pattern of members of the cAMP responsive element binding protein (CREB) family changed in myotubes, which was accompanied by increased binding to Cytochrome c promoter sequences. In conclusion, cAMP responsive element binding proteins, but not AMPK, seem to play a key role for the regulation of mitochondrial genes in the model of mitochondrial biogenesis.

## N5-010P
## 137Cs and mitochondrial oxidation in myocardium

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137Cs accumulates both in the cytosol and mitochondrial matrix of the myocardium [1–3]. In this work we investigated parameters of tissue respiration of myocardium slices of rats fed with 137Cs containing forage up to incorporation level of 170, 300, 1000 and 3700 Bq/kg (appropriate doses 4, 8, 17 and 460 μGy). We applied the method of polarography with Clark electrode. A substantial increase (30–60%) of oxygen consumption rate, when using endogenous substrates, was observed in all groups of animals. Respiratory activity of myocardial slices in groups with 170 and 300 Bq/kg of cesium incorporation increased more than twice when glutamate was added. At the greater level of incorporation (1000 and 3700 Bq/kg) this parameter grew less considerably. Exogenous succinate stimulated the respiratory activity of a myocardium of experimental animals to a lesser degree compared with glutamate. Inhibitory analysis (with amytal, malonate, and 2,4-dinitrophenol) of electron-transporting chain activity in mitochondria of myocardium testifies about changes in coupling of oxidative phosphorylation and possible change of a quantitative ratio of oxidized substrates. We conclude that low dose irradiation from incorporated 137Cs is a risk factor in the development of pathology in myocardium.

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## N5-011P
## Influence of Cs-137 on mitochondrial oxidation in liver and some biochemical parameters of rat blood plasma

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High sensitivity of mitochondrial oxidation of the myocardium and skeletal muscles to small quantities of incorporated Cs-137 was shown earlier [Gritsuk et al., 2002]. Cs is known to be actively deposited in liver mitochondria [Wellard et al., 2002]. The influence of this radionuclide on the parameters of mitochondrial oxidation and biochemical parameters of blood is not clear. We studied parameters of mitochondrial oxidation of liver tissue sections and the number of biochemical parameters in blood of rats who were fed with Cs-137-polluted forage. The tissue respiration of the liver was investigated with polarography method using Clark's electrode. At Cs-137 incorporation in the amount of 3700 Bq/kg the rate of oxygen consumption in endogenous substrates and also respiratory activity of liver increased once amytal and malonate had been administered. The labilization of the coupling of oxidative phosphorylation was observed. In blood plasma the contents of alpha-1-globulines increased, the contents of albumin and gamma-globulin was reliably reduced which was caused by the decrease of total protein amount in plasma. Concentrations of the uric acid and cholesterol increased, glucose level was reduced. The respiratory activity of liver sections remained reliably high while other parameters of mitochondrial oxidation remained stable after Cs-137 incorporation up to 30 000 Bq/kg. The amount of albumins, total protein and gamma-globulins remained reduced, and the level of alpha-1-globulines was high. The data obtained allow to assume that Cs-137 probably influences biochemical parameters of blood through the influence on mitochondrial oxidation in liver.

## N5-012P
## Cloning, expression and characterization of the human mitochondrial β-ketoacyl synthase: complementation of the yeast CEM1 knockout strain

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Failure in mitochondrial function has been implicated in the pathogenesis of a number of late developing neurodegenerative disorders, yet, surprisingly, the roles of many mitochondrial proteins are still unknown and the etiology of these diseases is poorly understood. Deficiencies in complex 1 are thought to be a common cause of mitochondrial encephalomyopathies and the discovery that one of its subunits is an acyl carrier protein (ACP) has stimulated interest in the possibility that a pathway for fatty acid synthesis may play an important role in mitochondrial function. To date, three components have been characterized, the ACP, a malonyltransferase and an enoyl reductase. We have now identified, cloned and characterized a fourth component, namely a β-ketoacyl synthase. Sequence analysis indicates that the protein is more closely related to free standing counterparts found in prokaryotes and chloroplasts than it is to the β-ketoacyl synthase domain of the human cytosolic fatty acid synthase. Like the other human mitochondrial FAS components, the full-length nuclear-encoded 459-residue protein includes a ~38 residue N-terminal mitochondrial targeting sequence element. The enzyme can elongate acyl-chains containing 2–14 carbon atoms with malonyl moieties attached in thioester linkage to the human mitochondrial ACP and is able to restore growth to the respiratory-deficient yeast mutant CEM1 that lacks the normal mitochondrial ß-ketoacyl synthase and exhibits lowered lipoic acid levels. The substrate specificity and complementation data for the ß-ketoacyl synthase suggest that, as in plants and fungi, in humans this pathway may play an important role in the generation of octanoyl-ACP, the lipoic acid precursor, as well as longer chain fatty acids that are required for optimal mitochondrial function.

## N5-013P
## Accumulation of long chain fatty acids decreases the cardioprotective effect of KATP channel openers

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The oxidation of fatty acids serves as the main energy source for cardiomyocytes. However, in ischemic tissue fatty acid oxidation is inhibited, resulting in accumulation of long chain fatty acids and their CoA derivatives. In this study, we investigated the influence of fatty acids on the effects of KATP channel openers on isolated rat heart mitochondria. Mitochondrial respiration rates were recorded by the means of Clark-type oxygen electrode in the isotonic KCl medium (37 °C), using pyruvate and malate (6–6 mM) or fatty acids - palmitoyl-L-carnitine (9 mkM) and malate (240 mkM) or palmitoyl-CoA (5 mkM; + L-carnitine (2 mM)) and malate (240 mkM) as substrates. The results showed that KATP channel openers diazoxide (300 mkM) and pinacidil (300 mkM) similarly increased (by ~100%) the state 2 respiration rate of mitochondria, oxidizing either pyruvate and malate or fatty acids. In contrast, when palmitoyl-CoA (2.5 mkM) was not oxidized (medium devoid of carnitine), it suppressed the uncoupling effect of KATP channel openers in mitochondria, respiring on pyruvate and malate. Thus, during ischemia, the effects of KATP channel openers could decrease due to accumulation of physiological inhibitor of adenine nucleotide translocase - palmitoyl-CoA. Since uncoupling is proposed as one of the mechanisms of pharmacological cardioprotection, our results suggest that higher concentrations of KATP channel openers might be required to protect cardiomyocytes from ischemic injuries.

