Yeast perfume factory: metabolic engineering of Saccharomyces cerevisiae for plant isoprenoid biosynthesis

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A1 – Ageing

A1.01

Abstract withdrawn

A1.02
BDNF Mediated Angiogenesis Potential is Decreased Associated with Aging

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The mechanism of age-related decrease of angiogenic potential in myocardium is still unclear. Cardiac microvascular endothelial cells (CMECs) play a key role in cardiac angiogenesis. In this study, using the CMECs which are isolated from young and old hearts, we found that the migration and proliferation capacity of old CMECs were diminished. BDNF was able to increase the migration and proliferation of CMECs no matter in young and old CMECs, however, the effects in old CMECs was less potent compared with the young CMECs. *In vivo* study showed that delivery of BDNF in young heart in ischemic situation was able to increase the vessel numbers in both infarct and border zone significantly, but not in young heart in non-ischemic situation. The microarray results showed that 84 genes were up-regulated, while 81 genes were down-regulated upon BDNF treatment. The functional annotations of genes are cell migration, blood vessel morphogenesis, angiogenesis, regulation of proliferation, cell cycle regulation, etc, which have been shown the strong potential effects in migration, proliferation and angiogenesis. The results of present study revealed that BDNF-TrkB pathway play an important role in angiogenesis of myocardium. Although CMECs express BDNF consistently, however, BDNF might not initiate the angiogenesis in heart individually *in vivo*. BDNF-mediated angiogenic potential might depend on the cross talk with focal micro-environment. Importantly, senescence of CMECs was able to impair the BDNF-mediated migration and proliferation capacity. It might contribute to age-related decrease of angiogenic potential in myocardium and poor regenerative capacity seen in aged heart.

A1.03
Memory enhancing effects of saffron in adult & aged mice are correlated with the antioxidant protection: *In vitro* and *in vivo* studies

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Oxidative stress is implicated in senescence and age-related pathologies, with memory deficits as the commonest manifestations. Herbal ingredients are sought to forestall/reverse those deficits as dietary components/or supplements. The effect on cognitive function of a 7-day, intraperitoneal administration of saffron was examined in healthy adult and aged mice by step through test. Results showed that saffron-treated mice exhibited significant improvement in learning and memory. Experiments in whole brain homogenates revealed that saffron administration resulted in significantly lower brain lipid peroxidation (malondialdehyde, 44–63%) and higher antioxidant parameters (glutathione, ascorbic acid, total antioxidant power). Salt- and detergent-soluble AChE activity was significantly decreased only in adult mice. Thus, the significant cognitive enhancement conferred by saffron administration in adult and aged mice, is closely related to the antioxidant reinforcement; AChE inhibition (in adult mice) plays also a minor role. Studying further the antioxidant potential, the effect(s) of saffron and crocetin (main crocin metabolite), were examined against H_2O_2-induced toxicity in SH-SY5Y and HEK293 cells. Cell viability and scavenging of free radicals after co-treatment with H_2O_2 (250–750 μM) and the tested compounds (1–250 μg/ml saffron, 1–125 μM crocetin) were determined with MTT and DCF assays. Results showed that saffron and crocetin provide strong protection in rescuing cell viability and repressing ROS production in the SH-SY5Y cells; moderate effects in HEK293 cells. Considering, thus, earlier metabolic studies, crocetin appears to be responsible for the *in vivo* effects.

A1.04
Protective effects of triphlorethol-A against formaldehyde-induced oxidative damage and apoptosis: role of mitochondria-mediated caspase-dependent pathway

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The toxicity of formaldehyde (HCHO) has been attributed to its ability to form adducts with DNA and proteins. Triphlorethol-A, derived from Ecklonia cava, was reported to exert a cytoprotective effect against oxidative stress damage via an antioxidant mechanism. The aim of this study was to examine the mechanisms underlying triphlorethol-A ability to protect Chinese hamster lung fibroblast (V79-4) cells against HCHO-induced damage. Triphlorethol-A significantly decreased the HCHO-induced intracellular reactive oxygen species (ROS) production. Triphlorethol-A prevented increased cell damage induced by HCHO via inhibition of mitochondria-mediated caspase-dependent apoptosis pathway. Triphlorethol-A diminished HCHO-induced mitochondrial dysfunction including loss of mitochondrial membrane action potential (Ψ) and adenosine triphosphate (ATP) depletion. Furthermore, the anti-apoptotic effect of triphlorethol-A was exerted through inhibition of c-Jun NH2-terminal kinase (JNK) which was enhanced by HCHO. Our data indicate that triphlorethol-A exerts a cytoprotective effect in V79-4 cells against HCHO-induced oxidative stress by inhibiting the mitochondria-mediated caspase-dependent apoptotic pathway.

A1.05
Antioxidant effect of Jeju water containing vanadium component

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The aim of this study was to examine the antioxidant effect of Jeju water containing vanadium component (20–25 ppb). Cells were incubated for 10 passages in media containing deionized distilled water (DW group) and Jeju water (JW group). DW and JW groups did not show to scavenge 1,1-diphenyl-2-picrylhydrazyl radical. Electron spin resonance spectrometer data showed that JW group significantly scavenged superoxide radicals induced by Fenton reaction ($H_2O_2 + FeSO_4$), and hydroxyl radicals induced by xanthine/xanthin oxidase system as compared to DW group. Furthermore, JW group significantly scavenged intracellular reactive oxygen species in human Chang liver cells as compared to DW group, which are measured by using fluorospectrometer, flow cytometer, and confocal microscope after staining 2',7'-dichlorodihydrofluorescein diacetate. These results suggest that Jeju water containing vanadium component showed antioxidant effect via scavenging radicals.

A1.06
Effect of aging and oxidative stress on elongation factor-2 in hypothalamus and hypophysis

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The hypothalamic-hypophysis system (HHS) is a major part of the neuroendocrine system. The output of this unit regulates several body functions. One common feature of hormones secreted by this system is that they are peptides whose size range from 9–56 amino acids. As the organisms age, a considerable diminution of the protein synthesis takes place in several tissues. Among the possible causes of the decline of translation in old animals are the modifications of elongation factor-2 (eEF-2). We studied whether the level of this protein was affected in the HHS in old animals. The effects of aging are compared to those of an oxidant compound (cumene hydroperoxide) administered to young rats. To test this, eEF-2 levels, adduct formation with both malondialdehyde (MDA) and 4-hydroxynonenal (HNE), and two oxidative stress markers were compared in old rats versus young rats treated with cumene hydroperoxide (CH), a compound that has been used in experimental models to induce lipid peroxidation. The results indicate that oxidative stress could be involved in the alterations of eEF-2, which forms adducts with MDA and HNE. The alterations of eEF-2 levels, secondary to lipid peroxidation and adduct formation with these aldehydes could contribute to the suboptimal hormone production from these tissues during aging. Besides eEF-2, proteomic analysis shows that several other proteins are affected.

A1.07
Analysis of ageing and stress resistance in natural clones

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In organisms that propagate by agametic cloning, the parental body is the reproductive unit and fitness increases with size of the colony, why such metazoans have despite lack of experimental data been considered potentially immortal. However, most clonal organisms derive evolutionary from sexually reproducing ancestors, why they may have inherited ageing. By analyzing asexual propagation rate as a measure of fitness or performance, and telomerase activity and telomere length as molecular senescence markers, in old asexual strains of a colonial ascidian and in their recent sexual progenies, we have for the first time investigated the possibility of long term molecular senescence in lineages of an asexual metazoan. The results present a novel explanation to the unsolved problem why sexual reproduction despite its costs persists relative to asexuality, and why asexual metazoans commonly undergo occasional cycles of sexual reproduction in the wild. The possibility of non-ageing was also investigated in a clonal starfish. Here comparative analyses of whole animal performance, telomere dynamics and antioxidant defense were analyzed in clonal versus sexually reproducing populations of the same starfish species. We emphasize the importance of natural clones as novel model systems for longevity research given that their solutions have undergone natural selection. Evolutionary and mechanistic ideas of how longevity may be achieved in clonal species will be presented.

A1.08

Abstract withdrawn

A1.09
Mathematical models of damaged and aggregated proteins in yeast *Saccharomyces cerevisiae*

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Nascent proteins have to be properly folded to become functionally active while unwanted and damaged proteins are continually degraded back to amino acids. These processes are precisely regulated to ensure proper balance among different proteins. However, several conditions, such as age, mutations and oxidative stress can impair such phenomena leading to protein damage and misfolding. Misfolded proteins are prone to form accumulation with other molecules in the cell which can trigger apoptosis and contribute to various neurodegenerative diseases such as Alzheimer's (AD), Parkinson's (PD) and Huntington's (HD). This study concerns about the effects of damaged and aggregated proteins on specific phenotypes of yeast including lifespan, cell size, generation time, carbonylation level and system robustness. Mathematical models are developed to simulate those phenotypes at different levels of damaged and aggregated proteins. The models suggest that the increase of aggregates has a toxic effect on

the cells and can cause replicative senescence especially in the mother cells where the agedness and retention of old and damaged materials are the main concern. The importance of protein segregation is also demonstrated to be a beneficial mechanism to decrease clonal senescence.

