

A1-Protein Function and Ageing

A1-001

The genetics of human longevity

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An overview of the results of our association studies on candidate genes for human longevity performed in Italian centenarians will be presented. Many genes gave negative results but others showed a positive or negative association with human longevity. Among the last ones a particular attention will be paid to the genes involved in inflammation (IL-6, IL-10, TGFbeta, TLR-4,PPARgamma2), Insulin/IGF-1 signalling pathway and lipid metabolism (Apolipoproteins, CEPT). The data obtained in centenarians and in younger control subjects will be compared with those obtained (on the same polymorphisms) in patients affected by age related diseases (myocardial infarction, Alzheimer's disease and type 2 diabetes). The data which suggest a strong role

of mitochondrial DNA (mtDNA) in human longevity and an interaction with nuclear genes will also be reviewed, with particular attention to mtDNA haplogroups and the C150T mutation. The identification of new longevity genes in a region of Chromosome 1 very rich of Alu sequences using a novel inter-Alu PCR approach will also be illustrated. Finally the strategy which will be adopted by the EU Integrated Project Genetics of healthy ageing (GEHA) for the identification of longevity genes in 90+ sib-pairs (genome scanning and mtDNA re-sequencing) will be presented. On the whole the data we obtained until now are compatible with the hypothesis that a major characteristic of the ageing process is the development of a chronic inflammatory status we proposed to call INFLAMM-AGING, and with the hypothesis that the genetics of human longevity is quite peculiar being a post-reproductive genetics where antagonistic pleiotropy can play a major role and where genes can have quite different biological role and effects at different ages.

A1-002**Longevity and survival factors implicated in human ageing and longevity**

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Ageing and longevity are two multifactorial biological phenomena whose knowledge at molecular level is still limited. We have cloned several senescence-associated genes including ApoJ, a novel survival factor. ApoJ is found over-expressed *in vitro* under a variety of stress conditions and *in vivo* in patients suffering from various age-related diseases as well as in tumours which confer chemotherapeutic drug resistance. In addition, it has been demonstrated that inhibition of endogenous ApoJ expression by RNA interference sensitizes cells to cytotoxicity by activating the cellular apoptotic machinery (*Cancer Res* 2004; **64**: 1834–1842). We have also studied proteasome function in replicative senescence of human fibroblasts. We have observed reduced levels of proteasomal peptidase activities coupled with increased levels of both oxidized and ubiquitinated proteins in senescent cells. We have found the catalytic subunits of the 20S complex and subunits of the 19S regulatory complex to be down-regulated in senescent cells. This is accompanied by a decrease in the level of both 20S and 26S complexes. Inhibition of proteasomes in young cells caused by treatment with specific inhibitors induced a senescence-like phenotype. Stable over-expression of $\beta 5$ subunit in various cell lines resulted in elevated levels of other β -type subunits, in higher rates of assembled proteasomes and in increased levels of all three proteasome activities. Functional studies have shown that these “proteasome activated” cell lines confer enhanced survival following treatment with various oxidants. Finally we have found that stable over expression of the $\beta 5$ subunit delays senescence in human fibroblast cultures (*J Biol Chem* 2003; **278**: 28026–28037, *J Biol Chem*, in press, 2005).

A1-003**Ageing intervention, prevention and maintenance of proteomic integrity.**

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Ageing is characterized by a progressive accumulation of molecular damage at the level of nucleic acids, lipids and proteins. The main reason for age-related accumulation of damage is the failure of maintenance, repair and turnover pathways, such as nucleic acid repair, antioxidant defences and proteasomal and lysosomal activities. Therefore, the ideal approach for ageing intervention and prevention is to stimulate these biochemical pathways by physical, chemical and biological means. One such approach, termed hormesis, is to make use of the homeostatic/homeodynamic stress response ability of cells and organisms by challenging them with low doses of different stresses. In a series of experimental studies we have shown that repetitive mild heat shock (RMHS) has beneficial and anti-ageing effects on human skin fibroblasts and *Drosophila*. We have reported the hormetic effects of RMHS at the levels of maintenance of stress protein profile, reduction in the accumulation of oxidatively and glycoxidatively damaged proteins, stimulation of proteasomal activities for the degradation of abnormal proteins, enhanced cellular resistance to ethanol, hydrogenperoxide, sugars and UV-B, and increased levels of various antioxidant enzymes. We have also observed the hormetic maintenance of phosphorylation and dephosphorylation states of ER-, JN- and MAP-kinases as a measure of cellular responsiveness to mild and severe heat stress.

Further studies are in progress to determine the effects of repeated mild stresses (heat, sugars and mechanical) on the maintenance of the proteomic integrity in terms of post-translational modifications of stress proteins, cytoskeletal components, proteasomal subunits and protein synthesis factors in mortal and immortalized cell lines.

A1-004**Cellular phenotypes with increased and reduced levels of mortalin protein**

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Mortalin, also known as mthsp70/GRP75/PBP74 is a heat uninducible member of hsp70 family of proteins. It is differentially distributed in cells with normal and immortal phenotypes. It has been assigned to various subcellular sites and has multiple binding partners and functions. The lifespan of human foreskin fibroblasts (HFF5), cultured under standard *in vitro* conditions (including ambient atmospheric oxygen tension), was extended slightly by expression of exogenous mortalin (mot-2)/mthsp70/Grp75, but not by the catalytic subunit of telomerase, hTERT. Together, mot-2 and hTERT permitted bypass of senescence, a substantial extension of lifespan, and possibly immortalization demonstrating that mot-2 and telomerase can cooperate in the immortalization process. Cells compromised for mortalin expression by hammerhead ribozymes, antisense and siRNA showed growth arrest suggesting that a threshold level of mortalin is essential for cell proliferation. Knock-down of mortalin expression by siRNA expression plasmid in human transformed cells resulted in apoptosis suggesting that mortalin-targeting may be employed for cancer therapy.

A1-005**T-lymphocytes activation, lipid rafts and aging: links for immuno-senescence**T. Fulop¹, A. Larbi¹, A. Khalil¹, N. Douziche¹, C. Fortin¹ and G. Dupuis²*¹Centre de Recherche sur le vieillissement, Dept. de Médecine, Service de Gériatrie, Université de Sherbrooke, Sherbrooke, Québec Canada, ²Centre de Recherche Clinique, Dept. de Biochimie, Université de Sherbrooke, Sherbrooke, Québec Canada. E-mail: tamas.fulop@usherbrooke.ca*

Ageing is associated with an increased susceptibility to infections, cancer, auto-immune disease. Adaptive immunity especially T-lymphocytes are the most affected by aging and this is mainly explained by impairments in T-cell receptor (TcR) signaling. Recent findings suggest that cholesterol-enriched microdomains called lipid rafts act as a platform in the initiation of T-cell activation by formation of the initial complex of signal transduction. Since the age-related immune deficiencies are accompanied by defects in TcR signaling, our laboratories sought to determine the links between lipid rafts and immune senescence. We studied lipid rafts composition in CD4+ and CD8+ T-cells from young and elderly donors. We found that CD4+ T-cells activation completely rely on lipid rafts polarization whereas that of CD8+ T-cells did not need lipid rafts polarization. We also found that resting CD8+ T-cells already possess triggered lipid rafts. Moreover CD4+ T-cells signaling is severely impaired in aging while CD8+ T-cells respond to stimulation when compared to young donors. The age-related increase in cholesterol content of lipid rafts is accompanied with a decline in rafts fluidity. Studies on HDL-driven cholesterol transport indicate that the pool of

cholesterol in lipid rafts is differentially extracted with aging suggesting defects in this process of membrane cholesterol regulation. Overloading T-cells with cholesterol induced a decrease in signaling molecules phosphorylation (Lck, LAT, Akt) following CD3 and CD28 ligation. Both CD4⁺ and CD8⁺ T-cell cholesterol content is increased in aging however, CD8⁺ T-cells did not rely on lipid rafts for their activation explaining why changes in rafts properties (cholesterol content, fluidity, signaling molecule composition) did not have such consequence on activation as observed in the case of CD4⁺ T-cells.

A1-006

Glycoprofiling of N-linked serum protein: an aging biomarker?

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In humans, the aging process seems to be primarily under genetic control. Age-dependent diseases develop on this background as a consequence of other factors. Due to the rapidly increasing number of elderly people in many countries, there is a need for innovative treatments for age-related diseases. Therefore, in addition to studying aging mechanisms, the identification of candidate aging biomarkers to measure age-related changes may be of great value not only to gerontologists, but also to people in general, by preventing age-related diseases through development of anti-aging medicines. It is well known that the N-linked glycans of glycoproteins play important biological roles by influencing the functions of glycoproteins. Although many studies reported the importance of the structural changes of glycans during development, little information is available on these changes during aging. Accordingly, age-related alterations of the glycans are relevant to the understanding of the physiological changes found in aged individuals. In this study, we demonstrated that the serum concentrations of N-linked sugar structures changes during aging in human beings and mouse. These changes of N-glycans in serum are independent of the modification of Ig glycosylation. Moreover, the serum N-glycoprofiling is species dependent, with age related peaks that are specific for a defined species. Thus, N-glycoprofiling could be used as an aging biomarker to predict the condition of human and animal health. In a similar way, the N-glycan profile may be especially interesting for testing the effects of dietary compounds and/or medications on the global health status of an animal, including humans.

A1-007P

Yeast growth stimulation and suppression of arginaza and enzymes of proline biosynthesis with the help of herbal extracts

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In our research we have used extracts of some herbs as – mother wort (*Artemisia absinthium*), St.Johns wort (*Hypericum perforatum* L.) and milfoil absint (*Achillea millefolium* L.), as stimulators for the *Candida guilliermondii* yeast growth. This brought about

biomass increase 2.5–3 times. A strongly pronounced inverse correlation between the accumulation of yeast biomass and the content of free proline in it is established. The scientific work carried out at our laboratory based on a number of research objects (bean harricot butterfly, pea shoot, infusorian, rat lactic gland) confirm that the intensively growing plants and animal cells oxidize the free proline at a maximal rate. By fractionation of extracts of wheat shoot on Sephadex G-150 the active fraction, containing stimulators of yeast growth was revealed. Suppression of some enzymes and their isoenzymes activity was observed, in particular that of arginaza and enzymes of proline biosynthesis, and glutamate dehydrogenase of yeasts *Candida guilliermondii*. The activity of high molecular and low molecular arginaza isoenzymes is suppressed under the influence of St. John's wort extracts 3 and 6 times respectively. The activity of glutamate dehydrogenase decreases about 1.5–2 times. The herbs which are studied are successfully used to cure diabetes, kidney and digestion system diseases and some others. The herbs contain proline in considerable amount. The content of the active fraction is recrystallized and subjected to X-ray structural analysis. The preliminary results revealed 1-prolineamin, butilene ether, N-methylproline and other compounds. The three-time increase of biomass is detected in yeast growing in the presence of the active fraction. The situation is the same in the presence of wheat shoot extraction.

A1-008P

Identification of metal-containing proteins in soybean milk by size-exclusion-reversed-phase chromatography and electrospray Q-TOF mass spectrometry

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Soybean possesses many medical qualities. This fact can be explained by the contrast between the acid character of most proteins and the high alkaline-bearing salts present in soybean, which can be regarded as a curative diet. The Chinese culture make a copious consumption of soybeans considered as a highly healthy food, which has been corroborated by recent studies from European and American laboratories. The great variety of soybean products commercially available and their growing use have prompted the development of analytical methods for their quality assurance. Among the techniques used to separate soybean proteins, high-performance liquid chromatography (HPLC) is the most widely used in different modes, namely, size exclusion (SEC), ion-exchange (IEC), reversed phase (RPC) and perfusion chromatography (1). The characterization of metalloproteins is the key to numerous studies related to the role that many elements play in life. Presence of metals in the biological systems is crucial for cell signaling, gene expression, enzyme action and other fundamental (bio)processes. As a consequence, interactions between metals and organic biomolecules have been the focus of many chemists and biochemists, who realized that selection of chemical elements by cells exhibits a great degree of sophistication and involves a variety of paths for each element in any organism. In this way, a new and promising –omics field related to the characterization of metal bound to proteins (metalloproteomics) (2) is emerging. The goal of this work is to identify and characterize metalloproteins in soybean milk and their quantification using a soybean protein isolate as external standard for calibration. High-performance size exclusion chromatography (HPSEC) directly coupled to diode array (DAD) and inductively coupled plasma-mass spectrometry (ICP-MS) was used for this purpose. The tryptic digest of protein fractions isolated by size-exclusion

chromatography was analyzed by reversed phase HPLC/ICPMS. For the identification of metalloproteins electrospray Q-TOF mass spectrometry has been used.

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A1-009P

Aging regulates neuronal nitric oxide function in rat mesenteric artery: role of gender

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This study examines how gender influences the effect of aging on the neuronal nitric oxide (NO) function in rat mesenteric arteries. For this purpose, endothelium-denuded mesenteric arteries from young and old female (in estrous phase) and male Sprague Dawley rats were used to analyze the vasomotor response to: (i) electrical field stimulation (EFS); (ii) NO donor sodium nitroprusside (SNP), and (iii) the cGMP analogue, 8Br-cGMP. EFS induced frequency-dependent contractions in arteries from all of the rat groups. In arteries from male rats, the NO synthase (NOS) inhibitor N^w-nitro-arginine-methyl ester (L-NAME) increased EFS-elicited contraction only in arteries from young rats. In nor-adrenaline- (NA) pre-contracted segments, SNP induced a vasodilator response, which was similar in segments from young and old male rats. In arteries from female rats, L-NAME increased the EFS-elicited contraction in arteries from young and old female rats to a similar extent. In NA-pre-contracted segments from female rats, SNP induced a vasodilator response, which was greater in segments from old than young rats. Pre-incubation of female segments with superoxide dismutase enhanced the response to SNP only in arteries from old female rats. In NA-pre-contracted segments from female rats, 8Br-cGMP induced a greater relaxation in arteries from old than from young female rats. These results indicate that aging: (i) decreases the neurogenic NO release induced by EFS in male rats, while does not modify it in arteries from female rats; (ii) increases the NO metabolism only in arteries from female rats; and (iii) increases the sensitivity to NO of vascular smooth muscle in arteries from female rats.

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A1-010P

The local structure for the binuclear (type 3) copper sites of hemocyanins, as investigated by X-ray absorption spectroscopy

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XAS studies for the hemocyanins are of primary importance due to the high molecular weight of these proteins that cause a lack of crystallographic data and the unfeasibility of NMR experiments. We have studied (1) the solution structure of the binuclear Cu(II) site of the met- and met-azido derivatives of two Hcs, from the mollusc *Octopus vulgaris* and the arthropod *Carcinus*

aestuarii. Comparative studies on ligand binding reactions with molluscan *O. vulgaris* and arthropod *C. aestuarii* Hcs, at different conditions of pH, are of particular interest to understand both the peculiar organization of the protein chain and the structural rearrangement in the active site region. The few Protein Data Bank codes for native oxy-forms from different species show that the site is more rigid and less accessible in arthropod than in molluscan proteins. In both cases, the two copper ions, Cu_A and Cu_B, are not equivalent and the Cu_A is the more exposed. Our results (2, 3) have shown how it is possible to extract quantitative information in the case of a binuclear centre. I will show how it is possible, by the XAS characterization in the low energy region with the help of the multiple-scattering analysis, to refine the structure of the site in order to select different contributions for the local structure of the two copper centres. The ultimate aim of this study is to disclose the structural differences, which allow the protein from the mollusc *O. vulgaris* to exhibit tyrosinase-like activity and the catalase activity present in the met form.

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A1-011P

Kinetics of formation of hydrophobic domains in fibrillar amyloid beta-protein

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Based on diphenylhexatriene (DPH) interaction with fibrillar amyloid beta-protein (A β), we have reported recently that A β forms hydrophobic domains during its fibrillization. The interaction of DPH and its charged analogue trimethylammonium (TMA)-DPH with fibrillar A β (fA β) did not change the emission spectra of DPH or TMA-DPH. This interaction was time-dependent for DPH while it was immediate for TMA-DPH, and it exhibited saturation kinetics with respect to concentration of DPH as well as TMA-DPH. The partition coefficients of DPH and TMA-DPH into fA β 40 were 2.41×10^7 and 2.01×10^6 respectively. Sonication of the fA β /DPH and fA β /TMA-DPH showed that packing of A β 42 is different from that of A β 40. While sonication of A β 40 fibrils did not affect the fluorescence intensity of DPH or TMA-DPH, the fluorescence of fA β 42/DPH or TMA-DPH increased with increase in sonication time. These results indicate that the hydrophobic domains formed during fibrillization of A β 42 are not completely accessible to DPH or TMA-DPH initially, and become fully accessible upon sonication. DPH interaction with fA β 40, fA β 42 and brain phosphatidylcholine liposomes with respect to temperature showed that fluorescence intensity decreases with increase in temperature during incubation of DPH with A β 40 /42 or liposomal membrane. However, the slope of decrease in fluorescence was higher in case of fA β as compared to that in liposomes. These results demonstrate that (a) fA β forms hydrophobic domains, (b) folding of fA β 42 is different from that of fA β 40, and (c) there is similarity between interaction of DPH with biological membrane and fA β , but this interaction is more pronounced with fA β . In conclusion, DPH or TMA-DPH can be used to measure the fibrillization of A β and to understand the physical packing of the amyloid fibrils.

A1-012P**Nanog changing in mouse kidneys with age**

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Nanog has been discovered that is essential for mouse and human embryonic stem cells (ESC) pluripotency and self-renewal. It is also expressed in several adult murine tissues by using reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. However, human Nanog transcripts have been isolated from adult bone marrow (EST, BF893620). Here, we study the Nanog gene expression profiling in the isolated mouse renal papillary cells that were confirmed by assessment of expression by Northern blots, RT-PCR. Mice renal cells whole RNA was got from fresh renal tissues, Phosphate Buffered Saline (PBS) infusion renal tissues, and the isolated mouse renal papillary cells respectively, as well as the renal papillary tissue from 18.5 days post-coitum (d.p.c.) fetal, 1–2 weeks young, 1–8 months adult and 24 months old. Our analysis shows that, a very low expression level were detected in mouse renal tissues, and the renal papillary cells express more than other tissues with northern blot and RT-PCR. This data suggest that kidney has its own Nanog expression cells exclude that from bone marrow derived cells, and Nanog expression loss in an age-dependent manner in the kidney, either due to developmental factors or aging, particularly in renal papillary tissue.

A1-013P**Therapeutic angiogenesis: searching for new paths to induce the production of new blood vessels**

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Introduction: Fibroblast growth factor (FGF)-1 is a potent angiogenic factor, able to induce the growth of new blood vessels, *in vivo*. For that reason, it is actively investigated as a possible candidate for gene therapy in ischemic heart disease. An alternative strategy to gene transfer is to boost the production and release of angiogenic factors in the ischemic heart. However, *in vivo*, FGF-1 secretion is active only under stress conditions. Therefore, to understand how to turn on the FGF-1 release pathway independently of acute stress would be a useful therapeutic approach to ischemic heart disease.

Methods and Results: Using two *in vitro* models of FGF-1 secretion – murine fibroblasts stimulated by heat shock and human melanoma cells stimulated by starvation – we studied the intracellular signaling regulating FGF-1 release. We demonstrated that inhibition of PI3-kinase/Akt mediated signal resulted in a significant attenuation of FGF-1 secretion. Moreover, in fibroblasts transfected with a constitutively active form of Akt (myr-Akt), FGF-1 was released in the medium even under conditions of no stress. We also noticed that these cells displayed higher levels of alphaB-crystallin and HSP70 as compared to controls.

Conclusions: The mechanism of release of FGF-1 is a stress-dependent event, which is regulated by PI3-kinase/Akt signaling. Activation of Akt results in an increased amount of angiogenic

factor released in the medium. What lays downstream Akt activation that is able to induce FGF-1 secretion is not known. However, heat shock proteins might be involved.

A1-014P**Characterization of potato (*Solanum tuberosum* L.) tuber ageing using physiological and proteomic markers (2D-PAGE).**

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The Physiological age of potato seed tubers greatly influences their agronomical performance. However, a reliable ageing index that could be used prior to planting is still lacking. In order to fill this gap, potato seed tubers (cv Désirée) were stored at 4 °C for 7 months and regularly sampled (10 time points) to assess and correlate both physiological and biochemical markers. Different physiological ageing parameters (Physiological Age Index [PAI], incubation period characterizing the duration between sprouting and daughter tubers production, measure of the longest sprout) were evaluated by recording the germination parameters of 40 tubers for each time point. Polynomial and linear models can readily be adjusted on PAI and incubation period data in order to define a robust frame of reference that could replace the chronological age in later studies. A complementary biochemical approach using two-dimensional polyacrylamide gel electrophoresis has then been set up. Two sample preparation methods using respectively SDS-containing extraction buffer and phenol-phase extraction were compared. The best profiles were obtained using the hot SDS extraction technique. For each time point, protein profile of 15 mixed sample tubers was assessed in order to discover protein markers of the ageing process and to correlate them with our germination-based physiological data. Preliminary results of extreme samples comparison (oldest vs. youngest sample) are shown.

A1-015P**The pro-inflammatory phenotype of senescent human cells *in vitro* and *in vivo*: the p53-mediated ICAM-1 over-expression**

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Most normal somatic cells after a certain number of divisions enter a state called replicative senescence, characterized by irreversible growth arrest. Moreover, they express a pronounced inflammatory phenotype that could contribute to the ageing process and the development of age-related pathologies. Among the molecules involved in inflammatory response that are over-expressed in senescent cells and aged tissues is intercellular adhesion molecule-1 (ICAM-1). We have recently reported that the transcriptional activator p53 can trigger ICAM-1 expression in an NF- κ B-independent manner (*Embo J* 2003; **22**: 1567–1578). Furthermore, p53 exhibits an increased transcriptional activity in senescent cells. Accordingly, we investigated whether p53

activation is responsible for the senescence-associated ICAM-1 over-expression. To this end, we used two model systems of cellular senescence: (a) human fibroblasts and (b) conditionally immortalized human vascular smooth muscle cells. Here, we present evidence from both cell systems to support a p53-mediated ICAM-1 over-expression in senescent cells that is NF- κ B independent. Furthermore, ICAM-1 seems to be critical in the development of atherosclerosis, an age-related, chronic inflammatory disease. So, we have demonstrated in atherosclerotic lesions the presence of cells co-expressing activated p53, ICAM-1, and stained with the senescence-associated β -galactosidase, a biomarker of replicative senescence. Collectively our data suggest a direct functional link between p53 and ICAM-1 in senescence and age-related disorders.

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A1-016P **CARF is a key regulator of p19ARF-p53-HDM2-p21WAF1 senescence pathway: biochemical and visual analyses**

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The INK4a locus on chromosome 9p21 encodes two structurally distinct tumor suppressor proteins, p16INK4a and the alternative reading frame protein, ARF (p19ARF in mouse and p14ARF in human). Each of these proteins has a role in senescence of primary cells, and activates pathways for cell cycle control and tumor suppression. We had previously identified a novel collaborator of ARF, CARF, from a two-hybrid interactive screen using p19ARF as bait (1). CARF is a nuclear protein, co-localizes and interacts with ARF in the perinucleolar region. It is co-regulated with ARF and cooperates with it in activating p53 (2). In the absence of ARF, CARF supports p53 function directly. It binds to p53 in the nucleoplasm and activates its transcriptional activation function. By employing a variety of approaches including overexpression of CARF, its suppression by siRNA and the use of protease inhibitors, we demonstrate that CARF not only regulates p19ARF-p53-p21 pathway by more than one way but it also interacts with another important player of this pathway i.e., MDM2, an antagonist of p53, and exerts another level of control.

A1-017P **Mitochondrial chaperones mortalin/mthsp70 and hsp60 are functionally distinct**

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Mortalin/mthsp70 and Hsp60 are heat shock proteins that reside in multiple subcellular compartments; mitochondria being the dominant one. We present biochemical evidence for their *in vivo* and *in vitro* interactions. With the use of Quantum dots (powerful tool used for simultaneous imaging of multiple proteins), we

visualized minute differences in subcellular niche of these two proteins in normal and cancer cells (1,2). Knock down of either of these two by shRNA expression plasmids caused growth arrest of osteosarcoma cells. Whereas an overexpression of mortalin extended *in vitro* lifespan of normal fibroblasts (TIG-1) (3), overexpression of hsp60 was neutral. Furthermore, an induction of senescence by expression of p14ARF in osteosarcoma cells involved down-regulation of mortalin only. Taken together, the study for the first time delineates (a) interactions of mortalin and hsp60, (b) their minute differences in subcellular distribution, (c) their involvement in tumorigenesis, and (d) functional distinction in pathways involved in senescence.

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A1-018P **Age and ethanol-induced oxidative stress: impact of exercise training on glutathione metabolism in rat myocardium.**

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The interactive effects of exercise training and ethanol on oxidative stress and free radical detoxification in the myocardium of young and old rats with special reference to glutathione metabolism was studied. Male wistar rats of younger (3 months) and older (18 months) age groups were trained as follows: 1) Sedentary Control (SC); 2) Exercise training (Ex) for 2 months; 3) Ethanol treatment (Et) (2 g/kg) for 2 months; 4) Exercise plus Ethanol treatment (Ex+Et) for 2 months. The activity levels of glutathione reductase (GR), glutathione peroxidase (GPx), glutathione-s-transferase (GST) and glutathione (GSH) content were estimated in the myocardial tissue under the ethanol and age-induced oxidative stress and with the interactive effects of exercise training by employing the standard methods. The rats exhibited significant changes in the specific activities of myocardial GSH content, GR, GPx and GST activities under the exercise and ethanol induced oxidative stress with reference to ageing. In the present study exercise training significantly inhibited the activities of these enzymes in both the age groups. Inhibition of GR and GSH indicates reduced synthesis of GSH during ethanol-induced oxidative stress. The increased activity of GPx during exercise training indicates enhanced detoxification of hydroperoxides, suggesting a protective role of this enzyme in reducing hydroperoxides and lipid peroxides. The stimulation of GST indicates involvement of multifunctional proteins in the detoxification processes. The present findings suggest that the biochemical changes due to ethanol-induced oxidative stress in the enzyme activities of glutathione metabolism are significantly altered with exercise training in both age groups of rats.

A1-019P**Coordination of base excision repair studied by photoreactive DNA probes and functional assays.**

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Cellular DNA is continuously damaged by endogenous and exogenous reactive species. The outcome of DNA damage is generally adverse, contributing to ageing and cancer. DNA-repair pathways represent multiprotein systems catalyzing transformations of DNA. A central goal of molecular biology of DNA repair is to understand the molecular basis employed by protein machines to fight against genotoxic stress. Photoaffinity labelling technique has been developed to study assembly of base excision repair (BER) proteins around DNA. Photoreactive DNA intermediates of BER pathways were created in cellular and nuclear extracts to identify proteins interacting with damaged DNA. The main target proteins interacting with branch point BER intermediate were identified as poly(ADP-ribose) polymerase1 (PARP1), flap endonuclease1 (FEN1), DNA polymerase b (Polb) and apurinic/apyrimidinic endonuclease1 (APE1). The results indicate that APE1 and PARP1 interact preferentially with nicked BER intermediate carrying photoreactive dNMP residue at the 3'-end and the 5'-sugarphosphate moiety, whereas intermediate with 5'-phosphate is less favourable interaction partner. Thus, PARP1 and APE1 can discriminate DNA intermediates of BER pathways to regulate the process. The efficiency of DNA repair synthesis catalyzed by Polb is modulated by the interplay between Polb, APE1, PARP1 and XRCC1. APE1 can perform stimulation of Polb activity and proofreading function. Our study further established that photoaffinity labelling combined to functional assay is a powerful tool to explore proteomic ensembles of DNA repair.

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A1-020P**The influence of BRCA1 mutation on the response of cells to chemopreventive substances**I. Misiewicz¹, K. Skupinska¹ and T. Kasprzycka-Guttman^{1,2}*¹Laboratory of Confocal Microscopy, National Institute of Public Health, Warsaw, Poland, ²Department of Chemistry, Warsaw University, Warsaw, Poland. E-mail: misiewicz@il.waw.pl*

BRCA1 protein plays a central role in cell maintenance, growth, cell cycle and apoptosis. Inherited BRCA1 mutations are responsible for hereditary breast and ovarian cancer. Most common BRCA1 mutations among polish population are: C61G, causing loss of ubiquitin ligase activity of BRCA1 protein, 3819 del 5 and 4153 del A (both in exon 11) cause the frame shift and probably formation of stop codon and termination of protein and 5382 INS C that alter transcriptional activity due to alteration of association with RNA polymerase II holoenzyme. In the study, the influence of single allele mutation on the response of cells to various isothiocyanates was evaluated. Isothiocyanates are the group of natural substances that prevent and block carcinogenesis. The alterations in cell cycle phases distribution, the changes in mitochondrial membrane potential and in cell membrane asymmetry, after isothiocyanate treatment of BRCA1 heterozygous cells was determined. Our results show that the cell cycle was altered variously in differently BRCA1 mutated cells, comparing to control non-mutated cells. Moreover the sensitivity of cells to apoptosis induction was differentiated depending on the

mutation type. The results indicate the strong impact of BRCA1 mutation type on cell maintenance and sensitivity to chemoprevention agents.

A1-021P**Enhanced proteasome-dependent degradation of the CDK inhibitor p27kip1 in immortalized lymphocytes from Alzheimer's dementia patients**

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Recent evidence supports the idea that dysregulation of cell cycle control plays a role in the pathogenesis of Alzheimer's dementia (AD), where postmitotic neurons display various cell cycle markers, prior to degeneration. Cell cycle disturbances are also observed in peripheral cells from AD patients. Previous work from this laboratory established a molecular link between decreased cellular content of the CDK inhibitor, 27^{kip1} (p27) and enhanced phosphorylation of pRb family proteins and cell proliferation of immortalized lymphocytes from AD patients upon serum stimulation. Calmodulin antagonists and the PPAR γ ligand 15d-PGJ₂ treatment to AD cells increased the levels of p27 and blocked the serum-mediated enhanced cell proliferation. This work was undertaken to evaluate the molecular basis involved in regulating the abundance of p27 in AD cells. It was observed that the half-life of p27 protein in serum-activated cells was reduced in lymphoblasts from AD patients as compared with that of cells from age-matched control individuals. Both, the calmodulin antagonist, calmidazolium, and 15d-PGJ₂ had no effect in control cells but increased the stability of the p27 protein in AD cells. The effect of these compounds was mimicked by the inhibitor of the proteasome MG132, suggesting an altered degradation of p27 by the 26S proteasome in AD lymphoblasts. The role of Ca²⁺/calmodulin signaling pathway and PPAR γ activation on p27 phosphorylation and ubiquitylation will be discussed. The distinct features of cell cycle control, by controlling the levels of key regulatory proteins, in peripheral cells from AD patients offer an invaluable, noninvasive, tool to investigate the etiopathogenesis and eventually for the early diagnosis and prognosis of this devastating disease.

A1-022P**Towards the elucidation of a physiological role of the AtNUDT4.1 protein, the *Arabidopsis thaliana* homologue of the mammalian GFG proteins**

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Mammalian GFG proteins, members of a Nudix family, are encoded by antisense fibroblast growth factor (FGF) mRNAs. In the human pituitary the GFG protein enhances prolactin expression and inhibits cell proliferation (1). In addition, it was shown that the rat GFG protein has antimitator nucleotide hydrolase activity since it can partially complement mutT mutation in a MutT-deficient *E. coli* strain (2). The *Arabidopsis thaliana* family of the GFG homologues consists of seven members. Similar to the mammalian proteins, they all possess conserved Nudix domains characteristic for a family of proteins which catalyze

mostly the hydrolysis of nucleoside diphosphates derivatives including: nucleotide triphosphates NTP, nucleotide sugars, NADH, NAD, FAD, coenzymeA, m7GTP-mRNA cap, dinucleoside polyphosphates (3). We have shown previously that, despite the homology to the GFG proteins, the *A. thaliana* AtNUDT4.1 protein does not complement MutT function in *E. coli* mutT mutator strain nor can it hydrolyze mutagenic 8-oxo dGTP, a main substrate of the MutT protein. Instead, using the reaction conditions typical for Nudix enzymes, AtNUDT4.1 was active mainly on ADP-ribose (4). To further elucidate a physiological role of the AtNUDT4.1 protein we have applied a pull-down method to search for its cellular partners. We have used the GST tagged AtNUDT4.1 protein as bait and *A. thaliana* cellular extracts as a source of protein partners. The results from two independent experiments indicate that the AtNUDT4.1 can cooperate within a cell with at least seven different proteins including: Hsp 70, GRF, LEA, WD-40, tubulin, ATP-synthase and methionine synthase. The experiments validating these results are in progress.

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A1-023P

The one-and-only calcium in neurodegeneration

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Introduction: Efforts to elucidate the pathomechanism of beta-amyloid peptide and its precursor protein in Alzheimer's disease and other factors in diverse neurodegenerative disorders have yielded an increasing pile of hypotheses. When analyzing thousands of scientific papers, the involvement of the central secondary messenger, calcium, becomes apparent.

Methods: Resting intracellular calcium concentration of neurons, glias, fibroblasts and lymphocytes were assessed utilizing comparative fluorimetric methods with or without treatment of cultures with beta-amyloid. Amyloid-precursor-protein levels and gene-expression profiles were determined using Western-immunoblot and DNA micro-chips. Medline search was performed to supplement and justify the involvement of calcium in various neurodegenerative disorders.

Results: Disturbed calcium homeostasis is present in all cell-types examined after beta-amyloid treatment. Medline-search points out the role of calcium dysregulation in several neurometabolic disorders, including schizophrenia, Parkinson's, Huntington's, amyotrophic lateral sclerosis, etc. Metabolites of the amyloid-precursor are strongly associated with calcium-induced cellular changes both at the proteomics and genetics level as confirmed by immunoblot and gene-chip analysis.

Discussion: Our results and data from Medline-search confirm that calcium imbalance might be a common underlying factor in brain pathologies. Disturbed calcium interferes with some of the many biochemical pathways characteristic of a certain disorder, determined by environmental and genetic factors, yielding disease-specific pathologies. Both calcium-mediated neuroprotection and neurotoxicity, therefore, is proposed in this study. By

targeting calcium, this new information promises to broaden our understanding of health and illness and the approaches we take to treating disease.

A1-024P

Complexation of supramolecular dye Congo red with immunoglobulins. The possible mechanism of dye-induced stabilization of antigen-antibody complexes.

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Congo red (CR) is commonly used as a specific ligand for amyloids. This dye, characterized by high self-assembling tendency, complexes to proteins by adhesion of the ribbon-like supramolecular ligand to polypeptide chains of beta-conformation. Complexation is allowed by local or global protein destabilization which can be caused by mutations or unfolding conditions, and can also result from structural constraints associated with biological function, as in case of antigen-binding derived torsional constraints in immunoglobulins. CR binding to antibodies significantly enhances the stability of immune complexes. The immunoglobulin light chain lambda was used as a model in studies of the mechanism of CR-antibody interaction. At elevated temperatures, it forms two distinct kinds of complexes with CR, easily differentiated as slow- and fast-migrating electrophoretic fractions, bearing four and eight-dye-molecule ligands, respectively. The slow-migrating complex is formed after displacement of the N-terminal polypeptide chain fragment. According to molecular dynamics simulations, binding of CR causes the disruption of beta structure in the V domain, increasing plasticity of the antigen binding site. Higher fluctuation of CDR loops can enhance antigen binding and allow even low affinity antibodies to form complexes with the antigen. Increased stability of antigen-antibody complexes in presence of CR red was studied using antibodies of different origin and specificity to agglutinate red blood cells. The effect was not observed for (Fab)₂ fragments, proving that CR binding can be induced only in complete immunoglobulins under constraints caused by simultaneous attachment to two antigenic determinants.

A1-025P

Lactadherin binds to arterial and dermal elastic fibers

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Lactadherin is a ubiquitously expressed, multifunctional protein. It consists of an epidermal growth factor-like domain with an Arg-Gly-Asp motif in the N-terminus and a 50 amino acid residue large fragment, called medin, in the C-terminus. This fragment is cleaved out and forms the most common form of amyloid, which is deposited in arteries. Earlier immunohistochemical work has

revealed that lactadherin-derived amyloid appeared in close association with elastic fibers. These findings encouraged us to study whether lactadherin interacts with tropoelastin, the main component of elastic fibers. Formalin-fixed and paraffin embedded human aortic and skin materials together with an anti-lactadherin-antibody were used for immunohistochemical and electron microscopical techniques. Results from these experiments show a clear labeling of the antibody close to elastic structures. For the first time lactadherin was demonstrated in the skin. An *in vitro* study, with recombinant tropoelastin and lactadherin in a solid phase binding assay, confirmed the interaction. Further characterization of the interaction by solid phase binding and surface plasmon resonance assays suggested that it is the median domain that binds tropoelastin. Lactadherin has been shown to bind to integrins on cells via its Arg-Gly-Asp motif. Lactadherin could organize elastic structures by linking them to smooth muscle cells and as a consequence this interaction might be of structural importance. Other studies have shown that murine lactadherin is an opsonin that links macrophages to apoptotic cells thereby promoting engulfment. Elastin is routinely degraded in skin and possibly lactadherin supports clearance by binding to elastin fragments, thus signaling for engulfment.

A1-026P

Impaired regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in aged rat liver: a new role for ROS

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With ageing, rat hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR), the key enzyme of cholesterol biosynthesis, becomes completely activated without any modification of its regulatory enzymes; cholesterol content is increased in the blood and the enzyme is slowly degraded. The HMGCoAR degradation, is strictly dependent on a correct arrangement of the membrane spanning portion, so a change of the degradation rate could represent a good signal of the changed structure of the membrane spanning domain of the enzyme (Shearer and Hampton, *Embo J* 2005; **24**:149–59). During ageing, a relationship between the presence of a low degradation rate and a full activation of the reductase has been suggested (Pallottini et al., *Mech Ageing Dev* 2004; **125**:633-9). One of the widely recognized causes of age-related metabolic modifications is the large increase of reactive oxygen species (ROS). Therefore, aim of this work was to study the effect of ROS increase on the activity and the regulation of the HMGCoAR. For this purpose, two different experimental models of ROS enriched tissue were used: liver from rats fed on diets deprived of Vitamin E or polyunsaturated fatty acids. The results show that in these models, compared to that of old rats, full activation the HMGCoAR is detectable while a different degradation rate is observable. Actually the use of these experimental models has shown that the increased ROS content is effective to increase the catalytic activity, but not the rate of the enzyme degradation; so, it is evident that a modified degradation rate is not always related to the HMGCoAR full activation. In conclusion, our data clearly support a direct correlation between ROS production and altered HMGCoAR activity, even if the definition of the underlying mechanism requires further investigations.

A1-027P

P-cadherin expression is involved in migration induction of MCF-7/AZ breast cancer cells

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P-cadherin (P-cad) expression in breast carcinomas has been associated with tumours of high histological grade and lacking estrogen receptor-alpha (ER), suggesting a link between these proteins. In a previous study, using the MCF-7/AZ breast cancer cell line, the inhibition of ER signalling with the antiestrogen ICI 182,780 (ICI) induced an increase of P-cad, which coincided with induction of *in vitro* invasion. Additionally, retroviral transduction of MCF-7/AZ cells showed the proinvasive activity of P-cad. In the present study, we investigated if the induction of cell invasiveness by ICI and by P-cad expression was a consequence of increased migration, and/or of other factors such as the upregulation of metalloproteinases (MMPs). In order to analyse cell migration, we performed a wound-healing assay for MCF-7/AZ cells treated with ICI, and for cells retrovirally transduced with P-cad (MCF-7/AZ.P-cad). We found that there were no significant differences between migration of cells treated with ethanol and cells treated with ICI. In contrast, P-cad-transduced cells migrated significantly faster than vector-transduced cells ($P = 0.013$). This difference in migration behaviour of ICI-treated and P-cad transduced cells might be due to the fact that the extent by which P-cad is upregulated by ICI may not be sufficient to promote motility as such or, alternatively, the growth inhibitory effect of ICI nullified the pro-migratory effect of P-cad in this assay. To analyse the gelatinolytic activity of MMPs in these cells, we performed gelatin zymography. Treatment of MCF-7/AZ cells with ICI led to a clear induction of MMP activity, as compared to solvent-treated cells. This higher MMP activity was not found in MCF-7/AZ.P-cad cells. In conclusion, whereas high levels of P-cad may be sufficient for induction of motility and invasion, ICI-induced invasion might require the synergistic action of multiple genes.

A1-028P

Architecture of interactions between human 8-oxoguanine-DNA glycosylase and AP endonuclease

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Human 8-oxoguanine-DNA glycosylase (hOgg1) is the main human base excision protein that removes a mutagenic lesion 8-oxoguanine (8-oxoG) from DNA. Since hOgg1 has DNA glycosylase and weak abasic site (AP) lyase activities and is characterized by slow product release, turnover of the enzyme acting alone is low. Recently it was shown that human AP endonuclease (hApe1) enhances the activity of hOgg1. This enhancement was proposed to be passive, resulting from hApe1 binding to or cleavage of AP sites after hOgg1 dissociation. Here we present evidence that hApe1 could actively displace hOgg1 from its product, directly increasing the turnover of hOgg1. We show that HAP1 forms an electrophoretically detectable complex with hOgg1 crosslinked to DNA by sodium borohydride. Moreover, such complex also formed when hApe1 was replaced with *E. coli* endonuclease IV (Nfo) or its yeast homolog Apn1, suggesting that the reported enhancement of

hOgg1 activity by Nfo cannot prove the passive enhancement model. Using oligonucleotide substrates with a single 8-oxoG residue located in their 5', central or 3'-terminal part, we show that hOgg1 activity did not increase, and the hOgg1-hApe1-DNA complex did not form, only for the first of these three substrates, indicating that hApe1 interacts with the DNA stretch 5' to the bound hOgg1 molecule. In kinetic experiments, hApe1 has been shown to enhance the product release constant but not the rate constant of base excision by hOgg1. Moreover, hOgg1 bound to a tetrahydrofuran analog of an abasic site stimulated the activity of hApe1 on this substrate. Using a concatemeric DNA substrate, we show that hApe1 likely displaces hOgg1 in a processive mode, with hOgg1 remaining on DNA but sliding away in search for a new lesion. Altogether, our data support a model in which hApe1 specifically recognizes a hOgg1/DNA complex, binds 5' to the hOgg1 molecule, and actively displaces the glycosylase from the lesion.

A1-029P

Sequence, structure and function of human placenta protein 23 (PP23) / SOUL protein

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Placenta protein 23 (PP23) was first isolated and physicochemically characterized in 1991 from term placentas which contain an average of 3 mg PP23. It was found to be a soluble, non placenta specific protein with a molecular weight of 28 kDa. We screened human placental cDNA library with anti-PP23 serum and the isolated cDNA clones were sequenced using an automated Genetic Analyzer. By databank search, PP23 turned out to be identical to human SOUL protein or Heme Binding Protein 2 (HEBP2), which was also confirmed by the amino acid sequencing of placental isolated PP23 with MALDI TOF MS and PSD. Analyzing the gene we found that it was localized on the long arm of chromosome 6 and consists of four exons and the 1 kb-long promoter region of PP23 contained transcriptional factors such as the c-Myb and AP transcription factor family. The expression pattern of PP23 was determined in different adult and fetal, healthy and tumorous tissues by Western-blot. PP23/HEBP2 was expressed for functional studies and examined by HPLC. Both the placental isolated and the recombinant protein had the ability to bind iron [Fe(II)] and heme. Using confocal microscopy, we examined the overexpression and subcellular localization of PP23-GFP fusion protein in NIH3T3 cells. NIH3T3 cells transfected with PP23 containing vector showed increased sensitivity to oxidative stress and cytostatic agents compared to controls. Further functional studies of PP23 are in progress. In summary, these results indicate that PP23 might play a role in different apoptotic pathways and also have a function in differentiation and the development of the fetus and placenta or in the formation of different tumors accentuating its oncogene function.