## N5-014P
## Rat liver mitochondrial carnitine palmitoyltransferase-I (CPT-I): starvation induced enrichment in mitochondrial contact sites

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Hepatic beta-oxidation of long-chain fatty acids provides acetyl-CoA that is either oxidized for energy production or converted to ketone bodies and is increased by high fat feeding, fasting and diabetes. The key enzyme in the overall process is mitochondrial CPT-I that is enriched in contact sites. The major determinants of the flux through CPT-I are changes in enzyme's kinetic properties, i.e., Vmax and Km for palmitoyl-CoA and carnitine and sensitivity to malonyl-CoA inhibition (Ki). The tissue content of malonyl-CoA, activated long-chain fatty acids and carnitine further impact the flux. The goal of this study was to elucidate the underlying kinetic mechanism for increased fatty acid oxidation during starvation. Sprague-Dawley, fed and 48 h fasted, rats were used for isolation of liver mitochondria, liver mitochondrial outer membranes and contact sites. The kinetic parameters of CPT-I were determined by measuring the maximal reaction velocity (Vmax), apparent affinities for palmitoyl-CoA and carnitine (Km), and the sensitivity of the enzyme to malonyl-CoA inhibition (Ki). We also determined the effect of starvation of CPT-I protein by immunoquantitation. In summary, 48 h starvation results in a significant enrichment of CPT-I in rat liver mitochondrial contact sites as well as in increased catalytic efficiency. In addition, there is a significant decrease in the enzyme's sensitivity to malonyl-CoA inhibition. These changes along with the decrease in cytosolic concentration of malonyl-CoA could explain the increased beta-oxidation flux observed during starvation. The data are consistent with the proposed role of contact sites in mitochondrial uptake of activated long-chain fatty acids.

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## N5-015P
## Comparative study of mitochondrial proteomes from rat liver and brain tissues

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Although the central roles of mitochondria is supply of cellular energy as ATP, it has other roles which may be related with the type of tissues. And it is related with many diseases and it is necessary to identify tissue-specific mitochondrial functions. Therefore, the mitochondria were purified by sucrose density gradient from brain and liver tissues and the proteome (Mito-B and Mito-L) were compared using 2-dimensional electrophoresis followed by MALDI-TOF. Mito-B and –L showed vast contamination from synaptosome and ER, respectively. The most abundant protein in both proteome is ATP synthase subunit. Besides the contaminants, there are significant differences between proteomes. TCA cycle proteins are rich in Mito-B, whereas metabolic proteins involved in urea cycle enzyme, pyrimidine *de novo* synthesis and etc. are abundant in Mito-L, relatively. Some of the differences seem to be unique to each tissue in this experimental condition. These results indicate that each tissue contains distinct proteome for mitochondria, which may have differential functions in tissue physiology and pathology.

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## N5-016P
## Modeling the biochemistry of Leber's hereditary optic neuropathy in *Escherichia coli*

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Leber's hereditary optic neuropathy (LHON), one of the most common mitochondrial disorders, leads to sudden loss of central vision, mainly in young adult males. The disease is inherited maternally as a consequence of mutations in mitochondrial DNA. Almost all known mutations affect mitochondrial respiratory chain complex I, and the three most common mutations (3460 in ND1, 11778 in ND4 and 14484 in ND6) account for 90% of the diagnosed patients. More rare mutations have hot spots in ND6 and ND1 genes. Only the 3460-mutation reduces complex I activity somewhat whereas the other mutations induce minor changes in affinities towards ubiquinone substrate and/or inhibitors. It remains unknown how mutations in different parts

of the enzyme result in a similar disease. We have generated LHON-mimicking mutations in six different positions of the ND1, ND4L and ND6 gene counterparts of the *Escherichia coli* complex I enzyme, including two out of the three common mutations. Enzyme activities (ubiquinone and hexammine ruthenium reductase) were assayed spectrophotometrically in cytoplasmic membrane vesicles. Complex I dependent growth ability was assessed in minimal medium with malate as the main carbon source. We found slightly altered affinities for decylubiquinone in some ND6 mutants, but not in all, and maximal enzyme turnover rates were decreased by up to 70%. Malate growth in ND6-Y59F was clearly affected suggesting impairment of energy conservation in this mutant. The small ubiquinone affinity changes in some mutants is probably insufficient to explain the pathogenesis of LHON. In order to find out whether there is a common pathogenic mechanism in LHON, we will analyze the proton pumping capabilities and superoxide production features in these mutants.

## N5-017P
## Docetaxel targets bioenergetic mitochondria by preventing oxidative phosphorylation and promoting opening of the permeability transition pore

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Docetaxel (taxotere), a taxol drug that stabilizes the microtubule network with consequent inhibition of its dynamics, has been utilized in the therapy of several malignances. However, it is not well stabilized whether the antitumor efficacy of docetaxel is due to an induction of apoptosis or another mechanism of cell destruction. In this work we studied the effect of taxotere in the oxidative phosphorylation system and permeability transition pore of isolated mitochondria. The results show that mitochondria energized in the presence of succinate have an increased respiratory rate (state 4) in the presence of taxotere, whereas the oxygen consumption is not affected by the drug under uncoupling conditions (presence of the protonophore FCCP). In contrast, mitochondria energized in the presence of glutamate/malate show a strong reduction of respiration (state 4) in both, presence and absence of FCCP. These observations indicate that complex I is strongly inhibited by taxotere (1–7 μM) whereas complex II, III and IV are not affected by the drug. Furthermore, we observed that the drug reduce transmembrane electric potential ($\Delta\psi$) of mitochondria energized with succinate by a protonophoretic action. The ATP synthase (complex V) did not appear greatly affected by the drug since insignificant alteration of the ATPase activity was observed in freeze-thawing mitochondria. We also observed calcium induced depolarization, increase swelling rate and decreased calcium accumulation in mitochondria treated with low concentrations (1–4 μM) of taxotere. These effects were prevented in the presence of cyclosporine A, suggesting that taxotere promotes the opening of the mitochondrial permeability transition pore. These findings indicate that taxotere may cause cell death by targeting primarily the mitochondrial bioenergetic machinary of the cell.

## N5-018P
## Metabolic control analysis provides proves for stimulation of ATP synthase by calcium ions

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Besides involvement to other signaling pathways, interaction of calcium ions with mitochondrial enzymes is important tool for tight regulation of cellular energy production and energy demand. Using metabolic control analysis, we earlier demonstrated that calcium (within the physiological range of concentration) had no significant direct kinetic effects on the adenine nucleotide translocator, although it stimulated the phosphorylation subsystem in heart mitochondria. Since the estimation of direct effects on the components of the phosphorylation subsystem is hardly achievable, we aimed to determine to what extent the stimulation of succinate oxidation in rat and rabbit heart mitochondria by calcium ions depends on the contribution of ATP synthase to the control over respiration at two different temperatures, 28 °C and 37 °C. Succinate oxidation in rabbit mitochondria was more sensitive to change in temperature and to increase in calcium concentration (from 5 nM to 1 M) than in rat mitochondria. State 3 respiration in rabbit mitochondria was more sensitive to temperature than state 2 respiration, and opposite was true for rat mitochondria. For both rat and rabbit mitochondria, the control coefficient of ATP synthase over state 3 respiration was determined by oligomycin titration, and the correlation between calcium stimulation and the control coefficient was estimated under variety of conditions used. The results showed statistically significant positive correlation ($r = 0.906$). Recently, Balaban's group presented complicated experimental evidence that calcium ions stimulate ATP synthase. Our data confirm their main conclusion but we derive it in more simple way.