A1.10
Down-regulation of protein kinase CKII induces the p53-p21Cip1/WAF1 pathway-dependent senescence in human colon cancer cells

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Protein kinase CKII plays a critical role in cell growth and proliferation. The expression level of CKII is greatly enhanced in a variety of tumor or leukemic cells. We have previously shown that the down-regulation of protein kinase CKII activity is tightly associated with cellular senescence of human fibroblast IMR-90 cells. Here, we examined the roles of p53 and p21Cip1/WAF1 in senescence development induced by CKII inhibition using wild-type, isogenic $p53^{-/-}$ and isogenic $p21^{-/-}$ HCT116 human colon cancer cell lines. A senescent marker appeared after staining for senescence-associated β-galactosidase activity in wild-type HCT116 cells treated with CKII inhibitor or CKIIα siRNA, but this response was almost abolished in p53- or p21Cip1/WAF1-null cells. Increased cellular levels of p53 and p21Cip1/WAF1 protein occurred with the inhibition of CKII. CKII inhibition upregulated p53 and p21Cip1/WAF1 expression at post-transcriptional level and transcription level, respectively. Rb phosphorylation significantly decreased in cells treated with CKII inhibitor. Taken together, this study shows that the activation of the p53-p21Cip1/WAF1-Rb pathway acts as a major mediator of cellular senescence induced by CKII inhibition.

A1.11

Abstract withdrawn

A1.12
Differences in ageing and stress resistance in clonal relative to sexual populations of the fissiparous starfish Coscinasterias muricata

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In organisms that propagate by agametic cloning the parental body is the reproductive unit, why such species have despite experimental evidence been considered potentially immortal due to presumed relocation of energy investment into body maintenance, rather than into gonad production. We have used the starfish Coscinasterias muricata, which can reproduce either fissiparous or sexually, to analyse if clonal animals are more stress-resistant than their sexually reproducing counterparts. To use C. muricata as a study organism is of high relevance, since the species naturally use both reproduction strategies, the starfish is easily maintained in the lab and their size makes the sampling simple. We have studied the animals on whole animal level, cellular and protein level. Since telomere length has previously been related to health and fitness in a variety of species, analysis of the relative telomere length is of high interest. To complement the telomere study, we have also studied differences in telomerase activity between these two groups. To verify if these two groups differ in ability to respond to stressors we have analysed different parameters of oxidative stress and used a robustness assay to measure their sustainability to physical exhaustion. In conclusion, we present experimental evidence for increased stress resistance in a clonal species. The results support the theoretical assumption long telomeres may be a potential mechanism for this.

A1.13

Abstract withdrawn

A1.14
Aging and oxidative stress in two populations of Atlantic cod fish: Effects of commercial fishing

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Sexual reproduction and ageing are closely related and regarded as opposite regulators of each other due to the costs of reproduction. Gender also plays a role in aging. We have addressed these relationships in wild cod fish populations where extensive fishing has caused genetic shifts in populations, resulting in sexual maturation at younger ages and smaller sizes. We have measured a number of oxidative stress parameters known to vary with age, in both adult fish tissue as well in eggs. Samples were analysed from genetically similar cod populations collected at two sites, Kattegat, a trawled region, and Öresund, a protected area. Fish ranged in age from 2 to 8 years. Our results indicate that male cod have significantly higher catalase activities in liver tissue than females, and that neither sex displays changes in CAT with age. Decreases in glutathione content (total and oxidized) correlates strongly with aging in males from both sites, but not females. GSH is also not affected in eggs. Fish from Kattegat had significantly lower levels of GSSG and CAT activity, indicating lower oxidative stress in these fish with early maturation. Protein carbonyls and lipid peroxides in liver tissue do not correlate with age, nor do these variables differ between genders. We do see a trend towards increasing protein carbonyls in eggs with female age ($p = 0, 083$), indicating a possible negative maternal affect with age. In conclusion, we observed gender differences in oxidative stress and potential negative maternal effects with age in wild Atlantic cod, and differences in oxidative stress between populations.

A1.15
Genetic association study between length of telomeres and healthy aging

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Inroduction: Telomeres are repetitive DNA sequences at the ends of linear chromosomes that consist of 5–15 kb pairs of multiple copies of TTAGGG sequences. Telomeres shorten with each cell division by 50–200 bp owing to the so-called "end replication problem". Although telomere length is known to play a critical role in cellular senescence, the relationship of telomere length to aging and longevity in humans is not well understood. Human population studies have correlated decreased telomere length in

peripheral blood leukocytes with higher mortality rates in individuals who are more than 60 years old. The aims of the study were to measure length of telomeres in three age groups and to evaluate the possible usage of telomere length as an informative biomarker of healthy aging.

Materials and methods: Three age groups were studied: individuals with age 18–40 years, individuals with age 65–75 years, and the centenarian group (with age above 90 years). DNA was isolated from leukocytes samples and telomere length was determined by telomere restriction fragment analysis.

Results: The mean length of telomeres in younger age group was 10.8 kb. The mean length of telomeres in 65–75 age group was 6.7 kb. Significant correlation between telomere length and age was observed in groups 18–40 and 65–75 years. Surprisingly, the mean telomere length in the centenarian group was slightly longer (7.6 kb) than in 65–75 age group.

Conclusion: The preliminary results of the present study in different age groups including centenarians found a positive link between telomere length and longevity.

A1.16
QPCR as standard test for determine programmed cell death and the response to different stresses in *Saccharomyces cerevisiae*

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The finding of the apoptotic marker *YAC1* in baker's yeast *Saccharomyces cerevisiae* a decade ago opened the possibility of study apoptosis in yeast as a model to understanding the programmed cell death (PCD) in higher organisms. However, apoptosis is not the only cellular death routine in eukaryotes; necrosis and autophagy are too. All these routines have different characteristics and several assays are routinely used to differentiate these pathways, such as co-staining of annexin-V (AnnV) and propidium iodide (PI) to discriminate between early apoptosis, primary necrosis and late apoptosis, TUNEL test for DNA fragmentation, ROS determination with dihydroetidium (DHE) and nuclear fragmentation and chromatin condensation observed with DAPI staining. These tests can assign the type of PDC of the cell but most of the time these tests are qualitative. The use of quantitative PCR (QPCR) for establishing the changes in expression levels during different stimulus and conditions could describe the differences between the PCD routines in a quantitative manner. In this work we test groups of genes whose transcription changes during unfolded protein response (UPR), apoptosis, necrosis, autophagy and general stress response in the baker's yeast *Saccharomyces cerevisiae* with the aim to establish a standard test for quantitative determination of activated death pathways.

A1.17
Phlorotannins isolated from eisenia bicyclis inhibit activity and expression of matrix metalloproteinase-2 in human fibrosarcoma

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Eiseniabicyclis (Kjellman.E.bicyclis) Setchellisaperennial brown alga, belonging to the family Laminariaceae. Fucofuroeckol A (FF) an deckol (EK) were isolated from *E. bicyclis*, and their antioxidant and matrixmetalloproteinase (MMP)-2 inhibitory effects were investigated. EK and FF showed significant antioxidant activities in several antioxidant assays, such as DPPH, hydroxyl, superoxide anion and peroxynitrite radicals scavenging activities using the electron spin resonance spectrometry technique and intracellular reactive oxygen species by DCFH-DA method. In MMP-2 inhibitory assay, FF and EK showed strong direct inhibition on MMP-2 dose-dependently. FF and EK also inhibited protein expression of MMP-2 in human fibrosarcoma cells. Therefore, these results suggested that FF and EK have remarkable antioxidant activities and strong potential as valuable natural MMP-2 inhibitor to develop cosmeceuticals for anti-wrinkle formation.

A1.18
Antiglycation activity of pyridoxal 5′-phosphate

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Glycation is a spontaneous chemical reaction, first discovered about a century ago by the French chemist Maillard. After him the reaction was called the Maillard reaction. In the last decades it has been recognized that the Maillard reaction is implicated in physiological processes such as senescence and ageing. In the glycation reaction carbonyl compounds such as reducing sugars interact with NH2-biomolecules including proteins, DNA and amino lipids, and thus impair their physiological function. The deleterious consequences of the Maillard reaction have prompted the active search for compounds capable to counteract the deleterious consequences of the Maillard reaction *in vivo*. In the present study we provide evidence that the vitamin B6 vitamer pyridoxal 5'-phosphate (PLP) exhibits carbonyl trapping activity. Under physiological conditions *in vitro* (37°C, pH 7) PLP interacts with the highly toxic dycarbonyl compounds 3-deoxyglucosone and methylglyoxal, the reaction reaching thermodynamic equilibrium after approximately 96 hours. Under the same conditions glycerol was also found to react with PLP while the model reaction of pyridoxal with 3-deoxyglucosone failed to give any detectable products. Based on electrospray ionization tandem mass spectrometry coupled to liquid chromatography and NMR spectroscopy we propose a structure for the reaction product of PLP with 3-deoxyglucosone. This product was detected also in the urine of rats with streptozotocin-induced diabetes. Data we provide in this study point to a novel physiological function of PLP. In addition to its cofactor activity PLP seems to play *in vivo* a role in detoxification of highly reactive dycarbonyl compounds.