A1-030P

Phytolectin wheat germ agglutinin can serve as a cytokine for phytosymbiont *Azospirillum brasilense*

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Cell to cell communication is important not only for multicellular organisms but also for symbioses of a macro-organism (plant, animal, etc.) with microorganisms. Our previous research has revealed that the phytolectin wheat germ agglutinin (WGA; protein excreted into the rhizosphere), which is known to be a mitogen for human lymphocytes, is active towards *A. brasilense* as well (1). The WGA binding to azospirillum cells results not only in the alteration of intracellular processes (1) but also in changing the growth parameters of the culture. A comparative investigation of the WGA influence on the growth of *A. brasilense* Sp245 using (i) total bacterium count and (ii) estimation of cell viability via colony forming units (CFU) revealed the following. The lectin did not affect the total number of cells in the culture; however, the number of viable cells sharply increased (up to 4-fold, as compared to the control). This, in turn, allows us to assume that WGA retards bacterial death (through necrosis and/or apoptosis) when the culture enters the stationary growth phase. Validation of this assumption is now under way. The finding obtained is in line with the data on the first bacterial cytokine (2). As WGA is available to rhizobacteria under the natural conditions, it is reasonable to assume that the ability of WGA to be a cytokine to the bacteria is one of significant functions of this multifunctional protein.

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A1-031P

Expression of Alzheimer-related genes in rat brain

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Permanent occlusion of bilateral common carotid arteries in rats (2VO) is a useful model for studying of ischemic-induced dementia. Alzheimer's disease (AD) is one of the most common types of dementia. Since the 2VO induces symptoms similar to those occur in vascular dementia as well as in AD therefore it may be used as a model for studying of AD-related issues. Several proteins have been found involving in the AD. This study aimed to investigate, *in vivo*, the expression of mRNAs encoding beta-amyloid precursor protein (APP), acetylcholinesterase (AChE), alpha7 nicotinic acetylcholine receptor (alpha7 nAChR), gamma-secretase and cyclo-oxygenase-2 (COX-2). Male Wistar rats received 2VO operation on day zero and brains were removed on day 2,

4, 7, 14, 35 and 112 for further total RNA isolation. Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) followed by gel electrophoresis were employed to measure the mRNA expressions. The results show that at day 4 after 2VO operation, the expressions of APP, alpha7 nAChR and gamma-secretase mRNAs were significantly greater than those in the sham group ($P < 0.05$). The AChE mRNA level tended to decrease after 5 weeks while the expression of COX-2 mRNA remained unchanged. This suggests that this model may be a useful model for screening of new compounds that possess potential effects on dementia or AD.

A1-032P

Analysis of plant telomere-binding proteins

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Telomeric proteins are important for telomere chromatin folding, capping and end-maintenance. A number of candidate plant telomere-binding proteins forming specific complexes with either single stranded or double stranded telomeric DNA have been identified by electrophoretic mobility shift assays or by database searches, but, apart from a few examples, little is known about their functions. An increasing number of proteins appear to bind telomeric DNA also indirectly, via association with pre-existing chromatin complexes. An example is human POT1 protein which binds either single-stranded telomeric G-strand overhang or can be recruited to the double-stranded part of telomere via TRF1 protein complex. Therefore, the study of DNA-binding proteins should be followed by searches for their binding partners i.e. proteins that are recruited to telomeres by protein-protein interactions. In our results, the ortholog of POT1 protein in *Arabidopsis thaliana*, AtPot1 (Acc. No. BT012568), seems to show a similar behaviour to its mammalian counterpart: (i) it binds directly and specifically the G-rich telomeric DNA strand; (ii) it interacts with AtTRB1 (AAL73123), a member of the single myb histone (Smh) family of plant proteins which bind specifically double stranded telomeric DNA *in vitro*. Our results thus suggest a possible role of AtTRB1 in recruiting AtPot1. A dual mode of binding of AtPot1 to telomere makes it plausible for AtPot1 to act as a terminal transducer of telomere length control.

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A1-033P

Phytolectins as biologically active substances for rhizobacteria of the genus *Azospirillum*

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Research in the last decade has shown that large oligopeptides and proteins can be important factors of extracellular regulation not only in mammals (growth factors, mitogens, hormones, etc.) but also in microorganisms (1). Earlier it has been found that the

phytolectin wheat germ agglutinin (WGA), being a mitogen for lymphocytes, is a biologically active substance (BAS) also for rhizobacteria of the genus *Azospirillum* (2). The effect of WGA on *Azospirillum brasilense* cells was found to be pleiotropic (2), similar to its effect on human lymphocytes. Among the described effects of WGA on the *azospirillum* cell, there are, stimulation of nitrogen fixation, promotion of ammonia excretion, induction of auxin biosynthesis, amplification of protein biosynthesis, induction of a surface-bound haemolytic factor. Reception of WGA on the bacterial surface also influenced the motility of *A. brasilense* Sp245, including social motility. Phytolectins that, like WGA, are specific to N-acetyl-D-glucosamine oligomers/polymers (STL – *Solanum tuberosum* lectin, UEA II – *Ulex europaeus* agglutinin), were active as BAS for *azospirilla*. In contrast, concanavalin A, having another carbohydrate specificity and binding to *azospirillum* cells, was not effective as a BAS to the bacteria. A putative receptor of WGA is haemagglutinin (surface-bound glycoprotein of *azospirilla*); its identification and isolation is now underway.

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A1-034P

Purification and some properties of glucose-6-phosphate dehydrogenase from sheep kidney cortex

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Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP+ 1-oxidoreductase, EC 1.1.1.49) is the first and key enzyme of pentose phosphate pathway. The pentose phosphate pathway is one of the important pathways because this pathway maintains the important proportion of reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) for biosynthetic reactions in the cytosol and production of ribose 5-phosphate for nucleotide synthesis by the de novo and salvage pathways for the cell, and interconversion of pentoses and hexoses. G-6-PD activity plays a critical role in cell growth and death by providing NADPH for cellular redox homeostasis. NADPH production is very important to reduce the oxidative damage. G-6-PD deficiency is a common genetic abnormality affecting an estimated 400 million people worldwide. In this study, glucose-6-phosphate dehydrogenase from sheep kidney cortex has been purified 1384 fold by 2',5'-ADP-Sepharose 4B affinity chromatography and DEAE Sepharose Fast Flow ion exchange chromatography with overall yield 16.96%. Previously, we used this procedure for the purification of glucose-6-phosphate dehydrogenase from bovine lens and lamb kidney cortex. The stability of the enzyme can be affected by several variables. Temperature and pH are the factors, which affects the enzyme activity. The effects of pH and temperature were studied. The enzyme was found to be stable at pH 6.5–10 and the optimal activity was at pH 7.4. The double reciprocal plots and product inhibition studies showed that the enzyme obeys 'Ping Pong Bi Bi' mechanism: Km NADP+, Km

G-6-P and Vm were found to be, 0.0147 ± 0.001 and 0.041 ± 0.0043 and 28.228 ± 0.858 , respectively by using non-linear regression analysis. The enzyme was stable at 4 °C for a week like lamb kidney cortex and bovine lens enzyme.

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A1-035P
Proteomic analysis of proteins specifically binding to potential LTR HERV-K regulatory element

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Background: By the use of novel approach of DNA-affinity chromatography [1] we purified 3 proteins with molecular masses of 60, 65 and 67 kDa [2] from a specific complex with potential regulatory region of long terminal repeat (LTR) of human endogenous retroviruses (HERVs), scattered in several thousand copies throughout the human genome and potentially capable of affecting the expression of closely located genes. Understanding of that might be extremely useful to elucidate the mechanisms of expression of these genes. These proteins were analyzed and characterized by proteomic analytical method.

Methods: Extract of nuclear proteins from ascite carcinoma cells was loaded on a heparin-agarose column to absorb a majority of DNA-binding proteins. LTR-binding proteins were eluted by double stranded oligonucleotide with sequence representing binding site of the protein(s) of interest, and detected by SDS-PAGE with coomassie staining. Then, each protein band has been cut out from the gel and analyzed by the MALDI-TOF-MS tryptic peptide mass mapping.

Results: Using the above approaches, three proteins from the LTR binding complex were purified. One of the constituent proteins was identified as a 70 kDa heat shock protein (HSP70).

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A1-036P
Calcium ATP ase activities in cremaster muscles and sacs

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Membrane transport proteins including channels, pumps and carriers are responsible from the maintenance of cellular calcium level, which has utmost importance. Membrane transport proteins including channels, pumps and carriers are responsible from the level of cellular calcium. While many conditions may affect the maintenance of cellular calcium, alterations in the regulatory mechanisms may also alter the cellular functions. Our previous studies have revealed that the cremaster muscles and sacs associated with undescended testis have been more vulnerable against parasympathetic tonus, and the levels of calcium ion in them have been affected. Therefore a study was planned to investigate and compare the activities of plasmalemmal and endoplasmic reticulum calcium ATP ase in cremaster muscle and sacs from boys and girls with inguinal hernia, and from boys with hydrocele or undescended testis. The activity of calcium ATPase has been determined spectrophotometrically. The results according to the sources have been compared through Mann–Whitney *U*- test and *P* values of <0.05 were considered significant. Calcium ATP ase activity in cremaster muscle and sacs associated with undescended testis have been found to reveal differences. Since sacs associated with undescended testis are vulnerable at more parasympathetic tonus, the increase may have reflected the increase in cytosolic calcium and/or the effects of G- protein linked signal transduction, or attempts at apoptosis.

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A2–Evolution of Protein Structure and Function

A2-001
Temporal changes in protein networks: from 90 minutes to 2 billion years

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Within the last 10 years, large-scale approaches delivered a wealth of data on molecular parts lists (e.g. genes and their expression) and there are an ongoing efforts to find associations between these (e.g. regulatory networks and protein networks). So far, the majority of these associations are done in 2D i.e. in form of networks that comprise nodes and edges. Here, I want to discuss a few temporal aspects of protein networks at different time scales, ranging from the yeast cell cycle via *Drosophila* development to the evolution of networks over long time periods.

A2-002
Structural studies of amyloid

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Numerous soluble proteins convert to insoluble amyloid fibrils having common properties. These fibrils are associated with neurodegenerative diseases, such as Alzheimer's and Parkinson's, and can also be formed *in vitro*. In the case of the yeast protein Sup35, conversion to amyloid fibrils is associated with a transmissible infection akin to that caused by mammalian prions.

A seven-residue peptide segment from Sup35 forms both amyloid fibrils and closely related microcrystals, which reveal the atomic structure of an amyloid spine. It is a double β -sheet, with each sheet formed from parallel segments stacked in-register. Side-chains protruding from the two sheets form a dry, tightly self-complementing steric zipper, bonding the sheets. Within each sheet, every segment is bound to its two neighbouring segments via stacks of both backbone and sidechain H-bonds. The structure illuminates the stability of amyloids as well as their self-seeding characteristic. Amyloid structure has also presented long-standing, fundamental puzzles of protein structure. These include whether amyloid-forming proteins have two stable states, native and amyloid, and whether all or only part of the native protein refolds as it converts to the amyloid state. We find that a designed amyloid of the well-characterized enzyme ribonuclease A contains native-like molecules capable of enzymatic activity. Also these functional molecular units are formed from a core ribonuclease A domain and a swapped complementary domain. These findings are consistent with the zipper-spine model for amyloid3 in which the fibrils are formed from 3D domain-swapped functional units, retaining native-like structure.

A2-003

Universal and lineage-specific trends in protein evolution

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Amino acid composition of proteins varies substantially between taxa and, thus, can evolve. So far, however, no universal trends in ongoing changes of amino acid frequencies have been reported, and it was generally assumed that any such changes would reflect evolution of nucleotide composition of the respective genomes and, consequently, would be unstable over long evolutionary times. Another widespread assumption regarding protein evolution is that proteins are in detailed equilibrium, i.e., reciprocal rates of amino acid replacements are equal. The availability of multiple sets of sequenced genomes of relatively close species allows direct testing of these assumptions. We compared sets of orthologous proteins encoded by triplets of closely related genomes from 15 taxa representing all three domains of life (Bacteria, Archaea and Eukaryota), and used their known phylogenies to polarize amino acid substitutions. The results of this analysis refute both of the above assumptions. The content of Cys, Met, His, Ser and Phe increases in at least 14 taxa, whereas Pro, Ala, Glu and Gly are consistently lost. This universal trend of protein evolutions holds also for the short-term evolution within human populations as shown by analysis of non-synonymous single-nucleotide polymorphisms. All amino acids with decreasing frequencies seem to be among the first incorporated into the genetic code; conversely, all amino acids with increasing frequencies, except Ser, were probably recruited late. Thus, expansion of initially under-represented amino acids, which apparently began over 3.5 billion years ago, continues to this day. In contrast, the same approach applied to the dynamics of insertions and deletions in proteins revealed lineage-specific trends apparently correlated with the evolution of genome size in the respective taxa.

A2-004

Predicting molecular details for protein interaction networks

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Protein interaction networks are central to most cell processes. Despite many attempts to elucidate them through high-throughput interaction discovery techniques, limited attention has been paid as to the molecular basis for how such interactions occur: who interact with whom is often known, but not how. Fortunately, there are a number of possibilities to predict how interactions are mediated based on similarities to protein three-dimensional structure, or by looking for sequence motifs likely to mediate protein binding. Ultimately, these can lead to models or proposed mechanisms for large parts of cellular machines or interaction networks.

A2-005

Trapping the building blocks of β -propeller proteins

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The superfamily of β -propeller proteins is characterized by an enormous diversity at the sequence level. However, all proteins share a common structural feature: they build their globular structure from repetitive units, the propeller blades. In the 50-odd structures of β -propellers in the Protein Data Bank, the number of blades ranges from four to eight. It is remarkable that nature can use such diverse numbers of structurally similar repeats to construct compact protein structures. How did this protein fold arise? One possibility is that single blades oligomerized and were only later combined to a single polypeptide chain. To evaluate this scenario, we tried to construct full-sized, oligomeric propellers from a single blade. This blade was derived from the consensus over a naturally occurring protein in which the individual blades are very similar to each other. We multiplied this blade to obtain larger building blocks and used these in assembly reactions. We determined the oligomeric status of the resulting complexes by gel-size exclusion chromatography and static light scattering. Using various building blocks in varying stoichiometry, we constructed oligomeric propellers with different numbers of blades. We also determined the molecular structure of a single blade in the context of such a propeller by an NMR spectroscopy. We conclude that β -propellers can be created by the oligomerization of smaller pieces. Depending on the number of blades (even/uneven) in each building block, protein oligomers of different size result. This indicates that β -propeller proteins may have evolved from single blades.

A2-006

Insertions/deletions in sequences of highly homologous proteins can infer targetable differences in their spatial structures

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Recent findings have shown that the protein elongation factor-1 α (EF-1 α) from the eukaryotic pathogen *Leishmania donovani*

possesses virulence properties. This was unexpected since it has >80% sequence identity with its human homologue. Given that EF-1 α is essential for cell survival, in principle, it can be considered as an attractive drug target. However, the challenge is to be able to selectively target the protein so as not to affect function of the human homologue. While a limited number of discrete differences were scattered throughout the sequence, most of the difference between these two homologues could be attributed to a 12 amino acid insert present in human EF-1 α and absent from the *Leishmania* sequence. We have modelled the spatial differences in structures of human and *L. donovani* EF-1 α inferred by this insertion–deletion (or “indel”). The protein models were used to develop antibodies directed specifically toward the deletion region of the pathogen protein. The strategy described allowed successful selective targeting of this putative *Leishmania* virulence factor while avoiding recognition of the highly similar human EF-1 α homologue. These findings may establish a new strategy for the development of antagonists directed against certain pathogenic targets having close human homologues.

A2-007P

PAB1725 is not what we thought: a contribution from a «post-genomic biochemistry» program

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Comparative genomics revealed a formidable task for biochemists: functional study of almost 2000 uncharacterized Clusters of Orthologous Groups (COGs) of proteins. Functional clues (inferred from genomic context, domains fusion, phyletic distribution, interaction networks, coexpression, copurification, structural consideration, etc.) combined with all-embracing views and prioritization of targets lead to successful approaches to assign new (or already known but missing) functions to many of these proteins. However, experimental studies are necessary for validation and sometimes may reveal a surprise. Two examples from our systematic «post-genomic biochemistry» program aimed at characterizing targets conserved in Eukaryota and Archaea but absent in Bacteria [1,2] will be discussed. The sequence of a human bifunctional enzyme involved in the two last steps of Coenzyme A (CoA) biosynthesis pathway was reported, fusion of two domains: PPAT and DPCK. The DPCK domain was identified from sequence comparison with its bacterial counterparts but not the PPAT domain. As COG1019 shows some similarities with the later domain, we determined whether a prototype, PAB0944 from *Pyrococcus abyssi*, is a PPAT (E.C. 2.7.7.3). Structural modeling indicates that, although only distantly related, archaeal/eukaryal (COG1019) and bacterial (COG0669) PPATs share ancestry and retain the same function. Although CoA holds a central position in cellular metabolism, many pieces involved in its biosynthetic pathway in Archaea are still uncharacterized. Archaeal orthologs of PAB1725 (COG0237) were annotated as carrying out the last step (DPCK). We could not detect any DPCK activity for this enzyme but a rather strong CMP/CDP kinase activity. The input of comparative genomics to identify candidates for archaeal CoA biosynthesis steps will be discussed, as well as a possible evolutionary scenario for this crucial central metabolic pathway.

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A2-008P

The renaturation kinetics of tortoise (*Kinixys erosa*, L.) muscle pyruvate kinase

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The study of the renaturation process of the denatured pyruvate kinase from tortoise, *Kinixys erosa*, was undertaken to understand the folding mechanism of the enzyme. *K. erosa* skeletal muscle pyruvate kinase was isolated and purified to homogeneity by a procedure which involved ion exchange chromatography on Carboxymethyl (CM) Sephadex and gel filtration on Sephadex G-200. The molecular weight of the active enzyme and its subunits were estimated to be $212\,333 \pm 2887$ and $49\,680 \pm 526$, respectively. The apparent Michaelis–Menten constant for PEP was 0.08 ± 0.02 mM and that for ADP was 0.67 ± 0.14 mM. The enzyme was denatured by 4 M guanidine-HCl. The denatured enzyme, on dilution into a buffer containing 10 mM PEP and 1 mM L-valine, exhibited a maximal recovery of up to 70–80% of the original activity. The renaturation in a buffer without PEP and L-valine was less than 30% of the non-denatured enzyme. The optimal protein concentration for renaturation of the enzyme was 80 μ M at 20°C. The kinetics of the renaturation process, which was first order with respect to the folding of the monomer, had a rate constant of 9.9×10^{-3} /min and a half-life of 69.6 min. The catalytically active renatured enzyme was a dimer, with a molecular weight of 100 000 da, even though it was kinetically similar to the tetrameric native enzyme. The mechanism of renaturation was proposed to involve an initial fast folding of the subunit followed by a slow rate limiting structural change to achieve its native conformation. The subunits would thereafter spontaneously reassociate to produce the catalytically active enzyme.

A2-009P

Superoxide dismutase activity in different tissues of vertebrates

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During the evolution, it was output the system of protection against oxidative damage that enables to keep the lipid peroxidation processes on a limited level. The superoxide dismutase (SOD) is an enzyme that decreases the concentration of superoxide-anion radicals in cell's media by disproportioning, being the first line of cell antioxidant protection from reactive oxygen species (ROS). Except of SOD, this system includes the whole complex of lipo- and water-soluble, enzymatic and non-enzymatic components (tocopherol, peroxidase, catalase, the system of glutathion, etc.). We isolated the brain, heart, liver and muscle of vertebrates: crucian carp (*Carassius carassius*), marsh frog (*Rana ridibunda*), Caucasian agama (*Stellio caucasicus*), and non-purebred white rats. Determination of SOD activity was done using two parallel methods based on ability of enzyme to brake the reaction of auto-oxidation of adrenaline in pH = 10.2 and to brake the photoreduction of nitroblue tetrasolium. Adrenokhrom concentration is measuring at 480 nm using the SF-46 (Russia) spectrophotometer; the inhibition of reaction with nitroblue tetrasolium is taking place at 560 nm. Our data had shown that biggest activity of SOD is achieved in brain tissue of all groups of experimental animals. There is expressed inversely proportional dependence between the level of the enzyme activity and degree of phylogenetic organization. The activity of SOD is much lower in heart, liver and muscle tissues. Especially interesting the very

low degree of SOD activity in myocard of amphibians and reptiles and its relatively high significance in skeletal muscles of white rats. This is an attractive fit to our earlier data about the changes of levels of oxidative processes in course of vertebrate evolution both for different organisms and their particular tissues.

A2-010P **Exploring protein evolution by saturation mutagenesis of the GST M2-2 active site residue 210**

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Glutathione transferases (GSTs) are a superfamily of enzymes that are found in a wide range of organisms. They function primarily as detoxication enzymes by inactivation of genotoxic electrophiles formed under oxidative processes in biological tissues. Inactivation is achieved by reacting the activated thiol of the tripeptide glutathione (GSH) bound in a conserved G-site of the enzyme with an electrophilic substrate in the more variable H-site. This variability accounts for the broad substrate diversity of GSTs. There are several classes of GSTs, the enzyme studied in this project belongs to the Mu-class and is designated GST M2-2. In the active site of GST M2-2, a mutation in position 210 to a serine residue has been found to increase the activity by up to three orders of magnitude with epoxide substrates in comparison with the wildtype threonine. Performing saturation mutagenesis, i.e. substituting the wildtype residue with all naturally occurring amino acids in the chosen position (210 in this case), gives an opportunity to study the molecular evolution of the GST M2-2 active site and also to demonstrate that point mutations can alter catalytic activities as well as substrate selectivity profiles. This is accomplished by determining the activities for the wildtype and all 19 resulting mutants with six different substrates, including three epoxides and the only known GST M2-2 specific substrate aminochrome. Differences in stability and expression of the mutants are also investigated. With the aid of multivariate analysis possible groupings based on specific activity data can be related to the different amino acids in position 210 and between the various alternative substrates.

A2-011P **Hidden messages in hidden subsequences: a study on collagens**

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All collagen sequences have a distinctive signature, described by the X-Y-Gly formula indicating that any amino acid might be present at X and Y positions, in many combinations, while the third position is fixed and invariably glycine. The unique periodic nature of these sequences makes it possible to perform a reliable statistical study on the physico-chemical properties of amino acids at X and Y positions. In this study, we have phase separated 20 different main human collagen sequences (classes) into three subsequences each. We have found that the X-Y-Gly formula is frequently corrupted by phase shifts caused by deletion of a glycine codon. The overall average charge of amino acids at X positions is always negative, while at Y positions it is always positive. No exception was found. This indicates the periodic nature of collagens even at X and Y positions and predicts a pattern-related interaction within and between collagen triple-helices. The first

and second letters in the genetic code of glycine are always guanine (G) while the third ("wobble") might be any nucleotide (A, U, G or C). The primary protein and nucleic acid sequences of large number of collagens are known. Therefore, collagens are ideal sequences to study the rules of base-selection at the wobble position. We will report that the wobble-base selection is clearly not random and there are hidden messages to discover.

A2-012P **Characterization of the non-structural protein encoded by the avian reovirus M3 gene**

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The avian reovirus M3 genome segment expresses two non-structural proteins of 70 and 60 kDa termed muNS and muNSC, respectively. The two proteins are recognized by anti-muNS-specific antibodies, indicating that they are protein isoforms containing common amino acid sequences. A Western blot analysis of recombinant muNS truncations expressed in transiently transfected cells revealed that muNSC lacks sequences from the amino-terminus of muNS. The results also suggested that muNSC originates by post-translational cleavage of precursor muNS, and not by translation initiation at an internal AUG codon. Immunofluorescence microscopy examination of the intracellular distribution of the recombinant muNS truncations showed that the inclusion-forming capacity of muNS resides in its coiled coil region comprising residues 448–605. Finally, immunoprecipitation of extracts from avian reovirus-infected cells that had been metabolically radiolabeled with [32P]orthophosphate demonstrated that the two M3-encoded protein isoforms are phosphorylated.

A2-013P **Enolase from *Klebsiella pneumoniae* – purification and comparative studies on molecular, catalytic and kinetic properties of bacterial and human muscle-specific enzyme**

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This report presents continued studies on comparative enzymology of glycolytic enzyme – enolase. In recent papers, we have presented purification and properties of enolase from fish and mammalian (including human) muscles. In present studies, there are included results for enolase obtained from the Gram-negative pathogen *Klebsiella pneumoniae*. In order to obtain electrophoretic-homogeneous protein from bacterial cells, the crude extract after ultracentrifugation was precipitated with ammonium sulphate, followed by gel-filtration on Sephadex G-100 and ion-exchange chromatography on DEAE-Sephadex A-50. In the end step of purification, we use preparative electrophoresis on Prep-cell 491 system (Bio-Rad, Hercules, CA, USA). Molecular and kinetic properties of *K. pneumoniae* enolase was similar to those from human muscle. The subunit molecular weight of bacterial enzyme was found 47 kDa. Functionally active molecule is a dimer with Mw 94 kDa, similar to that found in human muscle-specific enolase. Divalent cations were found obligatory for the bacterial enzyme activity. Maximal specific activity was achieved in the presence of Mg²⁺ ions, although the activating effect of Mn²⁺ and Zn²⁺ was observed at concentration lower than that of Mg²⁺. The interaction of bacterial enolase with inhibitors – phosphate and fluoride ions – was established. At high phosphate concentrations, a non-competitive, and at lower

concentration a competitive, inhibition with respect to 2-phosphoglycerate (glycolytic substrate) was found. Our investigations are helpful in the explanation of evolutionary chemistry and extend our knowledge about biochemical and phylogenetical dependences existing between the living organisms.

A2-014P Genomic and proteomic characterization of placental protein 25 (PP25)

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Soluble placental tissue protein 25 (PP25) was first isolated and characterized physico-chemically by Bohn et al. in 1991. It was described as a homopentamer consisting of 20 kDa subunits, from which an average human term placenta contains 10 mg. Recently, we used molecular biological methods to investigate its genetic, structural and functional characteristics. By SDS-PAGE/Western blot, using monospecific anti-PP25 serum, the highly purified PP25 antigen migrated in a 20 kDa band. By screening a human placental cDNA library, we isolated and sequence analyzed a 0.5 kb full insert-length cDNA encoding for a 144 aa protein of 16 kDa. By isolation and amino acid sequencing of PP25 antigen and the recombinant protein, we found their aa sequence identical to the protein encoded by the cDNA. We concluded the 20 kDa protein as a post-translational modified variant of the original 16 kDa PP25 transcript. By GenBank search, we found PP25 similar to an uncharacterized protein (HSPC034), and localized PP25 gene on chromosome 1. By Western blots, we found lower PP25 expression in different types of healthy human adult tissues and higher mainly in placenta, liver and adrenal gland. In different human fetal tissues and in some human tumors (neurogen tumor, liver adenocarcinoma, malignant melanoma), PP25 expression was elevated compared with matching adult tissues. PP25 could not be detected in sera. Using affinity chromatography, PAGE, MALDI-TOF MS and PSD, HSP90 was identified as protein specifically bound to PP25 in HeLa cells. We found that PP25 could bind to DNA, it was ribosylated by PARP enzyme, and furthermore it could be acetylated. PP25 cDNA was cloned into pcDNA3 vector and the transfected NIH3T3 cells had a proliferation advance detected by MTT test compared with controls. Further expressional, structural and functional analyses of PP25 are in progress.

A2-015P Molecular characterization of snRNAs/snRNPs in *Trypanosoma cruzi*

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Some important factors in functioning of the eucariotic cells are the small complexes of RNA and proteins; these particles of ribo-

nucleoproteins (UsnRNPs) have an essential role in the pre-mRNA processing, mainly during splicing. UsnRNPs present a common protein core associates between itself and with the snRNA, called Sm proteins, and specific proteins of each snRNP. Seven proteins (Sm B/B', -D1, -D2, -D3, -E, -F and -G) are joined forming ring-like structure into the snRNPs. Even though they are well defined in mammals, snRNAs/snRNPs are still not characterized in certain trypanosomatids as well as *Trypanosoma cruzi*, the causative agent of Chagas disease. Our preliminary results had demonstrated that the use of DEAE-Sephacell column allowed to concentrate these proteins of the nuclear extract from *T. cruzi* epimastigotes and after Western-blotting with antibodies anti-Sm bands, range of 15–60 kDa, were showed as expected. These proteins have been analyzed by two-dimensional electrophoresis to localize the common protein core, to be further characterized by mass spectrometry. Besides, other related studies are being carried through the laboratory with the purpose to characterize *T. cruzi* U1, U2, U4, U5, U6snRNAs using RT-PCR or PCR on genomic DNA with primers designed from *T. cruzi* genome database. Trypanosomatid sequences were aligned using BioEdit (version 7.0.0) and showed 80–85% of identity. Surprisingly, U5snRNA showed only 55% of identity. All secondary structures were predicted using RNA mfold (version 3.1). Studies are in progress in the lab comparing these sequences with UsnRNA human ones to search any therapeutic and/or autoimmunity response targets.

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A2-016P Sequence–structure–function relationships in R.NlaIV studied by modelling and mutagenesis

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Thus far, identification of functionally important residues in type II restriction enzymes (REases) has been difficult using conventional methods. Even though known REase structures share a common fold and marginally recognizable active site, the overall sequence similarities are statistically insignificant, unless compared among proteins that recognize identical or very similar sequences. NlaIV is a Type II REase, which recognizes the palindromic DNA sequence 5'GGNNCC and cleaves it in the middle generating the blunt ends. In straightforward comparisons, the NlaIV sequence shows no significant similarity to REases with known structures. However, the folding recognition studies showed the close relationship between NlaIV and EcoRV structures. The homology model of NlaIV was constructed and evaluated by the site-directed mutagenesis of residues predicted to be important for enzyme activity, DNA-binding and subunit dimerization. The restriction levels generated in the host strain by the wild type NlaIV and 11 mutants with single amino acid substitutions with alanine were measured by determination of the efficiency of plating of bacteriophage lambda. Lack of detectable restriction of mutants in the predicted catalytic amino acids confirmed modelling whereas different levels of restriction measured for the predicted DNA-binding amino acids were used to improve the structural model. The wt NlaIV and the three mutants conferring different level of restriction in the *in vivo* tests were overexpressed, purified and used in the *in vitro* assays. Results of the cleavage tests on lambda DNA and linear DNA fragment containing single recognition site paralleled *in vivo* test results. Based on these, it could be concluded that modelling of

the protein structure validated by *in vivo* assays could be a good alternative for the time consuming crystallographic method.

A2-017P

High expression of recombinant human Platelet Factor 4 in *Escherichia coli* and its bioactivities

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In order to improve the expression of human Platelet Factor 4 (hPF4) in *Escherichia coli*, we have constructed a prokaryotic expression vector pBV220-hPF4 by DNA polymerase chain reaction (PCR) and DNA recombinant technology, 3'-UTR of PF4 cDNA was deleted and TAG was mutated to TAATAA. The yield of recombinant hPF4 is 160 mg/L in shaking flask culture. The expression level has been improved by 80-fold compared with that of PT7-7 hPF4 expression system. After we washed, dissolved and renatured the inclusion bodies, inhibition experiment of blood vessel proliferation in chicken chorioallantoic membrane was carried out to determine the bioactivities of hPF4. The experimental result demonstrated that hPF4 prepared by our methods had the inhibitory activity against angiogenesis.

A2-018P

Identification of *Arabidopsis* mutants carrying T-DNA inserts in phosphoprotein phosphatase genes

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Although members of the phosphoprotein phosphatase (PPP) enzyme family are known to be involved in numerous essential cellular processes, their functions are poorly characterized in plants. We have exploited an *Arabidopsis* T-DNA insertion mutant collection to initiate functional analysis of PPP enzymes by reverse-genetics. Using a PCR screening technique described by Rios et al. (Plant J. (2004) 32, 243-253), we have identified T-DNA insertions in genes encoding PP7, PP1 (TOPP-1), PP6At1, and a plant specific phosphatase (the product of At2g27210). Analysis of homozygous insertion mutants revealed that none of the PPP genes studied are essential for viability, as all PPP insertion mutant lines display wild type phenotype under normal growth conditions. Study of various stress responses to osmotic and hormonal stresses, cadmium(II) salt, sugars, and photooxidative stress induced by paraquat showed no significant difference between wild type and most PPP mutants. However, the PP6 mutant displayed increased sensitivity to abscisic acid inhibition of germination. In response to blue light irradiation, the PP7 mutant exhibited a loss of hypocotyl growth inhibition. PP6 and At2g27210 plant specific phosphatase mutant plants showed a similar deficiency in blue light-mediated inhibition of hypocotyl elongation. These results suggest that PP6, PP7 and At2g27210 may play important roles in the regulation of growth responses by blue light.

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A2-019P

Mutational and crystallographic studies of RNase HIII from *Bacillus stearothermophilus*: importance of N-terminal domain on substrate binding

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Many organisms possess multiple RNase H genes in their genomes. For example, the *Bacillus subtilis* genome possesses three RNase H-related genes. We have previously reported that two of them encode functional RNases H, RNases HII and HIII (Bsu RNases HII and HIII), while the other one, *ypdQ*, encodes an RNase HI homologue with no RNase H activity. Amino acid sequence of Bsu RNase HIII is similar to those of Bsu RNase HII and *Escherichia coli* RNase HII, but is different from that of *E. coli* RNase HI. However, the enzymatic properties of Bsu RNase HIII are similar to those of *E. coli* RNase HI rather than those of Bsu RNase HII and *E. coli* RNase HII. Recently, we cloned the gene encoding RNase HIII from *Bacillus stearothermophilus* (Bst RNase HIII) and characterized the enzymatic properties of the recombinant protein. Bst RNase HIII highly resembles to Bsu RNase HIII in enzymatic properties. Purpose of this study is to understand the molecular mechanism of the enzymatic function of RNases HIII. Limited proteolysis of Bst RNase HIII produced two peptide fragments cleaved at Leu83-Ala84, suggesting that Bst RNase HIII consists of the N- and C-terminal domains. The N-terminal domain alone and the C-terminal domain alone of Bst RNase HIII were overproduced in *E. coli*, purified and characterized. Determination of the kinetic parameters for RNase H activity and the binding analyses of the proteins to RNA/DNA hybrid using BIAcore indicated that the N-terminal domain of Bst RNase HIII is involved in substrate binding. To understand the structural basis for these results, Bst RNase HIII was crystallized and the X-ray diffraction data sets of the native crystal and the heavy-atom derivatives were collected. Structure determination by MIR method is in progress.

A2-020P

The puzzling nature of cell wall anabolism: enzyme recruitment within and across pathways

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Enzyme recruitment is one of the most important mechanisms to originate new reactions and metabolic pathways from the pre-existing ones. The two original models, the "patchwork" and "stepwise", agree on the necessity of gene duplication as a prerequisite for the recruitment, but have two main differences: (i) the distance (number of reactions) among the recruited enzymes and, (ii) the chemical similarity of the reactions. Some major constituents that distinguish the bacterial cell wall from the eukaryotic and the archaeal counterparts are synthesized by the peptidoglycan (PG), folate (FL) and formyl-tetrahydrofolate (FTHF) anabolism, thus the study of these routes is important to understand the emergence of the three domains of life. In order to determine the amount and nature of the enzyme recruitment that have performed the assemble of these and other pathways, we have compared the chemical properties of their reactions, from a network

perspective; and the enzymes capable of catalyzing them, using a sequence/structure based approach. The obtained results indicate that diverse enzymes have been recruited across (for example, from FL to PG) and within (for example to conform four consecutive steps in PG) pathways. Patchwork appears to be the model with most examples, because of the topological nature of metabolic networks, however the stepwise seems to be more statistically significant. The recruited enzymes of these pathways tend to catalyze chemically similar reactions, as much in consecutive as in distantly steps.

A2-021P
Superoxide dismutase from the psychrophilic Antarctic eubacterium *Pseudoalteromonas haloplanktis*

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The antioxidant function of Fe- and Mn-containing superoxide dismutases (SOD) observed under constraints from extreme rather than mild cellular conditions could reflect an adaptive evolution to oxygen tolerance in the structural organization of this class of enzymes. For instance, the mitochondrial human Mn-SOD and the hyperthermophilic archaeal Fe-SOD from *Sulfolobus solfataricus* (SsSOD) share a similar structural organization. Further studies on members of this ubiquitous enzyme isolated from differently adapted micro-organisms could give useful information on possible adaptive mechanisms in the structure-function relationships of this SOD family. For this reason, this enzyme has been purified and characterized from *Pseudoalteromonas haloplanktis*, a psychrophilic eubacterium isolated from marine Antarctic sediments. Two chromatographic steps on DEAE-Sepharose and HTP allowed to purify SOD from *P. haloplanktis* (PhSOD) to homogeneity. The relative molecular weight of the purified enzyme estimated by SDS-PAGE is about 20 000. As SsSOD, also PhSOD shows a homotetrameric structure, as determined by gel filtration. PhSOD has an unusual thermal stability for a psychrophilic enzyme, as evaluated by its half-life of 10 min at 52 °C. Similar results were obtained by UV-melting curves. Enzymatic assays showed that PhSOD has a specific activity of 6500 U/mg. The enzyme is inactivated by hydrogen peroxide and it is inhibited by sodium azide, whereas PMSF, a specific inactivator of the archaeal SsSOD, has no effect. Future research plan includes the determination of the metal content and the cloning of the gene encoding PhSOD. To this aim, a molecular probe has been designed on the basis of the amino acid sequence of some fragments of the purified protein.

A2-022P
Structure and activity of different N-terminal domains from AAA-proteins

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AAA proteins are part of the large superfamily of AAA+ proteins, ring-shaped P loop NTPases, which display their function

by unfolding macromolecules in an energy-dependant manner. AAA proteins usually consist of an N-terminal domain, and one or two ATPase domains named D1 and D2. ATPase domains are relatively conserved within the family of AAA proteins and they are also thought to mediate the hexamerization of AAA proteins. N-terminal domains are important for substrate recognition and binding and, in contrast to the ATPase domains, they vary in their folds. Based on published data and additional bioinformatic analysis of AAA proteins, we selected several different N-terminal domains from archaeal AAA proteins for functional and structural characterization. All selected domains were expressed as recombinant proteins in *Escherichia coli*. Guided by prior knowledge that AAA proteins have a protein unfolding function, we used heat and chemical aggregation assays of different substrate proteins to assay N-terminal domains, or full AAA proteins, for possible chaperone activity. All constructs showed activity in inhibition of aggregation of protein substrates. Surprisingly, we found that the N-terminal domains could themselves play a role in the hexamerization of AAA proteins, a role previously assigned to the ATPase domains. The results of our study indicate that AAA ATPase N-terminal domains of AAA proteins containing different unrelated structures share a common function, namely intrinsic chaperone activity.

A2-023P
Structure of choline-binding protein E from *Streptococcus pneumoniae*, phosphorylcholine metallo-esterase activity as virulence factor

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The choline binding proteins (CBPs) are pneumococcal specific surface-exposed proteins, non-covalently bound to choline residues located on teichoic and lipoteichoic acids. We report here the crystallographic structure of the phosphorylcholine esterase (Pce) catalytic domain, of CBPE, a virulence factor involved in pneumococcal nasopharyngeal colonization. As predicted from sequence analysis, the Pce structure harbors the metallo-beta-lactamase fold. New features of Pce active site show that this enzyme is unique among this superfamily of hydrolases. Indeed, the orientation and calcium stabilization of an elongated loop lied on top of the active site suggest that the catalytic cavity may be rearranged to accommodate natural phosphorylcholine-containing-substrate(s). Furthermore, the resolution of the Pce structure complexed with phosphorylcholine together with the characterization of catalytic iron ions in the active site led us to propose a reaction mechanism modeled upon the purple acid phosphatase, a mechanism that we have confirmed using site-directed mutagenesis. Similarity between Pce and structurally unknown class of proteins involved in DNA uptake, ComEC, is discussed. These structural data provide preliminary clues in understanding CBPE function related to the process of pneumococcal diseases.

A2-024P
A highly divergent MpgS accounts for the synthesis of mannosylglycerate in the gram-positive bacterium *Rubrobacter xylanophilus*

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The extremely radiation resistant bacterium *Rubrobacter xylanophilus* is moderately tolerant to salinity (<7% NaCl) and has an

optimum temperature for growth of about 60 °C (1). *Rubrobacter xylanophilus* is the only gram-positive bacterium known to synthesize the compatible solute mannosylglycerate (MG), which is commonly found in hyperthermophilic archaea and some thermophilic bacteria (2). Unlike the salt-dependent pattern of accumulation observed in (hyper)thermophiles, in *R. xylanophilus* MG accumulates constitutively. Two biosynthetic pathways for MG are known: one involves a direct conversion of GDP-mannose and D-glycerate into MG by MgS; in an alternative pathway, a phosphorylated intermediate, mannosyl-3-phosphoglycerate (MPG), is synthesized from GDP-mannose and 3-PGA by MpgS and converted into MG by MpgP (3,4). While the two-step pathway has been found in several (hyper)thermophilic prokaryotes, *Rhodothermus marinus* is the only known organism with the genes for both pathways that are differentially regulated by salt and thermal stresses (5). The synthesis of MG in *R. xylanophilus* was tracked from GDP-mannose and 3-PGA, but the genome sequence of the organism failed to reveal any of the genes involved in both pathways. The purification of the native enzyme was carried out and the N-terminal sequence was used to identify the corresponding gene in the genome of *R. xylanophilus*. The gene encodes a highly divergent MpgS whose biochemical properties are reported here. The physiological relevance of MG accumulation in *R. xylanophilus* and the evolution of MG biosynthesis in prokaryotes are discussed.

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A2-025P

Purification and characterization of prolyl hydroxylases modifying hypoxia inducible factors

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Deficiency of oxygen (hypoxia) contributes to the development of different widespread human disorders such as cancer, heart infarction and stroke. Recently discovered prolyl hydroxylases (PHDs) have been pointed out as sensors for molecular oxygen in the cell. The enzymes regulate the intracellular level of hypoxia inducible factors (HIFs) by post-translational modification. These transcription factors activate expression of a large set of proteins in adaptation to hypoxia. As long as molecular oxygen is present, HIFs are hydroxylated by PHDs and thereby targeted for degradation via the ubiquitin-proteasome pathway. The large diversity of different PHD isoenzymes and various HIF alpha-subunits raises questions about the significance of the individual protein forms and how they are related to each other. Further knowledge of their difference in specificities is important for understanding the regulatory mechanisms at hypoxia as well as how to design appropriate drugs to hypoxia related diseases. In addition, such data will contribute to the field of post-translational modifications. Isoenzyme PHD3 was expressed with a histidine tag in *Escherichia coli*. No full-length protein was obtained using a C-terminal tag, but 200 mg enzyme per liter medium was achieved when expressed with an N-terminal tag. Low protein solubility is a problem, but the condition of purification is being

improved. Fluorescence spectroscopy and radiometric assays are being set up in order to study binding characteristics and catalytic properties of the purified PHD isoenzymes.

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A2-026P

Active TEM-1 β -lactamase mutants with random peptides inserted in three surface loops

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With the purpose of creating an allosteric-binding site into the TEM-1 β -lactamase, we have generated libraries of potential-binding sites for proteins or small molecules by engineering three surface loops close together in the structure. Random peptide sequences were genetically introduced into these loops. The tolerance to insertion was assessed by determination of the percentage of active mutants. The loop bordering the active site hardly accepts any insertion. Two loops approximately 20 Å away from the active site are rather tolerant but although in apparently symmetrical situations, they display very different behaviours. Four libraries featuring insertions of six-to-nine residues in replacement of a dipeptide connecting the N-terminal α -helix and the first β -strand presented a very low percentage of active clones. Following the observation that active mutants exhibited a cysteine at each end of the insert, new libraries were constructed with cysteine residues in these positions. A large increase in the percentage of active clones was noticed suggesting the requirement for a stabilizing disulfide bridge. On the other hand, insertion of six random residues in replacement of a residue of the loop connecting the last β -strand to the C-terminal α -helix affords libraries containing a satisfactory percentage of active mutants. The origin of this difference is analysed in terms of the difference in the stabilities of the concerned helices and in their interaction with the rest of the protein. The amino acid distribution in the engineered loops was also analysed and compared with distributions naturally observed in medium-size loops.