## N5-019P
## Staurosporine-induced caspase-independent cell death is abrogated by the Omi/HtrA2 inhibitor UCF101 by preserving mitochondrial trans-membrane potential

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Previously we have shown that staurosporine (1 M) can induce caspase-independent cell death in Z-VAD(OMe)-FMK (50 M) treated HL-60 leukemia cells in the forms of apoptosis or necrosis detected by morphology and flow cytometry after 8 h exposure (Mihalik et al., *Cell Death Differ* 2004; 11: 1357). Recently, the role of Omi/HtrA2 protease activity in the regulation of caspase-independent cell death was emerged. Omi/HtrA2 is situated in the mitochondrial intermembrane space and released into the cytosol in case of caspase-dependent apoptosis to counteract the inhibitory function of XIAP in caspase activation; while retained in the mitochondria in case of caspase-independent cell death and possibly processed intramitochondrial targets (Blink et al., *Cell Death Differ* 2004; 11: 937). In this study we found that pre-treatment with UCF-101 (30 M), a potent inhibitor of HtrA serine proteases, protected cells from both forms of staurosporine-induced caspase-independent cell death significantly. Mitochondrial transmembrane potential detected by DiOC6(3) (10 nM) was reduced moderately in apoptotic, while notably in necrotic cells that was also prevented by UCF-101. UCF-101 was slightly cytotoxic to HL-60 cells by 8 h exposure (6%) that was inhibited by the Z-VAD(OMe)-FMK treatment. In conclusion,

Omi/HtrA2 protease activity can contribute to the mitochondrial break-down in caspase-compromised cells opening pathways to both apoptotic and necrotic forms of cell death.

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## N5-020P
## The microarray of beta-carotene regulated gene expression in human endothelial cell progenitors.

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**Introduction:** The epidemiological studies revealed that intake of carotenoids decreases risk of cancer and coronary artery disease (CAD) incidence and increases immunologic resistance due to stimulation of the NK cell activity. Surprisingly two big clinical studies, which aim was undertaken to prove the efficacy of beta-carotene (BC) in decreasing the incidence for CAD, showed that administration of beta-carotene or vitamin A may increase the risk of lung cancer especially in smokers. Tumor growth and invasion is closely related to the angiogenesis. Unsaturated fatty acids and their metabolites in concert with retinoids - metabolites of BC regulate the activity of transcription factors (PPARS; RAR/RXR) and expression of genes participating in metabolic processes as well as genes promoting cell (including endothelial) differentiation.

**Aim:** Aim of the study was to analyze the influence of beta carotene (BC) on expression of genes related to angiogenesis (proliferation, chemotaxis and differentiation) in endothelial cells.

**Results:** BC was accumulated by HUVEC in time and concentration-dependent manner. BC and AA activate gene expression related to cell proliferation, adhesion, cell-cell signaling, chemotaxis, when expression of genes related to differentiation and apoptosis was inhibited.

**Conclusion:** However, in *in vitro* the cell proliferation and tubulogenesis is not influenced by beta-carotene, the induction of genes related to early phase of angiogenesis and inhibition of apoptosis argue for priming of endothelial cells to enter this process.

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## N5-021P
## Kidney morphological changes and mitochondrial function in diabetic Goto-Kakizaki rat

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Renal disease is a common complication of diabetes mellitus. The pathogenesis of diabetic nephropathy is not well understood but hyperglycemia seems to be a crucial factor. Recent evidence indicates that the overproduction of reactive oxygen species, observed in both clinical and experimental diabetes, and mitochondrial dysfunction are key factors in the pathogenic process. The objective of this investigation was to test the hypothesis of whether hyperglycemia could affect kidney morphology and mitochondrial bioenergetics as well as susceptibility to oxidative stress. Diabetic GK rats, a model of type 2 diabetes mellitus and Wistar rats were used in the study. Kidney mitochondrial respiration and changes in membrane potential were estimated simultaneously with Clark-type and TPP+ sensitive electrodes. Oxidative damage injury was induced *in vitro* by the oxidant pair ADP/$Fe^{2+}$ and the extent of membrane oxidation was assessed by oxygen consumption and thiobarbituric acid reactive substances (TBARS) formation. Coenzyme Q and β-tocopherol contents were determined by high-performance liquid chromatography. There were no significant differences in the kidney respiratory function and phosphorylation capacity between GK and Wistar rats. Mitochondria from kidney of diabetic rats were equally susceptible to *in vitro* oxidative damage as those from normal rats, while coenzyme Q and β-tocopherol concentrations were similar in both types of preparations. However, the kidney of GK rats presented in most glomerulus a capillary basement membrane thickening with mesangial widening, in evolution to segmental glomerular sclerosis and in some interlobular arteries, excessive deposition of periodic acid – schiff (PAS)-positive material at the tunica intima. The results show that the mild prolonged hyperglycemia and the kidney structural changes observed in GK rats are not sufficient to cause renal dysfunction and functional changes in mitochondria.

## N5-022P
## Analysis of metallothionein expression levels in rotenone-induced mitochondrial NADH: ubiquinone oxidoreductase deficient rat tissue

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Mitochondrial NADH: ubiquinone oxidoreductase (complex I) carries out a number of well defined functions required for cell physiology. Deficiencies of complex I lead to multi-system disorders that include several well known phenotypes such as diabetes, Alzheimer's disease as well as less known phenotypes such as MELAS, Leigh syndrome and MERRF. It was recently identified that ROS-sensitive proteins, metallothioneins (MTs), are expressed in complex I deficient cell lines and have a protective effect against ROS-related pathology. It is still not clear if isoform-specific MT expression occurs in this disease and if it plays a significant role *in vivo*. We investigated the expression of different MT isoforms in rotenone-treated Sprague Dawly rats, an *in vivo* model that has been used to study cell biological responses of mitochondrial complex I deficiency. The different MT isoform expression levels were analyzed in various tissue types by using real-time PCR. Five different housekeeping genes (GAPDH, beta-actin, beta-2-microglobulin, RNA polymerase II and 18S rRNA) have also been evaluated for their suitability to be used as internal controls for normalization. We report the tissue differential expression of MT-I to IV isoforms in complex I deficient brain, liver, heart and skeletal muscle. We hypothesize that with a complex I deficiency, an increase in ROS production will ultimately lead to an increase of MT

expression and that this underlines the importance and contribution of nucleus-mitochondrial communication in the responses to mitochondrial disease.