A1.19
Boolean model of yeast apoptosis

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Programmed cell death (apoptosis) is mediated through different pathways based on different stimuli and like most biological processes it is the result of sequential activation /inhibition signals acting as input to downstream components. In the simplest possible way this input/output feature of any cellular process like apoptosis can be represented by a discrete model called Boolean

model in which the state of one node, which can be a gene or a cellular function, is determined by all inputs to that node.
Based on extensive literature study we have developed a yeast apoptosis network. By converting a schematic network into the Boolean model several steady states were identified. Each steady state was tested with corresponding stimuli which was expected to activate the associated pathway. Less complex genetic network and conservation of apoptotic mechanisms among eukaryotes provide the possibility of including genes from different organisms into yeast apoptotic network. Based on these facts we selected three crucial players of human apoptotic pathway and insert them into the pre-existing yeast apoptotic network. Such 'humanized yeast' (which are, or can be also created experimentally) will demonstrate model functionality according to experimental data. The other expected outcome of our model is the estimate of quantitative effect of each node in the network which is achieved by dynamic simulation from steady states of the network.

A1.20
Phenolic compounds in the turkish table olive cultivars

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Olive oil is the fat of choice in the Mediterranean area, where the diet has been associated with a lower incidence of coronary heart disease and certain cancers. Phenols in extra virgin olive oil are responsible for its peculiar pungent taste and for its high stability. Recent findings demonstrate that olive oil phenolics inhibit oxidation of low-density lipoproteins (the most atherogenic ones) and possess other potent biological activities that if demonstrated *in vivo*, could partially account for the observed healthful effects of diets that include high-quality olive oil and other foods rich in flavonoids and phenols. There is increasing interest in olive phenolic compounds because of their biological properties as well as their contribution to the colour, taste and shelf life of olive products. In the Spanish and Californian procedures, olives are treated with a diluted aqueous NaOH solution, that brings about several changes in the susceptible classes of compounds in the fruit. Note, however, that the composition of the triglycerides remain unaffected by these procedures. After the lye-treatment the olives are rinsed to remove the alkali, and the fruit is then left to ferment in brine for several months. During the fermentation process phenols diffuse from the pulp into the brine. In this study, levels of phenolics, that have antioxidant activity, such as á-tocopherol, caffeic acid, ferulic acid, and tyrosol of raw and processed Turkish table olive oils have been determined seperated by high-performance liquid chromatography (HPLC).

A1.21
The changes of the chemical composition during processing three Turkish table olive cultivars (Olea europea L.)

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Different olive varieties subjected to the same processing method react differently, depending on their varietal, chemical and physical characteristics. In the Californian method the olives are processed by successive treatments using 1 to 2% (w/v) concentrations of sodium hydroxide solutions (lye) that penetrate the fruit to the pit. At the end of each lye treatment the olives are washed with water and aerated. This aerobic alkali treatment tends to cause dramatic changes in the texture of the flesh. This leads to a softening which makes the end product less marketable. The objective of this study was determine fatty acids and mineral content to monitor changes in the composition of table olives after the lye treatment. Domat, Edremit and Gemlik varieties crude and processed table olive samples were considered for their fatty acid and mineral compositions. The mineral contents of three olive varieties were determined by ICP and found to be excellent. Olives were found to be rich in Ca, Fe, K, Mg, Na and P minerals. Also, K, Na and P contents of the Gemlik variety were found higher than those of other varieties. Fatty acids methyl esters (FAME) analysis of olive samples were determined by GC. Oleic acid (% 73.63) was present in the highest concentration, followed by palmitic (16.85%), linoleic (16.01%), stearic (2.82%) and linolenic (0.61%) of the Domat variety. In all processed olive samples, mineral and fatty acid compositions has affected by alcaline treatment, negatively.

A1.22
Anti-proliferative effect of papaverine in HepG2 cells

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Plants of genus Papaveracae with many valuable secondary metabolites have been used for different purposes in traditional medicine. This study is focused on papaverine effects on growth rate of HepG2 cells as a model for hepatocarcinoma. LD50 concentration of papaverine in this cell line was measured equal to 130 μM using neutral red uptake and MTT cytotoxicity methods. Growth rate and population doubling time of the cells under long-exposure to papaverine at two different concentrations corresponding to LD10 (defined as the concentration of papaverine which causes 10% reduction of cell viability) and 10 fold lower equal to 5 and 0.5 μM respectively, for 48 hours in successive passages were evaluated by using cell counting after trypan blue staining. TRAP (Telomerase Repeat Amplification Protocol) assay was used to compare immortality of the 48 hours treated cells to untreated controls. Data collected showed reduced cell growth in HepG2 cells exposed to 5 μM papaverine for 48 hours per passage over 41 days. The number of doublings in control cells over this period was 23.2, while the papaverine-treated cells passed only 15.7 doublings. Doubling time was increased to 62.58 hours (47% longer) comparing to 42.39 hours for untreated control. TRAP assay indicated a 55% reduction of telomerase activity in treated cells at LD50. Real time RT-PCR showed diminished hTERT expression in the treated cells to 65% of untreated cells. In conclusion papaverine shows strong growth limiting effect in HepG2 cell line and probably is a valuable compound against cancer.

A1.23
Cell senescence induction by Chelidonine in MCF7 cells

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Chelidonine, a tertiary hexahydro-benzophenanthridine alkaloid of Chelidonium majus and one of the alkaloids of Ukraine, has been shown to induce apoptosis in cell culture. Although Ukrain is known as an anticancer drug, the mechanism of action of the components still remained to be well understood. This study has

focused on immortality and growth of MCF7 cells as a model for breast cancer after treatment with chelidonine. LD50 of chelidonine in MCF7 cells after 48 hour treatment was measured 37 μM by neutral red uptake and MTT cytotoxicity tests. Growth rate of treated cells under long exposure to sub-apoptotic concentrations of chelidonine was estimated by cell counting after trypan-blue staining. In every passage the cells were treated only for 48 hour, and followed by normal medium. Treated cells exhibit strong growth inhibition after four times treatment with 0.2 μM chelidonine, so that the cell growth curve reached plateau and the treated cells failed in re-plating. At this time, growth of the treated cells shows almost 60% decline comparing to controls while cell viability was not affected. The number of cell doublings in treated cells was eight, while untreated controls passed 18 doublings. The treated cells morphologically appear to be aged with a large cell volume and high cytoplasmic to nuclear ratio. Induction of senescence in long-time treated cells was shown by β-galactosidase activity, a commonly used biomarker for cell senescence. Expression level of some genes related to cell senescence is under estimation.

A1.24
Lipid peroxidation damage of retinal pigment epithelium contributes to the pathogenesis of age-related macular degeneration

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Age-related macular degeneration (AMD) is the leading cause of legal blindness in developed countries, and prevalence will increase rapidly due to demographic changes. Progressive dysfunction of the retinal pigment epithelium (RPE) is considered central to the pathogenesis of AMD. In particular, lysosomal dysfunction induced by lipid peroxidation products, like malondialdehyde (MDA) or 4-hydroxynonenal (HNE), seems to play a pivotal role. We found that lipid peroxidation-related protein modifications on photoreceptor outer segment (POS) proteins inhibit their lysosomal degradation in RPE cells. Lipid peroxidation products exerted striking inhibitory effects on lysosomal cysteine proteases. Feeding of RPE cells with HNE- or MDA-modified POS resulted in an 8-fold increase in cellular autofluorescence, indicating lipofuscinogenesis. In polarized RPE cells we observed apical-to-basolateral transcytosis of undegraded HNE- or MDA-modified POS, which, *in vivo*, may contribute to sub-RPE deposit formation and drusen biogenesis, a hallmark of AMD. Autophagy activity, measured as 3-methyladenine-sensitive turnover of radiolabeled engogenous proteins, was reduced by pretreating the cells with lipid peroxidation-modified POS by 40%. In conclusion, lipid peroxidation products generated in the outer retina due to its unique physiological characteristics, such as high tissue oxygen concentration, intense light exposure and high abundance of polyunsaturated fatty acids, severely affect RPE lysosomal function, resulting in lipofuscinogenesis, extracellular deposition of undegraded material and reduced autophagy, finally leading to senescence and degeneration of the RPE, as seen in AMD.