A2-027P

Structure–function studies of thyrotropin using site-directed mutagenesis and gene transfer: development of new agonists and antagonists

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Thyrotropin (TSH) and the gonadotropins (FSH, LH, hCG) are a family of heterodimeric glycoprotein hormones composed of two non-covalently linked subunits, a and b. The hTSH heterodimer was converted to a biologically active single-peptide chain (hTSHbCTPa), by fusing the common a subunit to the carboxyl terminal end of hTSHb subunits in the presence of a ~30 amino acid peptide from hCGb (CTP) as a linker. Ligation of the CTP to the carboxyl-end of hFSH resulted in increasing the biological activity and longevity *in vivo*. In the present

study, the hTSHbCTPa was used to investigate the role of the N-linked oligosaccharides of a and b subunits on secretion and function of hTSH. Two deglycosylated variants were prepared: one lacks both oligosaccharide chains on a subunit (hTSHbCTPa1+2), and the other lacks also the oligosaccharide chain on b subunit of the single chain [hTSHbCTPa(deg)]. The single-peptide chain variants were expressed in CHO cells and they are secreted into the medium. Absence of the N-linked oligosaccharides on a or b subunits and the O-linked oligosaccharides on the CTP does not affect the secretion of the variants. However, the absence of N-linked oligosaccharide chain on b decreased the secretion rate of the single-peptide chain. These results indicate that the signal for the secretion exists in the single peptide chain and is independent of the oligosaccharides. hTSH variants lack of the oligosaccharide chains is less potent than hTSHbCTPa on cAMP accumulation and T3 secretion in human cultured thyroid follicles. Both deglycosylated variants compete with normal hTSH and hTSI in a dose-dependent manner on TSH receptor-binding site. Maximal concentration of hTSHbCTPa1+2 (200 mU/mL) decreased significantly the hTSH and hTSI-stimulated levels of cAMP and T3 secretion. Moreover, hTSH variant inhibits TSH activity in animal model. Thus, this variant behaves as potential antagonist, who may offer a novel therapeutic strategy in the treatment of Grave's disease, the most common form of hyperthyroidism.

A2-028P

Study of the biodiversity of filamentous fungi isolated from different plants from a Mediterranean ecosystem

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To assess the biodiversity of the filamentous fungi populations in plants from a Mediterranean ecosystem, Natural Park of Serra da Arrábida, two zones with different microclimate characteristics were chosen, Mata do Solitário and Fonte do Veado. Sampling was performed mainly on leaves from three plants common to both zones (*Quercus faginea*, *Pistacea lentiscus* and *Cistus albidus*), as well as from one specific of each one (*Acer monspessulanum* – Fonte do Veado and *Ostrya quadripartita* – Mata do Solitário). Phenotypic and molecular methods were applied to characterize the isolated fungi and to compare fungal diversity in sampled plants and microclimate-distinct zones. This study is focused in *Cladosporium*, the dominant genus of filamentous fungi isolated in all the samples. After the phenotypic identification based on classical methods (colony characterization and morphology of reproductive structures), M13 PCR fingerprinting was used for genomic clustering of isolates. Identification of species was also assessed by Amplified Ribosomal DNA Restriction Analysis of Internal Transcribed Spacers (ITS-ARDRA), using endonucleases FokI and MvaI. So far, two species have been identified, *Cladosporium herbarum* and *Cladosporium cladosporioides*. Shannon–Weaver and Simpson indexes were applied to evaluate intra-specific diversity and to compare sampled plants and ecosystem zones in terms of fungal biodiversity. When the chi-squared analysis of contingency tables was used to test statistical independence, no significant associations could be found among M13 clusters and sampled plants or ecosystem zones. A similar analysis will be applied to the clusters defined by a com-

posite approach based on M13 PCR fingerprinting and ITS-ARDRA profiles obtained with a larger set of endonucleases.

A2-029P

On the catalytic role of conserved active site residues D256 and E190 of *Escherichia coli* Phosphofructokinase-2, a member of the ribokinase family

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Escherichia coli Pfk-2 is a homodimer that exhibits hyperbolic kinetics with respect to the substrates, and allosteric regulation by MgATP, which causes inhibition of the enzyme activity at low concentrations of fructose-6-P and promotes tetramer formation. Sequence alignment of ribokinase family members identifies two strictly conserved residues; an aspartate which acts as the catalytic base and a glutamate that interacts with a Mg²⁺ ion, as suggested by the solved structures of some ribokinase family members. In Pfk-2, these residues correspond to D256 and E190, respectively. In order to evaluate the proposed role for these residues in Pfk-2, we performed site-directed mutagenesis of D256 and E190 for asparagine and glutamine, respectively. The D256N mutant shows a 10 000-fold decrease in the k_{cat} value and no significant differences in the K_m values for both substrates, supporting the role of this residue as the catalytic base. Mutation of E190 to glutamine lowers the k_{cat} value by 400-fold at 1 mM-free Mg²⁺ and, in contrast with the wild type enzyme, presents a dependence of the k_{cat} value with free Mg²⁺ concentration. While for Pfk-2, the K_m value for fructose-6-P diminishes with high Mg²⁺ concentrations (30 mM), for the E190Q enzyme this value remains unchanged. Inhibition of Pfk-2 by MgATP is reverted by an increase in either fructose-6-P or free Mg²⁺ concentration. Nonetheless, the E190Q enzyme is not inhibited by MgATP. The results suggest a catalytic role for the E190 residue through interactions responsible for Mg²⁺ binding at the active site and interaction between this ligand and the allosteric site.

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A2-030P

Functional analysis of the yeast actin-binding protein (Abp1p) in *Saccharomyces cerevisiae*

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Yeast Abp1p is an actin-binding protein involved in the actin cytoskeleton regulation and endocytosis. It is important for the activation of the Arp2/3 complex, which plays a key role in actin cytoskeleton organization. Homologous of this protein have been identified in mammalian cells. Abp1 contains three domains including and N-terminal actin-depolymerizing factor homology domain, a proline rich region and a C-terminal Src Homology 3 (SH3) domain. A number of proteins associated with the cortical actin cytoskeleton contain SH3 domains, suggesting that these domains may provide the physical basis for functional interactions among structural and regulatory proteins in the actin cytoskeleton. The function of the SH3 domain is to mediate specific protein–protein interactions, which it achieves by binding to PXXP-containing sequence motifs in target proteins. We ana-

lyzed the *in vivo* effect of point mutations in the SH3 domain of Abp1p in yeast using different yeast gene deletion backgrounds, where ABP1 is required. We found that specific point mutations have different effects on growth depending on the strain background analyzed, suggesting that the SH3 domain has multiple functional roles. Also, we show by deletion analysis that the SH3 domain of Abp1p is more important for the *in vivo* function of the protein than other domains, such as the actin-binding domain. Our study provides a correlation between the binding affinities of mutants measured *in vitro* and changes in functional activity *in vivo*. Finally, we have demonstrated that Abp1p, similar to its mammalian homologues, is a phosphoprotein and the proline-rich region is required for its phosphorylation.

A2-031P

Conserved networks and the determinants of protein topology

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Protein structures are network systems which exhibit small-world, single-scale and to some degree scale-free properties (1). The application of network principles to protein structures provides a means of rationalizing the robustness in the three-dimensional-fold of proteins against mutations and an alternative avenue to investigate the mechanism of protein folding and stability (1). We propose that the critical determinants of the native topology are encoded by a conserved network of interactions between select amino acids in geographically important positions (2). We test this hypothesis by identifying a consensus network of long-range interactions within a set of model proteins, which share a common Greek-key topology, but differ in secondary structure, sequence and function. Computational studies involving the application of network principles and molecular dynamics simulations provide support for the proposed role of the conserved interactions in governing the network and dictating the common protein topology.

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A2-032P

Site-directed substitution of the key residue at the subunit interface of GST P1-1 by S-alkylcysteine residues sustains glutathione binding and catalytic activity of the enzyme

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The lock-and-key motif is responsible for a highly conserved hydrophobic interaction in the subunit interface of the dimeric glutathione transferases (GSTs) of the Pi, Mu, and Alpha classes. The aromatic key residue (Tyr50 in human GSTP1-1) in one subunit is wedged into a hydrophobic pocket of the other subunit. In addition to its contribution to dimer stability, revealed by Tyr50 mutants and the heterodimer GSTP1/Y50A, Tyr50 plays an essential role in GSH binding that influences the rate of catalysis. Conventional site-directed mutagenesis can mutate Tyr50 into 19 different amino acid residues only, but aided by chemical modifications additional residues can be introduced. In the pre-

sent study, site-directed chemical modification of Tyr50 to Cys was done in an otherwise Cys-free mutant of hGSTP1-1. The mutation of Tyr50 to Cys decreased the specific activity 3000-fold, from 15 to 0.005 units/mg protein. Furthermore, KMGSH for Y50C increased with 500-fold in comparison to the Cys-free mutant. Treatment with cysteine modifying reagents such as *N*-ethylmaleimide, 1-chloro-2,4-dinitrobenzene, 2,2'-dithiodipyridine, benzylbromide, and 2-bromopyrimidine did not reactivate Y50C by more than fourfold. On the other hand, a homologous series of 1-iodoalkanes reactivated the mutant to different degrees, depending on the chain length of the alkyl group. The highest specific activity (2.5 units/mg, 17% of the parent enzyme) was obtained with iodobutane. Furthermore, the modification of Y50C with iodopropane, iodobutane and iodopentane restored the KMGSH value of the mutant.

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A2-033P

Combined X-ray approach for studying metalloproteins function/misfunction : a powerful approach to metallogenomics

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The explosion of genome sequences have posed serious challenges to the structural biology community worldwide. Despite major high throughput structural biology initiatives, particularly in Japan, USA and Europe, the structure/function paradigm on “genome wide basis” has not yet major progress. The situation is even more acute in the case of metalloproteins, which quite often are not amenable to high throughput expression approaches for a variety of reasons including the fact that many of them require a specific “metal chaperone” which lower organisms may lack. Metalloproteins are expected to make up at least a third of the genome and worldwide effort is beginning to take shape for what has recently been referred to as “metallogenomics”. A variety of X-ray techniques [Protein Crystallography, Solution X-ray Scattering and X-ray Absorption Fine Structure (XAFS)] have proved very powerful in studying not only structure/function relationships in metalloproteins but also are proving unique in understanding misfunction of these proteins which quite often results in debilitating disease.

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A2-034P

Protein kinase PDK1 mechanism of interaction with substrates suggests a model for the propagation of proteins with non-physiological conformations

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The phosphoinositide-dependent protein kinase PDK1 is a Ser/Thr protein kinase, which phosphorylates and activates a number of other protein kinases, including protein kinase PKB (also called AKT), protein kinase C (PKC) isoforms, serum and glucocorticoid-stimulated protein kinase (SGK), p70 ribosomal S6 kinase (S6K), and p90 ribosomal S6 kinase (RSK), among others. We here discuss the model by which PDK1 recognizes its substrates. PDK1 only recognizes and interacts with substrates, which are in an inactive conformation, which exposes a C-terminal hydrophobic motif. Upon interacting of the C-terminal motif with PDK1 "PIF-binding pocket", PDK1 becomes activated and phosphorylates the substrate protein kinase. Upon phosphorylation, the conformation of the substrate protein is stabilized in an active conformation where the C-terminal hydrophobic pocket docks into its own catalytic domain and is not available for interaction with PDK1. In the absence of PDK1, the substrates of PDK1 remain in inactive conformations. Although this has no effect on some substrates, others, such as PKC isoforms, become unstable and can aggregate or are degraded. Based on this model, PDK1 regulates other protein conformations by using a key regulatory site, the hydrophobic PIF-binding pocket. Our model suggests a molecular mechanism by which an unfolded-inactive protein kinase substrate of PDK1 could bind to the PIF pocket, block the docking site for substrates and prompt the appearance of more inactive protein conformations with the ability to aggregate. We conclude that analogous mechanisms could participate in the propagation of proteins with specific conformations in conformational disorders.

A2-035P

Exploring the importance of an active site residue on the stereo and regio selectivity of Mu class glutathione transferases

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Glutathione transferases (GSTs) are a family of multifunctional enzymes that utilize the tripeptide glutathione (GSH) to detoxify a wide variety of electrophiles. The GSTs are grouped into classes based on protein sequence similarities. Although members of a class have high sequence identity, they often display different substrate specificities. One example is the human Mu class where the isoenzyme M1-1 is efficient in catalyzing the conjugation between GSH and a number of epoxide substrates, whereas the isoenzyme M2-2 has low activities with these substrates. Epoxides are formed during the oxidative biotransformation of xenobiotics, and many of them are well known carcinogens such as epoxides of polycyclic aromatic hydrocarbons. Epoxides are also important substrates in the chemical industry due to the versatil-

ity of the oxirane function. The GST-catalyzed reaction with epoxides starts with binding and deprotonation of GSH, followed by a nucleophilic attack on either of the carbons of the oxirane function, resulting in opening of the ring structure. We have previously demonstrated that a Ser to Thr substitution of the active site residue 210 between GST M1-1 and GST M2-2 to high extent is responsible for the differences in catalytic efficiencies with epoxides. The interaction between Ser210 and the epoxide substrate has been proposed to occur through second sphere interactions, or through a direct (or water mediated) hydrogen bond to the oxirane oxygen in the ring-opening step. We have constructed alanine mutants of GST M1-1 and GST M2-2 to gain more information about this interaction and investigated the effect of the mutations on the catalytic parameters and stereo and regio selectivity using epoxide substrates with different configuration of the oxirane carbon.

A2-036P

Structural double-mutant cycle analysis of a hydrogen bond network in Ketosteroid Isomerase: relationship between structure and function

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Ketosteroid Isomerase (KSI) catalyzes an allylic isomerization reaction at a diffusion-controlled rate. A hydrogen bond network, Asp99-Water504-Tyr14-Tyr55-Tyr30, connects two critical catalytic residues, Tyr14 and Asp99, with Tyr30, Tyr55 and a water molecule in the highly apolar active site of the *Pseudomonas putida* KSI. In order to characterize the interactions among these amino acids in the hydrogen bond network of KSI, double-mutant cycle analysis was performed and the crystal structure of each mutant protein within the cycle was determined, respectively, to interpret the coupling energy. The ΔG values of Y14F/D99L KSI, 25.5 kJ/mol for catalysis and 28.9 kJ/mol for stability, were smaller than the sums (i.e., 29.7 kJ/mol for catalysis and 34.3 kJ/mol for stability) for single mutant KSIs, respectively, indicating that the effect of the Y14F/D99L mutation was partially additive for both catalysis and stability. The partially additive effect of the Y14F/D99L mutation suggests that Tyr14 and Asp99 should interact positively for the stabilization of the transition state during the catalysis. The crystal structure of Y14F/D99L KSI indicated that the Y14F/D99L mutation increased the hydrophobic interaction while disrupting the hydrogen bond network. The ΔG values of both Y30F/D99L and Y55F/D99L KSIs for the catalysis and stability were larger than the sum of single mutants, suggesting that either Tyr30 and Asp99 or Tyr55 and Asp99 should interact negatively for the catalysis and stability. These synergistic effects of both Y30F/D99L and Y55F/D99L mutations resulted from the disruption of the hydrogen bond network. The synergistic effect of the Y55F/D99L mutation was larger than that of the Y30F/D99L mutation since the former mutation impaired the proper positioning of a critical catalytic residue, Tyr14, involved in the catalysis of KSI. Our study can provide insight into interpreting the coupling energy measured by double-mutant cycle analysis based on the crystal structures of the wild-type and mutant proteins.

A2-037P**Proteomics analysis of polypeptide pattern in *Olea Europea* (C.V. Zard) following transformation with P5CS gene**F. Hadi¹, F. R. Jazii¹ and N. Motamed²¹Laboratory of Biochemistry, Department of Biology, University of Tehran, Tehran, Iran, ²Laboratory of Biochemistry, Department of Biochemistry, National Institute of Genetic Engineering & Biotechnology, Tehran, Iran. E-mail: fara_hadi@yahoo.com

Olive is an ever-green tree which high adaptive nature towards different climatic condition and is cultivated in different parts of the world like Iran. High salt density in soil is dangerous for cell from different aspects. In order to increase salt tolerance in this very important plant, the P5CS gene delta1-pyrroline-5-carboxylate synthetase is a regulatory enzyme in proline biosynthesis (X, S, E) which has already been constructed and cloned in a binary vector PBI121 was transformed in olive embryo through agrobacterium. Following the transformation and plantlet formation the effect of transformation on plantlet response to the presence of salt was studied by analyzing plant proteome with the help of two-dimensional gel electrophoresis (2DE). Total protein was extracted from both transformed (with construct S) and non-transformed or negative control plantlets. Equal quantity of total protein from both two samples was subjected to 2DE and protein profile of transformed plantlets was compared to non-transformed control plantlets. Appearance, disappearance, up-and-down regulations of polypeptides were interpreted as response of plantlet to the induced stress and the difference in polypeptides profile of transformed vs. control plantlet was also considered. Our result indicated that both salt stresses induced expression of new polypeptide and down regulation of others. Along with the above observation a significant difference in specific polypeptides pattern of transformed plants was observed with that of control that indicates the effect of transformation on gene expression.

A2-038P**Molecular analysis of the β -fructosidases in yeast *Saccharomyces***

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Invertase (β -D-fructofuranoside hydrolase, ES 3.2.1.26) hydrolyses readily sucrose, raffinose and slowly inulin. The yeast *Saccharomyces* expresses invertase both in glycosylated external form located in the periplasmic space and cytosolic non-glycosylated internal form. Both enzymes are encoded by the same structural gene (SUC), but are translated from different start codons (Carlson, Botstein, 1982). In infer the molecular evolution of *Saccharomyces* yeast by analysis of β -fructosidase SUC genes, we have cloned and sequenced a 1600 bp PCR-amplified fragment of SUC gene from *S. cariocanus* and deduced the amino acid sequence for encoded protein fragment (532 residues). Sequence similarity of β -fructosidases within the genus *Saccharomyces* has been determined. The proteins of *Saccharomyces cerevisiae* and its five sibling species (*S. bayanus*, *S. cariocanus*, *S. kudriavzevii*, *S. mikatae*, *S. paradoxus*) have high degree of identity – 90–97%. The invertase of *S. bayanus* is the most divergent among the proteins studied. Multiple-sequence alignment of *Saccharomyces invertases* revealed several most conserved regions, which are completely coincident with the areas of local similarity of β -fructosidases of bacteria, plants and fungi. The Asp, Asn, Glu and Cys residues of the conserved regions are con-

sidered as components of active center of β -fructosidases (Reddy, Maley, 1990, 1996; Pons et al., 2004). The results obtained are discussed in the light of general evolution of the genus *Saccharomyces*. The genomes of *Saccharomyces* species, in particular their β -fructosidases SUC genes, represent the interesting model for studying evolution and classification of yeast species.

A2-039P**Isolation and partial characterization of protease producing bacteria from food samples**

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The bacterial strains isolated from meat, fish and egg samples were screened on milk agar media for their ability to produce extracellular proteases. Among thirty-one bacterial isolates from meat samples, nine isolates produced extracellular protease at different temperature and pH. The most potential producer (unidentified) was further examined for their extent of extracellular protease production and characterization. Maximum enzyme activity was achieved at pH 5–9 over 25 °C. Among 20 bacterial isolates from fish samples, five isolates produced extracellular protease at different temperature and pH. The most potential producer, identified as *Serratia marcescens*, was further examined for their extent of extracellular protease production and characterization. Maximum enzyme activity was achieved at pH 8.5 over 15 °C. Among 13 isolates from egg samples, two isolates produced extracellular protease at different temperature and pH. The most potential producer, identified as *Staphylococcus* sp., was further examined for their extent of extracellular protease production and characterization. Maximum enzyme activity was achieved at pH 9.0 over 40 °C. Thus, it may be concluded from the present study that the selected isolates could be applied in various industrial and biotechnological applications due to their maximum enzyme activity at different temperature and pH. Further studies are under progress to purify and characterize the enzyme and to explore the potential of these isolates in industrial and biotechnological applications.

A2-040P**Secondary structure prediction and 3D-modelling of mammalian mono-ADP-ribosyl hydrolases**

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Mono-ADP-ribosylation, like phosphorylation, is a protein modification regulating protein functions. Mono-ADP-ribosyl transferases (ARTs) catalyse the transfer of the ADP-ribose moiety from NAD⁺ to an acceptor amino acid of the target protein. Mono-ADP-ribosyl hydrolases (ARHs or ADPRHs) can reverse this modification. Using BLAST searches we identified two homologues of the known ARH1 in all completed mammalian genomes (designated ARH2 and ARH3). The human ARH1, ARH2 and ARH3 proteins have lengths of 357, 354 and 363 amino acids, respectively. ARH1 and ARH2 are more closely related and show 44% sequence identity, while ARH3 has 19% sequence identity to the other paralogues. The predicted secondary structures showed significant resemblance to only one structure in the protein structure database, protein mj1187 from the archaean *M. jannaschii* (pdb 1t5j). This protein is a putative

ADP-ribosyl hydrolase. The ARH-proteins evidently form a new protein fold family. Using the structure of mj1187 as a template we performed 3D-modelling of the mammalian ARHs by threading. Folding of the protein core is conserved between the archaean and the mammalian ARH proteins. Amino acid residues coordinating the catalytically important magnesium ions in mj1187 are conserved in mammalian ARH proteins and located at the end of four central alpha-helices. Interestingly, ARH-homologous proteins of the jellyfish *T. cystophora*, that have been recruited as eye lens crystallins, lost all magnesium coordinating residues. Proteins of the ARH-fold family seem to have evolved to fulfill different functions in animals. We cloned and expressed all 6 ARHs from man and mouse in order to test their enzymatic activities. Insights gained from the 3D models will be used for structure function analyses.

A2-041P

The proteomic analysis of red yeasts *Rhodotorula glutinis* and *Sporidiobolus salmonicolor* grown under exogenous stress

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The main objective of proteomics is the systematic and quantitative identification of all proteins expressed in a cell or tissue. However, proteins really present in the cell, do not respond directly to those coded by genes. Quantitative and qualitative changes in a cell protein complement can be induced by the environment, stress and other factors. Thus, identification of metabolic markers characteristic for certain events provides important insight into the mechanisms of pathways occurring in the organism and can lead to the production of some industrially significant metabolites. Especially in microorganisms is production of metabolites strongly influenced by series of physical, chemical and biological factors. Environmental stress surrounding yeast cells evokes various changes in their behaviour in order to survive under unfavourable conditions. Under stress, various specific compounds are overproduced (e.g. glycerol, trehalose, carotenoids, etc.). In this work, protein profiles of carotenogenic yeasts *Rhodotorula glutinis* and *Sporidiobolus salmonicolor* grown in optimal conditions and under exogenous osmotic and oxidative stress were obtained and compared. For this purpose all the procedures leading to complete protein identification, including protein extraction, precipitation, 2D electrophoresis separation and mass spectrometry analysis were developed and optimized. Since the peptide mixtures deriving from proteolytic digests of total yeast cell extracts are highly complex, gel-free techniques using RP-HPLC/ESI-MS were used. Simultaneously, production of carotenoids and ergosterol as specific stress metabolites were measured using LC/MS. Under osmotic as well as oxidative stress more than 100 proteins were overproduced in both studied yeast strains.

A2-042P

Diverging substrate specificities from a glutathione transferase library analyzed by multivariate methods

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Glutathione transferases (GSTs) are a large family of well-studied enzymes that are able to perform a wide variety of functions.

The most extensively investigated role is their function as detoxication enzymes where they catalyze the nucleophilic attack of the tripeptide glutathione on electrophilic substrates. Many of these compounds are environmentally and endogenously derived substrates that are potential cytotoxins, mutagens and carcinogens. A library of 384 variants was made through DNA shuffling of Mu class GSTs M1-1 and M2-2 as parents in order to evolve enzymes with new catalytic properties. All variants were screened with nine different substrates that represent two kinds of reaction, addition and substitution reactions. Each mutant in the library is represented by a point in a multidimensional activity space, and the isolated variants were analyzed with multivariate methods such as principal component analysis, K-means clustering, dendrograms and canonical variate analysis. Different groups of mutant enzymes that have related catalytic activities with one or several substrates are recognized and some individuals were chosen for further characterization. By this approach, parents for the next generation can be identified and a new cycle of mutations performed by DNA shuffling or other methods of stochastic DNA mutations. In this way, evolution is driven towards desired functional properties, and enzymes with new activities are obtained. These methods highlight how directed enzyme evolution can be optimized in order to get recombinant GSTs with potential applications in medicine, organic synthesis and biotechnology.

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A2-043P

The guards take the lead: genome “dialect”, DNA repair, and evolutionary variation

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Several species-specific characteristics of genome organization that are superimposed on its coding aspects were proposed earlier, including genome “signature”, genome “accent” and “compositional spectrum”. These notions could be considered as representatives of “genome dialect”. We measured within the proteobacteria some genome dialect representatives: The relative abundance of dinucleotides, or “genome signature”; the profiles of occurrence of 10 nucleotide “words” (compositional spectra) and the profiles of occurrence of 20 nucleotide words, using “degenerate” two letters alphabet (purine–pyrimidine compositional spectra). Here, we show that the evolutionary distances between enzymes involved in DNA repair and recombination, are highly correlated with purine–pyrimidine compositional spectra, and genome signature distances. All the enzymes of the nucleotide excision repair system belong to this group. Other (control) protein groups have significantly lower correlations of their evolutionary distances with the purine–pyrimidine compositional spectra, and genome signature distances. We hypothesize that the high correlation of the former group with genome dialect is resulted from coevolution of genome structure and DNA repair-recombination enzymes and discuss the mechanisms that might be responsible for this coevolution.

A2-044P**Secondary structure and binding propensities of calpastatin**D. Kovács², R. Kiss¹, P. Tompa² and A. Perczel¹¹Department of Organic Chemistry, Eötvös Lóránd Univ., Budapest, Hungary, ²Biol. Res. Ctr. Inst. Enzymol., Hungarian Acad. Sci., Budapest, Hungary. E-mail: rgida@ludens.elte.hu

Calpastatin is the specific inhibitor of calpain, the intracellular Ca²⁺-dependent cysteine protease, which is composed of five domains. Four of these sequences are capable of inhibiting a calpain molecule on its own. These inhibitory domains contain three short subdomains (A, B and C) of about 20 amino acids in length, which are important elements of calpain recognition. In preliminary examination calpastatin is fully disordered, but in closer view its subdomains are transiently ordered, with β -turn preference established for B and α -helical preference for A and C, given the structural disorder of calpastatin and the induced folding entailed by its binding. Our aim is to explore this residual structure and relate it to the mode of calpain binding by the inhibitor. In order to test if pre-formed helices are needed for the effective interaction of calpastatin with calpain, we generated a range of mutants, which either increases or decreases helical propensities within subdomains A and C. The effect of these mutations on the kinetics and thermodynamics of binding has been tested. The binding of calpastatin and its mutants to calpain are investigated by three independent methods, calpain activity measurements, surface plasmon resonance in order to determine the apparent second order rate constant, and isothermal titration calorimetry. The solution of structure and dynamics of wild-type and mutant A and C subdomains are also studied by multidimensional NMR techniques including relaxation experiments.

A2-045P**Novel antibacterial protein produced by *Streptococcus sanguis* 10556 is implicated for use in prevention and treatment of dental caries**J. Kaewsrichan¹, T. Chuchmo¹, R. Teanpaisan²¹Faculty of Pharmaceutical Sciences, Pharmaceutical Chemistry, Prince of Songkla University, Hat-Yai, Songkhla Thailand,²Faculty of Dentistry, Stomatology, Prince of Songkla University, Hat-Yai, Songkhla Thailand. E-mail: jasadee.k@psu.ac.th

Streptococcus sanguis is a pioneer bacterium colonizing the oral cavity, and plays an important role in maintaining oral micro-ecological balance. Deng et al. (2004) have reported the production of a bacteriocin-like substance by *S. sanguis* with an approximate molecular mass of 65 kDa. The mentioned substance was found to inhibit the growth of putative periodontopathogenic bacteria. Our results, however, have shown that the antimicrobial compound produced by *S. sanguis* 10556 is obviously smaller. Its molecular mass is estimated to be less than 5 kDa, and the compound dominantly affects the growth of *Streptococcus mutans*, rather than those strains reported by Deng et al. Its activity is lost by incubating with proteinase K, suggesting its proteinaceous nature and the fact that it is a bacteriocin-like substance. This new evidence would make *S. sanguis* 10556 attractive in biotechnological application as an antimicrobial agent in prevention and treatment of dental caries.

A2-046P**Inhibition of acetylcholinesterase and butyrylcholinesterase mutants by the pyridinium oximes 2-PAM and HI-6**

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2-PAM [2-(hydroxyiminomethyl)-1-methylpyridinium chloride] and HI-6 [(1-(2'-hydroxyiminomethyl-1'-pyridinium)-3-(4''-carbamoyl-1''-pyridinium)-2-oxapropane dichloride)] are reversible inhibitors of acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8). 2-PAM and HI-6, as strong nucleophiles, are also efficient reactivators of phosphorylated AChE and BChE. In attempting to determine the amino acid residues within the active site gorge involved in the interaction with the oximes, selective mutants of mouse AChE were subjected to inhibition by 2-PAM and HI-6 and their affinities were compared with wild-type AChE and BChE. Mutations in the choline binding site (Y337A) or combined with acyl pocket mutations (F295L/Y337A, F297I/Y337A) were employed to enlarge active site gorge dimensions and to mimic BChE active site. Enzyme-oxime dissociation constants (K_i) for the catalytic site were evaluated from the apparent dissociation constants as a function of the substrate concentration (0.05–1.0 mM acetylthiocholine). Dissociation constants for AChE w.t. were 150 and 47 μ M, for BChE w.t. 320 and 23 μ M for 2-PAM and HI-6, respectively. Introduced mutations lowered oxime binding affinities for both oximes, and K_i were 590 and 87 μ M for Y337A, 650 and 110 μ M for F295L/Y337A, and 1700 and 180 μ M for F297I/Y337A, for 2-PAM and HI-6, respectively. Despite introduced mutations in AChE, which correspond to residues found in BChE active site, affinities for the oximes did not approximate BChE affinities. This might imply that binding of HI-6 and 2-PAM did not include their stabilization with residues 337, 295 and 297. However, from the calculated change of free energy of binding, it seems that mutations Y337A and F297I affected binding with a cumulative effect, since the calculated change of energy was nearly doubled for F297I/Y337A relative to the single mutation Y337A, or to F295L/Y337A.

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A2-047P**Mutagenesis and kinetic study of human alpha-L-fucosidase**S. W. Liu¹, S. S. Chang¹, C. C. Lin² and Y. K. Li¹¹Applied Chemistry Department, National Chiao Tung University, Hsin-Chu, Taiwan ROC, ²Institute of Chemistry, Academia Sinica, Taipei, Taiwan ROC. E-mail: ykl@cc.nctu.edu.tw

Alpha-L-fucosidase (3.2.1.51), an exo-glycosidases unique to family 29, is capable of cleaving L-fucose residues from glycoconjugates involved in a variety of biological processes. In particular, the determination of L-fucosidase activity can be used to predict the development of several carcinomas, whereas the deficiency in this enzyme causes fucosidosis, a well-known lysosomal storage disorder. Serum alpha-L-fucosidase activity is now considered as a marker of hepatocellular carcinoma (HCC). Besides, a fair amount of AFU activity can be detected or purified from the seminal fluid and the plasma membrane of sperm. It has been proposed that AFU may relate to the interaction of sperm and oocyte. All of these findings strongly indicate the biological importance of AFU, which has not yet been extensively studied. The main drawbacks on study of hAFU are likely due to the

unavailability of native enzyme and/or lacking of a successful expression technique for recombinant protein, as well as the difficulty in synthesizing useful substrates. The hAFU was found to be a labile enzyme, which is unstable even in the neutral condition (pH7.0) at 4 °C. With careful control on the condition of bacteria growth, we are able to overexpress hAFU in *Escherichia coli*. The protocol of protein purification has been established. Also, substrates with various leaving phenols were successfully synthesized. With amino acid multi-alignment as well as X-ray structure inspection (*Thermotoga maritima* fucosidase), residues of Glu-70, Glu-135, Asp-158, Asp-225, Asp276, and Glu-289 are likely to present in the active site and part of them may function as the essential groups of hAFU. Site-directed mutagenesis studies on all of these residues revealed that Asp-225 is the essential nucleophile residue of hAFU. The preliminary results also showed that Glu-289 is likely to be general acid/base residue. Kinetic investigations and LC/MS analysis on wild type and mutant enzymes are carrying out for enzyme mechanistic action.

A2-048P

Tryptophan residues participate in forming of alpha1-acid glycoprotein binding site

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Alpha1-acid glycoprotein (AGP) is the acute phase protein which concentration is increased in different diseases. A very important function of this protein is transfer of different charged ligands in blood plasma. At the different pH in blood plasma AGP is undergone conformational changes, which decrease or increase the binding possibility of AGP. Using cationic fluorescent probe Quinaldine Red (QR) we studied the binding properties of AGP at different pH. Analyzing our data we concluded that at range of pH between 9.0 and 9.5 conformational changes are descended because QR produced high fluorescence. We also assayed binding of QR at other pH marked different changes in probe fluorescence as different state of AGP: AN changes (acid-normal) and NB ones (normal-basic). Detail studies are established that several amino acids from the surface of the glycoprotein participate in the conformational changes at basic conditions forming more hydrophobic binding region or binding site. To study conformational changes in native molecule of AGP we used the fluorescent probe Calcofluor White (CW), which is suitable acceptor of energy for tryptophan residues from binding site of AGP. Thus we can see position of the tryptophan residues in the binding site analyzing efficiency of energy transfer between Trp and CW at different pH. After number of experiments we calculated R_0 at pH 7.0 and 9.0 that was 23 nm and 29 nm, respectively. To take in consideration the increasing of the own Trp fluorescence we can say that Trp residues go to the inner area of the binding site. We concluded that at basic pH in range 9.0–9.5 pH-dependent conformational changes in alpha1 acid glycoprotein are occurred changing its binding properties for the cationic drugs presented in human blood.

A2-049P

Protein synthesis of yeasts *Candida* under some extremal conditions

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Alive organisms are distinguished with high adaptivity to the persistent changing environmental conditions. Even in extreme conditions

the organisms survive working out the definite protective mechanisms, particularly – synthesizing new proteins. The existence of some definite proteins has been proved that named as “proteins of thermoshock”, which are produced in organisms surviving in conditions of high temperature. The extreme factors firstly influence on nucleic acids and proteins, which realize the transmission of genetic information and the basic structural and functional characteristics of organism. The investigation of protein fraction composition of yeasts *C. guilliermondii* WKM U-42 in normal conditions and after nitrogen starvation was realized. It has been shown that the nitrogen starvation lead to decrease of water- and salt-soluble proteins, and to increase of alkali-soluble fraction. So, if the water-soluble protein fraction of non-starved yeasts is $52.3 \pm 2.2\%$, and the alkali-soluble fraction – $24.8 \pm 1.3\%$, then in case of nitrogen starved yeast they are $28.3 \pm 0.26\%$ and $51 \pm 0.5\%$ accordingly. The protein fractions content of yeasts *C. guilliermondii* NP-4 also suffer some changes under influence of X-rays. It has been shown that after X-radiation of these yeast cells the quantity of water-soluble fraction was decreased, and the alkali-soluble fraction – increased approximately in 6%. We have investigated the protein synthesis of yeasts *C. rugosa* WSB-925. It has been shown that in high temperature conditions the “proteins of thermoshock” with molecular mass of 74 and 79 kD are synthesized. These proteins probably carry out the direct protection function as well as have an important role in formation of cell resistance.

A2-050P

Characterization of some presumptive *Cerastoderma glaucum* populations from the Romanian Black Sea coast by means of total protein SDS-PAGE

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Total protein from hemolymph and different tissue types (gill, muscle, mantle) of presumptive *Cerastoderma glaucum* individuals from several stations scattered along the Romanian Black Sea coast were analyzed by means of SDS-PAGE. This was done in order to rule out the presence of the congeneric *Cerastoderma edule* at the targeted sites. The total protein electrophoretic patterns, correlated with the morphological characteristics of the sampled individuals, strongly suggest that at the studied locations only one species of *Cerastoderma*, namely *glaucum* is present. The inferred protein variability stands only for the normal, intraspecific protein polymorphism. Further preliminary 18S rDNA based RFLP analysis sequencing results uphold the above-mentioned conclusions.

A2-051P

Despite its high similitude with monomeric arginine kinase, muscle creatine kinase is only enzymatically active as a dimer

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Guanidino kinases are related enzymes which share important sequence and three-dimensional structure similitude but exist with different quaternary structures. Whereas arginine kinases are mostly monomeric, creatine kinases can be either exclusive dimers (cytosolic homo- or heterodimers MM, BB and MB) or

interconvertible dimers and octamers (mitochondrial isoforms). Muscle creatine kinase (MM CK), one of the best-known members of this family, is a symmetric homodimer with one active site per subunit. These active sites are far apart and direct evidence of cross-talk between subunits has not yet been reported. A functional asymmetry of the monomers has been envisaged, but this issue remains very controversial. We will present results obtained with wild type MM-CK, and with site-directed interface mutants, which confirm that dimeric state is very important for tertiary structure stability and show that it is indeed required for expression of enzymatic activity. These creatine kinase results will be compared with those obtained with a monomeric arginine kinase.

A2-052P

The hemocyanin from the shamefaced crab (*Calappa granulata*): functional characterization and subunit heterogeneity

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The hemocyanin (Hc) of *Calappa granulata* was purified from hemolymph and separated into two components by Sephacryl S-300 chromatography. A gel filtration on a pre-packed Superose 6 HR column allowed to assess the molecular weight of the two fractions that correspond to the dodecameric (900 kDa) and hexameric (450 kDa) aggregation states; the former, accounting for 90% of the total, showed a large Bohr effect ($\log P_{50}/pH = -0.95$) and a cooperativity almost constant ($n_{50} = 2.7 \pm 0.2$) in the 6.9–9.6 pH range. The hexameric molecule displayed a lower Bohr effect ($\log P_{50}/pH < -0.3$) and a decrease of cooperativity (n_{50} values = 1.5 ± 0.3) with respect to the dodecamer. Subunit heterogeneity was determined by 2D-electrophoresis and different MS approaches. Urate, a byproduct of purine catabolism, which is known to increase the oxygen affinity of various crustacean Hcs, did not affect at all the oxygen-binding properties of *C. granulata* Hc. L-lactate, the main end-product of anaerobiosis in Crustacea, increased the oxygen affinity of *C. granulata* Hc ($\log P_{50} = 0.55$), in agreement with other decapod Hcs. This effect has been related to the increased oxygen requirements that *in vivo* occur during muscular exercise. Calcium ions are needed for the structural stability of many arthropodan and molluscan Hcs. On the contrary, dodecameric *C. granulata* Hc resulted to be stable, at physiological pH, also in the absence of calcium ions. In contrast with other Hcs previously characterized, its oxygen affinity was unaffected at low calcium concentrations, but it largely increased at calcium concentrations higher than 10 mM, thus indicating only the low affinity calcium-binding sites to be operative in this Hc.

A2-053P

The thioredoxin system in the archaeon *Sulfolobus solfataricus*

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The thioredoxin system is a powerful redox machinery widely distributed in nature involved in several cellular functions. Usually,

this system is constituted by the flavoenzyme thioredoxin reductase (TrxR) and its protein substrate thioredoxin (Trx). TrxR catalyses the NADPH-dependent electron transfer to the active site disulphide of oxidized thioredoxin (Trx) to form a dithiol. While Trx functions as a monomeric protein, TrxR is organized as a homodimer and can be classified into two structurally unrelated groups. Type I high molecular mass TrxR (55–58 kDa per subunit), isolated from higher eukaryotes and type II low molecular mass TrxR (around 35 kDa per subunit) isolated from lower eukaryotes and prokaryotes. Studies on the archaeal thioredoxin system have been started only recently. In particular, we have isolated and characterized a TrxR from the hyperthermophilic archaeon *Sulfolobus solfataricus* (SsTrxR-B1). Because in the genome of this archaeon the gene coding for another TrxR (SsTrxR-B2) and for two Trx (SsTrx-A1 and SsTrx-A2, respectively) have been putatively identified, we decided to clone these genes in prokaryotic expression vectors. In this communication we report the purification of the heterologous expressed proteins, their biochemical characterization and the determination of the crystal structure of SsTrxR-B1. Preliminary results indicated that SsTrxR-B2, differently from SsTrxR-B1, was not isolated as a flavoprotein and did not show a thioredoxin reductase activity using heterologous substrates. We are currently attempting to use SsTrx-A1 or SsTrx-A2 as homologous substrates for either SsTrxR-B1 or SsTrxR-B2 activity to clarify the specific function of these enzymes.

A2-054P

Structure–function relationship of a ribosome inactivating protein from a Himalayan hemiparasitic plant

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This is the first report on the structural studies of a novel ribosome inactivating protein (RIP) obtained from the Himalayan mistletoe (*Viscum album*) (HmRIP). HmRIP is a type II heterodimeric protein consisting of a toxic enzyme (A-chain) with an active site for ribosome inactivation and a lectin subunit (B-chain) with a well-defined sugar-binding site. The crystal structure of HmRIP has been determined at 3.8 Å resolution and refined to a crystallographic R factor of 0.228 (R_{free} = 0.271). A comparison of this structure with other type II RIPs reveals distinct structural features present in the active site of the A-chain and in the 2γ sugar-binding site of the B-chain. The conformation of Tyr110 side chain which is a conserved active site residue in the A subunit is strikingly different from those observed in other mistletoe RIPs indicating its unique preference for substrate binding. The deletion of two important residues from the kink region after Ala231 in the 2γ subdomain of B-chain results in a significantly different conformation of the sugar-binding pocket. Distinct architecture of the sugar-binding site might be associated with the unique sugar binding properties of the HmRIP. A ribosome recognition site has also been identified in HmRIP. The site is a shallow cavity with the conserved residues Arg51, Asp70, Thr72 and Asn73 involved in the binding. The conformations of the antigenic epitopes of 1-20, 85-103 and 206–223 residues differ from those observed in other type II RIPs resulting in the distinct antigenicity and pharmacological properties of HmRIP.

A2-055P**Structural analysis of dimeric dUTPases from *Campylobacter jejuni* and *Leishmania major***

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Deoxyuridine triphosphate nucleotidohydrolase (dUTPase) catalyzes the hydrolysis of dUTP to dUMP. This decreases the intracellular concentration of dUTP, preventing incorporation of uracil into DNA. Most dUTPases belong to a family of homologous enzymes with an all- β fold and five conserved motifs contributing to the active site; they function either as monomers or as trimers (e.g., the human enzyme). However, a distinct group of dUTPases shows no sequence or structural similarity to this family. The characterized members of this group are the dimeric dUTPases from the protozoan parasites *Trypanosoma cruzi* and *Leishmania major*, and the food-borne pathogenic bacterium *Campylobacter jejuni*. The dimeric dUTPases could be candidate drug targets against these pathogens because they appear to be unrelated to other dUTPases, including the human one, and thus, inhibiting them is unlikely to damage the host organism. The structure of the first representative of this family, the *T. cruzi* dUTPase, has been determined previously. Here we present the X-ray structures of the *C. jejuni* and *L. major* dUTPases determined in complex with a non-hydrolysable substrate analogue, dUPNP and magnesium ions. These structures reveal a novel all- β fold. Sequence and structural analysis of these proteins, along with the recently released crystal structure of *Sulfolobus solfataricus* protein SSO12199, allowed us to delineate a new superfamily of all- β NTP pyrophosphatases with potential "house-cleaning" functions and to propose an evolutionary scenario for dimeric dUTPases. We discuss the possible mechanism of dUTP hydrolysis by dimeric dUTPases.