## N5-023P
## Metallothionein expression and its role in rotenone-induced NADH:ubiquinone oxidoreductase-deficient HeLa cells

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Deficiency of mitochondrial oxidative phosphorylation is a common pathology in several debilitating inherited and acquired metabolic diseases. Due to their debilitating consequences, current investigations into potential protective agents against these deficiencies are becoming more important. NADH:ubiquinone oxidoreductase (complex I) deficiencies are the most frequently encountered among this group of diseases. Complex I deficiencies are known to result in, amongst other, high levels of reactive oxygen species (ROS). These lead to various destructive consequences, including the induction of apoptosis. Metallothioneins are a family of metal-binding proteins with unique structural characteristics providing them the ability to bind and reduce ROS, as well as metals. We report the selective expression of metallothionein type 2A (MT-2A) in rotenone-induced complex I deficient HeLa cells. We also present data that suggest that MT-1B and especially MT-2A overexpression significantly increases cell viability and protects against the disease-related production of ROS as well as apoptosis in complex I deficient HeLa cells. We believe that investigating the expression of metallothioneins may not only provide better insight into responses to mitochondria-related diseases, but as metallothioneins can be induced in several ways, also provide exciting new possibilities to the limited therapeutic options that are currently considered for this disease.

## N5-024P
## Effect of magnesium on calcium-induced depolarization of mitochondrial potential

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We have investigated the effect of magnesium on calcium-induced depolarization of mitochondrial potential. Addition of 25 M calcium to suspension of mitochondria isolated from rat heart led to the collapse of succinate driven mitochondrial potential. Both binding of calcium by EGTA and inhibition of mitochondrial calcium uptake by ruthenium red (RuR) did not have any effect on repolarization of potential. In the presence of 5 mM magnesium, collapse of potential was achieved by addition of calcium to the concentration of 125 μM, however, rapid repolarization was observed after addition of either EGTA or RuR. In the presence of both magnesium and ADP, addition of 125 μM calcium led to the significant but not complete depolarization of potential, which was fully restored after addition of either EGTA or RuR. In the absence of magnesium, calcium-induced collapse of mitochondrial potential was prevented by pre-treatment of mitochondria with cyclosporine A suggesting that collapse of potential is caused by opening of mitochondrial permeability transition pore (mPTP). On the other hand, magnesium and ADP are influencing significantly the rate of mitochondrial calcium uptake and capacity of mitochondria to bind calcium. Based on this, we suppose that magnesium affects calcium-induced depolarization of mitochondrial potential mainly through the competition with calcium for the binding to unknown target involved in the process of mPTP opening and modulation of mitochondrial calcium transport. In addition, effect of magnesium on mitochondrial oxidative metabolism involved in transmembrane potential generation cannot be excluded. Our results indicate important role of magnesium in the process of calcium-induced dysfunction of mitochondria.

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## N5-025P
## Opening of mitochondrial permeability transition pore in oxyphilic thyroid tumors

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Oxyphilic tumors of thyroid gland are characterized by mitochondrial accumulation, alterations in activity of mitochondrial respiratory complexes, increased production of reactive oxygen species and disturbances in apoptotic processes. The aim of our study was to analyze the opening of the transitory mitochondrial permeability pore in thyroid carcinoma with cell oxyphilia. Our analysis of mitochondrial permeability transition pore (mtPTP) showed that higher concentration of arachidonic acid in XTC-1 oxyphilic cell line was needed for induction of mtPTP in comparison with non-oxyphilic B-CPAP control cell line. These results may indicate that XTC-1 cell line is resistant towards apoptosis in comparison with non-oxyphilic cell line. The addition of uncoupler (FCCP) induced decrease in fluorescence in both cell lines, indicating similar affinity towards agonists of mtPTP. Cyclosporine A inhibited the effects of stimulated mtPTP pore opening without significant differences in both cell lines.

## N5-026P
## Characterization of fatty acylcarnitines in rat hepatocytes *in vitro*: valproylcarnitine formation and a new perspective on fatty acid oxidation

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**Background:** The role of carnitine in fatty acid metabolism is crucial either to the transport of long-chain fatty acyl-CoA's into the mitochondria as carnitine esters, or to their export, especially when they accumulate in the matrix. Valproylcarnitine (VPC) has been detected in urine of patients under treatment with valproic acid (2-*n*-propylpentanoic acid, VPA).

**Aims:** The elucidation of the mechanisms underlying the interaction of VPA with carnitine was the objective of this study. A differential study on the acylcarnitines (AC) profile of permeabilized hepatocytes (PHep) incubated with VPA or valproyl-Coenzyme A (VPA-CoA) was undertaken.
**Methods:** Rat hepatocytes were obtained, permeabilized with digitonin and incubated (10 min/37 °C) with L-carnitine, [$^{13}$C]-palmitoyl-CoA and 0.5 mM VPA or VPA-CoA (0–100 μM). The quantification of AC was performed using ESI-MS-MS.
**Results:** In PHep incubated with VPA an unequivocal increase of the C3 and C5-AC was observed as compared with control cells. The increasing concentrations of VPA-CoA induced an increase of the levels of C3, C5 and C8-AC in PHep.
**Discussion:** The formation of VPC in PHep incubated with VPA-CoA is clearly confirmed by the corresponding increase in C8-AC, and its β-oxidation is evidenced by the accumulation of C3-AC in these cells. Our results strongly suggest that VPA enters the mitochondria not only by passive diffusion but also using the carnitine shuttle. This new insight on VPA biotransformation may have important implications for endogenous fatty acid oxidation in the light of the mitochondrial dysfunction associated with the drug.
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## N5-027P
## Effect of ischemia on mitochondrial function in rabbit atrium and ventricle fibers

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In order to investigate whole heart energetics and changes of mitochondrial function during human heart surgery mostly atrial appendage fibers are used. However it is not clear whether the ischemia-caused changes in atrium reflect the same situation in the ventricle. The aim of our study was to compare the ischemia-induced changes of mitochondrial function in rabbit cardiac fibers obtained from atrium and ventricle. Total ischemia was induced by autolysis (37 °C, 1 h). The respiration of mitochondria was measured in saponin + collagenase (50 μg/ml and 3 mg/ml) skinned atrium and ventricle fibers with glutamate + malate and succinate as substrates. We found, that glutamate-dependent state 3 respiration rate after 1 h ischemia decreased by 41% and 46% in both, atrium and ventricle fibers as compared to control ($p < 0.05$). Succinate-dependent maximal respiration rate was clearly reduced too (–28% and –30%). State 2 respiration rate increased by 17% and 15% indicating the ischemia-caused increase in permeability of mitochondrial inner membrane. Respiratory control index was also clearly reduced (–56% and –48%). Addition of cytochrome c stimulated state 4 respiration rate to the similar degree in both cases (by 34% and 45%, $p < 0.05$). This indicates that ischemia causes the injury of the outer mitochondrial membrane leading to the loss of cytochrome c. It is well known, that cytochrome c acts as a signal for inducing apoptosis. In conclusion, our results show that mitochondria from both, atrium and ventricle of rabbits are equally sensitive to 1 h ischemia. Investigation of mitochondrial function in atrium by using skinned fiber technique may give useful information for the prognosis of ischemia-induced changes in the cell bioenergetics of ventricle.