A2 – Molecular Immunology

A2.01
Isolation of a novel nanobody against HER-2/neu using phage display technology

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Camelid serums contain functional antibodies without light chains. The variable domain of heavy-chain antibodies is named as VHH. They have some biological, medical and biotechnological advantages over conventional antibodies. Nanobodies are well expressed in microorganisms (*Escherichia coli*, fungi and yeast) with high stability, good solubility and easy production in large quantities. In this study, we identified a nanobody that recognizes extra cellular domain of human epidermal growth factor receptor 2 (HER-2/neu) that over expressed in a number of various solid tumors are associated with over expression of erbB-2. Our nanobody (SR-87) has been isolated from immune phage nanobody repertoires. The soluble antibody was purified following immobilized metal affinity chromatography (IMAC) and characterized by SDS-PAGE, Western -blotting and ELISA methods. SR-87 was characterized and showed good affinity (10–10 M-1) and specificity towards HER-2 in comparison to murine monoclonal antibodies. This single domain antibody (14 KD) may be useful for targeting HER-2 marker on the surface of tumor cells. SR-87 was conjugated to gold – silica nanoshells and applied them to SK-Br-3 cell line which over expressed HER2 and HelaS3 cell line which didn't has any HER2 receptors. The cells were irradiated with NIR laser and evaluated for nanoshell binding and viability. The photothermal therapy was generated enough heat to destroyed SK-Br-3 cells while controls with no nanoshells or the nonspecific antibody binding, show no therapy.

A2.02
Blood serum levels of IL-1â, IL-6 and TNF-á in patients on maintenance hemodialysis

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Introduction: Dialysis provides effective and safe treatment of ESRD, but patients who are maintained on chronic dialysis are at risk for cardiovascular disease. One major risk factor for cardiovascular disease in adult patients with ESRD is chronic inflammation. Cytokines are essential mediators of immune response and inflammatory reactions. During a hemodialysis (HD), cytokines are released mainly by monocytes activated by endotoxin-type compounds in dialyzer fluid, Complement factors and direct contact with dialyzer membrane. Aim of this study was to examine effects of the duration of HD therapy upon systemic profile of the pro-inflammatory cytokines (IL-1 â, TNF-á and IL-6) in patients on regular maintenance HD.
Methods: The study included 43 CRF patients, aged 59.32 ± 14.43 years, on regular HD maintenance therapy for mean 26.44 ± 41.29 months and 43 age and sex matched healthy controls. It was designed to assess serum levels of inflammatory cytokines: IL-1â, IL-6 and TNF-á in CRF patients on regular maintenance HD.
Results: The serum IL-1â, IL-6 and TNF-á level were statistically significantly higher in patients than in the controls. There were statistically significant positive correlations between the duration of HD therapy and serum levels of the inflammatory cytokines.
Conclusions: Elevated serum IL-1â, IL-6 and TNF-á levels in our CRF patients on regular maintenance HD indirectly confirm importance of HD in amplification of the chronic inflammation substantially depend on the duration of dialysis treatment.

A2.03
Anticardiolipin antibody in acute myocardial infarction

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Background: Antiphospholipid (aPL) antibodies – both the lupus anticoagulant and anticardiolipin antibodies – are closely associated with arterial and venous thrombosis. The purpose of the present study was to determine whether the presence of aPL antibodies, namely, anti-cardiolipin (aCL) antibodies, are a risk factor for acute myocardial infarction (MI).
Methods: This case control study was carried out on 45 patients with acute myocardial infarction and 45 age, sex and MI risk factors matched healthy persons (control group) referring to peymanieh hospital of Jahrom between 2006 March to 2007 February. Using commercial enzyme-linked immunosorbent assay (ELISA) kit, the presence of anti-cardiolipin (aCL) IgG in the patients' and the controls' sera was determined.
Results: The prevalence of aCL IgG in the patient 62.29 ± 13.245 years (including 68.89% men and 31.10% women) and in the control group 61.71 ± 12.297 years (including 53.30% men and 47.70% women), was 18.60% and 11.60% respectively ($p = 0.366$).
Conclusion: This study shows no significant association between presence of aCL IgG and acute myocardial infarction. Future larger studies may be required to determine the precise role of aCL IgG in the pathogenesis of different subtypes of ischaemic heart diseases and Myocardial infarction.

A2.04
The Interleukins IL-13 and IL-18 in the primary breast cancer tumor tissue

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Some recent literature data suggest possible role of interleukins in the pathogenesis of breast cancer. The aim of this study was to investigate the presence and the expression levels of the IL-13

and IL-18 in the primary breast cancer tumor in relation to the unchanged breast tissue and pathohistological factors (lymph node status, tumor size, histological grade), estrogen and progesterone receptor status. The expression levels of IL-13 and IL-18 in the primary tumor tissue and unchanged surrounding tissue in 50 breast cancer patients and in breast tissue in 20 patients with benign breast diseases were determined using three-step immunohistochemical staining, as well as the hormones receptor status. IL-13 and IL-18 were present in breast cancer tumor, surrounding tissue and breast tissue in patients with benign breast disease. The expression of these interleukins was significantly higher in breast cancer tumor compared with surrounding tissue ($p < 0.05$). In addition, the IL-13 expression was significantly higher in breast cancer tumor compared with breast tissue in patients with benign breast diseases ($p < 0.01$), whereas IL-18 expression was not. No significant differences between IL-13 and IL-18 expressions were noticed considering the lymph node status. In relation to pathohistological factors no significant correlations in both interleukins expression were found, excluding significant correlation between IL-13 expression and tumor size in patients with lymph node-negative breast cancer ($p = 0.05$). However, expression level of analyzed interleukins in tumors in lymph node-negative patients was inversely correlated to hormone receptors, but not statistically significant.

A2.05

Abstract withdrawn

A2.06
Autoactivation of MASP-2: Role of exosite interactions

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The complement system is a key element of innate immunity in vertebrates. A cascade of enzyme reactions, triggered by a recognition protein complex, results in opsonization and destruction of the pathogen cell. The recognition complexes of the classical and lectin pathways of complement consist of structurally related proteins and act analogously. MASP-2, a modular serine protease of the recognition complex of the lectin pathway is responsible for the first proteolytic event of the cascade: its autoactivation. Our aim is to explore the structural background of the narrow substrate-specificity as well as autoactivation of MASP-2 and other related enzymes in atomic details. We report the structure of the active form of the catalytic fragment of MASP-2 crystallized in a new crystal form. The structure was refined to 2.5 Angstrom resolution. In the structure there is enzyme-product relationship between two symmetry-related molecules. In addition to the contacts corresponding to a canonical serine protease-peptide interaction there are extended exosite interactions as well between the two MASP-2 molecules. Exploring these exosite regions should help us to understand the high selectivities and high autoactivation rates of MASP-2 and C1r, two related activation-initiating enzymes of the lectin and the classical pathways, respectively. Support from EMBL and Hungarian Scientific Research Fund (OTKA) grants F67937 and K68408 is acknowledged.

A2.07

Abstract withdrawn

A2.08
Electrostatic allostery – A novel mechanism for neutralization of protein antigens by antibodies

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The binding of antibodies usually causes steric hindrance of functionaly important sites on their target molecules. In the present study by using theoretical and experimental approaches, we demonstrate a unique role for protein electrostatics in neutralization of the coagulation factor VIII (FVIII) by a human pathogenic antibody – BO2C11. Kinetic and thermodynamic analyses of BO2C11 binding to FVIII indicated that this interaction is characterized by an ionic strength dependency that is uncommon for other protein-protein interactions. By using continuum electrostatics calculations, we further demonstrated that BO2C11 binding to FVIII induces long-distance perturbations in the electrostatic potential and in the local electrostatic parameters (degree of ionization, proton affinity and electrostatic energy) of charged residues in the C2 domain of FVIII. The effects were not consecutive of structural alternations in C2. The distant changes in the electrostatic parameters were not delocalized, but affected predominantly the residues that constitute a binding site for von Willebrand factor (VWF) – a protein essential for FVIII stability and half-life in the circulation. Replacement of the in silico predicted electrostatic hotspots by alanine by site directed mutagenesis of FVIII resulted in considerable decrease in the binding to VWF. Thus, the allosteric perturbation of surface electrostatics at a VWF binding site on C2 could explain the pathogenic effect of the BO2C11 in preventing FVIII binding to VWF. Our findings suggest that some antibodies modify their targets by alteration of protein surface electrostatics at a long-distance from the binding site.

A2.09
Different molecular mechanisms of alternative complement pathway dysregulation result in common glomerular endothelial damage and contribute to the pathogenesis of the atypical hemolytic uremic syndrome

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Complement is a major innate immune defense against pathogens, tightly regulated to prevent host tissue damage. The atypical hemolytic uremic syndrome (aHUS) is characterized by endothelial damage leading to renal failure and is highly associated with abnormal alternative pathway regulation. We characterized the functional consequences of 4 aHUS-associated mutations in Factor B (FB) and C3 (forming the alternative

C3-convertase) and also 4 mutations in the key regulator Factor H (FH) (n = 2 N- and n = 2 C-terminal). FH depleted serum was used as a model for complement deficiencies. Three of the mutant proteins (in FB and C3) formed hyper-active C3-convertase. All mutations affected the C3-convertase regulation. The convertase formed by FB mutations was resistant to decay by FH. The C3 mutations led to decrease binding to normal FH and FH mutations resulted in decreased binding to normal C3b. Irrespective of the molecular mechanism of the defect, complement deposition on the surface of alternative pathway activator cells was enhanced. We demonstrated for the first time that all these mutations lead to increased C3-fragments deposition on TNF/IFNgamma activated adherent endothelial cells (HUVEC and glomerular), together with the formation of sC5b-9 complexes and enhanced tissue factor expression. The same results were obtained when the endothelial cells were incubated with normal human serum in presence of inhibitory anti-FH N- and C-terminal antibodies. These results could explain the link between the mutations and the disease, since excessive complement deposition on endothelial cells and induction of a pro-coagulant phenotype are central events in the pathogenesis of aHUS.