A2-056P**Effect of accumulation of amino acid substitutions on the hemadsorption character and antigenicity of influenza AH3 hemagglutinin protein during evolution**

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In order to clarify the effect of accumulation of amino acid substitutions on the hemadsorption character of influenza AH3 hemagglutinin (HA) protein, we introduced 335 single-point amino acid changes in total into the HA1 domain of the HA protein of A/Aichi/2/68 (A/Aichi/68) and A/Sydney/5/97 (A/Sydney/97) strains by a PCR random mutation or site-directed mutagenesis method. The mutant HA cDNAs inserted in an expression vector were expressed in the COS cells and hemadsorption activity was studied using human red blood cells. These changes were classified as positive or negative according to their effect on hemadsorption activity. We observed the following results: (i) The rate of positive substitutions was about 50% in both strains. (ii) Out

of 44 amino acid changes that were identical in both strains with regard to both the substituted amino acids and their positions, 22% of changes that were positive in A/Aichi/68 were negative in A/Sydney/97 and 27% of changes that were negative in A/Aichi/68 were positive in A/Sydney/97. The discordance rate of hemadsorption character of amino acid substitutions was suggested to be related to the number of amino acid substitutions between A/Aichi/68 and A/Sydney/97, and one amino acid substitution increased the discordance rate by 0.4%. A similar discordance rate was also observed in the antigenic regions. The above results suggested that the accumulation of amino acid substitutions in the HA protein during evolution promoted irreversible structural changes and, therefore, antigenic changes in influenza AH3 HA protein may not be limited.

A2-057P**The monomeric porin from *Porphyromonas asaccharolytica*-isolation and characterization of the encoding gene**

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Porphyromonas asaccharolytica strain ATCC 25260 is a Gram-negative anaerobic pathogenic bacteria, which is resistant to a large number of the beta lactam antibiotics. Beta-lactamases were not found, so the resistance could be because of the low permeability of the outer membrane. Only one monomeric porin was isolated from the outer membrane of *P. asaccharolytica* and it was revealed that this porin existed in two forms – "open" and "closed". The "closed" conformation of monomeric porins exhibits a property of heat-modifiability. In its denatured form this conformation of the protein migrated on SDS-PAGE as a protein with a molecular of 41 kDa, while without heating its molecular weight was 37 kDa. The "open" form was resistant to heating and thus did not change the speed of migration on SDS-PAGE after boiling. Cloning of this porin revealed an open reading frame of 1098 bp encoding a protein of 366 amino acids. Sequence comparison of the monomeric porin with other outer membrane proteins demonstrated a clear homology with porins of the Bacteroides family. Secondary structure prediction indicated that the "open" conformation of the porin contains 12 transmembrane beta-strands, whereas the "closed" conformation of the protein spans the outer membrane 8 times with amphiphilic beta-strands. It seems that only the "open" conformation serves as an open channel for the passage of organic solutes.

A2-058P**GH97 is a new family of α -glucosidases**

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Recently, a new family of glycoside hydrolases, GH97, has been formed (http://afmb.cnrs.mrs.fr/CAZY/GH_97.html). It included two α -glucosidases SusB from *Bacteroides thetaiotaomicron* ATCC29148 and *Tannerella forsythensis* ATCC43037, as well as a group of 16 conserved hypothetical proteins. Homology search of the updated sequence databases allowed us to reveal additional members of the family. In total, GH97 family contains 42 proteins. The majority of them have been annotated in several recent bacterial genome projects. Members of GH97 family are

represented only in a limited number of bacteria from phyla *Actinobacteria* (one genus), *Bacteroidetes* (three genera), *Planctomycetes* (one genus), and *Proteobacteria* (two and four genera from α - and γ -classes, respectively), as well as in a unique archaea. However, many of these bacteria have several paralogous genes. The most interesting case is that of *B. thetaiotaomicron* ATCC29148, which has α -glucosidase SusB and 9 putative paralogues. Many genes, encoding proteins of the GH97 family, are located in clusters with genes of glycoside hydrolases and other carbohydrate-active enzymes. Phylogenetic analysis allows to distinguish five subfamilies in the GH97 family with at least two known members in each of them. Among environmental samples from the Sargasso Sea we have found 60 sequences homologous to proteins of GH97 family. Iterated Blast searches demonstrate that GH97 family has a distant relationship with GH27, GH31, and GH36 families of retaining glycoside hydrolases, which belong to the α -galactosidase superfamily. It allows us to assume that glycoside hydrolases from GH97 also can have retaining mechanism of the glycoside bond hydrolysis and a similar (β/α)₈-barrel type fold of their catalytic domain.

A2-059P

The presence of four iron-containing superoxide dismutase isozymes in Trypanosomatidae: characterization and subcellular localization in *Trypanosoma brucei*

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Superoxide dismutases (SODs) form part of a defense mechanism that helps protect obligate and facultative aerobic organisms from oxygen toxicity and damage. We report the presence in the trypanosomatid genomes of four SOD genes: Fesoda, Fesodb1 and Fesodb2 and a newly identified Fesodc. All four genes of *Trypanosoma brucei* have been cloned, sequenced and overexpressed in *Escherichia coli* and shown to encode active dimeric FeSOD isozymes. Homology modelling of the structures of all four enzymes using available X-ray crystal structures of homologs showed that the four TbSOD structures were nearly identical. Subcellular localization using GFP-fusion proteins in procyclic insect trypanosomatids shows that FeSODB1 is mainly cytosolic, with a minor glycosomal component, FeSODB2 is mainly glycosomal with some activity in the cytosol and FeSODA and FeSODC are both mitochondrial isozymes. Phylogenetic studies of all available trypanosomatid SODs and 106 dimeric FeSODs and closely related cambialistic dimeric SOD sequences suggest that the trypanosomatid SODs have all been acquired by more than one event of horizontal gene transfer, followed by events of gene duplication.

A2-060P

Fold-recognition, homology modeling and mutagenesis of restriction enzyme Bsp6I

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Identification of functionally important residues in type II restriction enzymes (REases) has been difficult using conventional methods. Even though known REase structures share a common

fold and marginally recognizable active site, the overall sequence similarities are statistically insignificant, unless compared among proteins that recognize identical or very similar sequences. Bsp6I is a Type II REase, which recognizes the palindromic DNA sequence 5'GCNGC and cleaves between the cytosine and the unspecified nucleotide in both strands, generating a double strand break with 5'-protruding single nucleotides. There are no solved structures of REases that recognize similar DNA targets or generate cleavage products with similar characteristics. The Bsp6I sequence shows no significant similarity to REases with known structures. However, using a protein fold-recognition approach, we have identified a remote relationship between Bsp6I and the structure of PvuII, which allowed us to construct a homology model of Bsp6I and use it to predict functionally important regions and residues in Bsp6I. The model of the Bsp6I structure was built using the "Frankenstein's monster" method and tested by the characterization of the effects of single amino acid substitutions of residues predicted to be directly involved in cleavage, DNA-binding and dimerization. The endonuclease activity of an extensive panel of mutants was tested *in vivo* using the bacteriophage lambda-plating assay. All mutations in residues predicted as catalytic, involved in DNA binding dimerization decreased the restriction level to less than 1% of the wild type (wt) activity. A subset of mutants exhibiting different levels of reduction of the *in vivo* activity was recloned into an expression vector, overexpressed, purified and tested in an *in vitro* cleavage assay. The results agreed with the *in vivo* analyses, thus corroborating the model-based predictions. Our study represents an example of how the computational protein fold-recognition followed by model-based identification and experimental validation of functionally important residues can be used to reduce the "white spaces" on the structural map of a protein superfamily by providing links between known structures and the sequences of their remote homologs. Confident identification of a protein fold, which is very difficult in the case of restriction enzymes, is important for the selection of targets for high-resolution studies. Completing the picture of sequence-structure-function relationships in protein superfamilies becomes an essential task in the age of structural genomics and our study may serve as a paradigm for future analyses.

A2-061P

The guards take the lead: genome "dialect", DNA repair, and evolutionary variation

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Several species-specific characteristics of genome organization that are superimposed on its coding aspects were proposed earlier, including genome "signature", genome "accent" and "compositional spectrum". These notions could be considered as representatives of "genome dialect". We measured within the proteobacteria some genome dialect representatives: The relative abundance of dinucleotides, or "genome signature"; the profiles of occurrence of 10 nucleotide "words" (compositional spectra) and the profiles of occurrence of 20 nucleotide words, using "degenerate" two letters alphabet (purine-pyrimidine compositional spectra). Here, we show that the evolutionary distances between enzymes involved in DNA repair and recombination, are highly correlated with purine-pyrimidine compositional spectra, and genome signature distances. All the enzymes of the nucleotide excision repair system belong to this group. Other (control) pro-

tein groups have significantly lower correlations of their evolutionary distances with the purine–pyrimidine compositional spectra, and genome signature distances. We hypothesize that the high correlation of the former group with genome dialect is resulted from coevolution of genome structure and DNA repair-recombination enzymes and discuss the mechanisms that might be responsible for this coevolution.

A2-062P Functional and molecular diversity of enolase gene family

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Enolase (EC 4.2.1.11) – an enzyme of glycolytic pathway catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate. Enolase is a member of gene superfamily and performs several functions, i.e. an eye tau-crystallin protein, Myc-binding protein, surface receptor for the binding of plasminogen, etc. Three known isoforms of enolase: non-neuronal enolase (alpha or NNE), muscle-specific enolase (beta or MSE) and neuron-specific enolase (gamma or NSE) can be found in vertebrata. Enolase is a two-domain protein. N-terminal domain is shorter (residues 1–134) while beta-barrel domain (C-terminal) extends from residue 143 to end of molecule. A short unfolded region is present between these two main domains. This work was aimed at analysis of nucleotide and amino acid sequences of all enolase isoforms. Amino acid sequences were obtained from SwissProt and GenBank databases and aligned in ClustalX. Sequence alignments were analyzed in S.I.F.T. (Sort Intolerant From Tolerant) to predict particular positions of possible substitutions. Phylogenetic trees of enolase amino acid and nucleotide sequences were constructed in MEGA2 software. DIVERGE was used to find out a functional diversity of analyzed proteins. The overall phylogenetic tree clearly shows evolutionary linkage and functional diversity of three vertebrate isoforms: alpha, beta and gamma. Phylogenetic analysis suggests early gene duplication and that intergenic recombination among the enolase subtypes is not taking place. As expected, amino acid sequences analysis in S.I.F.T. shows uneven distribution of tolerated substitution sites among three isoforms. It is evident that C-terminal region is more susceptible for substitutions which might reflect a tendency to relaxation of amino acid sequence. Only NSE accumulated almost the same number of possible amino acid substitutions both in N-terminal (five substitutions) and C-terminal (six substitutions) region. Alpha enolase (NNE) accommodated much more possible substitutions than other isozymes. Gamma enolase (NSE) contains occasional substitutions in a few sites only – it suggests higher stability of this isoform, but also that it might be the youngest of vertebrate enolase isoforms. Residues playing critical role in enzyme activity such as active site (His 157) and ligands binding sites (Asp 244, Glu 292, Asp 317) remain unchanged.

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A2-063P

Sequence-structure-function relationships of a tRNA (m7G46) methyltransferase studied by homology modeling and site-directed mutagenesis

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The *Escherichia coli* TrmB protein and its *Saccharomyces cerevisiae* ortholog Trm8p catalyze the S-adenosyl-L-methionine-dependent formation of N7-methylguanosine at position 46 (m7G46) in tRNA. To learn more about the sequence-structure-function relationships of these enzymes we carried out a thorough bioinformatics analysis of the tRNA:m7G methyltransferase (MTase) family to predict sequence regions and individual amino acid residues that may be important for the interactions between the MTase and the tRNA substrate, in particular the target guanosine 46. We used site-directed mutagenesis to construct a series of alanine substitutions and tested the activity of the mutants to elucidate the catalytic and tRNA-recognition mechanism of TrmB. The functional analysis of the mutants, together with the homology model of the TrmB structure and the results of the phylogenetic analysis, revealed the crucial residues for the formation of the substrate-binding site and the catalytic center in tRNA:m7G MTases. Although the determination of the nuances of the catalytic mechanism will probably require the determination of a high-resolution crystal structure of a member of the TrmB/Trm8p family in complex with the tRNA, our analysis reveals that the protein-substrate interactions in tRNA:m7G MTases are made by different residues than in the case of mRNA cap:m7G MTases. We hope that our analyses will also stimulate structural studies and identification of catalytic residues in the family of rRNA:m7G MTases, which may present yet another independent solution to the problem of guanosine-N7 methylation.

A2-064P

Biochemical and structural characterization of human ECI and its complex with human carboxypeptidase A4

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The only endogenous protein inhibitor known for metallo-carboxypeptidases (MCPs) is ECI, a 25 kDa protein discovered in the rat brain. In the present work, the human ECI nucleotide sequence was amplified from human brain cDNAs and cloned into the prokaryotic expression vector pGAT2 of *Escherichia coli*. Recombinant ECI is a tight-binding, non-competitive inhibitor against vertebrate A/B-type MCPs. Inhibition studies based on pre-steady-state analysis yielded kinetic inhibition constants (K_i) values in the nanomolar range, indicative of very strong inhibition. These studies have also shown that ECI is unable to inhibit members of the MCP N/E class or an invertebrate A/B-MCP from the cotton bollworm, *Helicoverpa armigera*. In the isolated

state, ECI is an unstable, aggregation-prone protein, and its stability is being studied by NMR, circular dichroism and fluorescence methods. However, its complexes with target carboxypeptidases are stable entities, as is the case of the CPA4-ECI complex. Human CPA4 is a non-pancreatic A/B type CP whose expression is induced in prostate cancer cells after treatment with histone deacetylase inhibitors. Structural studies of both the zymogen form of hCPA4 and the CPA4-ECI complex unveil the determinants of the two inhibition mechanisms.

A2-065P

***Erwinia amylovora* outer membrane protein - characterization gene sequence and secondary structure**

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Erwinia amylovora is a Gram negative bacterium which is pathogenic to plants. An active porin (Omp-EA) was purified from the outer membrane of *E. amylovora* and was found to be a trimeric protein with a molecular weight of about 115 kDa (38 kDa of each of its monomers). The exclusion limit of Omp-EA channel for neutral sugars is estimated to be approximately 600Da. Permeability of neutral molecules is preferred by this porin over anionic charged molecules. The gene encoding for Omp-EA was cloned and sequenced. Each monomer of Omp-EA consists of 361 amino acids. When the first N-terminal 20 amino acids were sequenced and compared with other porins it was found that there is 100% homology with N-terminal of three known porins: OmpC, OmpF and PhoE of *E. coli* and only 92% homology with OmpN of *E. carotovora*. The identity of the overall sequence with the porins OmpC, OmpF and PhoE of *E. coli* is between 73 and 90%. By the prediction of the secondary structures the highest identity was found in their beta sheet sequences. In addition, the sequence of the outer loops of Omp-EA has only 30% homology with the loops of OmpC of *E. coli*. This difference may indicate the reason why *E. amylovora* is pathogenic to plants and not to humans.

A2-066P

A positive selection in vertebrate linker histone family

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A family of vertebrate H1 histones is composed of several developmentally regulated non-allelic variants with high primary amino-acid sequence conservation. The conserved regions that include a globular domain and assorted amino-acid sequence blocks in the N- and C-terminal tails are thought to be under negative selection. Nonetheless, certain sites in H1 histone variants might have been positively selected. Here it has been assumed that an adaptive evolution could be responsible for a functional divergence within vertebrate linker histone family. Using a Diverge program (Gu X and Vander Velden K., *Bioinformatics* 2002; **18**, 500–501) it has been shown that Θ values for a cluster of H1^o/H5 subtypes differ significantly from those of both amphibian and avian somatic subtypes, and from mammalian H1t variants as well. The latter also differed from the somatic H1s. However, no strong positive selection has been noted when avian somatic H1 histone C-terminal domains, which are known to be involved in the interaction with linker DNA, were analyzed against mammalian ones.

A2-067P

Molecular characterization and pheromonal properties of 19-kDa urinary protein in house rat (*Rattus rattus*)

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The olfactory cues in urine, vaginal fluid feces, salivary and scent glands are reported to be the source for pheromonal communications. The molecules involved in this process is called as pheromones and have been shown to influence in sexual attraction, evocation of aggression, territorial marking, mother – young interaction and individual identification. Recent reports vindicated that the pheromonal communication is involved with the help small non-volatile molecules called as urinary proteins, which is synthesized in the liver released in the blood plasma and excreted via the urine, which is 50% of the total protein concentration (Quantity one - Image analysis; Biorad USA) called as the major urinary protein (MUPs) in mouse (*Mus musculus*) and rat (*Rattus norvegicus*) called as the alpha 2u globulin. Therefore, the present study was carried out to identify the urinary protein in house rat (*Rattus rattus*) using SDS-PAGE. The examination of SDS-PAGE protein profiles shows that the 19,27,41,56,64,79, and 92-kDa respectively, which are all present in adult male rat, not in the pre-pubescent males. The 19-kDa is completely absent in the female system and if at all present only trace amounts. The variation in the expression pattern of 19-kDa is due to the multi hormonal control. Hence, the 19-kDa urinary protein is purified using Gel filtration column chromatography and analyzed its N-terminal amino acid sequence. The deduced amino acid sequence is consistent with large family of lipocalin protein. The tertiary structure of lipocalin protein family is having an eight and nine α barrel structure, which is having the tryptophan residue. It may play a paramount role to bind the pheromonal compound with the protein, and act as a carrier for pheromonal communication. The present study is undergone to determine the ligand binding ability between the protein and ligand. The exploration of ligand binding ability would pave the way to develop the pheromonal trap.

A2-068P

Nna1-like proteins are a new subfamily of metallo-carboxypeptidases

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Metallo-carboxypeptidases (CPs) catalyze the removal of C-terminal residues from proteins and peptides. These enzymes are widely distributed among organisms and perform a variety of functions, from digestive degradation to bioactive peptide processing, which requires a high specificity and strict control. Metallo-carboxypeptidases form the M14 family of peptidases, which consists of three subfamilies based on tertiary structure and zinc anchoring motifs: M14A (CP A/B), M14B (CP N/E) and M14C (gamma-D-glutamyl-(L)-mesodiaminopimelate). Nna1 is a putative carboxypeptidase, which gene is involved in axon regeneration

and was identified as the mutated gene in Purkinje cell degeneration (pcd) phenotype in mouse. By computational methods, we have identified proteins similar to nna1 throughout all currently sequenced eukaryotic genomes (except Fungi) and several bacteria. We propose to classify these carboxypeptidase-like forms as a new M14 subfamily. In order to define this new M14 subfamily we have done an exhaustive sequence study with all the putative members. The distinctive motifs of the new subfamily and its phylogenetic relationship with the other subfamilies were defined in this work. Structural models of several members were built confirming the suggested classification hypothesis. Annotation of chromosomal location were performed for human and mouse nna1-like peptidases. Bioinformatics analyses for the diversity and tissue specificity are also shown in this work.

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A2-069P **Evolutionary based structure-function relationships within the cysteine stabilized α/β motif-containing proteins**

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Invertebrate defensins, the most widely spread group of antimicrobial peptides, are characterized by the presence of the cysteine-stabilized α/β (CS α/β) motif signature. This structural scaffold is also present in several functionally diverse bioactive peptides, including various peptides from plants and the vast majority of scorpion toxins. Invertebrate defensins have been isolated from nematodes, arthropods and molluscs, in which they act as critical effectors of their innate immune systems. These features make the invertebrate defensins an interesting subject for evaluating their evolutionary relationship in the context of highly diversified innate immune systems. However, such a task is difficult due to the lack of significant sequence relatedness within this group. Similarly, the largest group of CS α/β motif-containing peptides, the toxins isolated from scorpion venoms, is divided into two main groups on the basis of their chain length. The short-chain toxins act as K^+ -channel blockers, whereas the long-chain toxins modulate the gating mechanism of Na^+ -channels. Individual peptides from both groups display some striking preferences for some ion-channels, making worthy the study of the molecular determinants of their specificity. Here we analyze the evolutionary history of CS α/β motif-containing peptides; we establish the paralogous nature of scorpion defensins and toxins, we analyze the phylogenetic relationships within each group of scorpion toxins correlating their structures and functions, and we are revising the current hypothesis regarding the evolution of the whole set of CS α/β motif-containing peptides.

A2-070P **Structural genomics of the biosynthesis of polyketide antibiotics**

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Polyketides form a large and diverse group of natural compounds produced mainly by microorganisms and plants. Among

these secondary metabolites are the anthracyclines, aromatic polyketide antibiotics produced by *Streptomyces* species. Anthracyclines are of particular medical importance, because some of the clinically most potent anti-tumor drugs are recruited from this class of compounds, for instance doxorubicin and daunorubicin. The complexity of the chemical structure of anthracyclines makes their chemical synthesis difficult, and combinatorial biosynthesis appears to be a promising competitive route towards novel anthracyclines with improved toxicity profiles. Genetic and biochemical studies of the underlying pathways and enzymes are therefore expected to provide the necessary insights required for the engineering of anthracycline biosynthesis either through the hybrid antibiotic approach or by redesign of biosynthetic enzymes. Our laboratories are engaged in a small-scale structural genomics project aiming at the structural and functional characterization of the enzymes of anthracycline biosynthesis. Out of approximately 50 target genes, we have at present cloned 11 genes and produced the corresponding enzymes in soluble form. Nine of these have been crystallized and the crystal structures of seven of the enzymes have been determined so far. All enzymes have been crystallized with bound ligands, which increase the mechanistic insights derived from the structure analysis. Mechanistic proposals have been verified by site-directed mutagenesis and other biochemical methods. These studies provided insights into the structural basis of substrate specificity of these enzymes. Binding and recognition of the anthracycline substrates is dominated by hydrophobic interactions, and specificity is controlled by the shape of the binding pocket rather than through specific hydrogen bonds. Several of the enzymes show novel mechanisms. For instance, the polyketide cyclase Snoal catalyses an intramolecular aldol condensation with a mechanism different from the classical aldolases, i.e. without the use of Schiff base formation or metal cofactors. The S-adenosyl-L-methionine dependent hydroxylase RdmB catalyzes a novel type of hydroxylation reaction, without metal ions or flavin cofactors. It is the first demonstration of the use of S-adenosyl L-methionine as a cofactor in an enzymatic hydroxylation reaction.

A2-071P **Prebiotic evolution in interstellar space**

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There is an interesting Goldanskii's hypothesis about the role of the molecular tunnelling in chemical and prebiotic evolution, occurring in interstellar space at very low temperatures [1]. The author has considered possible physical and chemical processes in interstellar dust clouds and comets with extended orbits, examining the radiationally stimulating low-temperature polymerization of formaldehyde at length, which should take place on a surface of dust grains by means of the molecular tunnelling process. Indeed, the modern models of dust diffuse and molecular clouds, and comets (for example, "bird's nest" model as a porous aggregate of inter-stellar dust for simulating of a comet nucleus) [2] suggest a presence of effective physical and chemical processes, resulting in the formation of simple and complex chemical combinations with mandatory participation of dust grains. Their temperature usually lies in the narrow temperature range 10–20 K. Consequently, there is an equilibrium exchange of energy between dust grains surface and thermal radiation. This circumstance allows us to apply the interaction model of thermal radiation with molecules at low temperatures [3] without resorting to use of wave properties of atomic and molecular particles, that is to exclude from consideration the molecular tunnelling process.

The conclusion reached removes all restrictions on masses of atoms and molecular groups responsible for the course of elementary chemical acts at low temperature. It leads to possibility of origin of not only complicated organic molecules, but also biologically active macromolecules in the interstellar space.

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A2-072P

Residue 234 in glutathione transferase T1-1 plays a pivotal role in the catalytic activity and the selectivity against alternative substrates

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Glutathione transferase (GST) T1-1 plays an important role in the biotransformation of halogenated alkanes, which are used in large quantities as solvents and occur as environmental pollutants. Many reactions catalyzed by GST T1-1 qualify as detoxication processes, but some reactions with dihalogenated alkanes lead to reactive products more toxic than the substrates. Murine GST T1-1 is particularly active with dichloromethane, which may explain the high carcinogenicity of dichloromethane in the mouse. Human GST T1-1 activity is considerably lower with halogenated hydrocarbons and some related substrates. Human GST T1-1 is polymorphic with a frequent null phenotype suggesting that it is advantageous, under some circumstances, to lack the functional enzyme, which catalyzes GSH conjugations that may cause bioactivation. The present study shows that amino acid residue 234 is a determinant of the differences in catalytic efficiency between the human and the rodent enzymes. The replacement of Trp234 in human GST T1-1 by Arg, found in the rodent enzyme, enhanced the alkyltransferase activity by an order of magnitude with a series of homologous iodoalkanes and some typical GST substrates. The specific activity of the alternative mutant Trp234Lys was lower than for the parental human GST T1-1 with many substrates, showing that a positive charge is not sufficient for increased activity. The enhanced activity of Trp234Arg with alkylating agents was dependent on the substrate tested, whereas no increase of the peroxidase activity with cumene hydroperoxide was noted. Residue 234 therefore is also involved in the control of the substrate selectivity of GST T1-1.

A2-073P

Tachykinin and tachykinin receptor of an ascidian, *Ciona intestinalis*: evolutionary origin of the vertebrate tachykinin family

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Tachykinins (TKs) are the most prevalent vertebrate brain/gut peptides. In this study, we originally identified authentic TKs and their receptor from a protochordate, *Ciona intestinalis*. The *Ciona* TK (Ci-TK) precursor, like mammalian preprotachykinin A (PPTA), encodes two TKs, Ci-TK-I and II, including the

DFXGLM-NH₂ vertebrate TK consensus. Mass spectrometry of the neural extract revealed the production of both Ci-TKs. and Ci-TK-I contains several Substance P (SP)-typical amino acids, whereas a Thr is exceptionally located at position 4 from the C-terminus of Ci-TK-II. The Ci-TK gene encodes both Ci-TKs in the same exon, indicating no alternative generation of Ci-TKs, unlike the PPTA gene. These results suggested that the alternative splicing of the PPTA gene was established during evolution of vertebrates. The only Ci-TK receptor, Ci-TK-R, was equivalently activated by Ci-TK-I, SP and Neurokinin A at physiological concentrations, whereas Ci-TK-II showed 100-fold less potent activity, indicating that the ligand selectivity of Ci-TK-R is distinct from those of vertebrate TK receptors. Ci-TK-I, like SP, also elicited the typical contraction on the guinea pig ileum. The Ci-TK gene was expressed in neurons of the brain ganglion, small cells in the intestine, and the zone 7 in the endostyle, which corresponds to the vertebrate thyroid gland. Furthermore, the Ci-TK-R mRNA was distributed in these three tissues plus the gonad. These results showed that Ci-TKs play major roles in sexual behavior and feeding in protochordates as brain/gut peptides and endocrine/paracrine molecules. Taken together, our data revealed the biochemical and structural origins of vertebrate TKs and their receptors.

A2-074P

Functional and structural characterization of the myoglobin from the polychaete *Ophelia bicornis*

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Ophelia bicornis body wall myoglobin was purified by ammonium sulfate precipitation, Sephadex G-100 gel chromatography and DEAE-cellulose chromatography. The primary structure, obtained from cDNA and protein sequencing, consists of 139 aminoacid residues. ESI-IT-MS analysis provided a mass value for the globin of 14357 ± 3 amu in good agreement with the theoretical average mass of 14359 amu, computed from the globin sequence. The alignment with other globin sequences showed that *O. bicornis* myoglobin misses the pre-A helix, the first six residues of the A helix, and possess a PheB10-GlnE7 heme distal residue pair. The measured oxygen affinity ($P_{50} = 0.85$ mmHg at 20 °C) is in agreement with the presence of the unusual heme distal residue pair. The autoxidation rate constant determined at 37 °C resulted equal to 0.28 h⁻¹, only slightly higher with respect to that of the sperm whale myoglobin mutant E7 His to Gln (0.21 h⁻¹) and to elephant myoglobin (0.1 h⁻¹), characterized by the same heme distal residue pair. Oxygen binding cooperativity was found absent in the 6.5–8.5 pH range. The resistance of *O. bicornis* myoglobin towards autoxidation seems to confirm the important role of part of the A helix in the stability of the globin. The higher pK of the acid-alkaline ferric transition of *O. bicornis* with respect to Asian elephant myoglobin, as well as the higher absorbance ratio of its ferric-form to the oxy-form measured in the Soret region (met/oxy) with respect to that of the African elephant myoglobin, suggested a stronger interaction between the distal glutamine and the water molecule at the sixth coordinate position.

A2-075P**Unique sugar affinity of four novel isoforms of a ribosome inactivating protein from *Viscum album* (L.) inhabiting NW Himalaya**R. S. Sharma¹, V. Mishra^{2,3}, S. Yadav³, C. R. Babu^{1,2} and T. P. Singh³¹Centre for Environmental Management of Degraded Ecosystems, School of Environmental Studies, University of Delhi, Delhi, India, ²Department of Botany, University of Delhi, Delhi, India, ³Department of Biophysics, All India Institute of Medical Sciences, New Delhi, India. E-mail: rads26@hotmail.com

Ribosome inactivating proteins (RIPs) having antitumor and immunomodulatory properties constitute the active principle of widely used mistletoe therapy in Europe. Himalayan *Viscum album* populations showed high morphological diversity therefore their RIPs (HmRIP) were investigated and four novel isoforms has been purified and characterized for the first time. HmRIP was purified by affinity chromatography and four isoforms were separated by ion-exchange chromatography. HmRIP 1, 2, 3 and 4 have isoelectric point of 6.6, 6.1, 5.2 and 4.7 respectively. Disulphide linked toxin and lectin subunits of HmRIP 1 and 2 isoforms have molecular weight of 28 and 34 kDa while that of HmRIP 3 and 4 have 28 and 32 kDa. The isoforms lacked blood group specificity. Lectin activity of HmRIPs remained unchanged for a wide range of temperature (0–65 °C) and pH (3–9). Unlike other type II RIPs, the HmRIP 1, 2 and 4 showed unique affinity towards L-rhamnose, meso-inositol and L-arabinose while HmRIP 3 has specificity to gal/galNAc. Sugar binding studies with 22 sugars also suggested that the C-4 hydroxyl of galactose might be the critical site involved in sugar binding of HmRIPs. Type II RIPs are known to be galactoside specific and do not have affinity for L-rhamnose and meso-inositol. However HmRIP 1, 2 and 4 having equal affinity for galactose and L-rhamnose does not strictly fit into any of the four structural classes of the lectins and represent a new class of type II RIPs and plant lectins.

A2-076P**Codes in the codons: codon-amino acid complementarity revealed in the structures of restriction enzyme – DNA complexes**C. E. Sansom¹, J. C. Biro^{2,3}, B. Benyo⁴ and Z. Benyo⁴¹Department of Crystallography, Birkbeck College, London, UK, ²Karolinska Institute, Stockholm, Sweden, ³Homulus Informatics, San Francisco, CA, USA, ⁴Department of Control Engineering and Information Technology, Budapest University of Technology and Economics, Budapest, Hungary
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In the early years after the elucidation of the DNA structure, there was controversy about whether there was any chemical rationale underlying the genetic code. In 1967, Carl Woese proposed that there was stereochemical affinity between amino acids and the base sequences that code for them. The alternative hypothesis proposed by Crick among others, that the exact genetic code was largely accidental, became largely accepted by the molecular biology community. We have now constructed a “periodic table” linking the chemical properties of the amino acids and the sequences of their associated codons. The amino acid table showed significant periodicity and indicated the importance of the central base in determining the chemical properties of amino acids. This adds support to Woese’s original hypothesis. If this stereochemical and structural affinity were true, we would expect interactions between amino acids and their associated codons to be favoured in DNA-protein complexes. We originally tested this hypothesis using known structures of restriction enzyme – DNA

complexes. We found that, not only were cleavage-site like base sequences found disproportionately often in the DNA sequences of restriction enzymes, but that, in the complex structures, the amino acids coded by those site-like sequences were found close to the restriction sites themselves. The average distance between the closest atoms in the codon and amino acid was significantly lowest when the amino acid involved was positively charged. We now update this work to include restriction enzyme – DNA complexes that have entered the PDB since December 2003.

A2-077P**Same fold but altered responsivity in the evolution of dUTPase homotrimer**E. Takács¹, O. Barabás^{1,2}, D. Svergun³, Z. Dubrovay¹, V. K. Grolmusz¹ and B. Vértessy¹¹Laboratory of Genome Metabolism and Repair, Institute of Enzymology, Hungarian Academy of Science, Budapest, Hungary, ²Theoretical Chemistry Department, Eötvös Loránd University, Budapest, Hungary, ³European Molecular Biology Laboratory-Hamburg Outstation, EMBL c/o DESY, Hamburg, Germany.
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dUTPase efficiently controls cellular dUTP/dTTP level. Lack of the enzyme leads to chromosome fragmentation and thymine-less apoptotic cell death. dUTPase inhibition therefore is a promising anticancer strategy. Most dUTPases are homotrimers where the C-terminal beta-strand is swapped between the jelly-roll forming subunits. An evolutionary highly conserved proline residue located at the hinge region, may facilitate this arm-swapping (1). In order to check the role of this proline in oligomerization, we examined alanine and glycine point-mutants and an arm-truncated mutant. In addition, we compared folding/unfolding characteristics of wild-type pro- and eukaryotic dUTPase representatives, which greatly differ in hydrophobicity of the threefold inner channel. We show that the physiological Mg²⁺ cofactor and three substrate analogues induce increment of the thermal melting temperature of the eukaryotic dUTPase only. In addition the more polar eukaryotic enzyme obviously possess a much lower stability against both denaturant and heat as compared to the prokaryotic enzyme (4). This sensible character supposedly contributes to enhanced fine-tuning of cellular pathways eventually required in the more developed organisms (2-3). Trimeric dUTPases unfold as a trimeric entity without dissociation preceding unfolding of monomers, and even the mutation of proline residue into Ala/Gly has no significant effect on the oligomerization state of the enzymes. However these proline mutations lead to a decrease in stability. Proline is therefore not indispensable for correct trimeric organization. We show that lack of the C-terminal arm, however prevents trimer formation arguing that arm-swapping is a major determinant of dUTPase oligomerization.

A2-078P**Ancient supramolecular complexes and new autonomous regulation of photosynthetic GAPDH. A paradigm for fine metabolic tuning in higher plants**P. Trost¹, F. Sparta¹, L. Marri¹, M. Zaffagnini¹, S. Fermani², G. Falini², A. Ripamonti² and P. Pupillo¹¹Molecular plant physiology, Department of Biology, University of Bologna, Bologna, Italy, ²Biocrystallography, Department of Chemistry, University of Bologna, Bologna, Italy.
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In oxygenic photosynthetic organisms the regulation of photosynthetic carbon metabolism in response to light/dark conditions

implies a specific role for thioredoxins, pyridine nucleotides and metabolites. GAPDH, the only dehydrogenase of the Calvin cycle, is strongly fine regulated in higher plants, but is not directly regulated in cyanobacteria and green microalgae. Regulation in these organisms is achieved through the action of an intrinsically unstructured protein (IUP) known as CP12. Under oxidizing (darkness) conditions, CP12 bears two internal disulfide bridges forming two peptide loops, which interact with GAPDH and PRK (a second member of the cycle) to form an inactive supramolecular complex. In the light, thioredoxin reduction of disulfides and CP12 displacement by ligands leads to disruption of the complex and enzyme activation. Higher plants have inherited this system and the expression of a CP12 gene in Arabidopsis is coordinately regulated with GAPDH and PRK. In addition, a second type of GAPDH (GapB) resulting from the fusion of a redox-insensitive cyanobacterial-type subunit (GapA) and the C-terminus of CP12 established itself in higher plants. This GAPDH isoform acquired autonomous regulation based on a CP12-derived regulatory mechanism. To understand this intricate regulatory network we have (i) cloned and heterologously expressed GAPDH, CP12 and PRK from Arabidopsis, and characterized the reconstituted supramolecular complex; (ii) solved the structure of regulated and non-regulated spinach GAPDH isoforms and dissected the regulatory mechanism by mutants. A comprehensive picture is emerging.

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A2-079P

Sequence correlation of proteins from different structural classes

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In this paper we analyze a set of 30 proteins belonging to different structural classes in order to reveal correlation in their amino acid sequences. We take into account different physical properties of lateral chains of amino acids by making a numerical correspondence between each amino acid and a physical property associated with it: the electric charge, the polar character and the dipole moment. For each series we determined the spectral scaling exponent, the detrended fluctuation analysis (DFA) scaling exponent, the Hurst coefficient and the correlation dimension. The values obtained for these coefficients revealed non-randomness in the great part of investigated series.

A2-080P

Pre-steady state analysis of the nucleoside hydrolase of *Trypanosoma vivax*. Evidence for rate-limiting product release

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The nucleoside hydrolase (NH) of the *Trypanosoma vivax* parasite catalyses the hydrolysis of the N-glycosidic bond in ribonu-

cleosides according to the reaction: β -purine (or pyrimidine) nucleoside + H₂O → purine (pyrimidine) base + ribose. The reaction follows a highly dissociative nucleophilic displacement reaction mechanism with a ribosyl oxocarbenium-like transition state. Here we describe the first pre-steady state analysis of the catalytic hydrolysis of a number of purine nucleosides. The NH exhibits burst kinetics. During the active site titration the maximum burst amplitude of product formation per enzyme subunit [$\pi/(e)$] is 0.52 mol/mol ± 0.04. The amplitude of the burst can be reduced by an internal equilibrium of the chemical step in the catalyzed hydrolysis. Considering that the *T. vivax* NH is a dimer, consisting of two identical monomers, a half-of-the-sites reactivity could also explain the reduced burst amplitude. The analysis suggests that the NH of *T. vivax* follows a complex multi-step mechanism in which a common slow step, different from the chemical hydrolysis is rate limiting. Stopped-flow fluorescence binding experiments with ribose indicate that a tightly bound enzyme-ribose complex accumulates during the enzymatic hydrolysis of the common purine nucleosides. This is caused by a slow isomerization between a tight and a loose enzyme-ribose complex forming the rate-limiting step on the reaction coordinate.

A2-081P

Molecular analysis of alpha-glucosidase isozymes from Japanese honeybees (*Apis cerana*)

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α -Glucosidase (EC 3.2.1.20) catalyzes the non-reducing terminal α -glucosidic bond and releases α -glucose from the substrate. We have been reported that there were three kinds of α -glucosidase isozymes (HBG I, II and III) in European honeybees (*Apis mellifera* L.). HBG I is in the ventriculus to digest sugar. HBG II is in the haemolymph to hydrolyze the sugar. HBG III is in hypopharyngeal gland to produce honey. We have also purified two α -glucosidases (JBG I and II) from *Apis cerana*, and their characterizations were investigated to be compared with HBG I, II and III. JBG I and II have been isolated as homogeneous proteins by salting-out chromatography (eluted at the high and low concentrations of ammonium sulfate respectively), ion-exchange, gel-filtration and hydrophobic chromatographies. The molecular weights were estimated to be about 82 000 (JBG I) and 76 000 (JBG II) on SDS-PAGE. Each enzyme was treated with endoglycosidase H, which gave the deglycosylated product of molecular weight smaller than that of intact enzyme, indicating that JBG I and II were glycoproteins. The N-terminal amino acid sequences of JBG II and I were similar to those of HBG II and I respectively. The properties, such as the effects of pH and temperature, and the substrate specificities of JBG I and II implied that JBG I and II seem to correspond to HBG I and II. We have isolated two candidates of cDNAs (1932 and 1862 bp) encoding JBG I and II, which contained the deduced amino acids of 577 and 579 respectively. The internal peptide sequences of JBG I and II were analyzed by in-gel digestion approach with MALDI-TOF-MS, confirmed that two cDNAs isolated were the genes of JBG I and II. The amino acid sequences of JBG I and II showed the high identity with those of HBG I and II (81 and 91% respectively). We have found the possible cDNA encoding JBG III. Currently we are searching the presence of JBG III protein.

A2-082P**Structural and functional analysis of the LysR-type Cbl transcriptional regulator from *Escherichia coli***

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The Cbl (CysB-like) protein, conserved in many Gram-negative bacterial genera, is an essential transcriptional activator of genes involved in assimilation of sulphur from organic sulphonates. In *E. coli* these genes are expressed in response to the strict absence of inorganic sulphate. We have found previously, that Cbl-mediated transcription initiation at the *ssuE* promoter in *E. coli* is abolished by adenosine 5'-phosphosulphate (APS) but not by sulphate itself. To elucidate the structural basis of Cbl function, the cofactor-binding domain of Cbl protein (c-Cbl, 88–316 aa) was cloned, purified and crystallized. The crystals belong to space group C222. X-Ray data, extending to 3.0 Å resolution, have been collected and the structure of the c-Cbl protein, using molecular replacement method, has been solved. As we assumed, there are four molecules of protein per asymmetric unit. Current R and R_{free} factor values, indicating the model reliability are 0.21 and 0.23 respectively. To improve the model of the c-Cbl protein, structural refinement calculations are under way. Structural investigations in connection with mutational studies will let us define the potential cofactor-binding site in the protein. We constructed several single residue substitutions: in c-Cbl (T102W, E150A, W166A and T202A), which result in constitutive activation of *ssuE* promoter *in vivo* and insensitive to APS transcription from this promoter *in vitro*. These residues surround the cavity formed by two α/β domains of c-Cbl and their role in accommodation of APS cofactor will be discussed.

A2-083P**Isolation, cloning and sequencing of transferrins from African ostrich and red-eared turtle**

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The aim of this study was to isolate, clone and sequence transferrins from African Ostrich (*Struthio camelus*) and red-eared turtle (*Chrysemus scripta elegans*). Until now the only known avian transferrin nucleotide sequence was from chicken (*Gallus gallus*). No reptilian transferrin sequence was published. Eggs and liver tissues were used as a material for protein and RNA isolation respectively. Proteins were identified by the N-terminal amino acid sequence analysis from the PVDF membrane. The purification

procedure for both transferrins included gel filtration on Sephacryl S-300 and anion exchange chromatography on MonoQ. The presence of individual proteins was monitored on the basis of molecular weight determination in SDS-PAGE. The purified transferrins were digested with CNBr and peptide mixtures were separated in SDS-PAGE. N-terminal amino acid sequence of selected peptides was determined. Two sets of primers were designed. First – on the basis of the determined ostrich and turtle transferrins partial amino acid sequences; second – on the basis of particularly conservative nucleotide sequences of different vertebrate - transferrins. As a PCR template cDNA synthesized from total RNA isolated from ostrich and turtle liver tissues was used. The purified PCR products were directly cloned into the pCR4-TOPO vector and sequenced. Gene-specific primers were designed on the basis of the determined nucleotide sequences. The use of 5'-RACE and 3'-RACE allowed to determine the full-length transferrin cDNAs. The sequences were placed in the EMBL Nucleotide Sequence Database with accession numbers: AJ786651 for African ostrich and AJ786650 for red-eared turtle.

A2-084P**Structure assay on PER 1 and its mediating function of carrying proteins translocating into liver cells for gene therapy**

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Structure prediction and assay on PER1 and its single amino acid mutant analogs. Based on the structures and functions of the PER 1, we utilize the peptides simulation system on the study of PER1 and its analogs, predict the conformations and the characteristics of PER 1, its electrostatic potentials, hydration characters and solvent accessible surface potentials. All PER1 and its analogs constructs described were cloned as in-frame C-terminal fusion protein to EGFP. Protein transduction domain of PER1 delivers proteins into mammalian cells with certain culture medium. Structure assay on the sequence derived from Per 1 and its mutant analog by Rosetta, an *ab initio* method. Calculation of electrostatic potentials and solvent accessible surface was calculated by delphi. Compare to the result of PER1 mediated peptides delivering into liver, to explain how PER1 deliver into cells by this mechanism. PTDs of all these proteins with penetrating ability are abundant with basic residues. The surface of electrostatic potential was observed in a high region in penetrated ability peptides. The solvent accessible surface of a molecule is defined as the surface area of the molecule exposed to solvent. We find some of physical properties of PER1 are very important for its penetrating function. There should be a certain length of its α -helix and an obvious strong positive electrostatic potential distribution region. A certain hydrophobic property of the molecule is for it to get over high-energy barrier between the environment of solution and lipid membrane.