## N5-028P
## Mutagenesis of polypeptide subunits of complex I from *Neurospora crassa* mitochondria

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The proton-pumping NADH dehydrogenase or complex I is a component of the mitochondrial respiratory chain. It catalyses electron transfer from NADH to ubiquinone, through a series of protein-bound prosthetic groups, coupled to proton translocation across the inner mitochondrial membrane. Complex I from *Neurospora crassa* contains at least 39 polypeptide subunits of dual genetic origin. The enzyme is likely regulated by transitions between active (A) and de-activated (D) forms. We have mutagenised specific polypeptide subunits of complex I and characterized the catalytic properties and the process of active/de-active transition of the resulting mutant strains. The results allowed the identification of proteins involved in the A/D transition phenomenon. On the other hand, mutations in complex I proteins have been implicated in several mitochondrial diseases. In order to develop disease models, we generated *N. crassa* strains harboring point-mutations in conserved amino acid residues of iron-sulphur subunits of the enzyme, equivalent to those found in the human diseases. Some of the strains also lack an alternative mitochondrial internal NADH dehydrogenase. Characterization of these mutant strains in terms of complex I structure and function will also be presented.

## N5-029P
## The kappa-opioid receptor specific agonist U50488H induces mitochondrial stress in cultured human cancer cells

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In this study, the cell death that was induced by a specific agonist of kappa-opioid receptor, U50488H, was investigated in a cultured human cell line (CNE2). A loss of cellular viability and a decrease of cell number were observed when cells were treated for twenty-four hours with U50488H (50–100 μM). Examination of these treated cells by electron microscopy demonstrated swelling of mitochondria without much evidence of chromosomal fragmentation. Consistently, the presence of a sub-G1 peak that was due to DNA-fragmentation was also only found in cells treated with staurosporine (serving as an apoptotic stimulus) but not U50488H. Only a small fraction of procaspase-3 had undergone limited proteolysis when cells were treated with U50488H. However, the uptake of the mitochondrial dye DiOC6 was tremendously reduced in cells treated with U50488H (in comparison to the control) suggesting that dissipation of mitochondrial potential had taken place. The decrease in the uptake of DiOC6 was accompanied by the release of cytochrome-C as shown by immunofluorescence experiments. Incubation of cells with U50488H in the presence of cyclosporine-A was able to prevent the release of cytochrome-C suggesting the involvement of permeability transition. It is proposed that the kappa-opioid receptor specific agonist U50488H might cause a type of cell death that was relatively independent of the activation of procaspase-3, but involving the development of mitochondrial stress as evident by the drop in mitochondrial potential and the release of cytochrome-C.

## N5-030P
## Modulation of mitochondrial transition pore components by thyroid hormone

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Thyroid hormone (TH) modulates metabolic efficiency by controlling the coupling of mitochondrial oxidative phosphorylation. However, its uncoupling mode of action is still enigmatic. Mitochondrial transition pore (MTP) is a nonspecific channel that may present itself in two modes: definitive and low conductance (LC). LC-MTP is innocuous and can be a good candidate for modulation of metabolic efficiency. We have previously demonstrated that triiodothyronine (T3) uncoupling was accompanied by MTP gating in isolated liver mitochondria. The aim of this study is to investigate the TH effect on LC-MTP gating in cells and to identify mitochondrial MTP components, induced or suppressed by *in vivo* T3 treatment that may be functionally involved in MTP gating by TH. Treatment of Jurkat or GH3 cells by T3 results in limited cyclosporine A sensitive mitochondrial depolarization, conforming to LC gating of MTP. MTP protein components modulated by T3 treatment were verified in T3 treated and hypothyroid rat liver mitochondria as well as in Jurkat cells. TH treatment resulted in induction of cypD and ANT2, but not of VDAC. However, overexpression of cypD resulted in mitochondrial hyperpolarization while overexpression of ANT2 resulted in cyclosporine A insensitive mitochondrial depolarization, thus indicating that MTP gating by TH was not accounted for by TH induction of these proteins. On the other hand, treatment of euthyroid or hypothyroid rats with T3 induced an increase in liver mitochondrial bax and bak and a decrease in bcl2. Overexpression of bcl2 protected Jurkat cells from T3 induced-depolarization, indicating that bax bcl2 proteins are key factors in mediation of T3 effect on MTP. Hence, TH induced mitochondrial uncoupling may be ascribed to LC-MTP gating, mediated by TH induced increase in mitochondrial proapoptotic combined with decrease in mitochondrial antiapoptotic proteins of the bax bcl2 family.

## N5-031P
## Yeast expression and functional reconstitution of brain-specific mitochondrial uncoupling protein UCP4

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The ability of mitochondrial uncoupling proteins to down-regulate mitochondrial production of reactive oxygen species (ROS) thus playing an "antioxidant role" is essential for the brain. Hence, the expression of three various mitochondrial uncoupling proteins UCP2 [1], UCP4 [2], and BMCP [3] (numbered as UCP5) should not be considered as excessive. In spite of indications of antioxidant properties of UCP2 [1], no biochemical or other characterization of UCP4 has been published since its discovery in 1999 [2]. That is why we have obtained rat UCP4 clone by RT-PCR of brain mRNA, inserted it first into the so-called entry vector, pDONR221 of the Gateway system (Invitrogen, Carlsbad, CA, USA) and transposed the rat UCP4 cDNA using the clonase reaction into the yeast expression vector pYES2-DEST52 of the Gateway system. The amplified vectors were electroporated into *S. cerevisiae* yeast strain JB516. UCP4 expression has been achieved by activation of a galactose promoter under selective conditions. Isolation and reconstitution of UCP4 into liposomes were performed by in principle identical procedure as for yeast-expressed UCP2 [4]. The common properties of mitochondrial uncoupling proteins UCP2 and UCP4 and detail differences are discussed within the context of their antioxidant roles in the brain.

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# N6 – IUBMB Symposium on the Education of Biochemistry and Molecular Biology: Visual Literacy in Biochemistry

## N6-001
## The importance of visual literacy and its assessment in biochemistry

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Visual literacy encompasses the ability to read (understand or make sense of) and write (draw or create) visual displays, including the ability to think, learn, and express oneself in terms of images. Since visual displays such as static diagrams and animated computer images are major tools for the teaching, learning and researching of biochemistry, it is crucial that we produce visually literate students. Although there is an extensive range of computer packages available for the visualization of biochemical phenomena, there has been little or no research done to assess their effectiveness in improving students' visualization and related conceptual understanding in biochemistry. The aim of this paper will be fourfold. Firstly, I shall discuss the meaning and importance of visual literacy and visualization in biochemistry. Secondly, I shall present a model we have developed of the factors affecting students ability to interpret and visualize diagrams and animations. Thirdly, I shall describe various quantitative and qualitative research methods, and type of questions we have developed for the assessment of visual literacy. This will include examples of our research data used to identify visualization difficulties and related misconceptions. Fourthly, I shall discuss various guidelines for improving the teaching and learning with, and design of, diagrams and animations that have emerged from our, and other, research findings. This will lead to a proposal as to how such knowledge might be used by educators, textbook writers and publishers to improve visual literacy in biochemistry.