A2.10

Abstract withdrawn

A2.11
Expression of endothelial selectin ligands on leukocytes following repeated dives in SCUBA divers

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Leukocyte cell surface adhesion molecule CD11b, decorated with CD15s, plays a critical role in the regulation of β2 integrin function during neutrophile endothelial transmigration. Hyperbaric oxygenation reduces neutrophil-endothelial cell adhesion, which is mediated by Mac-1 (CD11b/CD18) β2-integrin. This study investigated the expression of CD15 and CD15s, on leukocytes following repeated trimix (a mixture of oxygen, helium and nitrogen) dives in two series: in the first series seven divers performed six consecutive dives from 55–80 m, while in the second series seven divers performed three consecutive dives from 63–65 m. Five divers took part in each of the two series. CD15 and CD15s were determined before and after the 1st and the last dive. Leukocyte subpopulations were not elevated after either the first or last dives in series I. Only $CD15^+$ $CD15s^+$ granulocytes were significantly decreased after the 1st dive ($p = 0.006$). In the second series the monocyte proportion was increased ($p = 0.014$) and lymphocytes decreased ($p = 0.020$) within the total leukocyte population, while $CD15s^+$ monocytes and $CD14^+$ $CD15s^+$ granulocytes were elevated ($p = 0.019$, and $p = 0.018$, respectively) after the 1st dive. $CD15^+$ $CD14^+$ granulocytes were decreased after the 1st and the last dive in the second series ($p = 0.048$ and 0.017, respectively), while $CD15s^+$ granulocytes were decreased only after the last dive in the second series ($p = 0.006$). The current findings of decreased endothelial selectin ligand CD15s expression on $CD15^+$ granulocytes after certain dives point to the role of this subpopulation in the endothelial damage prevention.

A2.12
ER aminopeptidase 1 single Nucleotide Polymorphisms can influence antigenic peptide processing

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ERAP1 is an ER aminopeptidase that plays crucial roles in the generation and destruction of MHC class I-restricted antigenic peptides. Recently, large population studies have linked coding ERAP1 single nucleotide polymorphisms (SNPs) with predisposition to autoimmune diseases and virally induced cancer. We hypothesized that this link is due to ERAP1's role in antigenic peptide processing, through the aberrant generation or destruction of key antigenic epitopes that initiate or sustain autoimmunity or elicit anti-viral responses. To test this hypothesis we overexpressed and purified allelic versions of ERAP1 and tested their ability to generate antigenic peptides *in vitro*. We found that, for several but not for all of the epitopes tested, mature antigenic peptide generation rates were dependent on the ERAP1 allele used and in patterns that were also epitope dependent. Furthermore, the generation rate of specific antigenic peptides suspected to be linked with autoimmunity was highly dependent on the presence of the specific ERAP1 SNPs also linked with autoimmune disease. Our results suggest that ERAP1 SNPs may impose specificity changes in the enzyme. Furthermore, our findings provide support to the concept that antigenic peptide processing is the biochemical mechanism behind the link of ERAP1 SNPs and autoimmune disease predisposition.

A2.13
PolyCTLDesigner: A program for designing cytotoxic T-cell polyepitope immunogens

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T-cell epitopes are important tools for diagnosis and treatment of infectious, autoimmune or cancer diseases as well as for the development of novel polyepitope vaccines. Although immunogenicity of the peptide is known to be crucially determined by its MHC-binding affinity it was also shown to be dependent on amino acid residues which flank the epitope and affect efficiency of its proteasomal release and TAP-dependent transporting into endoplasmic reticulum. Here we present a program that tries to take these considerations into account when designing primary structure of cytotoxic T-cell immunogen. The PolyCTLDesigner software constructs polyepitope CTL immunogen selecting superior spacers for every pair of selected epitopes, choosing appropriate epitope matchings and selecting optimal arrangement of epitopes within designed construction using graph theory approach, thus increasing efficiency of polyepitope processing and favoring presentation of target epitopes. It also tries to minimize the number of "non-target" epitopes within desired polyepitope immunogen and is able to assist in collecting the set of peptides covering selected HLA repertoire with desired rate of redundancy using known genotypic HLA allele frequencies data together with either known

or predicted specificity of selected peptides towards different allotypes of HLA class I molecules. PolyCTLDesigner is integrated with previously created T-cell epitope prediction software named TEpredict. Both programs were written in Python programming language. They could be freely downloaded from TEpredict project site: http://tepredict.sourceforge.net.

A2.14
Studies of structural and functional properties of orthopoxviral CrmB proteins

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CrmB proteins of variola (VARV), monkeypox (MPXV) and cowpox (CPXV) viruses were produced in baculovirus expression system. Despite sharing high sequence identity, CrmB proteins of VARV, MPXV and CPXV differed in their efficiencies of inhibiting cytotoxic effect of human, mouse and rabbit TNFs in L929 mouse fibroblast cells. Of these CrmBs only VARV-CrmB was shown to have pronounced protective effect in the experimental model of LPS-induced shock in SPF BALB/c mice. Gel-filtration of the lysates of Sf21 insect cells infected with recombinant baculovirus containing the gene coding for either MPXV- or CPXV-CrmB revealed that TNF-neutralizing activity was mainly associated with fractions whose molecular weight was about 90 kDa, corresponding to homodimers of CrmB proteins. Whereas gel-filtrations of similar preparations containing recombinant VARV-CrmB protein revealed that TNF-neutralizing activity was predominantly associated with the fraction of high molecular weight (> 500 kDa), corresponding to large multimeric complexes of VARV-CrmB. CrmB proteins consist of N-terminal TNF-binding domain and C-terminal chemokine binding one. To study influences of these domains and their species-specific distinctions on biological activity and some physicochemical characteristics of VARV and CPXV-CrmB, we modelled spatial structures of these proteins and developed several mutant and truncated forms of these CrmBs. Designed mutant forms of VARV- and CPXV-CrmB were also produced in baculoviral expression system. And now properties of these recombinant proteins are being comparatively studied. The work was supported by Russian Foundation for Basic Research (grant #090400055a).

A2.15
Biochemical evidence for specific pairwise interactions of mouse NKR-P1B/D:Clr-b receptors engaged in lectin – lectin interactions

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Mouse NKR-P1B/D:Clrb receptor pair represents a recently discovered example of lectin – lectin interactions. In order to study this interaction by biochemical techniques, we have amplified the individual cDNA clones for the receptors by RT-PCR from B6/BL mice spleens and transferred DNA fragments coding for the extracellular ligand binding domains into pET-30 bacterial expression vectors. During expression proteins precipitated into inclusion bodies, from which they could be refolded *in vitro*. Using ion cyclotron resonance mass spectrometry, we have confirmed the quality of the refolding for Clrb checking the disulfide bonding. In order for the NKR-P1D to fold properly, the third cysteine which does not fit into the pattern usual for this family of receptors was substituted for serine. The resulting C118S NKR-P1D, just as the Clrb, was shown to be monomeric in solution. Moreover, we produced uniformly 15N-labeled variants of these proteins, and measured 1H/15N-HSQC spectra providing additional evidence for proper folding of these proteins. Using gel filtration and analytical ultracentrifuge we were unable to prove the interaction between Clrb and NKR-P1D in these monomeric forms. Using SPR technique a specific weak interaction was shown to occur only at pH = 4 while at physiological pH no interaction was observed. Further efforts to prepare the receptors in dimeric forms in which they appear on the membrane, and experiments to see if and under which conditions these forms interact will follow. Supported by grants from Ministry of Education of Czech Republic (MSM_21620808 and 1M0505), and from The Grant Agency of Czech Rep. (GACR 305/09/H008 and 303/09/0477).