A3 – Bioinformatics

A3-001

Correlations between quantitative measures of genome evolution, expression and function

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In addition to multiple, complete genome sequences, genome-wide data on biological properties of genes, such as knockout effect, expression levels, protein-protein interactions, and others, are rapidly accumulating. Many significant correlations between these variables have been detected, e.g., a positive correlation between the tendency of a gene to be lost during evolution and sequence evolution rate, and negative correlations between each of the above measures of evolutionary variability and expression level or the phenotypic effect of gene knockout. However, most of these correlations are relatively weak and explain a small fraction of the variation present in the data. We show that it is possible to explain a much larger part of the variability by using a single, composite variable, the gene's "social status in the genomic community". This variable contrasts phenotypic ("input") and evolutionary ("output") variables "High-status" genes, involved in house-keeping processes, are more likely to be higher and broader expressed, to have more interaction partners, and to produce lethal or severely impaired knockout mutants. These genes also tend to evolve slower and are less prone to gene loss across various taxa. "Low-status" genes tend to be weakly expressed, have fewer interaction partners, and exhibit a narrower (and less coherent) phyletic distribution. On average, these genes evolve faster and are more often lost during evolution than high-status genes. The "gene status" notion may serve as a generator of null hypotheses regarding the connections between phenotypic and evolutionary parameters associated with genes. Any deviation from the expected pattern calls for attention – to the quality of the data, the nature of the analyzed relationship, or both.

A3-002

Functional organization of transcriptional-regulatory networks

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Recent evidence indicates that potential interactions within metabolic-, protein-protein interaction-, and transcriptional-regulatory networks are differentially utilized according to the environmental conditions in which a cell exists. However, the topological units underlying such differential utilization have not been investigated. Here, we use the transcriptional regulatory network of *Escherichia coli* to identify such units, called orignons, representing regulatory subnetworks which originate at a distinct class of sensor transcription factors. Using microarray data, we find that specific environmental signals affect mRNA expression levels significantly only within the orignons responsible for their detection and processing. We also show that small regulatory interaction patterns, called subgraphs and motifs, occupy distinct positions in- and between orignons, offering insights into their dynamical role in information processing. The identified features are likely to represent a general framework of environmental signal processing in prokaryotes.

A3-003

Evolutionary history of the eukaryotic interactome

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Thousands of hypothetical protein-protein interactions in *Saccharomyces cerevisiae* were identified using two-hybrid system Y2H and mass spectrometry of coimmunoprecipitated protein complexes (Co-IP). Extracting biologically significant information from these data is an especially complex challenge. We have used the Filtered Yeast Intercatome dataset to study the evolution of yeast interactome by tracing when different modules of interacting proteins appeared. For this purpose we calculated phylogenetic profiles using simplified taxonomy containing the following kingdoms: Fungi, Animals, Plants, Eubacteria, Archea, and Viruses. It was assumed that two proteins have identical phylogenetic history if they have strong homologs in identical sets of systematic groups and at the same time no homologs in the rest of considered systematic groups. Such procedure creates sets of proteins certainly having similar evolutionary history. One can assume that interactions between such proteins are evolutionarily conserved and biologically significant. The proposed approach allows to detect parts of interactome appearing at a given stage of evolution. As a result separated islands of yeast interactome (modules) are obtained. Proteins from these modules are, in most cases, obviously functionally linked. Often, the modules contain interactions between different paralogous proteins. Such duplication events are detected by the proteins' presence in identical protein families (as guided by the results of PFAM analysis). The obtained network of interactions between co-appearing proteins during the process of evolution contains 379 proteins and 308 interactions. This network is extremely evolutionarily conserved – only one of these proteins has no homologue in the human genome (trehalose-6 phosphate). Visualization of the modules shows an obvious biological sense. The analysis of the modules reveals major steps in evolution of the eukaryotic interactome.

A3-004

Per residue characterization of protein-protein interfaces: a relationship between stability and functionality?

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Finding why protein-protein interactions (PPIs) are so specific can provide a valuable tool in a variety of fields. Complexes are frequently clustered according to their lifetime (transient or permanent), their composition (homodimeric or heterodimeric) and even the possibility (or not) of finding the unbound partners alone *in vivo* (non-obligate and obligate). Although this classification is arbitrary [1], a common feature for strong complexes is a somewhat more hydrophobic character in the interacting region than in the rest of the molecular surface. For soft interactions, on the contrary, it is difficult to find a similar correlation. Thus, it appears that, in general, transient complexes tend to involve the interaction between two regions at least equally polar than the rest of the surface. PPIs have to compete with

both the interaction between surface residues with water and the interaction between surface residues themselves in the unbound proteins. On the other hand, several works by Warshel and others have shown that the notion of active site electrostatic preorganization can be used to interpret the high efficiency in enzyme reactions (see, e.g. [2] for a review). It has been shown that this pre-organization can be related to the stability of the residues in this region. In some enzymes, in addition, conformational changes upon binding to other proteins lead to an increase in the activity of the enzymatic partner. These facts suggest that the evaluation of the stability of residues in a protein can be used to detect active site regions and eventually to assign functionality to orphan proteins. Following these arguments, we will try to extend the pre-organization theory to PPIs.

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A3-005

Protein structure prediction by combination of fold-recognition with *de novo* folding

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A new method for protein structure prediction was developed, which allows modeling regardless of the potential homology with any known protein structure. This method is a combination of the “FRankenstien’s Monster” approach for comparative modeling (CM) by recombination of Fold-Recognition models [1], and a new implementation of a Replica Exchange Monte Carlo (REMC) method for protein folding *de novo* or with restraints [2]. The sequence of a modeled protein is submitted to the GeneSilico structure prediction meta server (<http://genesilico.pl/meta/>), which is a gateway to a variety of methods for secondary structure prediction and fold-recognition (FR). FR alignments are compared and ranked and the most frequently reported folds are selected for further analysis. For each fold, the target-template alignments are used as a starting point for modeling using the “FRankenstien’s monster” approach [1]. Models are evaluated and fragments with best scores are used to derive spatial restraints. Secondary structure restraints are derived from the consensus of methods implemented in the GeneSilico meta server. Secondary and tertiary restraints are used to guide the REMC folding simulation using a high-resolution reduced lattice model CABS [2]. The conformations obtained in the course of CABS simulations are subject to the average linkage hierarchical clustering. For a representative structure from each cluster a full-atom representation is rebuilt. The performance of the new method will be discussed in the context of our successful predictions in the recent CASP-6 experiment and other modeling exercises.

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A3-006

PDB-ligand: an interactive clustering tool for protein-ligand complexes in PDB

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Understanding the protein–ligand interaction is very important in post-genomics biological science because many proteins require small molecular ligands or cofactors such as ATP or NAD. The first step for understanding protein–ligand interaction would be to analyze all the known protein–ligand complex structures in the Protein Data Bank (PDB, <http://www.rcsb.org>). Recently, we have developed a novel Web-based bioinformatics tool, called PDB-Ligand (<http://www.idrtech.com/PDB-Ligand/>), for the visual and interactive clustering of all the known protein-ligand complexes in PDB [1]. PDB-Ligand is a database and a tool that allows one to browse, classify, superimpose and visualize these protein-ligand binding structures. Currently, there are about 5390 types of small molecular ligands and more than 130 000 protein–ligand complex structures, experimentally determined as a complex with protein or DNA. One novel feature of PDB-Ligand is that it allows an interactive clustering of ligand-binding structures based on user-specific clustering criteria with flexible combinations of atoms or residues at the ligand-binding sites. PDB-Ligand, when using with other related ligand-binding structure analysis tools, will be a good resource for better understanding of ligand-binding structures, which may be critically important in many new drug discovery applications.

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A3-007P

Decision trees: a statistical method to explain the triggering mechanisms of acute splenic sequestration crisis in sickle cell disease

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Sickle cell disease (SCD) is widespread in the Caribbean islands, particularly in Guadeloupe. SCD is a homozygotic disease with a single nucleotide substitution in the 6 position of the beta-globin gene, leading to an abnormal, sickled hemoglobin chain (Hb S). The Hb S molecules polymerize, forming sickled red blood cells (sRBC). sRBC cannot normally traverse the microvasculature, leading to the occlusion of blood vessels. One of the most dangerous SCD complication is acute splenic sequestration crisis (ASSC), characterized by rapid sequestration of considerable amount of blood within the spleen. About 30% of children will experience an episode, usually between the age of 6 months and 3 years. ASSC may be quite severe, and severely affected children may progress rapidly to death. Treatment consists of transfusion, occasionally splenectomy, especially for patients with recurrent ASSC. The pathophysiological mechanism is still unknown. In this study, we wanted to know what the triggering factors explaining the two important notions in ASSC were: the severity

and the recurrence of the crisis. To better understand it, we used a statistical analysis: the decision tree. We studied a basis with different parameters (physical, hematological...) describing the patient and his crisis. Our preliminary work enabled us to isolate parameters explaining the severity or the recurrence of a crisis, using all the parameters of the basis. These first results offer encouraging perspectives for the use of mathematical systems to explain biological problems. This study will be further developed to exploit data on other kinds of SCD complications, especially to create a medical software to help make a decision and establish patients profiles.

A3-008P

Origin and development of the genetic code: Is there a code in the codon?

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The information carried by protein-coding nucleic acids is raptly three times as much as the information carried by the coded proteins: the Universal Genetic Code is degenerate. Accumulating number of evidence suggest that the information excess in the codons is not due to simple redundancy, but it has important biochemical role in determining the physicochemical properties of amino acids (Biro JC et al. A common periodic table of codons and amino acids. *BBRC* 2003; **306**: 408–415) and the nature of specific nucleic acid protein interactions (Biro JC & Biro JMC. Frequent occurrence of recognition site-like sequences in the restriction endonucleases. *BMC Bioinformatics* 2004; **5**: 30). We have provided evidence that the genetic code is the result of nucleic acid – protein coevolution (Woese CR 1967) and not simply a “frozen accident” (Crick 1968). The recent work will briefly review the history of discovering the genetic code, including George Gamow’s contribution (The “diamond code”, 1954). The intriguing geometry of the codons will be illuminated as well as its interesting connection with Gray codes and with the I Ching. Some fundamental questions will be asked and, hopefully, answered.

A3-009P

A C++ code library for metabolic modelling

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A code library for the manipulation of kinetic models of metabolism is being developed at our lab. The library is entirely coded in standard C++ making it portable across different platforms and compilers. The choice of the C++ programming language offers the benefits of an object-oriented language. The library developed aims at providing a common framework for the development of computational tools for the manipulation and analysis of metabolic models in the Biochemical Sciences context. The library offers high-level constructs to represent chemical species, compartments and rate equations. However, while it is possible to describe metabolic models directly in C++, using the facilities provided, the preferred method is to use a separate language specifically developed as part of the library. Having a separate language allows non-programmers to easily describe their models and use the library’s features. Facilities for the estimation of parameters using genetic algorithms are also provided.

A3-010P

Mitochondrial DNA phylogeny of *Androctonus Ehrenberg, 1828* (Scorpiones: Buthidae) and its bearing on taxonomy and biogeography in Tunisia

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The phylogeny of the *Androctonus* Ehrenberg, 1828 species in Tunisia was inferred by comparative sequences of a fragment of mitochondrial DNA (16S rRNA). The Neighbour Joining tree using Nei distances as an input matrix revealed three well supported main cluster within *Androctonus* genus: *A. amoreuxi* clade, *A. aeneas* clade and *A. australis* clade. Moreover, the study revealed a clear divergent phylogeny within the most dangerous and widely distributed *A. australis* in Tunisia: Two distinct monophyletic clades exist which are geographically separated by the chotteljerid in the central of the country: The “Northern clade” and the “Southern clade”. Moreover the morphology analysis of the number of pectinal teeth showed that males and females from the “northern” clade had respectively, significantly higher number than male and female from the southern clade. Only contact zone contain the two haplotypes. We propose that the derived lineages arose from both tectonic and paleogeography events which occurred in the Miocene period. The present contact zone is located around chotteljerid where the two mitochondrial haplotypes coexist. Based on molecular evidence, the putative subspecies *A. a. hector* is synonymized with *A. a. garzonii*.

A3-011P

A clustering solution for distributed data analysis in mass spectrometry – paOla

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Huge amounts of data are acquired each day in mass spectrometry. This data has to be analyzed in less than or equal time as it is recorded. Otherwise the volume of data will grow over time and will need more and more time to be processed. Reducing the processing time can be achieved by several ways: By applying high-performance hardware or by increasing the degree of parallelism; another option can be the tuning of the used algorithms, but this is rarely possible in cases of commercial software. Results will be presented combining the first two possibilities. We designed a clustered computer environment named “protein analysis on linux architecture” (paOla). This system implements a queuing architecture with a task submitting interface. The queue is implemented in a way ensuring every submitted task will have its turn (like a FIFO). A license mixer is integrated, ensuring commercial licenses are not exceeded. We will demonstrate how protein analyzing tasks are distributed in a transparent way over independent CPUs and machines in paOla, so that these tasks do not interfere or slow down each other. Job types are configurable, so adding new analyzing algorithms or even new task types can be done with nearly no effort. The key administration features of the queue like task submission, killing, stopping, viewing progress and status are all implemented in an easy-to-use web-based application that can be used even by unexperienced users. Advantages and limitations of the versatile protein analysis

platform paOla for mass spectrometry will be discussed including concrete examples and performance data.

A3-012P

Searching sequences in protein databases generated by overlapping translation

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The overlapping translation (OT) of nucleic acid is a novel method for generating virtual protein sequences from nucleic acid sequences (Biro JC. Overlapping translation of nucleic acid sequences for bioinformatics applications. *Medical Hypotheses* 2003; **60**: 654–659). The OT can be used for generating virtual protein databases. Many common tasks of bioinformatics can be more efficiently executed using this virtual protein database than using the traditional nucleic acid or protein databases (Biro JC, Benyó Z, Sansom C, Benyó B. In search of the nature of specific nucleic acid – protein interactions. *Acta Physiologica* 2005). The most commonly used sequence searching tool is the blast. The blast algorithms can be used without any modification in the virtual protein databases. This version of blast is called blastNP (Biro JC, Biro JMK. A novel sequence similarity searching and visualization method based on overlappingly translated nucleic acids: the BlastNP. *Medical Hypotheses* 2004). The blast tool calculates several scores featuring the result of the search. The occurrence of subsequent characters in the sequences generated by OT is not independent therefore the scores calculated by the traditional blast tool are not correct in the case of using virtual protein databases. In order to use blastNP instead of traditional blast tools, the blastNP must be able to calculate the same scoring parameters than the traditional blast tools. In this work we aimed to examine the applicability of different scoring systems and models in order to calculate similar scoring parameters by blastNP than those that are used in traditional blast tools.

A3-013P

Spatial limitations caused by ubiquitylation: analysis of 3D model of yeast peroxisomal citrate synthase–ubiquitin complex

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Ubiquitylation of peroxisomal citrate synthase from yeast (pCS-yeast) has been recently shown and data concerning particular ubiquitylation sites (Lys354 and Lys385) have been also obtained. It seems to be interesting to predict the influence of possible steric limitations, caused by ubiquitin attachment, on (i) active site accessibility; and (ii) oligomerization ability of target protein. We have carried out the multiple alignment of pCSyeast and known citrate synthases sequences to determine conservatism of ubiquitin acceptors as well as active site residues. Based on these data we have modeled 3D structure of pCSyeast using Swiss-Model service and analyzed spatial arrangement of these residues on the protein surface. We have found that the most conserved ubiquitin-modified residue, Lys385, is situated near the active site “pocket” and therefore ubiquitylation can influence

active site accessibility for substrates. Such co-localization of active site and conserved lysine residue is typical for the majority of known citrate synthases. We have also analyzed known 3D-structures of different dimeric citrate synthases to determine areas of contact between enzyme subunits. We have shown that the same acceptor lysine residue is placed within proposed area of inter-subunit contacts. As far as single ubiquityl moiety is a large modulator, it can raise spatial limitations affecting of pCSyeast native dimer formation. Consequently, we can hypothesize that both events described above can affect functional activity of pCS-yeast. And as far as ubiquitylation is a reversible modification, such mechanism of enzymatic activity regulation can play a significant role in cell metabolism.

A3-014P

Prediction of rotational orientation of transmembrane helices of membrane proteins using a new structurally derived scale

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It is estimated that more than 20% of genes in human genome code for integral membrane proteins (IMPs). These proteins control a broad range of events essential for the proper functioning of the cells. Most importantly, IMPs are targets for 60% of all drugs on the market. The determination of high-resolution three-dimensional structures of IMPs constitutes one of the most challenging problems in structural biology. In the absence of experimental evidence and taking into account the powerful constraint imposed by lipid bilayer, modeling the structures of IMPs can be viewed as a two-dimensional problem for which prediction of location, basic topology, relative depth and rotational orientation of helical transmembrane (TM) substructures are required. Predicting the location of the TM regions and their intra- and extracellular topology are usually accomplished with reasonable accuracy based on hydrophobicity and knowledge-based statistical methods. The rotational orientations of TM helices in the helical bundle are often determined by calculating the helical moment vector using some kind of scale. Application of scales based on residue hydrophobicities have been argued and shown to be poor indicator of the helical rotational orientation. Several methods have been proposed based on the statistical analysis of available known structures or sequences of IMPs to derive lipid exposure propensities of different residue types. Here we describe a method for predicting the rotational orientation of helical TM segments of IMPs based on a new scale derived from the structural analysis of a representative set of *c.* 1200 non-redundant protein structures taken from protein databank with no bias toward IMPs.

A3-015P

Large-scale analysis of patches of contiguous destabilizing residues in proteins of known three-dimensional structure: towards functional sites prediction

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We present a method to predict functional sites in proteins of known structure, based on the identification of spatially close

residues with an unfavorable contribution to the stability of the protein. The method only requires a three-dimensional structure of the protein and no other information from experiment, although such information can be taken into account if necessary. Contributions to stability are evaluated on the basis of physical first-principles: atom–atom interaction terms are considered, combined with a solvent accessible surface area-dependent empirical solvation potential. Groups of contiguous destabilizing residues are defined on the basis of a hierarchical clustering procedure, taking both the contribution to the stability of the protein, as well as the relative location of these residues and their neighbors into account. We validated the method on a set of functionally well-characterized proteins and subsequently applied it to a non-redundant set of 1941 proteins with very high quality structural data, taken from the Protein Data Bank. We statistically analyzed the patches obtained in terms of size, relative amounts of hydrophobic and polar residues, average destabilization contribution, and correlation with evolutionary conservation of the residues. A web interface to the program is under construction.

A3-016P

An analytical tool to detect and visualize residue co-locations in protein and nucleic acid structures

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The 3D structure of biological macromolecules (proteins and nucleic acids) is determined by intra- and inter-molecular interactions. The interacting residues of the sequences are close to each other, they are co-located. Structure databases (like Protein Data Bank, PDB and Nucleic Acid Data Bank, NDB) contain all information about these co-locations, however it is not an easy task for humans to penetrate this information. We have defined a procedure extracting information regarding the interacting, co-located residues from structure databases. The defined procedure has been implemented in a JAVA tool, called SeqX. SeqX is able automatically execute the information extraction and visualize the results in a user-friendly manner. SeqX requires three input fields: (i) the structure from PDB to analyze; (ii) an atom which is commonly present in every residues of the nucleic acid and/or protein structure(s) (iii) the distance from these atoms (3-10 Å). The SeqX tool detects every residue which is located within the defined distances from the defined “backbone” atom(s), provide a DotPlot-like visualization and calculate the frequency of every possible residue pairs in the observed structure. We have performed a series of tests to find optimal operating conditions, compared our results with data from the literature as well as with a large set of residue co-location data obtained only by analyzing PDB and NDB structures by human eye. SeqX was found to be a specific, reliable tool to detect residue co-locations. The tool is tested in the practice. The efficiency of SeqX tool is compared with the Swiss PdbViewer by benchmark-like examples published previously in the paper Biro JC & Biro JMK. Frequent occurrence of recognition Site-like sequences in the restriction endonucleases. *BMC Bioinformatics* 2004; 5: 30. The SeqX proofed to be much more efficient for these kinds of studies than the non-automatic Swiss PdbViewer.

A3-017P

Annotation of post-translational modifications in Swiss-Prot and their effect in cell context

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With accelerating progress in the field of proteomics, biological knowledgebases such as the UniProt Knowledgebase (www.uniprot.org), must cope with a huge wealth of information, in particular the protein modifications that play crucial structural and functional roles. Our challenge is to include this information in protein entries in a way that is consistent, logical, and meaningful in order to allow rapid understanding by scientists and easy retrieval by computer programs. We have recently standardized the feature annotations of protein modifications, that consist now of the name of the modified amino acid rather than that of the modification process, and defined a controlled vocabulary that is provided in the Swiss-Prot documentation. Standardization ended up improving the efficiency of proteomic tools that make use of the feature annotations, and by using an unequivocal name, permitted the resolution of a few ambiguities. At the moment we pay special attention to also inform users about biological effects of annotated post-translational modifications (PTMs). We published an article on the annotation of PTMs and we wish to develop a Swiss-Prot companion database aiming to provide users with chemical and biological information relative to protein modifications. This poster presents a selection of modified proteins in their cellular context, e.g. proteins bound to the membrane through lipid anchors, glycosylated proteins and amidated proteins, and the corresponding Swiss-Prot annotations. We encourage the scientific community (i.e. both individual researchers and database maintainers) to interact with us (www.expasy.org/sprot/update.html), so that we can continuously enhance the quality and swiftness of our services.

A3-018P

Bistability in caspase activation

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Caspases play crucial role in coordinating the onset of programmed cell death (apoptosis). All caspases are produced in cells as catalytically inactive zymogens and must undergo proteolytic activation during apoptosis. Caspases involved in apoptosis are generally divided into two categories, the initiator caspases and the effector caspases. Being so dangerous, caspases must be under a strict control that provides a stable inactive (off) state and also a rapid activation if it is needed. We have constructed mathematical models which describe the activation of caspase molecules. Starting with the simplest model of activation, we show possible ways for the wiring of the control system. Computer simulation and mathematical analysis of the models show that in order to satisfy the above mentioned requirements the caspase activation system requires a strong positive feedback loop, which makes the system to work as an irreversible switch. Furthermore by studying the model, we can suggest answers to the following questions. In what way dimerization helps caspases to have a stable on and off state? How can an inhibitor increase the activation threshold? Why are there two types of caspases: initiators and effectors?

A3-019P**Sequence comparison of high-affinity iron permeases (FTR1) from different zygomycetous fungi**

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Patients treated with the iron chelator deferoxamine have an increased susceptibility to invasive zygomycosis in consequence of the efficient uptake of deferoxamine-chelated iron by fungal cells. Siderophores and iron transporters have therefore been suggested to function as virulence factors. One of the transport proteins, high-affinity iron permease (FTR1), is also thought to be an important determinant of virulence. The aim of this study was a comparative analysis of the FTR1 genes of some zygomycetous fungi. Primers designed on the basis of the *Candida albicans* and *Rhizopus oryzae* FTR1 sequences [1] were used to amplify the homologous regions of 10 fungal strains from four different genera (two strains of *R. oryzae* and one strain each of *R. microsporus* var. *oligosporus*, *Rhizomucor miehei*, *R. pusillus*, *Mucor plumbeus*, *M. rouxii*, *M. circinelloides*, *M. racemosus* and *Backusella lamprospora*). The sequence analysis of the amplified regions revealed significant homology with other FTR1 sequences. In contrast with known fungal FTR1 genes, the resulting DNA fragments of *R. miehei* and *R. pusillus* contained two introns in the same position. The deduced protein sequences of the 11 zygomycetous strains were aligned, and phylogenetic analysis was performed by using the neighbor-joining method. Protein sequences from the different species demonstrated a degree of similarity ranging between 74 and 100%. In this tree, the strains of distantly related *Mucor* species formed a monophyletic group. *Backusella* was also located in this cluster. Surprisingly, the *R. oryzae* isolates formed a completely different group at a significant distance from the *R. microsporus* isolate.

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A3-020P**High-order structure probing for misbehaving proteins and high-throughput approaches**

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In the post-genome era, one of the most important tasks is to obtain structural information about proteins and their folding processes. Nuclear magnetic resonance (NMR) and crystallography are superior in resolution, but have high experimental requirements on protein behavior, which for many proteins cannot be fulfilled. For these “misbehaving” proteins, limited proteolysis in conjunction with mass spectrometry is a prominent tool for probing high-order structure, because of its rapid, easy use and its astounding sensitivity and accuracy in protein mass determinations. With this combination of methods, it is possible to screen for stable fragments (secondary structure),

map for domains (tertiary structure), probe protein-protein interactions (quaternary structure) or protein-DNA/ligands interactions. We have recently introduced a new evaluation approach, which rather provides carefully information about relative cleavage occurrences at certain cleavage sites of the target protein than to look at stable fragments only, thus giving a good and more detailed picture of tertiary structure organizations. Although the acquired data is very rich in information, the evaluation is tedious and time-consuming. To improve this situation, we have developed a computer-based data analysis tool, which performs virtual cleavage, peak picking, assignment and data processing, up to final results presented as time-course profiles of stable fragments or cleavage occurrences in 2D and 3D views. Taken together, the combination of limited proteolysis, mass spectrometry and our data analysis software tool provides proteome research with a valuable tool for high-order structure analysis and high-throughput approaches.

A3-021P**Using multi-properties of protein in predicting protein subcellular localization**

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As the number of new genomes has dramatically increased over recent years, a reliable and efficient system to predict protein subcellular location is urgently needed. In this study, support vector machine is used to predict protein subcellular localizations. The prediction is based on amino acid composition, hydrophobicity, instability index. According to hydrophobic character as the tendency to be found inside of a protein, five different scales of an residue in a protein were given as follow: (i) C; (ii) F, I, V, L, M, W; (iii) H, Y, A, G, T; (iv) S, P, R, N, Q, D, E; (v) K. Stability is another important physics chemistry property of protein, it is a key role in determining of the native structure. Guruprasad designed a dipeptide instability weight value according to their influence on the stability of protein native structure. So, every protein is represented as a vector in a 425 dimensional space. The first 20-components were supposed to be the occurrence frequencies of the 20 amino acids in the protein concerned, the later 400-components were the dipeptides instability index, and the last 5-components were the frequencies of the 5 types amino acids in the protein. Our method is tested on Reinhart and Hubbard's dataset, the total prediction accuracies is both 100% by self-consistency test, 90% for prokaryotic sequences and 76.2% for eukaryotic protein sequences under jackknife test, these are equal to the best results of other methods. The Matthews correlation coefficients are much higher than the results from existing algorithm.

A3-022P**UniProt & Swiss-Prot: providing a link between protein sequences and state of the art knowledge**

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The UniProt consortium (www.expasy.uniprot.org) provides the scientific community with a single, high-quality resource that links protein sequences with biological information. It combines the activities of Swiss-Prot, TrEMBL and PIR and provides

access to a sequence archive (UniParc), the UniProt Knowledgebase, and a Reference database (UniRef). The UniProt Knowledgebase combines manual annotation (Swiss-Prot) with high-quality computer annotation (TrEMBL), thus giving access to a maximum of different protein sequences. With a minimal level of sequence redundancy, Swiss-Prot (www.expasy.org/sprot) puts strong emphasis on experimentally verified evidence derived from the literature, databases and contacts with authors. It serves as a hub, providing links to numerous other databases and bio-informatics tools, thus facilitating the integration of sequence data with genetic, physiological and biochemical information. Swiss-Prot provides detailed information on protein function in health and disease, post-translational modifications, protein interactions, subcellular localization, tissue specificity, and annotation of polymorphisms and isoforms produced by alternative splicing or mRNA editing. Information derived from protein 3D-structure analysis combined with biochemical and genetic data permits the identification of active site residues, sites of interaction with cofactors, metal ions and other ligands. Integration of information derived from 3D-structures has high priority, both for the annotation of new entries and for adding valuable information to existing Swiss-Prot entries. Taking the example of nuclear hormone receptors and their interactions we present recent changes in Swiss-Prot, as well as useful tools and tricks.

A3-023P

Identification of genes taking part in siderophore biosynthesis in the genome of *Fusarium graminearum*

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Fungal non-ribosomal peptide synthetases (NRPSs) are responsible for the biosynthesis of numerous metabolites including host-specific toxins and siderophores which serve as virulence factors in several pathogenic interactions. The aim of our work was to investigate the diversity of these genes in a *Fusarium graminearum* sequence database using bioinformatic techniques. Our search identified 15 NRPS sequences, among which two were found to be closely related to peptide synthetases of various fungi taking part in siderophore biosynthesis. Besides sequence homologies, the sizes and domain structures of these genes were also similar to those responsible for ferrichrome biosynthesis in several fungi. Another NRPS gene was similar to that identified in *Aspergillus oryzae* which is possibly responsible for the biosynthesis of fusarinine, an extracellular iron-chelating siderophore. To our knowledge, this is the first report on the identification of a putative NRPS gene possibly responsible for the biosynthesis of fusarinine-type siderophores. A protein containing an AMP binding domain, a phosphopantetheine attachment site and a transferase domain, an ABC transporter protein, an amino acid transporter, an acetylase homologous to siderophore biosynthesis proteins of *A. oryzae*, and efflux pumps homologous to siderophore transporters of *A. nidulans* (MirB and MirC) were found to be closely linked to the putative NRPS taking part in fusarinine biosynthesis. Homologues of other genes, including L-ornithine-N-monooxygenase taking part in siderophore biosynthesis in other fungi have also been identified in the *F. graminearum* genome. Further studies are in progress to clarify the role of some of the identified NRPS genes in plant pathogenesis.

A3-024P

Heterologous expression and characterization of *S. cerevisiae* unknown proteins induced by hydrostatic pressure

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Microarray analysis of *S. cerevisiae* submitted to Hydrostatic Pressure (HP) revealed that this stress causes gene expression modifications, leading to a stress response profile. However, unknown genes were among the 10 highest expressed representing 45% of the induced total. Therefore, we selected nine of those genes for further functional and structural analysis. The up-regulation of *YER067W*, *YFL014W*, *YDR070C*, *YLR327C*, *YMR107W*, *YDL110C*, *YPR096C*, *YNL266W*, *YNL198C* was confirmed by semi-quantitative RT-PCR in two situations: 200 and 50 MPa for 30 min. To recognize the folding of these putative proteins and then, gain insights about the function and mechanisms involved in cell survival against HP, the cDNAs were inserted in pET28a plasmid and heterologous expressed in *E. coli*. Sequences were fusion to a N-terminal hexahistidine tag followed by a thrombin cleavage site. We carried out the expression of *Ydr070cp*, *Yer067wp*, *Ymr107wp* and *Yfl014wp*. After 4 h of IPTG induction, the soluble fraction presented a protein band with the expected molecular weight for all four putative proteins yielding 2.5; 2.9; 4.7 and 3.6 mg of purified protein/100 ml. In the purification step, thrombin digestion was used for elution of the proteins bound to Ni²⁺ affinity resin, avoiding possible structural interferences caused by histidine tag. Circular dichroism of *Yfl014wp* (*Hsp12p*) revealed that the secondary structure is in agreement with theoretical predictions. Further results involving NMR and fluorescence spectroscopy will unveil structural features for those unknown proteins that may play an important role for cellular stress resistance. Acknowledgment: This work was financially supported by CNPq.

A3-025P

Evaluation and analysis of the nucleolar proteome

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The nucleolus is an important subcellular component as has been underlined in recent years. Publications have linked important functions to the nucleolus such as cell-cycle control and transcriptional repression or activation in addition to its well-documented function as site for ribosome synthesis. Furthermore, proteomics has provided a high-throughput source of novel nucleolar proteins. Using neural networks we show that the nucleolar proteome is defined by protein features such as residue composition, protein motifs, and predictions of protein structure. A further aim of this work was to show that bioinformatics and integration of data from different sources can provide an additional high-throughput data source. We support our predictions by using independent data from protein-protein interaction databases and microarray expression data. Thus, our approach complements experimental techniques by providing additional candidates for the nucleolar proteome. Furthermore, this data-driven machine learning approach offers a solution to the problem of validating and cleaning up data from high-throughput proteomics studies.

A3-026P

3D-structure features of novel GDSL-enzyme by bioinformatic methods and mass spectrometry

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Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) catalyse hydrolysis and synthesis of lipids, depending on the reaction conditions. Their natural substrates are insoluble in water; therefore hydrolysis takes place on lipid–water interface. This property distinguishes lipases from classical esterases. The ability of these enzymes to stereospecifically catalyse various reactions on a broad range of substrates gives them significant biotechnological potential. Native extracellular lipase from *Streptomyces rimosus* (SrL) was purified and biochemically characterized. Also, the gene for this enzyme was cloned and primary structure of the protein deduced from nucleotide sequence. Although SrL was shown to be a true lipase by its activity, sequence homology searches showed no overall sequence similarity to other lipases in databases, and classified this protein in family II of bacterial lipolytic enzymes (GDSL-hydrolases). Similarity (~24%) was found with the *Streptomyces scabies* esterase (SsE), which is the only member of family II with solved three-dimensional (3D) structure. Secondary structure prediction was performed for SrL by various methods; consensus prediction was determined and compared to secondary structure of SsE. Other structural features were analyzed, such as active site and disulfide bridges. Catalytic triad residues were predicted, as well as forming of disulfide bridges. MALDI mass spectrometry (MS) was used to confirm the identity of catalytically active serine by analysis of lipase-inhibitor covalent complex. The disulfide bridges pattern was also determined with MALDI-MS. High degree of agreement between analyzed 3D-structure elements of SsE and SrL suggests that the latter enzyme might have 3D-structure different from other lipases.

A3-027P

The Plant Proteome Annotation Program (PPAP) of UniProtKB/Swiss-Prot

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Swiss-Prot (www.expasy.org/sprot), the manually annotated section of the UniProt Knowledgebase (www.uniprot.org), provides high quality annotation, a minimal level of redundancy and a high level of integration with other databases. With the completion of the first genome sequence of the model plant *Arabidopsis thaliana*, Swiss-Prot initiated in 2001 the Plant Proteome Annotation Program (PPAP): a program focused on the annotation of plant-specific proteins and protein families. Our major effort is currently directed towards *Arabidopsis thaliana*, but the completion of the *Oryza sativa* genome sequence prompted us to also start a new project specifically devoted to rice. Of special interest for plant biologists are the links to Gramene, a comparative mapping resource for grains, MaizeGDB, the maize genome database, MAIZE-2DPAGE, the maize genome 2D Electrophoresis database, SWISS-2DPAGE, a database of proteins identified on two-dimensional polyacrylamide gel electrophoresis, including one of *Arabidopsis* origin, maintained by the Geneva University Hospital, The *Arabidopsis* Information Resource (TAIR), and

GeneFarm, a database that gathers expert-curated annotations of *Arabidopsis* gene families. Protein families and specific proteins are regularly reviewed to keep up with current scientific findings, so that the wealth of information in our knowledgebase, and the numerous software tools provided on the ExPASy web site, help to identify and reveal the function of uncharacterized proteins. In the beginning of 2005 (release 45.5, January 4, 2005), Swiss-Prot contains 11 804 plant entries, distributed in more than 2700 plant species. The three more represented plants are *Arabidopsis thaliana*, *Zea Mays* and *Oryza sativa* with 3096, 513 and 438 entries respectively.

A3-028P

Prediction of protein functional sites by energetic stability calculations

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We have developed a method to predict functional sites in a given protein structure, based on the fact that single destabilizing residues in a protein structure are likely to be involved in protein function. No information from experimental biochemical methods is required, except the three-dimensional structure of the protein. We make use of energetic stability calculations based on physical interaction parameters, combined with a method of progressive distance-based clustering, to detect destabilizing regions on the protein surface or interior. The method has been extensively tested on a set of well-characterized proteins for which experimental information is available in abundance. Here we present the application of the method onto a set of fatty acid binding proteins involved in fatty acid metabolism. We show a conservation of functional patches in the protein structure rather than sequence, using information from homologous and hypothetical proteins, as well as molecular dynamics simulations of bound and unbound states of the Sterol Carrier Protein type 2. The information on calculated functional sites is correlated with related proteins in the β -oxidation cycle of fatty acid metabolism and the peroxisomal translocation of reaction substrates and products.

A3-029P

Statistical alignment of retro-pseudogenes and their functional paralogs via a common ancestor

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A model is introduced for the sequence evolution of a retro-pseudogene and its functional paralog from a common protein-coding ancestor. The model accounts for substitutions, insertions and deletions, and combines nucleotide- and codon-level mutation models. We give dynamic programming algorithms for calculating the likelihood of homology between two sequences in the model, for obtaining the most probable alignment, and for computing the posterior probability of ancestral codons. Our method has several advantages, which include the possibility of inferring evolutionary parameters, hypothesis testing, and quantitative assessment of the alignment. The algorithms were implemented in Java, and the performance of the methods are shown on analyzing the evolution of human cytochrome c.

A3-030P**NetPhosK – Prediction of kinase-specific phosphorylation from sequence and sequence-derived features**

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Objective: To increase performance of kinase-specific prediction methods by incorporating sequence-derived features.

Background: Prediction of kinase-specific phosphorylation sites has mostly been performed using primary sequence information, however pattern recognition methods based on structural information could possibly increase the predictive performance, since kinase-substrate interactions ultimately depend on the three-dimensional structure of the proteins involved. Sequence data alone and in combination with structural information were fed to standard feed-forward neural networks to predict new kinase-specific phosphorylation sites. Structural information was obtained by using the standard programs *sable*, *disembl* and *psi-pred*, that predict sequence-derived information about surface accessibility, protein disorder and secondary structure, respectively.

Results: The Matthews correlation coefficient performance measure is high for PKA, because of the highly conserved arginine-residues at position -3 and -2 from the phosphorylated central S/T residue. Thus, in the case of PKA, as expected, the enhancement in predictive performance is insignificant when combining sequence data and structural information. However, it is expected that the latter approach will yield higher predictive performances in the cases of e.g. PKC, PKG, *cdc2*, CKII and CaMII kinases than predicting on sequence alone. <http://www.cbs.dtu.dk/services/NetPhosK/>

A3-031P**Investigating lattice structure for Inverse Protein Folding**

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Inverse Protein Folding (IPF) has the potential to significantly impact future drug design by providing computational tools that aid in the development of novel proteins with specific structural properties. In its most primitive state, IPF is a method of determining an amino acid sequence which takes on a prescribed structure within a specified (natural) environment. IPF is known to be computationally complex: the hydrophobic-polar (HP) model proposed by Dill [1] is often used to simplify the problem. This model represents each residue as either hydrophobic or polar and the prescribed structure is approximated by attempting to maximize hydrophobic residue interactions. Each amino acid is treated as an individual unit that is placed at a single lattice point of a regular lattice structure. The choice of lattice plays a major role within this framework. Our previous research investigated the development of stable proteins in a 2D environment using the HP model [2] and we are now focusing on the study of plausible 3D lattice structures. We investigate attributes of lattices which make them more amenable to representation of known protein structures, identify lattices containing these attributes, and compare lattices using various metrics. Our investiga-

tions incorporate statistical and computational analyses of a large fraction of proteins from the Protein Data Bank to show that lattices which are regular, periodic, equilateral, distance preserving, and contain angles of 90 and 120° are most amenable to representation of known proteins. This research represents a first step in the development of a successful IPF methodology.

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A3-032P**Comparative genomics of transcription factors**

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Identification of all transcription factors (TFs) from genome sequence data is not a straightforward task because of wide varieties in number and kind of TFs from one species to another. Also, as manual assignments may be too tedious to work through for all species of known genome, we have developed a new method to automatically identify TFs and to classify them into TF families, taking domains or domain organizations of proteins into account. As a comprehensive collection of TFs is already available for eukaryotes, we have here focused on prokaryotes alone. DNA binding domains (DBDs) are good indicators for assigning TFs at the first place, and further refinements of the TF screening have been made by examination of other domains combined with DBDs. Our analyses discerned 53 kinds of DBDs in prokaryotes, based on the Pfam and SCOP databases. Once the empirical rules for screening TFs with domain combinations were set up, the procedure could be applied to any genomic data to automatically identify all TFs encoded. In this way, more than 18 000 TFs were identified from 154 completely sequenced prokaryotic genomes, classified into 68 TF families, and stored in a database called GTOP. It is found that the number of TFs per genome is roughly proportional to the genome size, but the number is significantly limited in archaea in comparison with bacteria: the number of TFs (or TF families) ranges 3–82 (3–17) in archaea, while 2–636 (2–49) in bacteria. All 17 but one TF families of archaea are commonly shared by bacteria, whereas additional 34 TF families are uniquely found in bacteria. This implies that the transcription regulatory system is more developed in bacteria than in archaea.

A3-033P**A statistical study on the distribution and pattern (structure) of 1200 different human repeat types using a newly developed tool (SeqRep) and a Compressed Repeat Database (CompRepDB)**

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Are the repeats (25% of human genome) just junk or well organized genomic structures carrying important genetic information?

To answer this question we developed a tool and a database. We downloaded the repeat map (i.e. the names and order of all repeats) of all completely mapped and annotated human chromosomes (NCBI Map Viewer, January 2005). We found about 1200 different types of repeats which were divided into 10 classes and 17 families. We reduced the volume of data into a manageable size and format by assigning an individual number to every repeat types and an individual letter to every repeat classes and families. This was done by our newly developed JAVA tool (called SeqRep) and resulted in a compressed repeat database (CompRepDB, ~15 MB). Neighbor analyzes (repeats following each other) showed that the repeat types belonging to different classes and families are varying independently of each other (except satellites) and, by that way, it was possible to apply the usual statistical and pattern analyzing methods to study the distribution and structure of repeats in the chromosomes. We are now able to provide evidences that the distribution of repeats is not random and numerous unique, conserved (statistically significant) repeat patterns do exist. We interpret these results in favor of a previously predicted genome-wide structure-forming and regulatory role of repeats. [Biro JC, Baroukh N. Repeats: Sequences Alike. Prediction of Genome-Wide Associative Regulatory Role of Short and Long Interspersed Nucleotide Elements (SINE and LINE). MHR 2,1, January, 2005].

A3-034P

Arrangement of sequences in the gamma-proteobacteria replication origin

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Bacterial chromosome replication usually starts at a single origin and two replication forks propagate in opposite directions. The *Escherichia coli* replication origin, called oriC, is well characterized; however, in others bacterial genomes key issues remain unsolved. The aim of this study is to contribute to the characterization of the sequence analysis and computational detection of gamma-proteobacteria replication origin. The coordinates have been predicted from data of the whole chromosome map, using GC skew, oligomers skew, and by the presence dnaA boxes and specific genes. Replication origin sequences from *E. coli*, *S. enterica* subs., *S. flexneri*, *V. vulnificus*, *X. campestris*, *X. citri*, *X. fastidiosa* and *S. oneidensis* have been aligned. These origins consist about 300 base pairs bearing DNA sequence elements that are highly conserved. The key sequences of the general arrangement are two series of short repeats: 13 base pair in the 5' region and repeats of a 9 base pair sequence. In *V. vulnificus*, lacks the sequence motif that binds the factor for inversion stimulation (FIS) between dnaA boxes R2 and R3. This observation points out whether FIS might play any regulatory role in *V. vulnificus*. In *Xantomonas* species only one dnaA box is shown. In *X. fastidiosa* the 5' region, which is rich in AT, the repeats of 13 base pair sequences were not identified. In *S. oneidensis* replication origin, the 3' extreme, including one dnaA box is located within the 5' regions of the mioC gene. This comparative analysis show the plasticity of the bacterial replication origin, and also that the *E. coli* structure is not general for whole bacterial genomes.