## N6-002
## The Education Committee of the International Union of Biochemistry and Molecular Biology

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The Committee on Education of the International Union of Biochemistry and Molecular Biology (IUBMB) is involved in a broad range of educational matters. The major aim of the committee is to enhance and improve the knowledge of biochemistry and molecular biology and the associated technical abilities among the scientists who work in these fields. The Committee also fosters activities to improve the educational skills of those whose responsibility it is to teach these subjects at the university or college level. The major focus of the committee is concentrated in those areas of the world, where biochemistry and molecular biology are not as yet well developed. To this end, the committee sponsors workshops in these regions, where participants can discuss modern education and related topics. The committee holds symposia on education at regional biochemical meetings and sponsors, organizes or helps to organize courses, laboratory or educational workshops and symposia. The committee also distributes annually complimentary issues of the specialized educational journal Biochemistry and Molecular Biology Education and other educational materials (such as books or CDs) to scientists and teachers around the world. A more detailed description of activities supported by the committee in the last few years and their impact, will be presented at the symposium.

## N6-003
## From cyberspace to real space: enhancing molecular visualization with physical models of proteins and other biomolecules

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Advances in rapid-prototyping technology and model design software allow creation of accurate physical models of biomolecular structures based on pdb files. For students just beginning to consider the invisible, non-intuitive molecular world, these physical models function as "thinking tools". Used in conjunction with computer visualization programs, the molecular image created and manipulated on a screen also becomes a tactile object to help students make predictions about structure-function relationships. This approach has been field-tested in several undergraduate classroom venues. Results show that physical models and computer visualization tools are synergistic; questions generated by students using the physical models are most easily addressed using computer visualization tools. We have introduced this at the high school level as well: students participating in our SMART team program (Students Modeling A Research Topic) work with researchers to create physical models of proteins under investigation in laboratories. To further the use of physical models to teach molecular literacy, we have established a Lending Library at the Milwaukee School of Engineering to provide models for the classroom. In addition we have introduced simple modeling tools ("Toobers®") that allow "hands-on" exploration of molecular structure and function.

**Acknowledgment:** This work was supported by grants from the National Institutes of Health and the National Science Foundation. A more complete description of this work can be found on the Center for BioMolecular Modeling website (www.rpc.msoe.edu/cbm).

## N6-004
## Designing visual literacy assessments in biochemistry: finding out what students know and do not know, as a prelude to effective instruction.

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In biochemistry, "visual literacy" usually refers to one's ability to recognize important chemical and structural details presented by images, especially 3-D images, of biological molecules. In a broader sense, "visual literacy" also includes other visual tasks, such one's ability to recognize important details presented by graphical representations of functional data. In fact, these two visual skills - structure recognition and graphical interpretation - go hand-in-hand, when defining the structure/function relationships of biomolecules. Because effective biochemistry instruction depends on student mastery of these basic visual skills, an instructor needs to know how "visually literate" students are when instruction starts. Since biochemistry students typically complete introductory chemistry, organic chemistry and introductory biology courses beforehand, it is tempting to assume that the requisite visual skills have been learned from these courses. To test this assumption, students in a year-long biochemistry course were assigned pre-assessment problem sets during the first week of instruction. These were designed to gauge student proficiency at i) recognizing basic structural features (e.g. double bond and, hydrogen atom placement, etc.) implied by typical images of organic molecules and ii) interpreting basic information from graphical representations of simple reversible binding reactions (e.g. weak acid/base titration plots, etc.). Highly varied and surprisingly poor student responses were found suggesting that biochemistry instructors may need to address the visual literacy skills of their students. Pre-assessments, like those described, may help instructors identify basic deficiencies and suggest remedies for improved instruction and student performance.

## N6-005
## 50 Years "Making Metabolism Meaningful, Wonder-full - and FUN"

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Every metabolic pathway from glycolysis onwards has been elucidated in my post-graduate lifetime and the first of my Metabolic Pathways Charts were created just 50 years ago. We will trace their evolution to MINIMAPS and ANIMAPS and thereby demonstrate that biochemistry really can be fun!

## N6-006P
## The teaching of concepts in protein topology using structured classes with the protein folder kit

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Protein Folder is a modular kit developed for the purpose of teaching concepts in protein three-dimensional structure.

Injection molded plastic components can be united in a very flexible manner so as to reproduce (in three dimensions) the cartoon-style representation commonly used in both the specialized literature and in standard biochemistry text books. The kit is faithful to standard stylized representations and therefore intuitive. The models are interactive in the sense that they can be readily assembled and dismantled. This brings with it the advantage that students can put together structures within the time frame of a typical lecture or practical class. Although pre-mounted models may also be of use during standard lecturing, this dynamic characteristic associated with their modular nature, appears to be their greatest advantage over alternative systems. With this in mind a series of exercises has been elaborated, each with the objective of emphasizing the teaching of a particular aspect of protein structure. This may be a particular architecture, topology rule, oligomerization interface, geometrical feature, topological analogy etc. A typical exercise consists of an introduction to basic concepts and biological relevance, followed by objectives and the exercise itself. The latter consists of a limited amount of structural information, which is sufficient to solve the problem at hand, by applying the basic principals already taught. Experience within the classroom setting shows the kit to be of great value in helping fix what otherwise might be rather abstract concepts. Post-use evaluation shows an approval rate of over 90% in almost all aspects evaluated and some of the models will be presented.

## N6-007P
## Peptide charge definition in teaching biochemistry: a graphical approach

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Studying protein biochemistry is based on understanding of physical and chemical properties of amino acids. One of the main characteristics of amino acid is their ionizing ability, that defines the protein properties as well. The graphical approach is based on the knowledge from school mathematics about function's extremum points. The task for the student is to find the ionizable structures in random oligopeptide, to put the appropriate pKa (logarithm of the acid dissociation constant), rolling as function's extremums along the pH axis and reveal the pH intervals with certain peptide charge. Students realize the role of pH in forming of the peptide charge. It is useful to analyze the behavior of the oligopeptide in the whole pH interval from 0 to 14, explaining which amino acid is responsible for the event of the total charge changing. The next task can be to define the direction of movement of peptide mix in the electrophoresis, simulating the situation of fractioning. Another task is to select appropriate buffer solution for better fractioning of the peptide mix. These exercises help the students to understand better the significance of pH gradient in digestion; the role of essential amino acids in nutrition; principles of peptide fractioning; acid-base balance in functioning of the enzymes and the whole organism; manifestations of some mutations, and action of some proteases (e.g. trypsin and carboxypeptidase B).