A2.16
Association of Fcγ receptor IIa (CD32a) with lipid rafts regulates ligand binding activity

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Binding of immunoglobulins to myeloid cells via Fc receptors is a key event in the control of innate and acquired immunity. Fcγ receptor IIa (CD32a) is a receptor for multivalent IgG expressed by myeloid cells and its association with microdomains rich in cholesterol and sphingolipids, termed as lipid rafts has been reported to be essential for efficient signalling. However, for many myeloid cell types, ligand binding to CD32a is suppressed by as yet undefined mechanisms. In this study, we have examined the role of CD32a-lipid raft interactions in the regulation of IgG binding to CD32a. CD32-mediated IgG binding was measured by flow cytometry using fluorescent-labelled IgG complexes in several cell types. We have introduced point mutations in the transmembrane and juxtamembrane region of CD32 and assessed the association of these mutants with lipid rafts by confocal immunofluorescence and extraction and analysis of detergent-resistant domains. Disruption of lipid raft structure following depletion or sequestration of membrane cholesterol greatly inhibited CD32a-mediated IgG binding. Furthermore, specific CD32a mutants, which show reduced association with lipid rafts (A224S and C241A) displayed decreased levels of IgG binding compared with wild type CD32a. In contrast, constitutively lipid raft-associated CD32a (GPI-anchored CD32a) exhibited increased capacity for IgG binding compared with the full-length transmembrane CD32a. Our findings clearly suggest a major role for lipid rafts in the regulation of IgG binding and more specifically, that suppression of CD32a-mediated IgG binding in myeloid cells is achieved by receptor exclusion from lipid raft membrane microdomains.

A2.17
Searching for new interaction partners and substrates of tissue transglutaminase in differentiated NB4 cells

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Tissue transglutaminase (TG2) is a member of the transglutaminase family of enzymes that covalently cross-link proteins in a $Ca2^{+}$ dependent manner. TG2 also has a guanosine triphosphatase (GTPase) activity, protein disulfide isomerase activity and protein kinase activity. TG2 is present in various cellular compartments including cytoplasm, nucleus, and extracellular matrix and involved in the terminal differentiation of immune cells. The NB4 is an acute promyelocytic leukemia cell line which could be differentiated into neutrophil granulocytes with all trans retinoic acid treatment. We have published that during differentiation process of NB4 cells the expression of TG2 was highly increased and contributed to the expression of GP91phox. Our working hypothesis is that TG2 could perform these distinct functions through its cross linking activity and/or mediate it by protein-protein interaction. Our aim is to identify new substrates and interaction partners in differentiating NB4 cells. For identification of substrates of TG2, differentiated cells were permeabilized and an artificial TG2 substrate (biotinilated pentylamine) was added to it. After cell lysis, the biotinilated proteins were purified using streptavidine beads, separated with 2D-PAGE and then identified with HPLC-MS/MS. With this method we could identify several new possible substrates of TG2. For identification of interacting partner proteins of TG2, after lysis of differentiated cells immunoprecipitation were carried out standard procedures. The precipitated proteins were separated with SDS-PAGE or with 2D-PAGE. Gels were stained with Sypro Ruby and protein band/spots were identified by HPLC-MS/MS analysis.

A2.18
Carboxylated calixarenes bind strongly to CD69 and protect CD69^{+} killer cells from apoptosis induced by tumor cell surface ligands

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CD69 is expressed at cell surface as homodimeric receptor belonging to C-type family. We have recently indetified carboxylated calixarenes as a new class of noncarbohydrate ligands for CD69 receptor. Binding activities of synthesized carboxylated calixarenes were tested using plate binding, plate inhibition, and plate precipitation assays using recombinant human CD69 protein. In direct binding assays we have employed the principle of fluorescence quenching. Human N-PBMC were isolated on ficoll-paque technique and lymphocytes from donors with more than 20% $CD69^{+}$ cells were further activated in the presence of PMA and ionomycin. The optained cellular fractions were used in cellular activation assays meassuring the production of inositol phospates and intracellular calcium. Proliferation of lymphocytes was meassured by a standard 3H-thymidine incorporation. Percentage of apoptotic cells was estimated using Annexin V-FITC/ Höechst 33250.Of the four compounds investigated here thiacalix[4]arene had the highest affinity in the direct binding assays, and proved to be the most specific inhibitor identified so far in receptor precipitations and cellular activation experiments. Moreover these compounds also proved effective at protection of CD69high lymphocytes from apoptosis triggered by a multivalent ligand SiaTnTRI2 or antibody crosslinking. Carbohydrated calixarenes investigated here set a new paradigm for noncarbohydrate ligands for CD69 making them attractive candidates for protection of killer cells in combine animal tumor therapies.This work was supported by Ministry of Education of Czech Republic (MSM 0021620808 and 1M0505), and by the Grant Agency of Czech Republic.

A2.19
Clearance of dying autophagic cells induces the inflammasome pathway in human and primed mouse macrophages

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Autophagy is now recognized as possible inducer of a distinct cell death mechanism happening under various circumstances (Mizushima N., Nature Reviews,2008). Clearance of dying autophagic (AU) MCF7 cells but not living or apoptotic ones can lead to pro-inflammatory response in human macrophages (Petrovski et al., Autophagy, 2007). These dying cells could induce activation of caspase-1 (IL-1β converting enzyme) as early as 1 hour after being co-incubated with human blood-born macrophages. Upon observations in human system, we decided to establish a mouse model and used the mouse Ba/F3 cell line (IL-3 dependent BM derived pro-B cells) as a possible AU cell clearance model. Ba/F3 cells have been shown to undergo death under IL-3 depletion with signs of autophagy (Wirawan and Vandenabeele et al., 15th Euroconference, ECDO, 2007). Our recent results show that AU Ba/F3 cells but not living cells can induce the IL-1 release from LPS primed mouse peritoneal macrophages. According to our results, Ba/F3 cells are partially dying after IL-3 depletion. Furthermore, rapamycin (m-TOR inhibitor) treatment trigger more cell death during IL-3 depletion compared to non-treated cells. On the other hand, LC3II levels are elevated upon IL-3 depletion with/without rapamycin treatment compared to living cells. These observations may indicate the importance of autophagy in cell death. Mechanisms behind these observations will be clarified by using knock out mice system to deduce the involvement of the members of the inflammasome pathway (e.g. ASC,NALP3) as well as to exclude the involvement of other inflammatory pathways (Myd88) in the process of this unusual immunogenic response.

A2.20
Apoptotic human cells inhibit migration of granulocytes via release of lactoferrin

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Apoptosis is a noninflammatory, programmed form of cell death. One mechanism underlying the non-phlogistic nature of the apoptosis program is the swift phagocytosis of dying cells. The objective of this study was to determine how apoptotic cells selectively attract mononuclear phagocytes and not granulocytes, the professional phagocytes that accumulate at sites of inflammation. In order to address this, Burkitt's lymphoma (BL), a non-Hodgkin's lymphoma, was employed as an in situ model of apoptosis. BL is characterised by a high rate of apoptosis and the selective infiltration of monocytes. However, no neutrophils are present in its stroma. In this study, we found that BL cells

as well as human cell lines of diverse lineages express lactoferrin, a 80 kDa pleiotropic glycoprotein, upon apoptosis induction. Lactoferrin was demonstrated to inhibit the chemotaxis of granulocytes but not mononuclear phagocytes, both *in vitro* and *in vivo*. This antiinflammatory activity of lactoferrin is independent of its iron-saturation status and does not alter intracellular calcium levels. Lactoferrin acts by preventing the acquisition of granulocyte activation status and morphology following agonist stimulation and affects granulocyte signaling pathways, such as phosphorylation of MAP kinases that regulate cell adhesion and motility. Together, our results identify lactoferrin as an antiinflammatory component of the apoptosis milieu and place it as one of the few till now identified molecules that act as negative regulators of granulocyte migration, eliciting in this way, tremendous therapeutic applications in the control of inflammatory conditions. J Clin Invest (2009)119(1):20–32.

A2.21
SKN-1 transcription factor required for pathogen resistance in *Caenorhabditis elegans*

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Oxidative stress is a major factor in aging, while antioxidant response is an important determinant of longevity. In recent years a relationship between oxidative stress and immunity has been revealed in humans and in various experimental models. Lately, oxidative stress during bacterial infection has been described in *Caenorhabditis elegans*. Reactive oxygen species are released both by invading bacterial pathogens and by NADPH oxidases from the *C. elegans* intestine. The mounting of oxidative stress response has been confirmed by the induction of antioxidant enzymes in worms during infection. Many of these enzymes are regulated by the stress inducible FOXO transcription factor, DAF-16. However, other antioxidative regulators such as the NRF2 ortholog SKN-1 transcription factor have not been investigated in *C. elegans* immunity. It is expressed both in ASI neurons and in the intestine of the nematode. Both our knock-out and RNAi knock-down experiments showed that in absence of all three isoforms nematodes displayed a highly elevated susceptibility to infection by *Pseudomonas aeruginosa*. To further investigate the role of SKN-1 in pathogen resistance, we employed oxidative stress (hydrogen peroxide pretreatment), which significantly enhanced the survival of worms against *P. aeruginosa*. The hormetic effect of oxidative stress was partially prevented in the absence of either SKN-1 or DAF-16. Moreover, the activation of SKN-1 during infection has been demonstrated by the induction of a SKN-1-dependent reporter. Thus, our data shows that SKN-1 is required for pathogen resistance and further strengthens the cross-talk between oxidative stress responses and immunity in *C. elegans*.