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A3-035P

Docking of the scorpion toxin α -KTx_{12.1} to K⁺ channels investigated by molecular dynamics simulations

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α -KTx_{12.1} is one of the several small proteins present in the venom of the Brazilian scorpion *Tityus serrulatus*. This toxin interacts with several K⁺ channels, usually producing neurotoxic effects, and can be used as a powerful tool for probing the structure and function of ion channels, providing new insights into the elucidation of their molecular mechanism of action. In order to study the molecular basis for this interaction, two homology models for the *Shaker B* and Kv1.3 K⁺ channels were built based on the available crystallographic data. Subsequently, the NMR-derived minimum-energy structure of the toxin was manually docked to the pore region of the K⁺ channel models. The stability and dynamical behavior of the complexes were studied by molecular dynamics simulations performed in explicit solvent. Based on these results, several mutants of the toxin were designed and the docked complexes were submitted to another cycle of molecular dynamics simulations, with the aim of testing the importance of specific interactions observed during the initial calculation. Since the identification of the molecular contacts between toxins and K⁺ channel, responsible for binding and specificity, constitutes the first step in the development of new molecules with improved efficacy and therapeutic properties, we believe this approach may provide valuable insights into the design of new compounds with biomedical applications.

A3-036P

Computer modeling of the aliphatic amidase structure

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Spatial organization of the proteins from nitrilase/cyanide hydratase family remains insufficiently studied. This family includes about 200 proteins, however, three-dimensional structure on the basis X-ray examination of the protein crystals had been described only for two proteins: for N-carbamyl-D-amino acid amidohydrolase from *Agrobacterium* and for NitFhit protein (nitrilase-fragile histidine triad fusion protein, IEMS code in PDB Data Bank) from nematode *Cenorhabditis elegans*. A three-dimensional model of the aliphatic amidase from *Rhodococcus rhodochrous* strain M8 was built by comparative modeling using crystal of the nitrilase subunit NitFhit protein from nematode *Cenorhabditis elegans*. Fragment of the amidase amino acid sequence (total length is 345 amino acid residues) from *Glu14* to *Asp192* had been used for modeling. Model had been optimized by the classical methods of the molecular mechanics and molecular dynamics. Final total energy is -8273.9 kJ/mol. The root square value (RMSD) between the model and the template is 0.26 Å for 166 C α atoms. Model predicts *Glu59-Lys134-Cys166* catalytic triad. *Cys166* plays a role of the active site nucleophile because of its sulfhydryl group. Theoretical model of the quaternary structure of the aliphatic amidase was built by docking method on the basis of the three-dimensional structure of the amidase subunit. This quaternary structure is a homotetramer.

There are 10 salt bridges between the subunits of the tetramer, which take part in the stabilization of the quaternary structure. Model of amidase quaternary structure had been used to prove the hypothesis, that indicates a role *Glu59* in the maintenance of quaternary structure of the aliphatic amidase.

A3-037P

QM/MM calculations on the rearrangement reaction of isobutyryl-CoA mutase

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Isobutyryl-CoA mutase (ICM) catalyses the reversible, coenzyme B12-dependent rearrangement of isobutyryl-CoA to n-butyryl-CoA, which is similar to, but distinct from, that catalysed by methylmalonyl-CoA mutase (MCM) [1]. Because both subunits of ICM (from *Streptomyces cinnamomensis*) exhibit high sequence homology to the functional large subunit of methylmalonyl-CoA mutase, homology modeling has been used for constructing the 3D structure of ICM. For alternative pathways of this rearrangement reaction B3LYP/6-31G(d,p) level *ab initio* calculations were carried out in Gaussian03W using properly truncated substrate/product radicals and ammonium ion which partially stabilize the carbonyl of the butyryl-CoA [2]. In this work we have evaluated these results by replacing the ammonium ion causing partial protonation to the carbonyl O of the CoA moiety with a properly positioned imidazol hydrogen bonded to the carbonyl O of the thioester (which refers to ICM's His235). In more sophisticated models other two aromatic moieties close to the S of the thioester (referring to ICM's Phe80 and Phe278) were also take into consideration. The performed ONIOM(QM/MM) calculations were in accordance with enzyme kinetics data with stereospecifically isotop-labelled substrates and also explained that isobutyryl-CoA can bind and react via two different conformations at the active site [3, 4].

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A3-038P

Computer analysis of glycosyl hydrolases

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Glycosyl hydrolases (EC 3.2.-.-) are a group of enzymes which hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate molecule. A classification of glycosyl hydrolases based on amino acid sequence similarities has been proposed earlier. So far there has not been a comparison of conformation of glycosyl hydrolases. We conducted the structural comparison of this enzyme group. The results of the comparisons are presented graphically. With this method five main regions were identified. The relation of this grouping to previous classifications is discussed.

A3-039P

Visualizing structural biology using DINO

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In the multidisciplinary research of structural biology, it is essential to have comprehensive visualization of the multitude of different types of data, such as atomic coordinates, molecular surfaces, or scalar fields. The realtime visualization tool DINO (<http://www.dino3d.org>) aims to visualize all this structural data in a single program and to allow the user to explore relationships between the data. There are five data-types supported: structure (atomic coordinates and trajectories), surface (molecular surfaces), scalar fields (electron densities and electrostatic potentials), topographs (surface topography scans) and geom (geometric primitives such as lines). No limit is placed on the number of different datasets that may be loaded nor the number of actual 3D objects displayed. Taking advantage of the powerful OpenGL 3D graphics library and the capabilities of current graphics hardware in normal desktop computers, the user can explore the datasets by manipulating complex, highly detailed, yet interactive scenes. At any time, a snapshot of the view can be exported as a raster graphic file or as a POVray (<http://www.povray.org>) scene. The latter allows additional application of sophisticated ray-tracing techniques to create photo-realistic renderings. DINO is currently in a beta stage and freely available for the platforms Linux-i386, OSX, IRIX, OSF1 and SunOS.

A3-040P

IntAct: an open source database system and analysis tools for protein interaction data

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IntAct (<http://www.ebi.ac.uk/intact>) is an open source database of Molecular Interaction which is freely available for local installation. As part of the International Molecular-interaction Exchange (IMEx) consortium, IntAct is a repository for large protein interaction data prior to publication. It is also added to by manual curation of published articles. Experiment details for interaction detection, participant detection, interaction type, interactor features are described using controlled vocabularies built in Directed Acyclic Graph (DAG). Cross-references to existing databases are added such as UniProt for Protein interactors, InterPro to describe the protein binding domains, Biological Resources Catalogues (CABRI) for cell lines, PubMed identifier and Gene Ontology (GO) to describe biological processes in which complexes are involved, their function and subcellular location. All structures of interacting proteins are automatically added into the IntAct database with the corresponding PDB file cross-referenced. All IntAct protein interactors have in the corresponding UniProt file a link to IntAct in Database cross-Reference line (DR). The high-confidence interactions are automatically exported from IntAct to UniProt files as a Comment line (CC) listing the gene name of the interactors. IntAct provides a web interface to search for interactions by several means, such as gene name or cross-reference identifiers. Interaction data can be displayed as detailed tables with hyperlinks or a graphical view of the local network. Selected data can be retrieved in Proteomics Standard Initiative-Molecular Interactions (PSI-MI) XML format via the web services or the URL-based interface. All data within IntAct is available in PSI-MI XML format at the EBI FTP site.

A3-041P**Residue contacts in protein structures and interfaces**

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Non-covalent contacts between amino acid residues are the basis for protein folding, protein assembly and protein–protein interaction. These contacts occurring within a protein chain stabilize the tertiary structure, whereas those occurring at the interface between protein chains stabilize the quaternary structure and protein–protein complexes. To see if there is any difference in preferential residue-pair interactions in different cases, we have generated databases of protein tertiary structures, protein–protein complexes, homodimeric proteins and monomeric proteins involved in crystal contacts, and then calculated in each case Pxy, the propensity of a residue X to be in the environment of Y (the two residues should have at least a pair of atoms within 4.5 Å). Using the similarity of the environment as a criterion the amino acid residues have been clustered into nine groups. For the dimeric interfaces, the (20 × 20) Pxy values have large values along the diagonal, indicating strong preferences for interactions between identical amino acid residues, as was also reported earlier. This is due to the existence of “self contacts” – the contact between the equivalent residues from the two subunits lining the twofold axis. Leu makes the largest contribution (13%) to the total surface area buried through self-contact. Interestingly, Leu, which is the most ‘sticky’ residue across the twofold axis is also the residue of choice in leucine zippers. The average percentage composition of residues to be involved in self contacts can be used to distinguish biological dimers from the twofold related contacts observed in monomeric protein crystals.

A3-042P**Enantioselectivity in *Candida antarctica* lipase B reaction: transition states calculated by QM/MM methods**

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In this study our aim was to rationalize the enantioselectivity *Candida antarctica* lipase B (CalB) reaction by comparing the possible transition states of the two enantiomeric forms calculated by sophisticated QM/MM methods. CalB exhibits high enantioselectivity in esterifications of secondary aryl methyl carbinols. The degree of enantioselectivity of CalB-catalyzed acetylations of 1-phenylethanol, 1-cyclohexylethanol and 1-(benzofuran-2-yl)ethanol was determined experimentally by chiral GC. Until now the most realistic calculations were performed by calculating the energy difference between tetrahedral intermediates for the two enantiomers [1]. For each enantiomer two transition states (one for the alcohol-acylenzyme complex and one for the enzyme-acyl ester complex) need to be calculated, the real energy difference can be evaluated by comparing the highest energy for the S enantiomer with the highest energy of the R enantiomer. For the calculation of transition state energies ONIOM(QM/MM) methods were used by optimizing a 10 Å area around the substrate while the substrate and the most important residues were calculated using *ab initio* and DFT methods.

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A3-043P**Enzyme–substrate interaction through docking stimulation – Part II. Binding properties of vasopectidase inhibitors studied in complexes with Zn(II) metallopeptidases involved in blood pressure regulation**

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Angiotensin-converting enzyme (ACE), neutral endopeptidase (NEP), and endothelin converting enzyme (ECE), are zinc metallopeptidases which all belong to the gluzincin family [Spyroulias GA et al. *Curr Top Med Chem* 2004; **4**: 403–429] and they share common structural and functional features. They all metabolize peptides, like angiotensin I and II (AI/AII), bradykinin (BK), atrial natriuretic peptide (ANP) and endothelins (ETs), which are essential for arterial tone and water-electrolyte homeostasis regulation. The design of these gluzincins’ inhibitors has, until recently, relied on structure–activity studies, based on thermolysin (TLN), and/or Carboxypeptidase A (CPA). However, the X-ray structures of ACE (Natesh R et al. *Nature* 2003; **421**: 551–554) and NEP (Oefner C et al. *J Mol Biol* 2000; **296**: 341–349) have recently been solved. Since ACE, NEP and ECE are involved in the metabolism of peptides participating in blood pressure regulation, regulation their activity by only one mixed inhibitor has been attempted. Mixed ACE/NEP inhibitors are termed vasopectidase inhibitors (VPIs) encapsulates both NEP and ACE inhibitory properties. However, few are known for the structure of enzyme complexes with such kind of molecules. Fosidotrilat, sampatrilat and omapatrilat are VPIs with the latter to be the most clinical tested one. Simulation of ACE/NEP-VPI complexes using enzyme’s X-ray structure models through Simulating Annealing or Genetic algorithms could provide some valuable information of common and/or different binding properties of VPIs to ACE and NEP. We are currently performing docking simulations using AUTODock software with the aim to gain insight for structure-based design of new bioactive compounds.

A3-044P**A method for enhancing the significance of short linear motif matches**

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The detection of conserved domains and motifs in protein sequences by profile- or HMM-based techniques is an important task in sequence analysis. Conventional methods fail when motifs are too short or too ill-defined to yield statistically significant match scores. This unfavorable situation is frequently observed for short linear motifs, including those that determine post-translational modifications or protein interactions. Existing linear motif databases, such as ELM, are useful for storing motif-associated information, but are of limited use when trying to find database sequences with biologically relevant motif instances. We present a method for systematically discerning biologically relevant motif instances from random hits by making use of motif conservation in orthologous sequences. In its simplest incarnation, the statistical significance of a motif match can be strongly improved if the motif is also conserved in a set of orthologs of the evaluated target protein, in particular if other similarly-sized motifs do not show this conservation. If motifs contain so little information that they can be expected to occur somewhere in

most (if not all) sequences, additional constraints are needed, e.g. the requirement that the orthologs do not just contain the motif but also carry it at an analogous position. Unfortunately, many biologically important motifs are embedded in a compositionally biased sequence context that can be very hard to align. In order to deal with those cases, we relax the requirement for positional correspondence by resorting to “landmark alignments”. Here, we do not require the motifs in the orthologs to align to each other but only to be located in a corresponding window between alignable landmarks.

A3-045P

Exploiting protein–protein interactions for automated discovery of promiscuous binding motifs

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Interaction between proteins can be mediated by short segments of sequences. Consensus motifs of these short sequence segments have been found in diverse proteins to mediate their interactions with multiple binding partners. Such promiscuous motifs are distinct elements in the signaling transduction pathway that allow proteins in the pathway to cross-talk with one another. Promiscuous binding motifs are also found in diverse proteins as localization signals to direct them to different cellular compartments. Finding these motifs is thus important given the roles they are playing in signaling and localization of proteins. However, elucidating them is a challenge. Mutagenesis studies are directed at finding consensus binding motif of a single protein and are not adapted to find motifs bind by multiple proteins. Current computational motif finding methods require manual grouping of sequences which is not suitable for finding promiscuous motifs that often occur in proteins with no palpable similarities. In our work, we exploit the availability of genome-wide interaction data and devise a novel Interaction-Driven Motif Mining (IDMM) approach that utilizes the inherent functional associations in the protein interaction data to mine for promiscuous binding motifs automatically. Work on genome-wide interaction data of *Saccharomyces cerevisiae* and *Caenorhabditis elegans* validate that IDMM is able to extract many known signaling motifs and nuclear localization signals without any prior biological knowledge. IDMM also reveal many novel promiscuous binding motifs that can guide further interaction studies.

A3-046P

Vectorial proteomics of biological membranes

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Vectorial proteomics is the methodology for differential identification and characterization of peripheral proteins and surface domains of membrane proteins exposed to the opposite sides of biological membranes. This approach determines cellular localization of the identified proteins, their membrane topology and post-translational modifications. We apply vectorial proteomics for detailed characterization of two membrane systems: (i) photosynthetic thylakoid membranes of plants and green algae and (ii)

caveolae vesicles isolated from plasma membranes of human fat cells. Proteolytic shaving of the isolated membrane vesicles and following mass spectrometry of the released peptides reveals the surface-exposed domains of membrane proteins. We specifically focus our studies on the identification of *in vivo* protein phosphorylation sites in the membrane and membrane-associated proteins in both systems. For the first time we performed mapping of protein phosphorylation sites and characterized dynamic changes of protein phosphorylation in the photosynthetic membranes during state transitions and during adaptation of green algae to limiting environmental carbon. We also identified novel protein phosphorylation sites in the membrane and peripheral proteins at the cytosolic face of caveolae membrane domains from adipocytes. The hormone-modulated changes in this protein phosphorylation network of fat cells are studied by stable isotope labeling of phosphorylated peptides shaved from the surface of caveolae. For this purpose we isolate the caveolae vesicles from the plasma membranes of insulin treated and untreated fat cells. Vectorial proteomics is becoming a powerful methodology for functional characterization of biological membranes, providing significant insights in cell biology.

A3-047P

Theoretical approach to the influence of macromolecular crowding on reaction rates

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A high total concentration of macromolecules is one of the characteristic features of the living cells. Typically, proteins, RNA and other macromolecules occupy about 20–30% of the total volume of the cytoplasm. Since no single macromolecular species is present at such high concentrations, but many different species taken together “exclude” certain part of the volume, such media like cellular plasma are referred to as “crowded”, not “concentrated”. Macromolecular crowding has been observed to influence interactions between many types of macromolecules, with consequent effects on the rates and equilibria of reactions occurring in it. Experimental results with different crowding agents have been obtained. They led to very interesting results that are often, however, very difficult to interpret quantitatively in terms of the influence of volume exclusion on reaction rates. The main reason of this seems to be the problem with choosing the crowding agent that is of proper molecular weight, is soluble in water in high concentrations, does not aggregate, consists of globular molecules. None of the crowding agents that have been used so far fulfills all the conditions mentioned. This is why creating a theoretical model of macromolecular crowding, allowing for prediction of its influence on biochemical reactions seemed to be so desirable. We have developed a Brownian Dynamics program designed for calculations of reaction rates in highly crowded solutions and performed several calculations with it. We started with simple spherical models of proteins in different concentrations and calculated self-diffusion coefficient of such molecules. Results of those computations are in agreement with results previously obtained by other authors. Then we switched to calculation of protein–protein condensation rates for simple models in several concentrations of crowding particles. Finally – atomic-level models of hen egg lysosyme and its antibody were used for calculation of influence of crowding on the rate of proteins mentioned. Results of these calculations, constituting an important step towards understanding mechanisms of the influence of crowding on reaction rates (and thus towards creating a theoretical model of macromolecular crowding), will be presented.

A3-048P**Interaction of endothelial cells with selected biomaterials causes changes in protein expression profile**

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Background: Endothelial cells play a key role in angiogenesis. In the case of reconstructive arterial or cardiac surgery the cells are in direct contact with an implant surface. A response of endothelial cells to this contact may be a high importance factor in respect to final success of the surgery.

The aim: Our study was devoted to check whether contact of endothelial cells with biomaterial surface can modulate protein expression.

Materials and methods: The EA.hy 926 cells were grown in the DMEM medium in 25 cm² T flasks. Samples of studied materials, used in a powder form, were applied into separate T flasks containing cell culture in a confluent stage. Cells were then cultured for 24 h in a standard condition. After that the cells were harvested with trypsin, washed with PBS, and suspended in a lysis buffer. Samples of extracted proteins were separated in 2-D electrophoresis with use of Multiphor II system. The proteins were visualized by silver staining and gels were documented with an optical scanner.

Results and discussion: Changes in protein pattern were detected after 24 h incubation, both in cells grown in the presence of medical steel and those cultured with nanocrystalline diamond (NCD), when compared to the control. The differences in a response of different cells to a contact with implants require an attention and additional research. Although a contact of endothelial cells with NCD coating results in expression of additional proteins, when compared to the control (not treated cells), an use of medical steel produces still much more changes in the protein pattern.

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A3-049P**Identification of the C-terminal signal peptides for GPI modification and prediction of the cleavage sites**

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Glycosylphosphatidylinositol (GPI)-anchored proteins represent a subclass of cell surface proteins found in all eukaryotic cells. Knowledge of a protein's GPI modification is very valuable, since it defines its subcellular localization and limits the range of possible cellular functions. The modification takes place in the endoplasmic reticulum and involves enzymatic removal of a C-terminal signal peptide (much like N-terminal signal peptides), followed by addition of the GPI moiety to the new C-terminal amino acid residue of the protein. A number of sequence based methods have been developed for the prediction of GPI-anchored proteins, most of which rely on defining a C-terminal consensus sequence for GPI modification. However, only about 40 proteins with experimentally verified cleavage sites are known to date. Many of the prediction tools currently available are therefore based on protein examples for which the GPI signal sequence and the cleavage site are either inferred from homology or predicted by previous *in silico* methods.

Here we present a new sequence based GPI-anchor prediction tool, based exclusively on experimentally verified data extracted from Swiss-Prot. The method is based on artificial neural networks – a machine-learning method able to capture non-linear context dependent patterns. The predictive performance of the method is better than all other methods currently available.

A3-050P**Towards an extensive proteome analysis of platelet membranes**

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Platelets are anucleate and discoid blood cells which are involved in thrombosis and hemostasis. Lacking almost any protein synthesis, they are well suited for a large scale proteome analysis. Since membrane proteins are involved in almost all important cellular processes, the elucidation of the platelet plasma membrane subproteome will lead to an improved understanding of the function of these cells, providing new and potentially important targets for pharmacological research. Therefore, starting from fresh platelet concentrates a membrane enriched fraction was prepared by means of ultrasonic disruption and sucrose density gradient centrifugation. Membrane protein samples were subsequently either separated by 1D-SDS-PAGE or by 2D-BAC/SDS-PAGE. Visualized protein spots were excised, washed, tryptically digested and analyzed via nano-LC-MS/MS. So far, more than 100 membrane and membrane associated proteins could be identified, including low abundant receptors like GPVI present at about 1000 copies per cell as well as transmembrane domain containing proteins of unknown function representing future research targets of major interest. Up to now, the total amount of identified membrane proteins is more than four times higher compared to former platelet proteome studies which mainly focused on common 2D-PAGE approaches which are known to be unsuitable for membrane proteins.

A3-051P**Biased purine frequency and polypurine tracts in mRNA sequences of the proteins belong to information processing: comparisons within Prokarya and Eukarya**

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The mechanisms of an organism's adaptation to high temperatures have been investigated intensively in recent years. It was suggested that the macromolecules of thermophilic microorganisms (especially proteins) have structural features that enhance their thermostability. It was already shown that the purine/pyrimidine (R/Y) ratio within the mRNAs of prokaryotic thermophiles is significantly higher than that of the mesophiles. We show here that also within mesophilic prokaryotes and eukaryotic species, the R/Y ratio of the mRNAs of histone-like proteins, DNA-dependent RNA polymerase subunits, ribosomal proteins, tRNA synthetases, heat-shock proteins, and some other stress related highly expressed proteins is higher than their average over coding part of the genome. We suggest that polypyrimidine, (R)*n* (with *n* ≥ 5) in the template strand, especially thymine tracts, might affect the speed and efficiency of the processing beyond the contribution of amino acids encoded by purine tracts to the protein stability, and the already postulated higher stability of the mRNAs that include polypurine tracts.

A4–Human Genomics and Diseases

A4-001

Genomic approaches to dissecting complex phenotypes

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Identification of the genes underlying common, genetically complex disorders is a central challenge of post-genome genetics. The availability of genome sequences and genome-scale technologies has enabled new strategies to be implemented for identifying genes underlying complex phenotypes and has greatly accelerated progress in this field. The high heritability of variation in gene expression has suggested that identification of the genetic determinants of gene expression may throw light on the molecular basis of complex traits. One justification for studying the genetics of gene expression is that transcript abundance may act as an intermediate phenotype between genomic DNA sequence variation and more complex whole body phenotypes. However as a tool for studying disease phenotypes, aside from a single study in F2 mice, the approach remains relatively untested. We have applied combined expression and linkage analysis to study the regulation of gene expression in the BXH/HXB panel of rat recombinant inbred (RI) strains, one of the largest available rodent RI panels and a leading resource for genetic analysis of the highly prevalent metabolic syndrome. In two tissues important to the pathogenesis of the metabolic syndrome, we have mapped *cis*- and *trans*-regulatory control elements for expression of thousands of genes across the genome. Gene expression for many of the most highly linked expression QTLs (eQTLs) are regulated in *cis* essentially as monogenic traits, and are attractive candidate genes for previously mapped physiological QTLs in the rat. By comparative mapping we generated a dataset of 73 candidate genes for hypertension that merit testing in human populations. Mining of this publicly available dataset is expected to lead to new insights into the genes and regulatory pathways underlying the extensive range of metabolic and cardiovascular phenotypes that segregate in these RI strains.

A4-002

The mystery of conserved non-genic (CNG) sequences

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The comparison of the sequences of human chromosome 21 with that of the syntenic regions of the mouse genome revealed a large number of conserved sequences (>100 nt in length and ≥70 % ungapped identity) that are not transcribed. We called these elements conserved non-genic (CNG) sequences. Most of these map in gene-poor regions of chromosome 21. A large majority of CNGs are also present in several mammalian species, indicating a conservation of more than 120 million years. The patterns of evolutionary conservation allow a sufficient separation of CNGs from both coding regions and non-coding RNAs. Furthermore, the evolutionary characteristics are independent of their position relative to protein-coding sequences. The overall level of conservation of CNGs is higher than exonic sequences and strongly suggests functional importance. We anticipate that mutations in CNGs may contribute to human disorders; a search for those is now in progress. The function of CNGs is largely unknown and

considerable effort is now devoted to the functional analysis of these genomic elements that may account for up to 1–3 % of the human genome. Some CNGs may be *cis*- or *trans*- regulatory elements of gene expression, others may be structural elements, and yet others may have a function totally unsuspected to date.

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A4-003

The ATP-binding cassette transporter ABCA3 is associated with the lung surfactant syndrome and also affects platelets and lymphocytes

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ABCA3 is strongly expressed in lung tissue and mutations of the gene have been shown to cause surfactant syndrome of full-term infants. Recently, we identified three new homozygous mutations in children with pulmonary insufficiency (case 1: IVS15-1 G>C; case 2: IVS21-1 G>A; case 3: Exon 31 4875 Gdel, 4877 Adel). Differences in onset and severity of the clinical symptoms could at least be partially correlated to the localization and type of the mutation. Immunohistochemical staining clearly localized ABCA3 to the limiting membrane of lamellar bodies in alveolar type II cells while the signal was diminished or absent in patients with homozygous mutations. In Western blots protein expression of lung tissue was accordingly decreased. On the cellular level ABCA3 seems to be involved in the apical secretion of surfactant lamellar bodies whereas ABCA1 mediates basolateral lipid efflux. Functional analysis and the clinical symptoms of one patient (case 1) with a homozygous ABCA3 mutation who survived the postnatal period due to successful heart-lung-transplantation indicated a role of ABCA3 in platelets and lymphocytes in addition to alveolar type II cells. Platelet aggregometry revealed a severely impaired response to ADP, collagen and epinephrine while the reaction to ristocetin and arachidonic acid was normal. Supporting the relevance of ABCA3 in hematopoietic cells, we were able to show protein and RNA expression in platelets and T-lymphocytes from healthy donors. Taken together, our results underscore the relevance of the ABCA3 transporter in the pulmonary system and reveal a novel role in platelets and lymphocytes.

A4-004

Toxic properties of mutant SOD1 aggregates in a neuronal cell model

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The appearance of protein aggregates is characteristic of many protein-misfolding disorders, including familial amyotrophic

lateral sclerosis caused by inherited mutations in Cu/Zn superoxide dismutase (SOD1). While SOD1 mutants form an array of misfolded and aggregated species, it remains unclear what role aggregate formation plays in neuronal toxicity. Here, we examine the structural and biophysical properties of SOD1 aggregates formed by two well characterized mutants, G85R and G93A. In differentiated PC12 mammalian neuronal cells and in human HeLa cells, either SOD1 mutant forms both diffuse and aggregated species. Furthermore, other soluble proteins retain the ability to freely diffuse through the aggregates, suggesting a permeable inert structure. As components of protein folding quality control (molecular chaperones and the proteasome) colocalize with SOD1 aggregates, we tested whether they are also freely mobile or sequestered within SOD1 aggregates. We report that the molecular chaperone Hsp70 interacts transiently with mutant SOD1 aggregates and retains its mobility. In contrast, the proteasome forms dynamic and immobile populations that closely parallel the heterogeneous populations of both SOD1 mutants. These data reveal that SOD1 aggregates are dynamic and form heterogeneous interactions with other cellular proteins. To relate SOD1 aggregate appearance to cell survival, individual PC12 cells were followed using time-lapse microscopy. We show that nearly all (90%) cells containing SOD1 aggregates die within 48 hrs, whereas 70% of cells displaying a diffuse mutant SOD1 localization survive. Our data suggest that, unlike the proposed cytoprotective role of other protein aggregates, the appearance of dynamic mutant SOD1 aggregates is not beneficial for neuronal survival.

A4-005

Improving specificity of DNA hybridization-based methods.

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Methods based on DNA reassociation in solution with the subsequent specific detection or PCR amplification of certain hybrid molecules, such as DNA microarray hybridization, coincidence cloning and subtractive hybridization, all suffer from a common imperfection: cross-annealing between various types of paralogous repetitive DNA fragments. Although the situation can be slightly improved by the addition of repeat-specific competitor DNA into hybridization mixture, the cross-hybridization outcome is a significant number of background chimeric clones in resulting DNA libraries. In order to overcome this challenge we developed the technique, called MDR (mismatched DNA rejection), which utilizes a treatment of resulting reassociated DNA with mismatch-specific nucleases. We examined the MDR efficiency using cross-hybridization of complex whole genomic mixtures derived from human and chimpanzee genomes, digested with frequent-cutter restriction enzyme. We demonstrate that both single stranded DNA-specific and mismatched double stranded DNA-specific nucleases can be used for MDR separately or in combination, reducing the background level from 60% to 4% or lower. The technique presented here is of universal usefulness and can be applied to both cDNA and genomic DNA hybridizations of very complex DNA mixtures. MDR is also useful for the genome-wide recovery of highly conserved DNA sequences, as we demonstrate by comparing human and pygmy marmoset genomes.

A4-006

Triosephosphate isomerase deficiency: relationship between enzyme mutation and neurodegeneration

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Triosephosphate isomerase (TPI) deficiency is a unique glycolytic enzymopathy coupled with neurodegeneration. Two Hungarian compound heterozygote brothers inherited the same TPI mutations (Phe240Leu and Glu145Stop codon), but only the younger one suffers from neurodegeneration. We have investigated their erythrocytes and lymphocytes to search the consequences of the genetic mutations at mRNA, protein, enzyme activity and metabolic levels; and the possible relationship of these hierarchic characteristics and neurodegeneration. The TPI-related metabolic pathway was analyzed experimentally and was modelled by using the measured activities of the glycolytic enzymes. We have shown in erythrocytes that (i) the activities of some key glycolytic enzymes other than TPI are enhanced in mutant cell; (ii) therefore the glycolytic flux is higher in mutant cells as compared to that of the control; (iii) the modelling data revealed extensive increase of dihydroxyacetone phosphate and fructose 1,6-bisphosphate levels in mutant cell; (iv) in the presence of tubulin which interact with TPI and its sequential enzymes, the compartmentation of glyceraldehyde-3-phosphate was assessed. These data explain why the ATP level is not reduced despite the low TPI activity. Since the TPI activity and the enzyme amount of the two brothers are similar in both cell types, we search other factors, which might be responsible for the neurological disorder in the younger brother. We found significant differences in the mRNA levels of prolyl endopeptidase by QRT-PCR in the case of the two brothers. The protease activity of the younger brother was reduced by 40 and 30% respect to the control and the healthy brother. Similar activity decrease of this protease was observed in other neurodegenerative diseases. We suggest that the TPI mutation cannot be alone responsible for the development of the neurodegeneration, but other factor(s), which could be interrelated to the reduced endopeptidase activity.

A4-007P

Role of podocalyxin in the regulation of platelet function

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Podocalyxin (PDX) is a strongly sulfated transmembrane sialoprotein found in the polyanion coating of the podocytes of the glomerular epithelial cells. This protein, that is essential for a normal glomerular function, is also expressed in vascular endothelial cells, megakaryocytes and platelets. The surface exposure of PDX following platelet activation and its structural homology to intercellular adhesion ligands suggests that it might play an important regulatory role not yet investigated. To elucidate its physiological role in platelets we analyzed: firstly, the effect of its specific platelet overexpression; secondly, we carried out functional experiments on Chinese Hamster Ovary (CHO) cells stably expressing mouse or human podocalyxin-green fluorescence protein (GFP). The transgenic animals were produced by microinjection of a construct comprising the human PDX cDNA followed

by the SV40 polyA whose transcription was directed by the platelet specific glycoprotein IIb promoter. The transgenic animals were identified by either PCR or dot-blot. The expression of PDX was verified by retrotranscription of platelet RNA. Moreover, in some experiments the transgen was a fusion of PDX cDNA with the GFP so that platelets were visualized microscopically. The visual inspection of the transgenic animals was normal as well as the functional parameters analyzed; however, an important feature of these mice was a marked shortening of the bleeding time *in vivo*. CHO cells stably expressing either mouse or human podocalyxin-GFP showed an increased adherence to platelets immobilized on fibrinogen and stimulated by agonists. The adherence onto endothelial cells induced the formation of long filopodia with PDX found at their tips and also at the edge of the lamellipodia.

A4-008P

Variable metallation of wild type human superoxide dismutase

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We have recently obtained atomic or near atomic resolution crystallographic data for the wild type enzyme in several metal-loaded forms. In the 'as-isolated' recombinant enzyme, which was expressed in yeast cells, the 1.24 Å resolution structure showed that the Cu-binding site in fact contains a mixture of Cu and Zn atoms. Recombinant apo-enzyme, re-metallated with Zn atoms only, gave a Zn-Zn SOD1 structure that was solved to 1.24 Å resolution. The native human enzyme was re-constituted with copper to give an enzyme containing only Cu atoms at the Cu-binding site (i.e. a fully metallated Cu-Zn enzyme). This structure was determined to 1.15 Å resolution and is the only atomic resolution structure for human SOD1. A detailed analysis of these structures and their use in Molecular dynamics calculations is being undertaken to shed important light on metal incorporation and associated structural changes.

A4-009P

Evidence for a link between sphingolipid metabolism and expression of CD1d and MHC-class II molecules: monocytes from Gaucher disease patients as a model

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Gaucher disease is an autosomal recessive inherited defect of the lysosomal enzyme glucocerebrosidase that leads to glucosylceramide accumulation in the reticuloendothelial system. Hereby, we show that glucocerebrosidase-deficient monocytes from Gaucher disease patients display higher levels of expression of CD1d and MHC-class II molecules at the cell surface than monocytes from healthy controls. The anomalies are specific for these lysosomal trafficking molecules because expression of other receptors, including MHC-class I molecules, was within control values. CD1d and MHC-class II were also upregulated in Gaucher disease patients undergoing enzyme replacement therapy, some of which presented overt imbalances in the percentage of CD4+, CD8+,

and Vα24+ T cells. Follow-up studies revealed that enzyme replacement therapy induced a decrease in MHC-class II expression by monocytes and partial correction of the CD4+ T cell imbalances. Finally, we show that treatment of monocytes from healthy controls with conduritol-B-epoxide, an irreversible inhibitor of glucocerebrosidase activity that results in glucosylceramide accumulation, induced upregulation of CD1d molecules at the cell surface. These studies reveal a new link between expression of MHC-class II and CD1d molecules and sphingolipid accumulation in monocytes, which may result in imbalances of regulatory NKT cell subsets. These immunological anomalies may contribute to the clinical heterogeneity in Gaucher disease.

A4-010P

Increased apoptosis and decreased p53 and Bax expression in colorectal tumours

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Colorectal cancer (CRC) is the second most prominent cause of cancer-related death in the Western world. Most cases arise sporadically, whereas environmental factors such as diet may lead to development of CRC. Absence of p53 and Bax expression in CRC tumours has been associated with High Microsatellite Instability and defects in the Mismatch Repair System. In this study, CRC patients followed an unbalanced dietary pattern, with high intake of saturated fat and cholesterol, low intake of vegetables as well as frequently elevated alcohol consumption. CRC tumour biopsies and normal mucosa from six patients were evaluated for apoptosis using the TUNEL assay. The results showed an increase in apoptosis from 0.3 to 2 positive cells per 40× field in normal mucosa to 1.5–19 apoptotic cells in tumours ($P < 0.05$). In addition, steady-state expression levels of p53, phosphorylated p53 and Bax proteins were determined by Western blot analysis of frozen tumour and normal tissue samples. p53 was undetectable in tumour tissue, while phosphorylated p53 was almost 2-fold decreased in adenocarcinomas and adenomas ($P < 0.01$). Bax expression was also decreased in tumour tissue compared with normal mucosa ($P < 0.001$). These results suggest that apoptosis in colorectal tumour tissue follows p53-independent pathways. Moreover, increased tumour cell apoptosis may be associated with improved prognosis. The significance of our findings needs to be further accessed by tumour molecular characterization to ascertain the role of apoptosis-related gene products on cell death and survival.
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A4-011P

Altered DNA methylation at 4q35 in FSHD patients

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Facioscapulohumeral muscular dystrophy (FSHD1, OMIM 158900) is an autosomal dominant neuromuscular disorder, with an incidence 1:20 000. The clinical symptoms as progressive

wasting of the facial and shoulder girdle muscles are highly variable; the age of onset and the clinical severity differ between and within the families. A contraction of the polymorphic D4Z4 repeat array located at 4q35 locus is associated with the disease. Healthy individuals carry 11–150 units of D4Z4 on both chromosomes 4, but individuals affected with FSHD1 carry 1–10 units of D4Z4 on one chromosome 4. Despite of extensive sequencing work there was no gene identified for causing FSHD1 phenotype. Several observations suggest an epigenetic etiology in FSHD1 that causes the transcriptional deregulation of genes close to D4Z4. van Overveld et al. tested this hypothesis by checking the methylation status of the proximal D4Z4 unit at 4q35. They found hypomethylation of D4Z4 units in DNA samples of FSHD1 patients isolated from peripheral blood lymphocytes. There were no tissue-specific methylation differences between muscle and blood lymphocyte samples [van Overveld et al. *Nat Genet* 35, 315-316 (2003)]. We asked if we can also detect hypomethylation of proximal D4Z4 units located at 4q35 in hungarian FSHD1 patients. In our study we included samples from healthy individuals and from patients of non-FSHD1 muscular dystrophy. Genomic DNA was isolated from lymphocytes and digested with methylation sensitive restriction endonucleases. Southern-blot analysis was performed using P32 labelled p13E-11 probe, specific for 4q35. The intensity of the signal was measured by an electric autoradiograph. Our results supported the observation, that D4Z4 proximal units are hypomethylated in samples of FSHD1 patients.

A4-012P

Proteomic analysis of inducible AML1-ETO expression leukemic cell line U937 with multidimensional chromatography combined with SELDI-TOF and MALDI-TOF-TOF

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Chromosome translocation t(8;21) (q21;q22), which produces leukemogenic fusion protein AML1-ETO, commonly occurs in acute myeloid leukemia (AML). It has been widely believed that AML1-ETO block differentiation of hematopoietic cells mainly by dominantly negative inhibiting the function of wild-type AML1 protein that is an important transcriptional factor for leukemic cell differentiation. However, little is known about the mechanisms by which AML1-ETO cause leukemia. In order to shed new light for the role of AML1-ETO, we tried to identify AML1-ETO-regulated proteins in an ecdysone-inducible AML1/ETO-expressing leukemic U937 cells by two independent proteomic approaches, that is, two dimensional gel electrophoresis (2DE) and liquid separation with multidimensional chromatography combined with SELDI-TOF technique, followed by MALDI-TOF-TOF analysis. Our results showed that dozens of proteins were elevated or suppressed when AML1-ETO was expressed in U937 cells. 57 spots were identified by mass spectrometry and they include proteins involved in cell cycle, apoptosis, mRNA splicing, RNA synthesis, differentiation and detoxication. In particular, some of them have been verified by independent means in our group and others, such as GST-Pi and cathepsin D. It has laid the foundation for further detailed functional studies to elucidate the role of AML1-ETO in the pathogenesis of AML. Our study also demonstrated the great potential

capability of multidimensional chromatography combined with SELDI-TOF as 2DE-independent separation workflow for protein. The use of liquid separation methods provides a powerful means interfacing with SELDI-TOF and MALDI-TOF-TOF, where accurate intact Mr values, differential profiling, peptide mapping or MS/MS can be obtained with higher speed, sensitivity and accuracy compared with 2DE.

A4-013P

Effect of steroid hormones on the *in vitro* growth and viability of *Entamoeba histolytica*

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The *in vitro* exposure of *Entamoeba histolytica* trophozoites to sex steroids 17 β -estradiol, progesterone, and dehydrotestosterone, had little effect on parasite viability or proliferation. However, treatment with the adrenal steroid dehydroepiandrosterone markedly inhibited parasite proliferation, adherence and motility, and to a certain dose induced lysis of trophozoites. The opposite effect on proliferation was found when the trophozoites were exposed to cortisol. Moreover, dehydroepiandrosterone decreased while cortisol increased the parasite's DNA synthesis determined by ³H-thymidine incorporation. Lysis of trophozoites by dehydroepiandrosterone appeared to be caused by a necrotic rather than an apoptotic process, as observed in propidium iodide and terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling assays. Two possible mechanisms of action were derived from experiments demonstrating that the activity of 3-hydroxy-3-methyl glutaryl CoA reductase detected in trophozoite extracts was inhibited in the presence of dehydroepiandrosterone and a specific inhibitor of the androgen receptor blocked the dehydroepiandrosterone effect. Contrary to its *in vitro* inhibitory effect, *in vivo* administration of dehydroepiandrosterone to infected hamsters, resulted in exacerbation of the amebic liver abscesses. These results demonstrated that androgen steroids act directly upon *E. histolytica* growth and viability, and may bring a new light to the understanding of some age and gender differences in disease progression, as well as find application on the drug treatment of amebiasis in humans.

A4-014P

Differences between the potentials and excitability properties in two simulated cases of demyelinating neuropathies

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Objective: The purpose of this study is to investigate the potentials (intracellular, extracellular, electrotonic) and excitability properties (strength-duration time constants, rheobases, recovery cycles) in the cases of uniform internodal and uniform paranodal demyelinations (70%) along the fibre length.

Methods: The internodally and paranodally systematically demyelinated cases (termed as ISD and PSD, respectively) are

simulated using our previous double cable model of human motor nerve fibres.

Results: In both demyelinated cases, the intracellular potentials are with significantly reduced amplitude, prolonged duration and slowed conduction velocity, whereas the electrotonic potentials show abnormally greater increase in the early part of the hyperpolarizing responses. The extracellular potential indicates increased polyphasia in the PSD case for the radial distance of 1 mm. The strength-duration time constant is longer in the ISD case and shorter in the PSD case than in the normal case. For both cases, the rheobases are higher than the normal case. In the recovery cycles, the demyelinated cases have greater supernormality and less late subnormality than the normal case. The recovery cycle in the ISD case has greater refractoriness (the increase in threshold current during the relative refractory period), whereas in the PSD case it has less refractoriness than the normal case.

Conclusions: The myelin thickness and paranodal seal resistance have significant effects on the potentials and excitability properties of the simulated demyelinated human motor fibres. The obtained abnormalities in the potentials and excitability properties in the ISD and PSD cases can be observed *in vivo* in patients with Charcot-Marie-Tooth disease type 1A (CMT1A) and chronic inflammatory demyelinating polyneuropathy (CIDP), respectively.

Significance: The study provides new information about the pathophysiology of human demyelinating neuropathies.

A4-015P

Xenobiotic metabolising enzymes: influence of polymorphism on oesophageal cancer susceptibility

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Squamous cell carcinoma of the oesophagus is one of the most common cancers among black male South Africans. The importance of genetic factors in determining individual susceptibility to cancer is becoming clearer. Xenobiotic metabolizing enzymes are an important part of cellular enzymatic defense against endogenous and exogenous chemicals, many of which have carcinogenic potential. These enzymes are classified into phase I and phase II. During detoxification of harmful substances, an imbalance between phase I drug metabolism and phase II detoxification may contribute to the development of several diseases and this imbalance is a result of genetic polymorphism. Genetic variants of phase I enzymes result in reduced, abolished or increased enzyme activity thereby impairing bioactivation, whereas polymorphic variants in phase II often result in impaired detoxification. Several detoxification genes were investigated for their possible role in the development of oesophageal cancer. These included CYP3A5, CYP2E1, ALDH2 and GSTs amongst patients and control individuals in South Africa. In comparing patients with controls, some allelic variants and genotype combinations were associated with either increased or decreased risk for esophageal cancer. The CYP3A5 homozygous mutated genotypes were associated with decreased risk while wild type CYP3A5 was associated with increased risk for the development

of squamous cell carcinoma of the oesophagus. Furthermore different South African population groups had different genetic profiles thereby having a different predisposition to oesophageal cancer.

A4-016P

N-acetyltransferase 2 gene polymorphism and age-related hearing loss

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N-acetyltransferase enzymes are involved in the metabolism and detoxification of cytotoxic and carcinogenic compounds as well as reactive oxygen species (ROS). ROS generation occurs in prolonged relative hypo-perfusion conditions such as in aging. The etiology of presbycusis is much less certain, however, a complex genetic cause is most likely. The effect of aging shows a wide inter-individual range; we aimed to investigate whether profiles of N-acetyltransferase 2 (NAT) genotypes may be associated with the risk of age-related hearing loss. We examined 68 adults with presbycusis and 69 healthy controls. DNA was extracted from the lymphocytes by high pure template preparation kit. NAT2*5A, NAT2*6A, NAT2*7A/B, NAT2*14A were detected by using LightCycler-NAT2 mutation detection kit by real time PCR with LightCycler instrument. Associations between specific genotypes and the development of presbycusis were examined by use of logistic regression analyses to calculate odds ratios (OR) and 95% confidence intervals (CIs). Gene polymorphisms at NAT2*5A, NAT2*6A, NAT2*7A/B, NAT2*14A in subjects with presbycusis were not significantly different than in the controls (p>0.05). Also the combinations of different NAT2 genotypes were not an increased risk of presbycusis (p>0.05). We could not demonstrate any significant association between the NAT2*5A, NAT2*6A, NAT2*7A/B, NAT2*14A polymorphism and age-related hearing loss in this population. This may be due to our sample size, and further studies need to investigate the exact role of NAT gene polymorphisms in the etiopathogenesis of the presbycusis.