## N6-008P
## Exploration of bacterial laccase in *Pseudomonas Stutzeri* and its application in bleaching the wood pulp

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Because of their capabilities of catalyzing the oxidation of phenols, laccases are receiving increasing interest as potential industrial enzymes in various applications such as delignification and detoxification. Identification of bacterial laccases for which genetic tools and biotechnological processes are well established may be of significant importance. The present study exploits the capability of laccase secreting bacterium, *Pseudomonas stutzeri*, isolated from a forest site (Roorkee, India) to bleach the wood pulp biologically. Bio-bleaching eliminates the use of chlorine in bleaching process of pulp mills and thus stops the generation of chlorinated toxic pollutants. This bacterium produced laccase as the predominant extracellular phenoloxidase. Among 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS), syringaldazine, veratryl alcohol and vanillic acid - syringaldazine (0.20 mM) were found as the best inducers for laccase induction. Laccase activity in the crude extracellular medium and purified sample was assayed by monitoring the oxidation of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) at 420 nm ($\varepsilon$ = 432 $cm^2$/mM). This enzyme was also found capable to oxidize vanillic acid, catechol and syringaldazine. The purified enzyme was identified as a glycoprotein with a molecular mass of 54 kDa. Use of laccase secreted by *Pseudomonas stutzeri* has been found effective to bleach the wood pulp to an extent of 8% brightness. Bleaching of wood pulp could be observed when unbleached pulp was incubated at 37 °C for two hours in the concentrated extracellular medium, containing enzyme and mediator, 1-hydroxybenzotriazole (HBT).

## N6-009P
## The use of molecular visualization freeware in an internet-based structural biology course

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The School of Crystallography at Birkbeck College has been running online courses in structural biology for almost a decade. We currently offer three one-year courses: principles of protein structure (PPS), techniques in structural molecular biology (TSMB) and protein crystallography (PX). Students may obtain a full masters degree with the successful completion of PPS followed by one of the other two courses. Over 30 students have graduated from the MSc programme since it started in 2001. They come from a variety of backgrounds and many have little or no previous experience of interpreting three-dimensional molecular structures. Molecular visualization is an essential topic within protein structure; we use visualization tools extensively in PPS and occasionally in the other courses. Our choice of visualization tool is crucial if our students are to understand three-dimensional structure and how it drives protein function. Our choice is limited by the fact that we choose not to restrict students' choice of platform. Initially we chose to use Rasmol almost exclusively,

making minimal use of Chime. We have now replaced Rasmol with the Java based plugin, Jmol. This has given us better control over and more flexibility in, customizing the displays that the students see and freed them from the need to learn commands. However, it does have disadvantages: it is memory hungry and can be slow to load. In this presentation, we will illustrate the use of these tools to teach specific concepts in protein structure and compare students' experience of these and other graphics packages.

# N7 – FEBS Symposium on the Education of Biochemistry

## N7-001
## Development of a protein purification practical: providing an integrated set of biochemistry laboratory skills.

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Increasingly, both time, economic pressures and the ever growing content of the biochemistry curriculum put pressure on the ability to deliver practical training. Yet, biochemistry is still fundamentally a practical subject with employers of graduates demanding high-level laboratory skills. One approach to this paradox is an integrated practical course that transmits a whole range of interconnected and modern biochemical laboratory skills. At Leeds we have developed a laboratory practical covering the purification of alkaline phosphatase from *E. coli*. Students experience initial crude enzyme preparation from cell paste, high speed centrifugation, ion exchange chromatography using salt gradient elution, microplate based protein and enzyme activity assays for location of active fractions, determination of enzyme kinetics using a conventional spectrophotometer and assessment of enzyme purity on SDS-PAGE mini-gels. All of this is covered in two (maximum) five-hour sessions using inexpensive commercial equipment (columns, gel rigs) or home made devices (gradient makers, peristaltic pumps). Since antibodies against alkaline phosphatase are widely available, the practical could also be extended to include immunoblotting. Write up is by pro forma with parallel specimen calculations of yield, specific activity and fold-purification, facilitating assessment. Finally, a key aspect to effective delivery of this practical class is to have sufficient postgraduate demonstrator support to troubleshoot and overcome common bottlenecks encountered. Student performance on the practical is good and feedback routinely positive.

## N7-002
## Enzyme kinetics for 21st century biologists – does it make any sense?

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Enzyme kinetics has been an inevitable tool in the field of biological chemistry for many decades. Its golden age in the mid-20th century had occurred as the fundamentals for this scientific discipline were established at the beginning of the last century. Enzyme kinetics has been developed as a powerful technique since that time because of its strength to research enzyme acting characteristics. Although essentially presented in each biochemistry comprehending textbook and/or curriculum it seems that the enzyme kinetics is out of fashion in today's –omic era. Consequently it is introduced to students more from historical point of view than as promising study material worth deep understanding. Enzyme kinetics and especially the analysis of experimental data demand a quantitative mathematical approach of thinking mostly missing in these days graduate biologists. Therefore, the right presentation of enzyme kinetics to a modern biology student together with good laboratory practical experiments could fill the gap between qualitative and quantitative thinking about dynamic molecular biological systems. Such practical and theoretical skills obtained by teaching enzyme kinetics could encourage future biologists to focus on advanced novel biology comprehending courses at the frontiers with other (quantitative based) scientific disciplines and see them as a challenge and not as a barrier in their career. Therefore teaching/learning enzyme kinetics in the 21st century makes sense but the appropriate question is how to do it? The construction of kinetic models describing transformed and raw progress curve experimental data as well as using of appropriate mathematical tools for data analysis could help to indicate the applications also in the other fields of modern biochemistry and molecular biology.

## N7-003
## Taking an experimental approach in basic science education

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Problem-oriented approaches are continuously infiltrating all fields and levels of education. The principles and methods of problem-based learning (PBL) are considered to be superior over those of traditional forms of education: PBL is thought to increase student creativity and motivation, and thereby the efficacy of the teaching/learning process. In basic natural sciences, in which experimentation has a central role, the understanding and memorization of facts and processes by the student can be enhanced by introducing practical and theoretical aspects of scientific research into the process of education. A course of molecular cell biology using such an experimental approach has been implemented and developed in the medical curriculum at Pecs University Medical School. The course includes laboratory practicals in which students perform simple experiments of cell and molecular biology, and PBL-based tutorial sessions to have students analyze experimental situations and interpret research data. In these sessions, various types of problem-solving exercises (such as figure analysis, designing experiments, multiple-choice question-based problem-solving tests) are employed and great emphasis is given to develop student creativity and independence. In addition to the facilitation of the learning process by PBL techniques, problem-solving tests have also been introduced into the course for the assessment of student performance. The main characteristics of this PBL course and experience gathered during its development will be presented.