A2.22
Cytokine assessment in chronic kidney disease by xMAP technology

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Background: Endothelial dysfunction represents the initiating event in the atherosclerosis process, playing a crucial role in the development of cardiovascular and renal diseases, as a pathogenic link between vascular and renal involvement. Chronic kidney disease (CKD) can determine metabolic changes that may lead to increased oxidative stress or/and an enhanced inflammatory state, changes that can determine endothelial dysfunction.
Aim: To evaluate and validate new investigation methods for early stages of vascular dysfunction using new cellular and molecular biology techniques.
Methods: Multiplex analysis of cytokine levels using xMAP technology was performed on serum samples from 20 CKD patients and 20 controls; IL-6, IL-10 and TNFα were analyzed on Luminex® 200™ (Luminex Corp., USA) using Milliplex™ MAP Human Cytokine/Chemokine Panel (Millipore, US). Multiplex data acquisition was performed using STarStation 2.3 (Applied Cytometry Systems, UK).
Results: IL-6 and TNFα serum levels were increased in CKD-patients (6.042 pg/ml ± 0.888 versus 3.163 pg/ml ± 0.473, $p < 0.01$, respectively 14.56 pg/ml ± 1.11 versus 7.463 pg/ml ± 0.883, $p < 0.0001$). IL-10 was decreased in CKD samples (4.528 pg/ml ± 0.984 versus 12.11 pg/ml ± 4.964, $p < 0.05$). IL-6 and TNFα increase with stage, while for IL-10 no trend was visible.
Conclusions: The use of multiplex xMAP technology made possible the simultaneous quantitation of serum levels for 3 relevant molecules in CKD. IL-6 and TNFα showed a good potential of prediction with ROC areas of 0.76 with $p = 0.02$ for IL-6 and 0,865 with $p = 0.001$ for TNFα.
Acknowledgment: The present work was supported from Grant 4.2-171/2008.

A2.23
Correlation of the changes of blood plasma albumin and t-lymphocytes phenotype to tumor stage in gastrointestinal pacients

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The original fluorescent probe ABM (an amino derivativeof benzanthrone) was used to characterize the membranes of lymphocytes and blood plasma albumin recovered from colorectal and gastric cancer patients with Stage II-IV. The fluorescence intensity of ABM in patients differ from the values seen from healthy control and reflected specific differences before and after medically indicated surgical treatment and corresponds to cancer stage. ABM fluorescence associated with select immunological parameters ($CD4^+$:$CD8^+$ ratios, lymphocyte counts etc.,) in the cancer patients. Surgical treatment elevates immune state. With progress of cancer stage, $CD4^+$, $CD4^+$:$CD8^+$ gradually decreased, while $CD8^+$ gradually increased. The preoperative immune state of patients is negatively related to cancer stage. An aim of these studies was to elaborate criteria for clinical interpretation (i.e. of any alterations in albumin physicochemical parameters and/or lymphocytes functional activity) using ABM as a analytical agent. There was a seemingly excellent agreement between changes in ABM spectral parameters and both clinical and pathological estimatesof the severity of disease in patients with solid tumors αA.
Acknowledgement This work was supported by the European Structural Funds (Project Nr. 2009/0205/1DP/1.1.1.2.0/09/APIA/VIAA/152)

A2.24
Glycoconjugates containing immunoactive LELTE peptide: Effect of glycosylation on cellular activation and natural killing

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CD69 is a widespread receptor of the immune system cells. Although a physiological ligand for this receptor is unknown, we have previously proved its affinity towards a range of ligands including calcium, carbohydrates, and charged compounds such as carboxylated calixarenes. Moreover, a pentapeptide sequence LELTE derived from a mycobacterial heat shock protein hsp65 has been recently identified as a ligand for CD69 representing a "danger" signal for the immune system. However, this peptide is not immunoactive *per se*, but only after its presentation within the multivalent environment of its parent protein, or after artificial dimerization using bifunctional reagents. Here we describe an entirely new way to present this peptide through attachment to a cyclopeptidic RAFT scaffold $(K\text{-}K\text{-}K\text{-}P\text{-}G)_2$ through the ε-amino groups of lysine residues, alone or in combination with a carbohydrate 1α-GalNAc. The ability of such scaffolds to precipitate the CD69 receptor or to activate CD69-positive cells is enhanced in compounds, which possess both peptide and carbohydrate epitopes. These compounds efficiently activate natural killer lymphocytes, but are inactive from the point of view of activation-induced apoptosis of lymphocytes. These unique properties make the combined peptide / carbohydrate RAFTs highly suitable for evaluation in animal tumor therapies *in vivo*, and predict them to be readily available and efficient immunoactivators. *Supported by the Univ. Joseph Fourier, CNRS, Ministry of Education of Czech Republic (LC06010, MSM_0021620808, and 1M0505), Grant Agency of the Czech Academy of Science and Czech Grant Agency.*

A2.25
Role of the mannose-binding lectin-2 X/Y (MBL-2 x/y) polymorphisms in patients with rheumatoid arthritis

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The mannose-binding lectin (MBL) pathway of innate immunity is part of the first line of defense against microorganisms. MBL recognizes and binds to carbohydrate patterns on the surface of microorganisms leading to complement activation by the MBL-associated serine protease2 (MASP-2). Rheumatoid arthritis (RA) is an immune disorder in which the immune system mistakes normal tissues for foreign ones and attempts to neutralize and rid the body of the perceived threat. Although some experts theorize that genetic and environmental factors play a role, the factors that lead to this self-attack and subsequent induction of the inflammatory process remain unknown. We aimed to investigate whether profile of MBL-2 X/Y genotyping may be associated with the risk of RA. The study population consisted of 59 patient with RA and 80 unrelated healthy individuals. Blood was collected in EDTA-containing tubes and DNA was extracted from leukocytes by High Pure PCR template preparation kit. Genotyping of MBL-2 polymorphisms were detected by using a LightCycler MBL-2 mutation detection kit in real-time PCR. No association was observed between the MBL-2 X/Y genotype and RA.The frequencies of XX, XY and YY genotypes were 50.8%, 44.1% and 5.1%; in cases and 56.3%, 38.8% and 5% in controls. Further studies on larger groups are needed to determine the prevalence of MBL-2 X/Y polymorphisms in patients with RA.

A2.26
Intravenous immunoglobulins as drug delivery system for target anticancer combined therapy

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Imunotherapy can be induces statistically significant inhibition of tumor growth, invasiveness, angiogenesis and prolongation of survival time. Its combination with anticancer destructing therapies (chemotherapy and photodynamic therapy) can be effective way for tumor destruction without next cancer recurrence. Therefore, we designed supramolecular multimodal system based on intravenous immunoglobulins for target transport of anticancer drugs and combined therapy. Their study in mice model showed its excellent anticancer affectivity.
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A2.27
Adiponectin limits experimental autoimmune encephalomyelitis by suppressing the differentiation of $CD4^+$ cells into Th17 cells

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Experimental autoimmune encephalomyelitis (EAE) has been identified as an important and most commonly used animal model for investigating multiple sclerosis (MS), which is an inflammatory disease of the central nervous system (CNS). Previous studies showed that leptin can worse the symptoms of EAE by increasing the production of pro-inflammatory cytokines. Since adiponectin is an anti-inflammatory adipokine which acts in an antagonistic manner to leptin, we hypothesize that adiponectin may reduce the symptoms of EAE and block the development of this disease. Our results showed that adiponectin knock-out mice are more susceptible to EAE development with higher clinical scores and disease incidence compared to wild type littermates. In addition, there are more inflammatory infiltrates into spinal cords in adiponectin knock-out mice. Multiple-cytokine

profiling of the splenic cells from both types of mice with EAE demonstrated that interleulin-17 (IL-17) plays an important role in this process. Accordingly, in the spinal cords of adiponectin knock-out mice, the gene expressions of IL-17 and its related cytokines were significantly elevated compared to the wild type littermates. Furthermore, our ex vivo study demonstrated that recombinant adiponectin can inhibit the differentiation of $CD4^+$ T cells into Th17 cells, resulting in reduced production of IL-17 and IL-6. In summary, these results demonstrate that adiponectin can limit EAE by suppressing the development of Th17 cells and the production of IL-17.

A2.28
New hybridoma technology based on antigen-specific immunoglobulin receptors

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There are two types of immunological responses in vertebrates for defense against invading substances and pathogens. B lymphocytes, known to generate antibodies that can specifically bind to foreign antigens, are able to mature and express antigen-specific immunoglobulin receptors on their surfaces after repeated antigen stimuli. To generate hybridoma cells continuously secreting monoclonal antibodies, B lymphocytes must be somatically fused with cancerous myeloma cells. For this purpose, the expressed receptors play pivotal roles in selecting B lymphocytes generating specific antibodies to target antigens. We have established a new hybridoma technology to yield specific monoclonal antibodies against antigens of interest with high specificity and selectivity. The new technology consists of three critical steps. Antigen-sensitized B lymphocytes are pre-selected in advance for antigens based on immunoglobulin receptors on B lymphocytes. The antigen-selected B lymphocytes are then combined with myeloma cells by exploiting strong and specific interactions between biotin and avidin. Finally, B lymphocyte-myeloma cell complexes are selectively fused by electrical pulses. This entire pathway could be successfully confirmed on the basis of immunofluorescence analysis. The new technology confers at least a 5–40-fold increase in efficiency over that obtained with the poly(ethylene glycol)-mediated method. The advanced technology may also be applicable for generation of human antibodies for medical purposes using transgenic mice and for selective production of stereo-specific monoclonal antibodies against native structural antigens using antigen-expressing myeloma cells.