A4-017P

Systems for endogenous detoxification in inherited metabolic diseases

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Systems for detoxification are mostly studied on xenobiotics derived from exogenous sources, like pharmaceuticals, food substances or environmental contaminants. It is generally accepted that endogenous substances, synthesized in secondary metabolic pathways, which become operative in metabolic diseases, might also have toxic effects, causing some of the clinical symptoms in such patients. Endogenous detoxification occurs if these substances are excreted as their conjugates.¹

Results: In this communication we present results on new conjugates of glutamic acid, found in patients with a deficiency in propionyl-CoA carboxylase. Solvent extraction, followed by gas chromatography-mass spectrometry or direct separation on a C18 HPLC column and detection by multiple reaction monitoring on tandem mass spectrometry (VG Quatro II)², was used to

identify conjugates formed in metabolic diseases. New conjugates, which we describe here are propionylglutamic acid and tiglylglutamic acid. A systematic comparison of conjugates previously detected by us, as well as other known conjugates, indicates that a system for detoxification in inherited metabolic diseases can be postulated.

Conclusions: The system for detoxification of endogenous substances which we postulate here, consist of a number of phases, which differ however from the phases of the system for detoxification of xenobiotics due to the difference between the biochemical nature and source of the endogenous substances and the xenobiotics.

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A4-018P

Regulation of iNOS expression by the MAPK pathway in human melanoma

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Activating mutations of N-ras and B-raf, components of the mitogen-activated protein kinase (MAPK) pathway, occur frequently in melanoma. Activation of B-raf, whether a consequence of gene mutation or N-ras activation, results in ERK phosphorylation and subsequent downstream transcriptional events. ERK phosphorylation has been shown to play a critical role in melanoma proliferation. Another protein supporting melanoma growth is the enzyme inducible nitric oxide synthase (iNOS). Tumor expression of iNOS predicts shortened survival for melanoma patients, and nitric oxide, the product of iNOS, is anti-apoptotic for melanoma cells *in vitro*. Evidence from several cell systems supports the regulation of the iNOS gene by the MAPK pathway, placing iNOS downstream of B-raf and ERK. Thus, we have hypothesized that activating mutations of N-ras and B-raf ultimately drive iNOS expression in melanoma. To test this hypothesis, a human melanoma cell line with constitutive iNOS expression was transfected with BRAF siRNA. The resulting decline in B-Raf protein led to inhibition of ERK phosphorylation and was accompanied by a decrease in iNOS expression, as well as by slowing of proliferation, as evidenced by BrdU uptake. The same cell line, when treated with the iNOS inhibitor S-methylisothiourea, displayed a similar reduction in BrdU uptake. Furthermore, twenty-one human melanoma tumors were examined for NRAS and BRAF mutations by PCR and DNA sequencing, and for immunohistochemical evidence of ERK phosphorylation and iNOS expression. A significant association was found among NRAS or BRAF mutation, ERK phosphorylation, and iNOS expression. We conclude that in human melanoma, activating mutations of N-ras and B-raf drive constitutive iNOS expression, which supports the malignant phenotype.

A4-019P

Functional exploration of liver genome by using cDNA microarrays in a model of estrogen-induced cholestasis

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Rats treated with Ethinylestradiol (EE) have been widely accepted as one animal model to elucidate molecular mechanisms of cholestasis. We have used cDNA microarray technology to identify mRNA differentially expressed after EE administration to intact male rats. Since it is well known that estrogen treatment markedly influences secretion of pituitary hormones, the possibility of pituitary influence in EE-induced gene expression profiles was also investigated in hypophysectomized (HYPOX) rats. mRNA levels of genes involved in bile acid synthesis as well as transport were markedly decreased after 24 h of EE administration. 6200 genes were monitored of which 1012 exhibited a change at one or two rat models. 268 and 744 genes showed significant changes in gene expression levels in intact and HYPOX, respectively. Functional gene annotation showed that little overlap is apparent between the effects of EE exerted in intact with those in HYPOX, suggesting that an intact pituitary is required for a normal response to EE. Correlation analysis showed that EE and GH exert significant overlap among their hepatic effects. EE still modulates the expression of 744 genes in HYPOX, suggesting that EE exerts qualitatively different effects on liver gene expression in the absence of pituitary hormones. Since ER mRNA level is drastically reduced in the absence of pituitary hormones, alternative mechanisms may contribute to the effects of EE in HYPOX. This analysis has identified a number of genes that heretofore have not been implicated in EE actions; such genes may provide new areas of research into the pathogenesis of EE-induced liver injury. Pituitary hormones contribute to EE effects on liver (FIS 1/1000, PETRI1995-0711, Pfizer CN-78/02-05045, MCYT SAF2003-02117).

A4-020P

The study of the biological function of the alternative spliced forms of BLM gene and their role in cancerogenesis

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Bloom syndrome (BS) is rare autosomal recessive disorder. The BS patients show strong genetic instability and a predisposition to the development of cancer. We found three BLM truncated forms resulted from the alternative splicing. The expressed sequence tags derived from tumors have been reported to be the splicing patterns which differ from the canonical mRNA sequence. Moreover, different types of cancer-specific splicing shifts disrupt tumor suppressor function. In BS affected individuals the development of different types of cancer might be the consequence of the dominant negative effect of BLM alternative spliced forms. This project is aimed to the elucidation of the functional role of the BLM variants in BS and normal cells. We investigated the nuclear transport pathway of BLM splice variants, their expression profile and to estimate the level of genomic

instability in response to up- or down-regulation of BLM truncated forms in various cell types.

A4-021P

Recruiting phage HK022 integrase for mammalian gene manipulations

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The Integrase (Int) protein of coliphage HK022 catalyzes the site-specific integration and excision of the phage into and from its *Escherichia coli* host chromosome. Int expressed from a plasmid in mammalian cells is localized in the nucleus. Site-specific recombination studies in human cells using plasmids with the proper att sites and the Green Fluorescent protein (GFP) as a reporter have shown that the wild type Int of HK022 can promote integration and excision in the human cells, both in the *cis*- as well in the *trans*- configurations. The integration and excision reactions in *cis*- works also when the att sites are located on the chromosome. These reactions take place without the need to supply any accessory proteins (IHF or Xis) that are required in the bacterial host. The nuclear localization of Int-HK022 and its activity in mammalian cells renders the system as a potential one for site-specific gene manipulations in mammals.

A4-022P

Inter-individual variation in *GSTM1* and *CYP2A6* genotypes in relation to lung cancer risk among tobacco smokers in Turkey

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Among the xenobiotic metabolizing enzymes, the CYP2A6 family is characteristic of its catalytic properties to metabolize nitrosamines and *GSTM1* is known to catalyze detoxification of reactive oxygen species. Our laboratory previously reported a lower risk for *CYP2A6* gene deletion (*CYP2A6**4C/*4C) in oral carcinoma among betel quid chewers in Sri Lanka (Topcu et al, 2002). In this study we investigated the relationship between inter-individual difference in *CYP2A6* (*1A/*1A, *1A/*1B, *1B/*1B, *1A/*4C, *1B/*4C, *4C/*4C) and *GSTM1* (wild type and null) genotypes in relation to lung cancer among habitual tobacco smokers in Turkey. Blood samples from a total of 350 tobacco smoker subjects with or without lung carcinoma (small cell, non-small cell, squamous cell and adenocarcinoma) were obtained and genotyping was performed with restriction analyses of PCR-amplified samples of isolated DNA. Our current results suggest that the genetic polymorphism of *CYP2A6* and *GSTM1*, when analyzed together, is significantly differing between two groups, which suggests the involvement of these enzymes in lung carcinoma.

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A4-023P

Two novel disease-causing mutations in the CYP 21 gene in Russian patients with 21-hydroxylase deficiency: affect on functional enzyme activity.

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Steroid-21-hydroxylase is the one of essential enzymes involved in adrenal steroid synthesis. Inherited defects of this enzyme cause impaired cortisol and aldosterone synthesis and result in excess of androgens. 21-hydroxylase deficiency accounts for 95% of congenital adrenal hyperplasia (CAH). This is a classical monogenic autosomal recessive disorder with a wide spectrum of disease manifestations. The molecular genetic basis of 21-hydroxylase deficiency has been thoroughly studied. This enzyme is a microsomal cytochrome P450 (also termed P450c21), which is encoded by two genes: CYP21B- an active gene, and CYP21A – a highly homologous inactive pseudogene, located in the HLA class III gene region on chromosome 6p21.3. The aim of the present study is to determine the affect of two novel missense mutations, G178R and R426C, found previously in Russian patients with CAH, on activity of steroid-21-hydroxylase. Mutant CYP21 cDNA with novel mutations were constructed and cloned in pcDNA3.1 (+) vector. After transfection of HEK293 cells the conversion of progesterone to 11-deoxycorticosterone and 17-OH-progesterone to 11-deoxycortisol was measured. Both mutations result in strong inactivation of steroid-21-hydroxylase, which is in agreement with location of both aminoacid substitutions in functionally important domains of the enzyme.

A4-024P

Proteomic investigation into the propagation and spread of infectious prions in non-neuronal cells.

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Prion diseases are transmissible neurodegenerative disorders including Creutzfeldt-Jakob disease in humans, scrapie in sheep, and bovine spongiform encephalopathy (BSE) in cattle. According to the protein only hypothesis, an abnormal isoform of the host encoded prion protein (PrP^C), referred to as PrP^{Sc}, is the sole or major component of the infectious and neurotoxic agent (the "prion"). However discrepancies between PrP^{Sc} and its relationship to prion infectivity and neurotoxicity indicate that alternative forms of PrP, distinct from infectious PrP and/or

unidentified cellular factors, are necessary for the acquisition of infectious properties and may be involved in the mechanisms of prion toxicity. Using a mouse adapted strain of human prions we have developed a cell based model of prion propagation which produces infectious prions, providing a means to investigate cellular factors involved in prion propagation. Previous *in vitro* studies suggest cells release prions in association with exosomes. Consistent with this idea, the culture medium of our infected cell line contains exosome associated PrP^{Sc}. When isolated from cell media, the exosomes from the infected cells transmitted infection to non-infected cells suggesting cellular co-factors necessary for generation of infectious PrP^{Sc} are contained within the exosomes. Due to the unique protein composition of exosomes we are using proteomic approach to examine the profile of expressed proteins of infected vs. un-infected exosomes to identify cellular factors implicated in the propagation and spread of infectious prions.

A4-025P

Analysis of differentially expressed genes in human leukemia HL-60 cells induced by diallyl disulfide using suppression subtractive hybridization technology

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Diallyl disulfide (DADS), an effectively active oil-soluble sulfur compound of garlic, has been known to exert potent chemopreventive activity and induced differentiation effect against cancers. Animal and *in vitro* studies of DADS had showed that it played a major role in inducing differentiation of human acute myeloid cell line HL-60. But its molecular mechanisms are still unknown. In this study, untreated HL-60 cells and DADS-treated HL-60 cells were used. We used suppression subtractive hybridization (SSH) technique constructed the differentiation subtractive cDNA library and obtain those genes correlated to differentiation of leukemia cells. Results demonstrated that subtractive cDNA library was constructed successfully and efficiently. Random analysis of 100 clones with restriction enzyme showed that about 84% clones contained 100~600bp cDNA inserts. Through homology analysis in PUBMED using BLASTN, up-regulated genes including CD11b, P21WAF1, STAT1, c-fos, c-jun and Calmodulin 1 gene, down-regulated genes including PCNA, STAT3 and c-myc gene were obtained. And some maybe novel genes need to be study further. The above results suggest that those differentiated expression genes, correlated to cell proliferation, differentiation, DNA synthesis, cell cycle arrest, cell factor, and so on, may played important roles in inducing differentiation of human leukemia HL-60 cell.

A4-026P

Analysis of epithelial-mesenchymal interaction in lung fibrosis using a cell-culture model

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Introduction: Fibrotic lung diseases are associated with a severe loss of functional alveolar epithelium and proliferation of fibro-

blasts. Mutual crosstalk was demonstrated in both cell populations, alveolar epithelial cells (AEC) and interstitial fibroblasts (iFB). In normal lungs AEC inhibit iFB proliferation and collagen production. Alterations of the normal gene expression profile of AEC and iFB, respectively, induce phenotypic changes and may represent a key event in the development of fibrotic lung diseases.

Methods: We establish a co-culture system for AEC and iFB including cells from healthy and fibrotic mice (induction by bleomycin treatment). We extract RNA from freshly isolated cells and after short-time co-culture to identify and characterize intracellular signalling profiles using real-time PCR and oligonucleotide glass arrays. Furthermore, we investigate the protein expression levels by immunohistochemistry and flow cytometry.

Results: Fibrotic AEC produce less surfactant proteins and lose their anti-proliferative influence on fibroblasts indicated by an upregulation of angiotensin system and downregulation of cyclooxygenase II. Under co-culture conditions we found a reduced AEC-dependent inhibition of iFB proliferation due to fibrotic AEC. These fibroblasts show an upregulation of the angiotensin system and matrix-metalloproteinase 2.

Conclusion: Our co-culture system is suitable to detect markers involved in epithelial-mesenchymal interactions which are relevant for development of lung fibrosis. We now analyze mRNA and protein expression profiles using oligonucleotide glass arrays and mass spectrometry to find additional candidate markers that may play an important role in the pathomechanisms involved in fibrotic lung diseases.

A4-027P

Gene and protein regulation after short exposure to hypoxia in the mouse lungs

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Introduction: Hypoxia causes oxidative stress and vasoconstriction which leads to pulmonary hypertension and vascular remodeling. Additionally hypoxia increases the level of reactive oxygen species (ROS) that are involved in signal transduction. Until now the mechanism underlying the hypoxic gene/protein regulation is not fully understood, therefore we seek to find genes and proteins regulated in the early time points by arrays and 2D-PAGE analysis.

Methods: Total RNA and proteins were isolated from lung homogenate of mice exposure to 24 h normoxia or hypoxia (FiO₂ = 0.1). Gene regulation was investigated by applying 30k glass arrays and analysed by "limma" in R 1.9.1. Protein regulation was defined by 2D-PAGE and subsequent MALDI-TOF analysis. Protein expression levels were confirmed by Western blot and cellular localization determined by immunohistochemistry.

Results: Array data showed 101 genes up and 32 down regulated. 2D-PAGE revealed 190 differentially regulated proteins from which 35 were identified by MALDI-TOF. The majority of regulated genes and proteins were responsible for metabolism, transport and ROS balance. Three proteins Eef1a1, Cat1 and HMGB1 were confirmed by western blot analysis. For these proteins, no regulation was detected on mRNA level.

Discussion: Even short exposure to hypoxia (FiO₂ = 0.1) induces many changes in gene and protein regulation in the lung parenchyma. Interestingly not many of the 35 proteins identified by MALDI-TOF were regulated at the transcriptional level. This study demonstrated that differences on mRNA and protein level do not always correlate. Therefore, only the combination of proteome and transcriptome analysis can reveal undergoing changes in complex tissue.

A4-028P

Hunter disease in a girl due to nonrandom inactivation of the X-chromosome

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Hunter disease (mucopolysaccharidosis II) is an inherited lysosomal storage disorder caused by deficiency of iduronate-2-sulphatase (IDS) activity. This enzyme is involved in heparan and dermatan sulphate degradation. Deficiency in IDS leads to accumulation and storage of these glycosaminoglycans (GAG) in virtually all tissues. Hunter syndrome is an X-linked recessive disorder, therefore, affecting primarily males. Females can be carriers of the disease but, in extremely rare cases, Hunter syndrome has been diagnosed in females with abnormal X-chromosome inactivation or chromosomal rearrangements. Here we report a case of a karyotypically normal girl diagnosed enzymatically for Hunter disease. Genomic DNA from the patient and its parents was analyzed by automated sequencing for mutation of the IDS gene. We revealed a previously described missense mutation A1568G resulting in the substitution of cysteine for thymine at position 523 (Y523C). Both, mother and patient are heterozygous for the mutant allele and father is a wild-type hemizygote at this locus. We examined the X-chromosome inactivation pattern in the patient and her parents. DNA was isolated from peripheral blood samples and the methylation status of the androgen-receptor (AR) gene was determined using the methylation-sensitive restriction enzyme HpaII. We identified a completely skewed pattern of X-inactivation in the girl-patient, namely, the paternal X-chromosome was inactive and the maternal allele was active. We conclude, that a skewed X-chromosome inactivation of the paternal gene and a point mutation in the maternal gene were responsible for the Hunter disease in the girl-patient. It is of our interest to determine the cause of the nonrandom X-inactivation in this case.

A4-029P

Suppression of HER2/neu by SV40 large T antigen exon 1 product in prostate cancer DU145 cells

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The HER2/neu gene amplification and/or its protein overexpression is found in approximately 30% of prostate cancer

patients and frequently associated with tumor metastasis and tumorigenicity leading to poor clinical outcome. Repression of HER2/neu gene expression and its signal transduction suppresses the tumor proliferation and metastasis, suggesting the HER2/neu is a good target for developing anti-cancer therapy. We have demonstrated that the N-terminal 1~178 a.a. residues of SV40 large T antigen (LT425) can act as a transforming suppressor of the HER2/neu oncogene. Here, by using the same model system, we show that the LT425-exon-1 only has the similar repressing effects on the HER2/neu oncogene. The LT425-exon-1-transfected prostate cancer line DU145, which expressed higher level of HER2/neu, was found to have reversed morphology, reduced HER2/neu protein expression, decreased growth rate, low anchorage-independent growth, and low motility and metastasis potential. Furthermore, the effect on the cell cycle after the interaction between the LT425-exon-1 and HER2/neu was also investigated. It seemed that LT425-exon-1 might prolong cell cycle at G2/M phase. These data provide an alternative way to the human prostate cancer gene therapy.

A4-030P

Genetic risk factors of Attention Deficit Hyperactivity Syndrome

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The dopamine D4 receptor (DRD4) gene has attracted increasing interest as a candidate gene in association studies of Attention Deficit Hyperactivity Disorder (ADHD). The most frequently investigated polymorphism of the DRD4 gene is the exon III 48 bp VNTR (Variable Number of Tandem Repeats). Hitherto only a few studies have assessed polymorphisms located in the promoter region of the gene, although many gene variants have been described in the 5' untranslated region of the DRD4 gene. Here we present an association study for four polymorphisms of DRD4 gene 5' upstream region (120 bp duplication, -616 C/G, -615 A/G, -521 C/T SNPs) and the exon III 48 bp VNTR. We genotyped and haplotyped 529 individuals (229 controls, 300 patients) applying the methods elaborated in our laboratory¹. Possible genetic association of the ADHD and the investigated polymorphic sites was evaluated both by the case-control and the Transmission Disequilibrium Test approach. An increased allele-frequency of the 1 × 120 bp variation was shown among ADHD children by the case-control approach ($P = 0.0375$). Moreover, a preferential transmission of the 1 × 120 bp ~ -616 C ~ -615 A ~ -521 T promoter haplotype was obtained ($P = 0.0278$). In this haplotype structure, the main effect of 1 × 120 bp ~ -521 T was shown in the preferential transmission to the affected offsprings ($P = 0.0482$). These haplotype effects were also confirmed by the case-control study. In conclusion, our results verified the role of the 1 × 120 bp ~ -521 T haplotype as a possible risk factor of ADHD. *In vitro* studies on the molecular effects of these promoter polymorphisms are underway.

Reference

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A4-031P**Gene expression profiling after treatment of acetaminophen in liver of mouse**

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DNA microarray has become a powerful approach for exploring the biological effects of drugs, particularly at the stage of toxicology and safety assessment. In this study, we investigated gene expression changes associated with the early stage of hepatotoxicity by acetaminophen using microarray technology. C57BL/6 mice were orally single dosed with acetaminophen (four doses and four time points) and hepatic gene expression was assessed using high-density oligonucleotide microarrays capable of determining the expression profile of > 14 500 well-substantiated mouse genes. We found that acetaminophen up- or down-regulated the genes encoding for stress responsiveness, metabolism, cell growth and maintenance, transport, signal transduction, cell death, and development. We used Hierarchical clustering to get the pattern of similar gene expression, and found that 22 type and 25 type clusters from dose- and time-dependent expressed genes, respectively. These expression profiling lead to a better understanding of the molecular basis of acetaminophen-induced liver injury, suggesting their potential importance in propagating or preventing further toxicity.

A4-032P**Metabolic profile of a genetic animal model of depression**O. Kotsovolou¹, D. H. Overstreet¹, M. Marselos¹, M. A. Lang¹ and M. Konstandi¹¹*Pharmacology, Medical School, Ioannina, Ioannina, Greece,*²*Psychiatry, Medical School, North Carolina, Chapel Hill, NC USA,*³*Biochemistry, Faculty of Pharmacy, Uppsala, Uppsala, Sweden. E-mail: mkonstan@cc.uoi.gr*

The metabolism of a drug is crucial in determining to a large extent its pharmacokinetic behaviour, the inter-individual variability and interactions with other drugs, all important parameters in drug therapy and pharmacotoxicity. On the other hand, stress and depression alter homeostasis of the body increasing the vulnerability of the exposed individuals in developing various diseases. In this study the metabolic profile of a genetic animal model of depression (the Flinders Sensitive line: FSL) was investigated. The data showed that hepatic total P450 content is higher in FSL rats compared to controls (Flinders Resistant Line: FRL). In particular, the constitutive expression of hepatic cytochromes CYP1A1 and CYP2E1 is higher in FSL than in FRL rats. In contrast, cytosolic ALDH3 and GST activities and GSH content are lower in FSL compared to FRL. After exposure to benzo(a)pyrene (B(a)P), the induction of CYP1A enzymes did not differ in FSL and FRL rats. In contrast, microsomal CYP2B1/2 and CYP2E1 expressions were higher in B(a)P-exposed livers of FSL compared to FRL rats. Again, GST activity and GSH content remained lower compared to non-exposed rats. In conclusion, important elements of drug metabolizing enzymatic systems are differently expressed in FSL and FRL rats. This in turn may explain, at least in part, the increased cancer incidence and the development of several pathologies in patients suffering depression compared to normal population.

A4-033P**CpG activated human primary B lymphocytes are readily transduced by lentiviral vectors**K. Kvell^{1,2}, T. Nguyen³, P. Salmon³, C. Favre², M. Barnet², D. Trono³ and R. Zubler²¹*Department of Immunology and Biotechnology, University of Pécs, Pécs, Hungary,* ²*Research and Development Unit, Department of Hematology, University Hospital of Geneva, Geneva, Switzerland,* ³*Department of Microbiology and Molecular Medicine, University of Geneva, Geneva, Switzerland. E-mail: krisztian.kvell@aok.pte.hu*

Lentiviral vectors are capable of transducing a large variety of target cells, including activated, non-dividing cells. However, the transduction of human primary B lymphocytes is still challenging. In some culture systems (like CD40L) B-cells are properly activated yet they remain poorly transducible. In others (like EL4-B5/B-cell co-cultures) B-cells are readily transduced but the nature of activation/survival signals is not characterized, seriously limiting downstream applications. Here we report a monoculture system for the efficient transduction of human primary B lymphocytes. Synthetic CpG oligonucleotides can activate B-cells through Toll-like receptor 9 (TLR9) which then become transducible by lentiviral vectors. By default, TLR9 is present on memory B-cells only, but Ig cross-ligation can upregulate TLR9 expression on naive B lymphocytes too. We have found that the transgenes introduced by bicistronic lentiviral vectors produce transgenic proteins that are fully functional. As proof of principle we have tested a secreted and an intracellular protein. The human IL4 gene was transduced into B-cells, which then secreted IL4 that could increase cell proliferation and promoted IgE switch in secondary cultures. When the molluscum contagiosum viral flice-inhibitory (vFLIP) gene was introduced to B-cells, vFLIP could potentially protect the transduced cells from apoptosis triggered by FasL. In conclusion human primary B lymphocytes activated by CpG oligonucleotides are efficiently transduced by lentiviral vectors. We have found that bicistronic vectors can introduce biologically active molecules that are fully functional allowing gene function research and perhaps the development of gene therapies in human primary B cells.

A4-034P**Expression and methylation analysis of some chromosome 3p "hot spots" genes in epithelial tumors**V. Loginov¹, E. Klimov², I. Pronina¹, D. Hodirev¹, G. Kilosanidze³, A. Malyukova^{1,3}, R. F. Garkavtseva⁴, G. E. Sulimova², E. R. Zabarovsky³ and E. A. Braga¹¹*Russian State Genetics Centre, Moscow, 117545 Russian Federation,* ²*Vavilov Institute of General Genetics, Russian Academy of Sciences, Moscow, 119991 Russian Federation,* ³*Microbiology and Tumour Biology Centre, Centre for Genomics and Bioinformatics, Karolinska Institute, Stockholm, 17177 Sweden,* ⁴*Blokhin Russian Cancer Research Centre, Russian Academy of Medical Sciences, Moscow, 115478 Russian Federation. E-mail: loginov7@genetika.ru*

Abnormalities involving the short arm of chromosome 3 are frequently reported in carcinomas of the kidney, lung, breast, ovary, cervix and other epithelial tumors. Detailed mapping of allelic deletions from 3p in five tumors (approx. 400 T/N DNA samples) revealed the new region in 3p21.31 (D3S2409-D3S3667, 600 kb). Frequencies of HD were found rather high in D3S2409-D3S3667 (3p21.31) region in comparison with other 3p "hot spots". SAGE analysis displayed tumor specific differential expression of some genes from the new D3S2409-D3S3667 region revealing features of putative oncogenes and potential TSG for the same gene.

Up- and down-deregulation of the same gene in various tumor cases was also found for RARbeta2 (3p22.3), SEMA3B (LUCA region, 3p21.31-p21.2) and for USP4 and DAG1 genes (D3S2409-D3S3667, 3p21.31) using RT-PCR and Real Time PCR studies. The same 3p genes were analyzed for promoter methylation. These six candidate genes were shown differentially methylated in tumor cell lines. The methylation of RAR-beta2, RASSF1A and SEMA3B in epithelial carcinomas was assayed in detail by means of methylation-specific PCR (MSP), PCR-based methylation-sensitive restriction enzyme analysis (MSRA) and bisulfite sequencing. RASSF1A gene was found highly (>60%) methylated in renal cell (RCC), breast (BC) and ovarian carcinomas (OC). Methylation of SEMA3B occurs in half (40–60%) cases of these tumors. However, methylation frequency of RAR-beta2 substantially differs between these tumor types, 60% in RCC vs. 27% in OC. At the same time methylation status of RASSF1A and SEMA3B was shown in significant positive correlation to tumor progression (stage, grade and metastases potential). Thus, diagnostics and prognostics markers can be designed.

A4-035P

Cryoprecipitate-removed plasma “cryo-removed plasma” as a source of factor IX in the treatment of haemophilia B

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Fresh frozen plasma (FFP) is the main source of factor IX (FIX) in the treatment of bleeding episodes of haemophilia B in the Philippines. Cryoprecipitate-removed plasma otherwise known in the Philippines as cryosupernatant, is a by-product of cryoprecipitate preparation. These blood products expire in storage or are just thrown-away because of less demand for clinical use. By theory, this product should have almost the same amount of FIX as in FFP, therefore can be used in the treatment of haemophilia B. There is no local data on the actual FIX content of the cryoprecipitate-removed plasma. Hence the authors established these data to support the use of this product. Eighty-three bags of cryoprecipitate-removed plasma received from three different blood banks in Manila, Philippines were tested for FIX activity using an activated partial thromboplastin time (APTT)-based one-stage FIX assay. The FIX content in each bag of cryoprecipitate-removed plasma was calculated by multiplying its volume in mL with that of FIX activity per mL of plasma measured *in vitro*. The total mean FIX content per bag was 212.20 U (± 88.98) exceeding the contents set by the American Association of Blood Banks (AABB, 70–90 U). The mean FIX activity per bag was 127.62% (± 38.23) with the mean volume of 164.28 mL (± 52.23). Statistically significant difference on volume ($P = 0.000$) was found across the three sources resulting to a significant variation of the actual FIX content ($P = 0.000$).

A4-036P

Identification of disease genes in genetically heterogeneous disorders through bioinformatic data integration

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Dissecting the molecular mechanisms underlying disease, is the source of large amounts of new information about the function of cells and tissues under normal conditions. Such information can be

used to develop novel diagnostic methods or even cures for the particular disorder studied, and lead to a better understanding of the molecular cell biology essential to the existence of us all. The key to this information is the gene in which mutations lead to a particular disorder. Once identified, a disease gene can be explored in a variety of experimental set-ups, for functional characterization. Due to the information that can be revealed by disease genes, finding them is a field of much interest. There is no standard procedure for disease gene finding, but after the publication of the first-draft of the human genome in 2001, sequence based methods have made this field accelerate dramatically. Most diseases can be linked to particular locations in the genome. However, pinpointing the correct gene in such a location can be excruciatingly hard work. Any method that could reduce the amount of real candidates, in a genomic region known to link to a disease, would be a breakthrough. Many diseases show characteristics, which makes it seem likely that interactions exist between the disease genes on a protein level. We have made a semi-automated disease gene finding pipeline based on this assumption. Central to this pipeline is identification of genes in model organisms, which resemble the human disease genes. This approach is followed by automatic large-scale protein interaction queries, and finally sequence alignment against linkage intervals. Using this bioinformatic pipeline, we have searched several disorders for disease genes, and have found a number of interesting candidates. These candidates have been reported to experimental collaborators, with access to patient material, in order to verify our predictions and establish proof of concept.

A4-037P

Screening for the 342-kb deletion of GJB6 gene in patients with non syndromic hearing loss from Jordan

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Hearing loss is a common congenital disorder frequently associated with mutations in the connexin 26 gene (GJB2). However, recent studies found a 342-kb deletion in another gene, connexin 30 (GJB6) that causes non-syndromic recessive hearing loss in either a homozygous monogenic inheritance of Cx30 deletion or digenic inheritance of Cx30 deletion and a Cx26 mutation. The objective of this study was to screen for the 342-kb deletion in Cx30 gene in patients with non-syndromic hearing loss from Jordan. Genomic DNA was extracted from blood samples obtained from 160 patient with non-syndromic hearing loss. Two different PCR conditions were used to detect the 342-kb deletion of connexin 30 gene by amplifying the deletion breakpoints using specific primers. None of the patients with non-syndromic hearing loss was found to carry deletion in connexin 30 gene indicating that the occurrence of this deletion is restricted to certain populations (Spanish, Caucasians, and Ashkenazi Jews).

A4-038P

Cytoplasmic retention of p53 in colorectal cancer cells

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Mutational inactivation of p53 gene product as one of the most common genetic events that occur in human cancers, highlighting

the central role of p53 as a tumor suppressor. As p53 functions as a transcription factor, localization of p53 to the nucleus plays a key role in regulating its activity. We analyzed colorectal carcinoma of common types with a different differentiation and stages as well as a normal colorectal mucosal samples from patients with benign/malignant tumors. Levels of p53 in cytosolic and nuclear fractions of colorectal cancer tissue were estimated by Western blot assay using anti-p53 antibody CM-1. Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated colorectal cancers but not in differentiated tumors, indicating that aberrant p53 sequestration in colorectal carcinoma is intimately associated with failure to differentiate and malignancy. Furthermore, since it is found in the earliest stages, the alteration appears to occur early in the tumor progression. In this case, the cancer cells might use an altered version of a physiological mechanism to retain p53 in the cytoplasm. While mechanism by which the wild-type p53 could be retained in the cytoplasm is not known, an interesting hypothesis derives from the fact that one of the nuclear localization signals is located between amino acid residues 316 and 321. Serine-315 can be phosphorylated by cdc-2-like kinase. It is therefore possible that a growth-regulatory signal, such as an active cdc-2-like kinase could alter p53 protein via phosphorylation and exclude it from the nucleus. Mutant p53 proteins with deletions in the C-terminal region show cytoplasmic localization.

A4-039P

3-HK induces differentiation in human NB4 and U937 leukemia cell lines

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Several leukemia diseases are characterized by a break-down in myeloid cell maturations. To restore the normal differentiation, such patients are treated with differentiation therapy. The therapeutic agents, however, have short lives and are accompanied by a variety of side effects. Therefore, it is worthwhile to search for new substances, which induce differentiation. Among plant-derived agents, diterpene esters have been proven to be potent differentiation inducers. In this study, we evaluated the differentiation efficiency of the new diterpene ester, 3-hydrogenkwadaphnin (3-HK), using NB4 and U937 cell lines. 3-HK has been isolated from *Dendrostella lessertii* (*Thymeleaceae*) and its chemical structure has been previously reported. Morphological (Wright-Giemsa) and biochemical (NBT reducing) studies revealed that 3-HK (<10 nM) induced 40% and 65% differentiation in U937 and NB4 cell lines, respectively. These effects were dose- and time-dependent, so that maximum differentiation was observed in 7.5 nM and after 72 h treatment. However, after 72 h the numbers of viable cells decreased as examined by trypan blue exclusion test. Cell cycle analysis of NB4 cells revealed that 3-HK (7.5 nM) induces 15%, 25% and 30% G1 cell cycle arrest at 24, 48 and 72 h, respectively. In addition, an increase in sub-G1 cell population was appeared after 72 h, suggesting that differentiated cells underwent apoptosis. DNA fragmentation and morphological studies with fluorescence microscopy also confirmed that like some of diterpene esters spontaneous apoptosis occurred in the drug-treated cells after drug-induced differentiation. According to current and our previous results, this new diterpene ester appeared to be a powerful candidate for leukemia therapy.

A4-040P

Genetic diversity and phylogenetic analysis of human immunodeficiency virus type 1 circulating in Iran

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Surveillance of HIV-1 subtypes has important implications for the development of candidate vaccines and understanding the possible differences in the transmission and natural history of different subtypes. In this study, molecular characterization of HIV and its heterogeneity was determined for in Iran. Whole blood was collected from HIV-infected persons coming to two referral centers for blood donations and AIDS-related illnesses in Tehran, the capital of Iran. Drug abusers and hemophilia patients were enrolled in this study as main and oldest groups of infected individuals in Iran, respectively. Proviral DNA were extracted from 16 positive sample and were subjected for amplification of 1.2 kb DNA fragments of HIV Env gene by nested polymerase chain reaction (PCR). In three cases, PCR products showed 2 different bands shorter or longer than predicted size. Multiple Clones were taken from each sample and totally 193 clones subjected for genetic analysis via determination of homologies in the V1-V3-V5 regions by heteroduplex mobility assay (HMA) as well as full length sequencing. All finds of obtained via Individually analysis of of Iranian Isolates sequence. Constructed Phylogenetic tree and HMA showed that the Subtypes A-1 (50%) and B (43.7%), have the most frequency among HIV-infected Iranians under studied and just one sample was determined as C subtype. In this regard all drug abuser were infected with A subtype of HIV-1 and subtype B were found in all infected hemophilia patients. The Majority of analyzed proviral DNAs specially B subtypes evolved multiple mutation, insertion or expanded deletion and illustrated noticeable value of inter and/or intra-heterogeneity based on computation of their sequences diversity as well as their mobility on gel electrophoresis.

A4-041P

Genetic variations in genes of lipid metabolism and the Metabolic Syndrome

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The Metabolic Syndrome, characterized by an impaired insulin secretion and action, adipositas, high blood-pressure and dyslipidemia, results from the interaction between genetic susceptibility and environmental factors. We investigate the associations between coding and regulatory single nucleotide polymorphisms (cSNPs, rSNPs) in more than 20 genes of the lipid metabolism and the outcome of the Metabolic Syndrome (MSX) using the Taqman allelic discrimination method. Candidate SNPs were selected from dbSNP (NCBI) and Celera's db according to their gene localization and minor allele frequency (>0.03) and verified

by direct sequencing using a cycle sequencing method and a high-throughput 96-capillary electrophoresis analyser (ABI PRISM 3700). As a result of SNP genotyping, performed in a nested case control study of 192 incident Type 2 diabetes subjects and 384 sex and age-matched controls taken from the EPIC-Potsdam cohort, we identified six genes with evidence for significant association ($P \leq 0.05$) with disease status: rSNP (OR = 0.63, 95% CI = 0.41–0.96) and cSNP (1.9, 1.0–3.5) in acyl-CoA binding protein, rSNP in intestinal fatty acid-binding protein (0.5, 0.26–0.9), cSNP in fatty acid binding protein 1 (1.6, 1.2–3.2), rSNP or cSNP in glucose-dependent insulinotropic peptide (0.54, 0.36–0.83) and its receptor (0.70, 0.49–0.99) and cSNP in microsomal transfer protein (0.5, 0.33–0.83). Functional analysis of selected SNPs and replication of these findings in other cohorts e.g. KORA with DNA of 1000 Caucasians are underway. Our results support the hypothesis that genes of lipid metabolism contribute to the polygenic basis of the MSX.

A4-042P

P600 is a cytoskeletal transducer that links endoplasmic reticulum and calcium signaling to microtubules for neuronal positioning in the developing cortex

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Microtubules serve as tracks for intracellular transport of organelles and cargoes, thereby modulating the shape of CNS neurons in accordance to their developmental stages and functions. The mechanisms by which MTs convey endoplasmic reticulum (ER) membranes, an essential compartment for calcium signaling, remain however largely unknown. Here, we report that Protein 600 (P600) is a novel developmentally regulated Microtubule-associated protein that stabilizes MTs for ER transport in neurons. P600 co-purifies with MTs and ER from mouse brain and the regions of association of P600 to MTs partially overlap with ER-directed regions. When expressed ectopically, specific fragments of P600 distribute in tubule pattern, indistinguishable from ER structure. P600 depletion by RNA interference in primary neurons results in scarce transport of ER, culminating in destabilization of MTs and neuronal processes. Consistent with a role for ER in calcium signaling, P600 co-operates with CaM/CaMKIIa in modifying MT dynamics in primary neurons. Furthermore, in utero electroporation of P600 RNAi in embryonic cortical brain alters the leading process of neurons, thereby impairing neuronal positioning. Thus, P600 is a unique cytoskeletal transducer that connects ER and Ca²⁺ signaling to MT dynamics during neuronal migration in the developing cortex.

A4-043P

Telomere length regulation in the proliferation of vascular smooth muscle cells

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Intimal hyperplasia, due to smooth muscle cell proliferation and migration is responsible for the pathogenesis of atherosclerosis

and restenosis occurring after balloon angioplasty. In eucaryotic cells, the telomere is found at the end of linear chromosomes and acts to preserve chromosomal integrity. Telomeres are maintained by the activity of the enzyme, telomerase, a ribonucleoprotein that synthesizes and directs the telomeric repeats onto the 3' end of existing telomeres using its RNA component as template. Telomerase activity is found to be increased in highly proliferative cells including vascular smooth muscle cells (VSMC) whereas inhibition of telomerase diminished their growth. The aim of this study was to investigate the role of telomere length regulation in the proliferation of VSMC. For this purpose, balloon angioplasty was performed in the iliac artery of the rabbits with a Fogarty 2.5 cm. balloon catheter and the injured and non-injured arteries were isolated in predetermined days (0, 2, 7 and 14 days) after the angioplasty. Therefore, isolated artery segments were homogenized for a PCR- and ELISA- based telomerase expression. We extended our research to cover measuring the length of telomeric repeats via restriction analyses of high molecular mass DNA prepared and digested with HinfI on Southern blots as initial experiments did not detect telomerase activity, which is found in some pathological conditions in the form of Alternative Lengthening of Telomeres (ALT). Our results are discussed in relation to possible recombinational events during the telomeric length regulation in the course of the proliferation of VSMC.

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A4-044P

Epigenetics and the molecular mechanisms of FSHD

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Facio-Scapulo-Humeral muscular dystrophy (FSHD) is an autosomal dominant neuromuscular disease with a prevalence of one in 20 000. The FSHD locus has been mapped to the telomeric region of the long arm of chromosome 4 (4q35) and has been shown to be caused by the deletion of an integral number of tandemly arrayed 3.3 kb D4Z4 repeat units. The disease gene involved in FSHD is not yet known, but current hypothesis favors the loss of transcriptional repression operating on adjacent genes including a putative FSHD-related gene FRG-2. Recent studies have shown that the D4Z4 repeat linked to the FSHD locus binds an activator-repressor protein YY1 (Gabellini et al., 2002). This protein was shown to be a component of the nuclear matrix, raising the interesting possibility that the D4Z4 repeats may be associated with the nuclear matrix, and that the decrease in the numbers of D4Z4 repeats may lead to changes in the large-scale chromatin structure of the D4Z4 domain (FSHD domain). We shall follow the organization of the D4Z4 chromatin domain in normal cells and in FSHD patients by monitoring domain size and nuclear matrix attachment specificity within the 4q35 gene domain. The interactions detected in normal cells and in FSHD patients will be studied further by isolation and analysis of specific proteins that participate in these interactions. We shall study the association of the D4Z4 repeats and FRG1/2 genes to with heterochromatin in normal cells and in FSHD patients by Fluorescent In Situ Hybridization (FISH). In FSHD patients we expect to see a de-localization of the FRG genes and the D4Z4 repeats on chromosome 4 that carries a deletion in the number of D4Z4 repeats from heterochromatic regions into the euchromatin. We have also found a strong transcriptional enhancer within the D4Z4 repeat and propose that the D4Z4 repeat may modulate transcription of neighboring genes. We shall study the capacity of D4Z4 repeats to modulate reporter gene transcription

by creating a series of subclones and measuring their effects in transient transfection assays in a myoblast cell line. The minimal effector element of the enhancer within the D4Z4 repeat will be determined. This will be followed by a determination of the proteins that interact with this minimal element, and by a search for potential target genes upstream and downstream of the D4Z4 repeat units on chromosome 4.

A4-045P **E-cadherin and beta-catenin expression patterns in malignant melanoma assessed by image analysis**

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Beta-catenin is bound to E-cadherin in adherens junction formation, but also functions as a signaling molecule in the wnt pathway. We investigated the expression of E-cadherin and beta-catenin in 70 malignant melanomas by immunohistochemistry. The slides were evaluated by Image analysis as staining density, i.e. light permeability (LP). Comparison of mean values of relative LP for both proteins in melanomas shows that values for E-cadherin are significantly greater than those for beta-catenin (25 967–116,23; $P = 0.000$). Since greater LP indicates less protein, levels of E-cadherin are lower than levels of beta-catenin in the same patients. The comparison of mean values of the relative LP of the E-cadherin in melanomas to the LP in the immediately adjacent normal skin, also shows significantly greater values in tumor tissue (25 606–16 987; $P = 0.000$). It is obvious that the expression of E-cadherin in tumor tissue is significantly lower than that recorded in the adjacent skin. Comparison of LP for beta-catenin in melanomas and adjacent skin did not show any differences (11 530–11 524; $P = 0.968$). The Clark stages of melanoma progression were not correlated with the expression of the E-cadherin. However, a significant difference in beta-catenin expression was noted in the different Clark stages (Hi2 = 12 854; $P = 0.005$). The presence of beta-catenin could visually be determined in the cytoplasm-30% of patients; in the cell membrane 24.2%; in both the cytoplasm and membrane-21%; in the membrane and nucleus 1.6%; in the cytoplasm and nucleus-4.8%, while in 17.7% of patients beta-catenin could not be observed. Our results demonstrate changes in E-cadherin and beta-catenin levels in melanoma and could be used as molecular markers of disease evolution.

A4-046P **Selective repression of HPV-16 E6 and E7 oncogenes by RNA interference in cervical cancer cells**

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Introduction: HPV infection is the main etiologic agent in cervical cancer. HPV-16 covers 50% of the cases and E6 and E7

oncoproteins have properties of cell transformation and immortalization. Prophylactic and therapeutic strategies had been developed against cervical cancer with limited results to patients. The targeted inhibition of E6 and E7 oncogenes in tumor cells infected with HPV may provide a rational approach towards the development of novel anticancer therapies.