## N7-004
## Maintaining subject interest through judicious choice of practicals

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Within many institutions introductory biochemistry courses/modules are pursued not only by students undertaking pure biochemistry, but also by those undertaking courses in disciplines such as biotechnology, environmental science, food science, as well as medical and allied sciences. Most such courses encompass a laboratory component, in which lecture-based material is reinforced by appropriate laboratory practicals. We have found that students are more enthusiastic, and generally display improved performance when at least a proportion of the specific experiments undertaken show direct relevance to their core degree area. For example, enzymes utilized to display basic principles of enzymology are often chosen on the basis of ease of assay. Alkaline phosphatase using *p*-nitrophenyl phosphate represents one such example. We have developed a phytase-based enzyme practical as an alternative. Because of its industrial applicability as an animal feed additive (to reduce phosphate pollution), it is of particular interest to students undertaking core programs in biotechnology and environmental science. In terms of teaching principles of antibody-antigen interaction, we have also developed a latex-based agglutination ''pregnancy detection'' assay, in which the students coat latex particles with anti-hCG antibodies and screen samples provided for the presence of hCG (which indicates pregnancy). Ideas for such applied practicals can often be obtained from selected websites, from publications such as pharmaceutical pharmacopoeia or often arising from informal discussions with relevant industry.

## N7-005P
## Mathematics as an indicator of molecular students performance in processes of science

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The successful practice of science is dependent not only on the knowledge of the scientist, but also on the scientist's abilities to practice the processes of science. These processes require skills in designing experiments and communicating data and findings based on analyses, inferences and conclusions derived from existing research. These are called ''science process skills.'' Throughout the training of a scientist, these skills are often not explicitly taught; they are taken as a given in the students by teaching staff. There is evidence that science process skills must be explicitly taught to students for them to be able to exercise them adequately. Some students develop these skills prior to tertiary education and others learn them through observation. Several students however do not have, or gain theses skills without teaching. At tertiary level, curricula are often overloaded and there is no time to teach science process skills. This paper will show that it is possible to predict students' abilities with the processes of science by examining their abilities in mathematics. Students at the University of Pretoria's Genetics Department were given a test instrument, which assessed their abilities in various science process skills and the use of mathematics in genetics research and communication. Data on their performance in mathematics at school exit level were available. It was found that students' abilities in the science process skills tested for were dependent on both their school exit scores in mathematics and the sub-score for mathematics in the test instrument. These findings are relevant for student selection for courses that require the practice of science process skills and where there is no time to explicitly teach the science process skills for the courses.

## N7-006P
## A model of biochemistry dry practical for learning liver functions and bilirubin metabolism in medical school; student feed back

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The biochemical learning issues of the ''Liver Structure and Functions Module'' ongoing in Dokuz Eylül University School of Medicine Problem Based Learning Program for the second year students are: liver and biliary tract functions, related enzymes, bilirubin metabolism, types of jaundice and related laboratory tests. In this module a biochemistry student practical ''interpretation of liver function tests'' has been carried out with 4 clinical case scenarios, with diagnosis pre-hepatic jaundice; hepatic jaundice; post-hepatic jaundice and cirrhosis. The ''practical guide'' consists of brief anamnesis and clinical findings, detailed laboratory test results, figures and schemes, and comprehensive discussion questions. The practical was effectuated interactively by the pre-trained tutors in groups with 7–10 students. This practical was evaluated by 231 students with ''student feed-back forms'' throughout 1999–2002. The percentages of the students ($n=231$) who evaluated all of the criteria as 5 (very good) were: clearness of the objectives (96.8%); clearness of the identification of the objectives (94.5%); efficacy of the practical period (96.1%); adequacy of the material (95.8%); benefit of the discussion (93.9%); organization of the practical (93.6%). Moreover the percentage of the students evaluating all of the criteria as 4 (good) or 5 was determined as at least 95% and the students expressed that they want that type of practicals in other learning issues as well. The results of the ''student feed-back forms'' clearly demonstrate that the biochemistry dry practical with clinical cases containing detailed laboratory test results and comprehensive questions is very effective for learning and the student gladness and benefit is high.

## N7-007P
## Teaching biochemistry at Technical University of Brno

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Technical University of Brno is the second biggest one in Czech Republic. Faculty of Chemistry was rebuilt in 1992. Nowadays this faculty realizes 4 study programs focused on food chemistry and biotechnology, customer chemistry, material chemistry and environmental chemistry. According to Bologna Process it was necessary to establish a new structure of teaching all the basic chemical branches, biochemistry as well. There is a course basic structural biochemistry for all undergraduate study programs, held in second year. This course is completed by enzymology and applied biochemistry. After basic course we provide practices in structural analysis of biopolymers for some branches. For some undergraduate specializations is provided Biochemistry II course held in third year. This course is focused on metabolism, bioenergetics and metabolic regulations. Simultaneously, for UG study program biotechnology basic principles of biotechnology are

introduced and afterwards the course of gene technologies is provided. Both of these courses are realized by combination of traditional form of readings and also by modern form of problem-based learning. Small groups of students work with academic staff member to solve some of problems. Courses of biochemistry and gene technologies have also practices. Teaching of biochemistry disciplines in following master programs is provided by traditional and problem-based learning form as well. Teaching is held for smaller group of students only, thing is, that there could be some problems to ensure sufficient number of skilled teachers and even rooms for teaching. There is a paradox we face at problem-based learning - students have negative opinions to be examined during running courses instead of examination at the end of semester.

### N7-008P
### Actin immobilization on chitin for purifying myosin II: an excellent model for teaching protein purification and immobilization

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The aim of this work was to propose an easy method for teaching protein chemistry to undergraduate students. We took advantage of the muscle contraction properties, such as actin-myosin interaction to propose a simple method for purifying myosin II by affinity chromatography. The proposed affinity chromatography method was based on the production of an affinity column, containing actin molecules immobilized on chitin particles through covalent bonding. For that the following steps were necessary: Chitin particles, from crab carapace, a waste of food industry, were prepared as suggested by Bon et al. (1984); actin was obtained from chicken breast muscle by the procedure of Pardee and Spudich (1982); myosin II was extracted from chicken breast muscle (Bremel and Webel,1975); and actin-F was covalently immobilized to active chitin as suggested by Bon et al. (1984). Then, actin-chitin particles (1 g) were transferred to 5 and 10 ml affinity chromatography columns and different amounts of myosin-enriched preparation (15 to 80 mg) applied to the affinity columns. Unbound protein molecules were washed out with 50 mM Tris buffer pH 7.0 and myosin molecules attached to actin-chitin were displaced and eluted with the same buffer containing 3 mM ATP and 5 mM $MgCl_2$, as detected by SDS-PAGE. Our experience, with this easy and low cost methodology, has been showing that it was an excellent model for teaching protein chemistry to undergraduate students of biological sciences. The students could integrate concepts of cell biology, biochemistry and protein chemistry, and they were able to deal with classical methods of protein purification, such as ultracentrifugation, chromatography and SDS-PAGE, as well as, with protein immobilization on solid supports.

# N8 – FEBS Conference on Science and Society

### N8-001
### Knowledge transfer and translation - major unmet challenges

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### N8-002
### Discussion

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