Objective: Using HPV-transformed cells as a model system, we analyzed whether RNA interference (RNAi)-mediated E6 and E7 oncogenes silencing can be employed in order to overcome the carcinogenesis process.

Methods: For this end, we generated two interfering RNA constructs (psiRNAE6 and psiRNAE7) in a DNA-vector, specifically directed against HPV-16 E6 and E7 oncogenes. SiHa cells (HPV-16) were transfected with these constructs to evaluate the effect on E6 and E7 oncogenes silencing by RT-PCR real time and western blot. To confirm the effect of E6 and E7 oncogenes silencing, we analyzed the C33A cells (HPV-negative) transfected with pSV2E6 and pSV2E7 expression vector and co-transfected with RNAi-constructs.

Results: Our results indicate a selective reduced expression of mRNA E6 and E7 oncogenes in HPV-positive cells as well as in HPV-negative cells that E6-E7-expressing. In addition, we performed the E6 and E7 oncoproteins expression analysis as well as functional effects in cell proliferation.

Discussion: Thus, these results suggest that HPV-16 E6 and E7 oncogenes can be repressed by RNAi strategy and this approach may represent an alternative of treatment against the cervical cancer development.

A4-047P **Structural characterization and functional promoter analysis of the human BM88 gene**

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Identification of promoter elements that control expression of genes involved in neuronal differentiation is of paramount importance for understanding the principles underlying their regulation, as well as for elucidating the developmental mechanisms of neuron generation. BM88 is a neuron-specific protein, widely expressed in the mammalian nervous system, which is involved in cell cycle exit and differentiation of neuronal precursors. BM88 is expressed at low levels in neuronal progenitors before terminal mitosis while it is up-regulated in their post-mitotic neuronal progeny. This tight correlation between BM88 expression and the progression of progenitor cells towards neuronal differentiation, suggests that BM88 is regulated by elements necessary for committing a neural progenitor cell to a neuron. Here we report the characterization and promoter analysis of the human BM88 gene. BM88 promoter lies in a CpG island and lacks TATA box and Initiator element. Transcription starts from multiple sites within a region of 75bp. Several DNA fragments tested for promoter activity were preferentially active in neural cells. Both positive and negative elements were identified, among which an intronic repressive element. Transient transfection studies performed in primary cultures from embryonic brain revealed a minimal promoter region of 87 bp, which is sufficient to confer specific transcriptional activity in primary neurons, but not in glial cells. Computer assisted search revealed multiple transcription factor binding sites, among them four consensus sites for Sp1. Mutational analysis showed that all four contribute to promoter activity while transactivation experiments demonstrated that Sp1 directly activates the BM88 promoter.

A4-048P**Latvian population and its Indo-European roots by analyses of mitochondrial DNA (mtDNA) and Y chromosome data**A. Krumina¹, L. Pliss², A. Puzuka¹, L. Timsa², V. Baumanis², K. Tambets³ and R. Villems³¹Riga Stradins University, Riga, Latvia, ²Biomedical Research and Study Centre, Riga, Latvia, ³Institute of Molecular and Cell Biology, Tartu, Estonia. E-mail: liana_@navigator.lv

A high-resolution phylogenetic analysis of Latvian population was undertaken in a comprehensive context, through use of maternally (mtDNA) and paternally (Y chromosome) inherited variation. mtDNA variation was investigated in a sample of 299 Latvians by analysing HVS-I and HVS-II regions and restriction polymorphisms in coding region, meanwhile Y chromosome analysis was performed in a sample of 140 males by analysing 10 biallelic markers. 113 mitochondrial haplotypes that belonged to 10 major haplogroups (H, HV, V, J, T, U, W, X, M, and I) were observed. However, haplogroup U4 that is particularly frequent in Volga-Uralic populations and Western Siberia is well present as well as diverse among Latvians (9.7%). A phylogeographic analysis of one of the predominant Latvian mtDNA haplogroups, H, covering almost half (44.5%) of the mtDNA variants, was undertaken by use of 12 mtDNA coding region markers. The vast majority of haplogroup H genomes belonged to H*, H1, and H5 that altogether encompassed 80% of haplogroup H haplotypes. The analysis of Y-chromosomal polymorphisms revealed the presence of two major (N3 and R1a, ~80%) and five minor haplogroups (K*, I, R1b3, E3b, and G). The high frequency of haplogroup R1a (40.3%) was also observed among Eastern and Northern European populations. In contrast, the high frequency of haplogroup N3 (39.7%) with its considerable diversity has been found among Finno-Ugric-speaking populations (20–50%). Interestingly, the proportion of this variant of Y chromosome dropped drastically in the geographic neighbours of Latvians and Lithuanians – among Poles (2%), Russians (11%) and in Northern Scandinavia (8%). Maternally inherited genes showed evidence of genetic homogeneity between Latvians and other neighbouring Indo-European populations, however, nearly half of their Y chromosomes (haplogroup N3) shared with most Finno-Ugric and Siberian populations.

A4-049P**Gene expression in septic patients manifest significant diagnostic signatures despite strong center-associated effects**M. Prucha¹, E. Moller², P. Deigner², S. Russwurm², V. Matoska¹, R. Zazula³, I. Herold⁴ and L. Sedlackova¹¹Clinical Biochemistry, Haematology and Immunology, Hospital Na Homolce, Prague, Czech Republic, ²SIRS-Lab GmbH, Jena, Germany, ³Department of Anaesthesiology and Intensive Care, Thomayer's hospital, Prague, Czech Republic, ⁴Department of Anaesthesiology and Intensive Care, Klaudivia's Hospital, Mlada Boleslav, Czech Republic. E-mail: miroslav.prucha@homolka.cz

Sepsis represents the systemic response to the infection. Improvements of early diagnosis, the differentiation between infectious and non-infectious origins of systemic inflammatory response, the inclusion of surrogate markers for the systemic inflammatory response, are all the targets of many studies. In a previous study we were able to identify that robust gene activity pattern correlated to the severity of sepsis. Therefore, the aim of the present study was to evaluate whether (i) center-dependent effects on the gene expression pattern exist, and (ii) whether significant diagnostic gene expression profile can be identified.

Materials and methods: 50 patients were randomized and enrolled from three Czech and one German hospital. The ACCP/SCCM consensus conference definition was applied to predict the severity of sepsis in ICU patients. As controls we used post spinal and bypass surgery patients without signs of inflammation. Gene expression analysis using a specialized medium-density microarray (5500 inflammation relevant genes, SIRS-Lab Jena, Germany) was made. Differentially expressed gene activities were selected with a mean expression change outside the thresholds and with a corresponding p-value of <5% for at least one comparison.

Results: Overall, 1236 of 5226 assessed gene activities showed strong center-dependent effects. However, 131 gene activities (2.5%) were differentially expressed in patients with sepsis as compared to the control group.

Conclusions: The present data indicates that microarray technology is suitable for systematically identifying those genes that underly the attenuated inflammatory response in sepsis. Gene expression profiles were able to distinguish between infectious and non-infectious systemic inflammatory response, despite a magnitude of center-associated effects. Therefore, the gene expression pattern was robust and has a potential impact for future diagnosis and treatment of sepsis.

A4-050P**The association between lymph node metastases and class I and class II antigens in gastric cancers**I. Pirim¹, B. Aydinli² and Y. N. Sahin³¹Department of Medical Biology, Ataturk University, Erzurum, Turkey, ²Department of General Surgery, Ataturk University, Erzurum, Turkey, ³Department of Biochemistry, Ataturk University, Erzurum, Turkey. E-mail: ibrahim.pirim@gmail.com

Gastric cancer is common malignancy throughout the world and is one of the leading causes of death in eastern region of Turkey. In the clinical setting, it is not easy to predict whether specific anticancer therapy will eradicate a tumor, and investigators have long hoped to identify useful predictors. Our result and some previous works hypothesized that a putative HLA antigens correlated with a low risk of lymph node metastasis could also be correlated with the response to cancer therapy. In the study we investigated relation between lymph node metastases and HLA association in gastric cancer. The PCR-SSP assay was used to examine Class I and Class II HLA antigens in 39 patients who underwent resection of gastric cancer. The incidence of HLA antigens in patients with or without lymph node metastasis was analyzed. HLA-A2, DR1 was significant associated with low risk of lymph metastases. It has, however, been found out that A24, B51 and DR4 antigens were associated with an increased risk of lymph node metastasis.

A4-051P**Genetic polymorphism of factor IX gene in Greek patients with thrombophilia**M. S. Iskas¹, R. M. Papi¹, P. E. Makris² and D. A. Kyriakidis¹¹Laboratory of Biochemistry, Department of Chemistry, Aristotle University of Thessaloniki, Thessaloniki, Greece, ²Haemostasis and Thrombosis Unit, AHEPA University Hospital, Thessaloniki, Greece. E-mail: kyr@chem.auth.gr

Factor IX (FIX) is a vitamin K-dependent plasma protein that plays an important role in both the intrinsic and extrinsic

coagulation pathway and in the process of hemostasis. It is synthesized by hepatocytes as a precursor protein of 461 amino acids. After several post-translational modifications the mature protein is activated by two enzymes, factor XIa (a serine protease), or by the tissue factor FVIIa complex. The activated factor IX forms a complex with calcium ions, membrane phospholipids and coagulation factor VIII to activate coagulation factor X. A defect or deficiency of FIX results in the X-linked bleeding disorder hemophilia B (Christmas disease). Recent studies shown that the activated FIX plays a key role in thrombin generation in the vicinity of platelets and that FIXa is the thrombogenic trigger after infusion of prothrombin complex concentrates. The exact locus of coagulation FIX was found to exist in the Xq26-q27 region of the X chromosome. The FIX gene spans 34 kb and contains eight exons. Over 300 different mutations have been identified in the FIX gene, all of them result in hemophilia B. Mutations in FIX gene could also be responsible for an increase in its coagulation activity, thus being the cause of thrombophilia syndromes. Genotyping of the FIX gene in Greek patients from unrelated families, with thrombophilia, was carried out for the first time. A total of 108 individuals were involved in this study. Each DNA sample was amplified by PCR with specific labelled primers for factor IX gene exons that have been described previously in the literature. PCR products were analyzed with LI-COR DNA sequencer and those that showed different pattern from the control (a non-thrombophilic individual) were finally sequenced. A correlation of the genotype and thrombophilia will be presented.

A4-052P

Improved molecular diagnosis of dystrophin gene mutations: Comparison of multiplex PCR-, cDNA probe- and multiplex ligation-dependent probe amplification methods in Hungarian DMD/BMD families

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Duchenne and Becker muscular dystrophy (DMD/BMD) are common X-chromosomal recessive disorders caused by mutations in the dystrophin gene. The majority (2/3) of recognized mutations are copy number changes in one of the 79 individual exons. Here we report the results of dystrophin gene analysis of Hungarian DMD/BMD patients performed by different molecular diagnostic techniques. Detection of deletion patterns in 103 male patients was first performed by multiplex PCR technique that enables simultaneous screening of 18 exons of the dystrophin gene. In 59 cases deletions were detected in the most commonly affected exons. Identification of female carriers was carried out by radioactive Southern blot hybridization using several cDNA probes. Out of the 32 female family members, where deletions were detected in the index patients, 17 proved to be carriers of deletions of the DMD/BMD gene. Recently, we introduced the newly developed multiplex ligation-dependent probe amplification (MLPA) assay. Until now, 10 male patients have been analyzed and the method developed for the agarose gel detection confirmed all previously recognized mutations and identified additional ones in four patients. Moreover, in one patient without any previous deletions, rare exon deletions were detected. To improve the MLPA assay, we also introduced quantitative fluorescent detection by a capillary electrophoresis system that will

allow us to detect additional duplications and to recognize female carriers. According to our preliminary data, the use of MLPA analysis will facilitate and improve the diagnosis of DMD/BMD patients in Hungary as the mutation pick-up rate will be increased significantly.

A4-053P

Amplification of the EGFR gene and the 7q31 locus are associated with metastasis formation of malignant melanomas

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There is a growing number of evidence demonstrating that alterations of different oncogenes play important role during the progression of malignant melanoma. By CGH others and we found frequent gains on chromosome 7p12 and high level amplification on 7q mainly covering the 7q31-qter region. In an effort to describe the copy number distribution pattern of chromosome 7, EGFR and 7q31 loci, we used interphase FISH on melanoma imprint preparations. In the present study 47 primary tumors were analyzed for EGFR and 8 for both loci by FISH. Based on disease progression tumors were grouped into two subgroups; 18 primary lesions did not developed metastases within 5 years after the surgery of the primary tumor, whereas 29 had metastases during the follow up period. Aneusomy for chromosome 7 was present in both subgroups, however the frequency of polysomy was significantly higher in tumors with metastatic behavior ($P = 0.05$). EGFR alteration was seen in both subgroups. Gain of EGFR signals in relation to chromosome 7, which is the measure of relative gene amplification, were seen in 33% of tumors without and 48% of tumors with metastases. Deletion of the EGFR gene was also observed in six samples, however subpopulation of cells with amplification was also noted in four of these cases. Amplification level of the 7q31 region was much higher (sometimes 50–60 copies/cell) compared to the 7p12 locus (4–10 copies/cell). High level amplification of 7q31 was associated with EGFR gene amplification in three primary tumors, all of these formed metastases within 1 year. Based on these FISH results we assume that chromosome 7 aneusomy, simultaneous amplification of the EGFR and the 7q31 loci are associated with metastases formation of malignant melanoma. Quantitative analysis of these loci by FISH may improve prognostic assessments in malignant melanoma, because it allows detection of highly amplified malignant cell subpopulation on a cell-by-cell basis.

A4-54P

Novel functional ability of MDM2 confirms p53 independent mechanisms

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The human homologue of a gene that was originally detected in murine double minute chromosome is a cellular protooncogene called *mdm2*. This gene is often amplified or overexpressed leading to abnormal levels of MDM2 oncogenic protein expression in human cancers. Though MDM2 was initially identified as a potent negative regulator of p53, a tumor suppressor protein,

recent reports suggest that it may regulate normal and abnormal (neoplastic) cell growth through p53-independent mechanisms as well. Therefore, amplification and/or overexpression of *mdm2* oncogene are widely regarded as an alternate mechanism responsible for tumor growth initiation irrespective of the *p53* gene status. Furthermore, aggressive tumors need to induce formation of new blood vessels, a process widely known as tumor angiogenesis, that permits rapid tumor growth and metastasis. The angiogenic process that is necessary for cancer metastasis involves several growth factors, among which the vascular endothelial growth factor (VEGF) is shown to have greater influence. We investigated the expression of MDM2 and VEGF in breast (GI-101A and MCF-7), leukemic (HL-60), ovarian (A2780/CP70) and prostate (LNCaP) cancer cell lines. Interestingly, we have detected the coexpression of MDM2 and VEGF in all the above listed cancer cell lines. Additionally, we investigated the effects of MDM2 inhibitors such as chalcone and an antisense oligonucleotide (AS5) on MDM2 and VEGF expressions. Our results indicate that the aforementioned inhibitors are able to significantly decrease the expression of both MDM2 and VEGF in p53 intact as well as p53 null cells. Furthermore, comparison of VEGF expressions in *mdm2* gene transfected and non-transfected cell lines (LNCaP) clearly reveals that MDM2 may have a direct role in regulating VEGF expression at the transcription level in cancer cells. In conclusion, our results confirm that MDM2 has p53 independent mechanism that may directly impact cancer growth and metastasis. The actual pathway linking MDM2 and VEGF remains to be elucidated.

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A4-055P

Malaria in pregnancy: Serum enzyme and electrolyte levels in pregnant malarial patients in Lagos, Nigeria.

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A total of fifty pregnant women were recruited for this study. Twenty-seven were found to be positive and show symptoms of malaria while twenty-three were negative and do not show any symptom of malaria. The results of this study show an association between the levels of Serum Glutamate Oxaloacetate Transaminase (SGOT), Serum Glutamate Pyruvate Transaminase (SGPT) and Alkaline Phosphatase (ALP) and malaria in pregnancy. The mean concentrations of SGOT, SGPT and ALP were found to be 11.03 ± 4.22 , 11.33 ± 2.37 and 29.0 ± 8.60 U/L respectively in pregnant malarial patients (PMP) while in the pregnant patients without malaria (PPWM) it was found to be 10.17 ± 1.52 , 8.40 ± 1.47 and 19.90 ± 7.52 U/L respectively. The levels of sodium (Na^+), potassium (K^+) and bicarbonate (HCO_3^-) in PMP were found to be 128.59 ± 5.96 , 3.04 ± 0.60 and 19.93 ± 2.75 mmol/L respectively while in the control (PPWM) it was observed to be 127.76 ± 6.56 , 2.82 ± 0.43 and 18.70 ± 1.66 mmol/L respectively. There was a significant difference in the mean levels of ALP and SGPT in the two groups at $P < 0.01$ while there was no significant difference in the mean levels of serum electrolyte (Na^+ , K^+ and HCO_3^-) between the two groups at $P < 0.01$. The results also show significant difference in the mean level of the enzymes at different trimester of pregnancy in PMP.

A4-056P

Activin – growth suppressor of ovarian cancer

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While activin, a member of the TGF beta superfamily of ligands, is an important regulator of normal reproductive function and have been implicated in the development of ovarian cancer, its exact role in this disease is poorly defined. We are, therefore, investigating the underlying molecular mechanisms by which it contributes to the pathogenesis of ovarian cancer. Of 16 ovarian cell lines treated with activin, 10 are unresponsive, with the remaining 6 cell lines either moderately ($n = 4$) or strongly ($n = 2$) growth inhibited by activin, a finding that is contrary to the published literature. Activation of the intracellular SMAD2/3/4 pathway occurs in one of the cell lines strongly growth inhibited by activin. This leads to the transcriptional induction of the cyclin dependent kinase inhibitor *p15 (INK4B)*, and is probably involved in the suppression of *MYC* mRNA levels. These events most likely account for the growth suppression seen in this line, by arresting the cells in the early G1 phase of the cell cycle. By microarray analysis, we have found a number of other genes important in cell proliferations that are down regulated by activin, such as the Inhibitors of Differentiation (*IDs*). A number of novel targets for activin mediated transcriptional regulation, such as *BMP7*, were also identified. This further raises the possibility that other signaling pathways may be modulated by activin. Through this work, we have demonstrated a growth suppressive effect of activin on ovarian cancer cells. This finding suggests a new role for activin as a negative regulator of ovarian cancer, and warrants further exploration of the role of this molecule in the development of the disease *in vivo*.

A4-057P

Inhibition of PTHrP expression by BMP-2 and its implication in osteoblast differentiation

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Bone morphogenetic proteins (BMPs) constitute a family of multi-functional growth and differentiation factors related to transforming growth factor-beta. They are potent inhibitors of myoblast differentiation and inducers of osteoblast differentiation, both *in vivo* and *in vitro*. In this study we have identified the parathyroid hormone-related peptide (PTHrP) as a target gene regulated by BMP-2. PTHrP was originally described as a tumor-derived agent responsible of hypercalcemia in patients with malignancy. Here we show a reduction in the expression of PTHrP mRNA after 2 hours of BMP-2 addition to the cell culture. Assays with the PTHrP mouse promoter using luciferase as reporter gene, and the degradation of PTHrP mRNA observed in presence of BMP-2 and actinomycin D reveals that this effect of BMP-2 occurs at the transcriptional level. As PTHrP is an important regulator of several processes in bone such as chondrocyte differentiation, osteoclast activation and bone resorption we suggest a possible role of this peptide in the transdifferentiation of myoblast cells into the osteoblastic phenotype promoted by BMP-2. We have analyzed different osteoblast markers in C2C12 cells stimulated with BMP-2 when adding exogenous PTHrP (1–34) to the culture. The induction of the alkaline phosphatase activity was reduced by the

addition of PTHrP (1–34) as well as the expression of osteocalcin mRNA. This data suggest that BMP-2 decreases PTHrP expression as a permissive mechanism of osteoblast differentiation.

A4-058P

Evaluating the severity of Turkish Hex A mutation for the new therapeutic approaches

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Deficiencies in heterodimeric Hexosaminidase A (alpha/beta) leads to a severe inborn error of metabolism known as Tay-Sachs disease. GM2 ganglioside accumulates in the lysosome of the neuronal cell and a wide spectrum in clinical severity of neurodegeneration appears. It has been estimated that as little as 10% of normal Hexosaminidase A activity is necessary to prevent GM2 storage. Two pharmacologically based therapeutic strategies have been proposed for mutations that allow some residual Hex A to be produced; substrate deprivation and pharmacological chaperones. Estimating the severity of the mutation's effect on the translated protein's ability to fold and exit the ER is thus important in identifying candidates for these emerging therapeutic approaches. One approach to assessing the degree to which a mutation has interfered with the folding of the alpha-subunit is to express the mutation in the aligned position of the beta-subunit. Therefore, the severity of the most common Tay-Sachs mutation in the Turkish population, which causes four amino acids deletion (Try366-Gly369) in the protein was analyzed by reproducing it in beta-cDNA and expressed the mutant beta-subunit in CHO cells. Western Blot analysis of permanently transfected CHO cells expressing the mutant detected only the pro-form of the beta-subunit coupled with the total lack of detectable Hex B activity. The absence of mature beta protein is a good measurement of stability as intracellularly the pro-beta chain is only found in the ER. These data indicate that the beta-deletion mutant, like the alpha-subunit mutant, is probably misfolded and retained in the ER and does not undergo lysosomal processing. Thus this is a severe mutation that is not likely to be treated by the emerging chemical chaperone and/or substrate depletion therapies.

A4-059P

Molecular evolution of genes from the POLR2J family on human chromosome 7

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Four independent genes encoding different variants of the hRPB11 subunit of Homo sapiens RNA polymerase II were revealed in human chromosome 7. Three genes (POLR2J1, POLR2J2, and POLR2J3) form a cluster of total length of 215Kb in the genetic locus 7q22.1 on the long arm of chromosome 7 (contig NT_007933). The fourth gene (POLR2J4, 31 Kb) was localized in the cytogenetic locus 7p13 of the short arm of chromosome 7 (contig NT_007819). The genes contain from 4 to 16 exons, most of them were not previously described. An analysis enabled us to refine dissimilar experimental data on the mapping of the hRPB11 subunit gene on chromosome 7. In particular, the presence of three sites of its localization according to data on hybridization with fluorescent-labeled probes (the FISH method) was explained. It was established that, upon the expression of the four human POLR2J genes, at least 17 types of mature mRNAs encoding somewhat differing hRPB11 isoforms can be synthesized. Fourteen of these mRNAs were revealed (as

full-length copies or clearly identifiable fragments) in the available databases of expressed sequence tags and cDNAs. The most probable scheme of origination of the multiple genes of the POLR2J family as a result of three consecutive segmented duplications increasing in size was proposed and substantiated. On the basis of the scheme, some assumptions on the pathways of evolution of separate human genes and the mechanisms of generation of protein diversity in higher eukaryotes were made.

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A4-060P

Evaluating the severity of Turkish Hex A mutation for the new therapeutic approaches

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Deficiencies in heterodimeric Hexosaminidase A (alpha/beta) lead to a severe inborn error of metabolism known as Tay-Sachs disease. GM2 ganglioside accumulates in the lysosome of the neuronal cell and a wide spectrum in clinical severity of neurodegeneration appears. It has been estimated that as little as 10% of normal Hexosaminidase A activity is necessary to prevent GM2 storage. Two pharmacologically based therapeutic strategies have been proposed for mutations that allow some residual Hex A to be produced, substrate deprivation and pharmacological chaperones. Estimating the severity of the mutation's effect on the translated protein's ability to fold and exit the ER is thus important in identifying candidates for these emerging therapeutic approaches. One approach to assessing the degree to which a mutation has interfered with the folding of the alpha-subunit is to express the mutation in the aligned position of the beta-subunit. Therefore, the severity of the most common Tay-Sachs mutation in the Turkish population, which causes four amino acids deletion (Tyr366-Gly369) in the protein was analyzed by reproducing it in beta-cDNA and expressed the mutant beta-subunit in CHO cells. Western Blot analysis of permanently transfected CHO cells expressing the mutant detected only the pro-form of the beta-subunit coupled with the total lack of detectable Hex B activity. The absence of mature beta protein is a good measurement of stability as intracellularly the pro beta-chain is only found in the ER. These data indicate that the beta-deletion mutant, like the alpha-subunit mutant, is probably misfolded and retained in the ER and does not undergo lysosomal processing. Thus this is a severe mutation that is not likely to be treated by the emerging chemical chaperone and/or substrate depletion therapies.

A4-061P

Activity, but not expression of OGG1 glycosylase is decreased in tumors of lung cancer patients

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Tobacco smoking, the main cause of lung cancer, induces chronic lung inflammation and oxidative stress. Using semi quantitative

RT-PCR, we investigated the mRNA level of genes involved in the repair of oxidative DNA damage, *hOGG1*, eliminating from DNA 8-oxoguanine (8-oxoG), and AP – endonuclease (*hAPE*), incising DNA at AP-sites and increasing OGG1 turnover on damaged DNA. We also measured OGG1 activity by the nicking assay. A positive correlation between OGG1 repair activity and its expression in tumor and non-affected lung tissue was observed. However, while the repair activity of 8-oxoG was lower in tumor than in normal surrounding, *hOGG1* mRNA level was only slightly decreased in tumor, and this difference was statistically non-significant. The loss of heterozygosity (LOH) in *hOGG1* locus in tumor, was found in only 3 out of 34 patients tumor tissues. *hAPE* mRNA level showed a tendency to increase in tumor. So other factors than LOH in *hOGG1* locus and lack of cooperation between OGG1 and APE are responsible for the decreased OGG1 activity in tumor. We also searched for polymorphisms and/or mutations of the *hOGG1* gene by the multi-temperature PCR-single strand conformation polymorphism (MSSCP) analysis. *hOGG1 Ser326Cys* variant was found in 7 out of 34 patients. *hOGG1Cys/Cys326* genotype was associated with a reduced enzyme activity in normal and tumor lung. In one case, a new genetic change was found in exon 1, resulting in Arg46Glu, and this lowered the enzyme activity, probably due to disrupting of hydrogen bonds with His179, which could affect 8-oxoG binding.

A4-062P

Construction of differentially expressed cDNA library in human gastric cancer treated by diallyl disulfide

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Aim: To construct subtracted cDNA library of differentially expressed genes in human gastric cancer MGC803 cell line treated by diallyl disulfide (DADS).

Methods: Differentially expressed cDNA species induced by DADS in human gastric cancer MGC803 cell line was determined through suppression subtractive hybridization (SSH). Then these cDNA species were directly inserted into T/A cloning vector to set up the subtractive library. Amplification of the library was carried out with transformation of JM109. One hundred positive clones were randomly picked and identified by PCR.

Result: all clones contained 100–600 bp inserts. A subtracted cDNA library of differentially expressed genes in human gastric cancer MGC803 cell line treated by DADS was constructed successfully with SSH and T/A cloning techniques.

Conclusion: The library was efficient and lays solid foundation for screening and cloning new and specific tumor associated genes of human gastric cancer, and provide a new idea for further exploring the mechanism of DADS effecting on cancer cell.

A4-063P

Intracellular measurement of tripeptidyl peptidase I activity with a fluorogenic substrate

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The neuronal ceroid lipofuscinoses (NCL) are lysosomal diseases characterized by progressive neurodegeneration resulting

in loss of vision, seizures, mental regression, behavioral changes, movement disorders and premature death. One of the most frequent of these diseases is classical late infantile NCL caused by mutations in the CLN2 gene and is related to defects of lysosomal tripeptidyl peptidase I (TPP-I). We have developed a new fluorogenic substrate to measure the *in vivo* activity of TPP-I in various cell lines. This substrate is a membrane permeable non-fluorescent bisamide derivate of rhodamine 110. After cleavage of the tripeptides the released rhodamine 110 becomes fluorescent and can be measured by fluorescent flow cytometry or can be visualized by fluorescent microscopy. K562 cells overexpressing TPP-I showed more than 10-fold higher activity than K562 wild type cells and were less sensitive towards a TPP-I specific peptide inhibitor. Colocalization of the rhodamine signal with the lysosomal marker lamp-1 revealed the specific lysosomal activity of TPP-I. In primary mouse neurons TPP-I activity was present in lysosomal as well in other compartments. This newly designed substrate might be a valuable tool to study the neurodegenerative pathomechanism underlying classical late infantile NCL.

A4-064P

Proteoglycan expression in cancerous human laryngeal cartilage

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Introduction: Larynx is one of the most important structures in the upper aerodigestive tract. Its skeleton is comprised from four kinds of cartilage. It was recently showed that the main component of extracellular matrix of healthy human laryngeal cartilage is the large hyaluronan-binding proteoglycan (PG) aggrecan. Several other PGs like versican, decorin, and biglycan were also identified. In cancerous human laryngeal cartilage alterations in the concentration and structure of PGs were observed. The amounts of all the above referred PGS, aggrecan, versican and decorin, were substantially decreased in cancerous samples in comparison to those of healthy. In the present study we examined whether these alterations were in metabolic or expression level.

Methods: RNA was extracted from healthy and cancerous samples and then was reverse transcribed for aggrecan, decorin and versican. Resulting RT-PCR products were analyzed by electrophoresis on 2% agarose gels.

Results and Discussion: A decrease in mRNA of aggrecan was observed in cancerous samples in comparison with the healthy ones, contrary to decorin mRNA which was increased in cancerous samples. On the other hand, versican mRNA was traced only in cancerous samples. The results suggested that the observed alterations of PGs in laryngeal cancer were due to both metabolic and expressional alteration.

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A4-065P**Comparative Cytochrome P450-2C, -2E1, -3A5 and -2A6 expression in oral tissue samples of the individuals with habitual tobacco smokers**D. Sarikaya¹, I. Chiba², C. Bilgen³, G. Altan⁴, P. Guneri⁵, M. Fujieda⁶, T. Kamataki⁶ and Z. Topcu⁷

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The development of oral squamous cell carcinoma (SCC) shows a positive correlation with the carcinogen exposure that occurs during tobacco and alcohol use. Metabolic activation of numerous exogenous and endogenous chemicals are catalyzed by cytochrome P 450 enzymes (CYPs). In this study an RT-PCR based expression profile using *CYP 1A1, 1A2, 2A6/7, 2B6, 2C, 2D6, 2E1, 3A3/4, 3A5, 3A7, 4B1* and *2A6*-specific primers were analysed in homogenized oral buccal tissue samples from seven individuals with habitual tobacco smoking, after their informed consents were obtained. An RNA competitor of known copy number covering the primer sequences necessary to amplify all the object *CYPs* within a single molecule was used as the reference. Our results demonstrated a consistent expression of mRNA for the *CYPs -2C, -2E1, -3A5* and *-2A6*. Based on our previous report on the reduced oral cancer risk in homozygote deletion genotype (Topcu et al, 2002), *CYP2A6* expression is of particular significance as this enzyme is characteristic of its catalytic properties to nitrosamines. Our results are discussed in quantitative ratios of the expressed CYP members in relation to xenobiotic activation in the etiology of squamous cell carcinomas.

Acknowledgements: This study was supported by the State Planning Organization of Turkey (Grant number 04 DPT 005), and was approved by the ethics committee of Ege University, Izmir, TR.

A4-066P**Prognostic significance of BCL2L12, a new member of the BCL2 family of apoptosis-related genes, in breast cancer**H. Thomadaki¹, A. Arnavanidis², M. Talieri³, D. Kletsas⁴ and A. Scorilas¹

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Programmed cell death and apoptosis are highly regulated physiology processes. We have recently cloned a new member of the BCL2 (Bcl-2) apoptosis-related genes, BCL2L12, which was found to be highly expressed in mammary gland (Scorilas et al., *Genomics* 2001; **72**: 217–221). In the present study, we explored

the research on the prognostic value of BCL2 and BCL2L12 together, as breast cancer biomarkers. Fifty five specimens from patients with, histologically confirmed, epithelial breast carcinoma were analyzed for BCL2 and BCL2L12 gene expression by RT-PCR. Actin was used as a control gene. Their gene expression profile was associated with clinicopathological parameters and survival analysis regarding to relapse and death were evaluated by constructing Kaplan-Meier curves and developing a Cox proportional hazard regression model. Overexpression of BCL2 gene was found in patients belonging to the age groups 55 years ($P = 0.027$), as well as in estrogen receptors (ER) positive and BCL2L12 overexpressed tumors. Our results indicate that BCL2L12 positive breast tumors are mainly of lower stage (I/II) ($P = 0.04$). In addition, BCL2 and BCL2L12-positive patients are almost 4 times less likely to relapse ($P = 0.022$ and $P = 0.014$) or die ($P = 0.021$ and $P = 0.031$) in comparison to BCL2 and BCL2L12-negative patients respectively. Multivariate analysis indicated that BCL2 and BCL2L12 could be used as independent biomarkers of favorable prognosis in breast cancer.

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A4-067P**A novel mechanism between type II diabetes mellitus and procalcitonin gene expression**

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Objective: Procalcitonin (PCT) was originally described in 1984 as a 116-aminoacid protein with a molecular weight of 14.5 kDa. The PCT gene, referred to as Calc-1, is located on chromosome 11p15.4 and was sequenced in 1989. The promoter has sites for basal transcription factors but more interestingly, also has sites for NF λ β (Nuclear factor λ β) and AP-1 (Activator protein-1), factors induced under inflammatory conditions. Type II diabetes mellitus is associated with oxidative stress and elevation of advanced glycation end products (AGEs). AGEs are produced by a non-enzymatic, Maillard reaction between reducing sugars and either proteins or lipids. AGE's interacts the receptor for advanced glycation end products (RAGE) and, RAGE activation is caused by elevation of transcriptional factors NF λ β and AP-1. These factors induce procalcitonin gene expression. The aim of this study was to determine whether or not procalcitonin is a specific marker in patients with type II DM.

Research design and methods: Eighteen type II DM patients were studied along with age and sex matched normal non-diabetic subjects (10 males and 8 females) Blood samples were taken for measurements of procalcitonin. Serum procalcitonin levels were analyzed with kryptor analyzer. Kryptor –PCT is a kit designed for kryptor automated immunofluorescent assay of procalcitonin in serum. Procalcitonin levels were elevated in type II diabetic patients when compared with normal non-diabetic subjects. There was a statistically significant difference in serum procalcitonin of type II diabetic patients vs. normal subjects ($P < 0.02$).

Conclusion: Our study revealed a raise in serum procalcitonin in type II diabetic patients. Type II diabetes associated with increased circulating concentrations of procalcitonin may be a mechanism which explains many of the clinical and biochemical features of type II diabetes and its complications.

A4-068P**Novel mutation of the CYP17 gene, enzyme activity and molecular modelling studies**

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The CYP17 gene, located on chromosome 10q24-q25, encodes the cytochrome P450c17 enzyme. Mutations of this gene cause the 17-alpha-hydroxylase/17,20-lyase deficiency. Approximately 40 different mutations of the CYP17 gene have been described. In this study we present the clinical history, hormonal findings, and mutational analysis of two patients from unrelated families, who were evaluated for hypertension, hypokalemia and sexual infantilism. Analysis of the CYP17 gene by polymerase chain reaction amplification and direct sequencing demonstrated a novel homozygous mutation of codon 440 from CGC (Arg) to TGC (Cys) in both patients. The effect of this novel mutation on 17-alpha-hydroxylase/17,20-lyase activity was assessed by *in vitro* studies on the mutant and wild-type P450c17. These studies showed that the mutant P450c17 protein was produced in transfected COS-1 cells, but it had negligible 17-alpha-hydroxylase and 17,20-lyase activities. In addition, three-dimensional computerized modeling of the heme-binding site of the P450c17 enzyme indicated that replacement of Arg by Cys at amino acid position 440 predicts a loss of the catalytic activity of the enzyme, as the mutant enzyme containing Cys440 fails to form a hydrogen bond with the propionate group of heme, which renders the mutant enzyme unable to stabilize the proper position of heme. Based on these findings we conclude that expressing the CYP17 gene with functional analysis, combined with three-dimensional computerized modeling of the heme-binding site of the protein provide feasible tools for molecular characterizing of functional consequences of the novel CYP17 mutation on enzyme function.

A4-069P**Determination of CYP2C9 and CYP2C19 genetic polymorphisms in Behcet's disease**

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Behcet's disease is a chronic inflammatory disorder of unknown aetiology characterized by chronic relapsing oral-genital ulcers and uveitis. Multiple systemic associations including articular, gastrointestinal, cardiopulmonary, neurologic and vascular involvement are also observed. CYP2C9 and CYP2C19 are xenobiotic-metabolizing enzymes that metabolize foreign compounds such as clinically used drugs, and other xenobiotics such as environmental chemicals. It is possible that environmental factors and/or genetic polymorphisms in xenobiotic-metabolizing enzymes may contribute to the development of Behcet's disease. The aim of the present study was to investigate whether association between Behcet's disease and genetic polymorphism of CYP2C9 and CYP2C19. 53 Behcet's disease patients and 107 healthy control subjects were enrolled in the study. Blood was collected in EDTA-containing tubes and DNA was extracted from the lymphocytes by high pure template preparation kit

(Roche diagnostics, GmbH, Mannheim, Germany). CYP2C9*2, CYP2C9*3, CYP2C19*2, CYP2C19*3 alleles were detected by using LightCycler- CYP2C9 and CYP2C19 mutation detection kits by real time PCR with LightCycler instrument. There is no significant statistical association between CYP2C9 gene polymorphisms and Behcet's disease. We investigate that CYP2C19*2 heterozygous genotype was an increased risk of developing Behcet's disease (OR: 2.763, % 95 CI: 1.29-5.88) in comparison with that of the control group. We couldn't find gene polymorphisms of CYP2C19*3 in patient group with Behcet's disease and control group. As a result of this study we conclude, CYP2C19*2 gene polymorphisms may be a determinant role in susceptibility to Behcet's disease.

A4-070P**Biological significance of antibodies specific to the HA2 gp, the conserved part of influenza A hemagglutinin**

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The most variable surface antigen of influenza A virus, hemagglutinin (HA), is composed from two glycopolypeptides, HA1 and HA2 gp. The HA2 gp is responsible for the fusion of virus and cell membranes during endocytosis. It represents the conserved part of the HA. In contrast to HA1-specific antibodies (Ab), which are mostly virus-neutralizing, the biological significance of HA2-specific Abs is unclear. Therefore we followed the effect of HA2-specific monoclonal antibodies (MAbs), that recognize 4 distinct antigenic sites, on the *in vivo* infection and on the *in vitro* replication of influenza virus. BALB/c mice were infected intranasally with 1LD50 of influenza virus after the intravenous administration of MAbs. Two of the four MAbs tested (CF2, FE1) improved survival of mice to 100% and one MAb (IIF4) to 87.5% in comparison to the control group in which 50% mice survived. Virus was eliminated from lungs on the 6th day in mice immunized with MAb CF2, while in the control group with irrelevant MAb, the titer only dropped below the detectable level on the 8th day post infection. The CF2 MAb, which was specific to the fusion peptide of HA2 and inhibited the fusion activity of hemagglutinin, reduced the virus replication *in vitro* by 40 % in plaque assay or by 20–40% in cell radioimmunoassay. The antibodies specific to the HA2 gp conferred the partial protection to infected mice and lowered the *in vitro* replication of virus. It can be supposed, that HA2-specific antibodies contribute to the more effective recovery from the influenza infection. As HA2-specific Abs are crossreactive within subtypes and even between subtypes their effect on the course of infection is of special significance in the case when the disease is caused by a new antigenic variant of influenza virus.

A4-071P**Endogenous detoxification as a basis for heterogeneity in phenotypic manifestations of metabolic diseases**

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Clinical phenotypes in inherited metabolic diseases vary from severe to clinically benign. This heterogeneity generally relates to

diversity of defects on the gene level. Our analysis of urinary organic acids and their conjugates with tandem mass-spectrometry indicate a benign phenotype might also be related to very effective detoxification of primary and secondary metabolites formed in metabolic diseases.

Results: The first case was an adult presenting with a chronic fatigue syndrome. Low levels of 3-methylcrotonyl-glycinuria and 3-methylcrotonylcarnitine indicated 3-methylcrotonyl-CoA deficiency with a mild clinical profile, probably due to sufficient detoxification. A unique second case was a child with mild clinical symptoms, but diagnosed as classical maple syrup urine disease (branched chain 2-keto acid dehydrogenase complex activity <1.0%), which mostly has severe clinical symptoms. Unique biochemical findings were (i) low urinary amino acids secretion (e.g. 160 mmol leucine/mol Cr, vs. 1350 - 2300 in other patients); (ii) low concentrations of branched chain 2-keto acids (e.g. trace amounts of 2-ketocaproic acids vs. 960 - 1480 mmol/mol Cr in other patients) and (iii) very high concentrations of amino acid conjugates (e.g. 484 mmol N-Ac-leucine vs. 2,5 - 20), indicating efficient detoxification.

Conclusions: We postulate that detoxification of endogenous substances in patients with metabolic diseases is important in understanding the phenotype of these diseases. Phenotypic variation apparently relates to at least three factors: (i) the primary enzyme defect, (ii) variability in detoxification of endogenous primary and secondary metabolites, and (iii) variability in the nature and concentration of formation of secondary metabolites.

A4-072P

Inhibition of calf purine-nucleoside phosphorylase by formycin B and its aglycone – is there a cooperativity?

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Interactions of calf Purine-Nucleoside Phosphorylase (PNP), an enzyme responsible for severe inherited immunological disorders, with inhibitors Formycin B and its aglycone [pyrazolo(4,3-d)pyrimidine-7-one] were studied by means of kinetic analysis and fluorescence titrations. Both compounds are close analogues of the natural substrates inosine and hypoxanthine. Formycin B is a weak and noncompetitive inhibitor of PNP in the phosphorolytic pathway, and its complexes with PNP are fluorescent, which allows independent estimation of the binding constant (~100 μM). The aglycone inhibits strongly the sythetic pathway

of the reaction catalyzed by PNP, but in the phosphorolytic pathway shows mixed behaviour and clear evidence of negative cooperativity (Hill coefficient <0.5). Its complexes with PNP are nonfluorescent, which may indicate different tautomeric form than that found in Formycin B/PNP complex. The above findings will be analyzed in terms of the reaction mechanism.

A4-073P

Evaluation of antitumour activity of some novel platinum(II) complexes in breast cancer and glioma cells

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Cisplatin is a potent anticancer drug for the treatment of testicular and other germ-cell tumors, but its clinical use is limited by the diminished activity against a number of cancers, the acquired resistance developed by many tumors and severe side effects. Therefore, the search continues for an improved platinum antitumor agent and in this search the clinical inactivity of cisplatin was considered up to recently a paradigm for the classical structure pharmacological activity relationships of platinum drugs. However, to this end several new analogues of cisplatin, which exhibit a different spectrum of cytostatic activity including activity in tumor cells resistant to cisplatin have been identified. The success of cisplatin in cancer chemotherapy is thought to originate from its ability to covalently bind DNA leading to important changes in the helical structure. During the last decades new Pt(II) complexes have been designed, however, few analogs with properties better than cisplatin We have recently synthesized novel platinum(II) coordination complexes containing a 1,2-bis(diphenylphosphino)-ethane (dppe) and 1,2-bis(diphenylphosphino)-methane (dppm) as ligands, [Pt(dppe)SA₂, Pt(dppm)SA₂, Pt(dppe)FU₂], in order to obtain an agent with more favorable therapeutic indices than cisplatin. In this study, the new platinum(II) complexes were tested for their cytotoxicity, by MTT assay, on various human cancer cell lines also including different cisplatin-resistant cells endowed with different mechanisms of resistance, MCF-7 (Human Breast Cancer) cells and C6 (Rat Glioma) cells. Cisplatin [cis-diamminedichloroplatinum(II)] and Pt(NH₃)₂ I₂ were used as reference agents. The *in vitro* antitumour activity of these novel Pt compounds were evaluated with IC₅₀ values determined using different concentration of drugs in different time intervals. The obtained IC₅₀ values were compared to the values of the clinically used cisplatin drug.