A2.29
Dimeric thiourea linked GlcNAc are molecular switches that trigger the antitumor potential of natural killer cells due to a sequential cooperative engagement of activating receptor CD161 linking innate and adoptive immunity

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Activating lectin-type receptors on natural killer (NK) cells such as CD161 (NKR-P1) have been shown to react with N-acetyl-D-glucosamine (GlcNAc) conjugates resulting in partial protection against tumors in animal models. We describe here optimized compounds linking two GlcNAc residues to alkyl via thiourea bonds. GlcNAc decyl dimers can efficiently precipitate the A isoform of NKR-P1 in both rat and mouse NK cells, and activate NK cells at concentrations as low as 10–10 M. When administered into melanoma bearing mice, GlcNAc dimers can provide a permanent protection in 70% of animals. This is due to activation of NKT cells, and subsequent tumor infiltration by active $CD8^+$ T cell. The exceptional signaling efficiency of GlcNAc dimers is explained by sequential cooperative engagement of the target receptor leading to large signaling complexes of about 20 MDa containging G proteins, β-arrestin, phosphorylated dynamin, Src tyroxine kinases, Vav, Rac1, Grb2 and Ras. Supported by grants by Ministry of Education of Czech Republic (MSM_21620808 and 1M0505), by the Institutional Research Concept for the Institute of Microbiology (AVOZ50200510), by Czech Science Foundation (303/09/0477 and 305/09/H008), and by the European Commission (Project Spine 2 Complexes, contract LSHG-CT-2006-031220).

A2.30
Conformation dependent continuous antigenic epitopes

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Continuous, or linear, antigenic epitopes are common to proteins and peptides. The accessibility of continuous epitopes often depends on protein/peptide conformation and its proximity to disulfide bridges. Temperature dependence of the equilibrium binding constants and the kinetic rates were studied for mAb 106.3 and mAb3-631 by means of fluorescence spectroscopy. This antibody recognizes a relatively short amino acid sequence in the loop between cysteines 10 and 26 of human B-type natriuretic peptide (BNP) which is a cardiac hormone that regulates blood pressure and vascular water retention. Thermodynamic parameters including changes in the free energy, enthalpy and entropy measured at equilibrium are in a good agreement with the parameters calculated from kinetic data. The differences in thermodynamic parameters measured for the two antibodies under study support structural data obtained by NMR and X-ray crystallography.

A2.31
Biotin-kodecytes – novel function-spacer-lipid (FSL) modified cells capable of being recovered from the circulation after 3 days

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The ability to modify a population of blood cells with both an antigen of interest and a recovery label, infuse them into the circulation of an animal, and then visualize or recover a sample of the infused cells some days later for analysis, is now possible through the use of FSL (function-spacer-lipid) constructs. Murine kodecytes bearing both blood group A antigen (1) and biotin (A + biotin-kodecytes) were created by incubating murine red cells with a solution of FSL-biotin and FSL-A. These A + biotin kodecytes were then infused into the circulation of laboratory mice. Blood was sampled (0.05 ml) at specific time points post transfusion and using the secondary reagent, avidinAlexfluor, the infused kodecytes could be identified in blood films for periods of up to 96 hours in naïve mice. When the same A + biotin-

kodecytes were infused into laboratory animals with circulating anti-A (stimulated by immunization with A substance), the A + biotin-kodecytes were observed to have significantly reduced survival times. Control kodecytes with either FSL-biotin, or FSL-biotin plus an innocuous FSL antigen (e.g. GB3), gave normal survival times in immunized and naïve animals. By using avidin coated microparticles, biotin-kodecytes could be purified from whole blood samples and subjected to further *in vitro* analysis. The results of this work demonstrate a novel technique for both determining *in vivo* cell survival and for the recovery of cells that have been exposed to the circulation for several days.

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Reference:

1. Frame T et al *Transfusion* 2007; **47:** 876–882.

A2.32
Significant difference in antiviral unit of different interferon in stimulating expression of interferon stimulated gene 15

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Interferon stimulated gene 15 (ISG15), an ubiquitin cross-reactive protein, could conjugate to target proteins. Unlike ubiquitination, protein ISG15 modification did not target protein for degradation, but enhanced the cellular response to interferon, which played a key role in antiviral response. In this study, western blot and/or immunocytochemistry were performed to explore minimum antiviral units of interferon-α, -β, -τ in stimulating saturation expression of ISG15 by explants of bovine endometrium and mammary gland, as well as Madin CDarby bovine kidney (MDBK), endometrial and mammary cells. Western blot indicated differential minimum antiviral units among recombinant human interferon-α (rhIFN-α, 100 IU/ml), rhIFN-β (1000 IU/ml) and recombinant bovine interferon-τ (rbIFN-τ, 10,000 IU/ml) in stimulating saturation expression of free and ISG15 conjugated proteins by MDBK cells, endometrial and mammary explants. The above results were further confirmed through immunocytochemical analysis by use of MDBK, endometrial and mammary cells. The expression patterns of ISG15 conjugated proteins by different explants were various at the same antiviral unit of the same interferon. In conclusion, there were 10 to 100 fold differences in minimum antiviral units of rhIFN-α, rhIFN-β, and rbIFN-τ in stimulating saturation expression of ISG15, and the different expression patterns of ISG15 conjugated proteins by different tissues might lead to different antiviral response on different tissues with the same interferon.

A2.33
The role of tyrosyl-tRNA synthetase in heart failure development

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Background: Autoantibodies (auAbs) directed against the aminoacyl-tRNA synthetases are associated with myositis, arthritis, Raynaud's phenomenon, fever and interstinal pneumonia, systemic lupus erythematosus and rheumatoid arthritis. N-terminal catalytic module of tyrosyl-tRNA synthetase (YRS) can function as a cytokine and act as a factor that stimulates angiogenesis. It is very promising in terms of new cardiotropic drugs development, important for the treatment of common cardiovascular diseases such as myocardial infarction and heart failure.

Materials and methods: We developed of monospecific polyclonal anti-YRS antibodies directed against of full-length form of YRS using original immunization procedure. The level of specific anti-YRS autoantibodies were examined in sera of patients bearing of dilated cardiomyopathy (DCM) as chronic stage of heart failure progression in comparison with normal ones. The level of YRS expression in DCM-affected human hearts were identified by Western-blot analysis in comparison with normal samples. The time course changes of YRS expression were studied by immunoblotting in mouse hearts with experimental DCM-like autoimmune damage of myocardia.

Results: The increased level of YRS expression (both full-length enzyme and truncated N-terminal module) have been observed in cardiomyocytes from DCM-affected heart in comparison with normal ones. These changes accompanied by significant increase of anti-myosin autoantibodies level detected in DCM patients sera as well as in mouse model sera.

Conclusions: This results show a potential role of YRS in heart failure development and could be a real base for new diagnostic tools development.

A2.34
Optimization of recombinant expression of human NK cell receptors NKRP1 and LLT1 in HEK293 cells

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Natural killer cells are an intensively studied part of immune system due to their ability to directly kill cancer cells. Recent research in their C-type lectin-like receptors repertoire has shown that ligands of some of these previously orphan receptors are lying within their own family, describing a lectin-lectin interaction. This is also the case of human inhibitory receptor NKRP1 and its ligand LLT1. It was shown that overproduction of LLT1 in cancer cells or lower production of NKRP1 in NK cells is connected to cancerous manifestations. Previous efforts to study this system on a structural level via recombinant expression in *E. coli* have shown that the proteins aggregate to inclusion bodies and their refolding is rather impossible. Moreover, the presence of glycosylation might be required for lectin-lectin interaction. Here, we present successful expression of human NKRP1 and LLT1 in eukaryotic expression system based on transient transfection of HEK293 cell line. Both proteins were produced in small scale, purified by IMAC affinity chromatography followed by gel filtration to homogeneity and correct fold was verified by mass spectrometry. Next, we optimized suspension cultivation of HEK293T and 293-6E cell lines in different media and their transfection conditions using easily quantifiable markers, secreted alkaline phosphatase (SEAP) and green fluorescent protein (GFP). This should lead to large scale production of human NKRP1 and LLT1 or other NK lectin-like receptors and eventually to structural and biophysical studies of these proteins. This work is supported by the European Commission (Integrated project SPINE2-COMPLEXES, contract No. 031220).

A2.35
Anti-inflammatory effect of 5′-nitro-indirubinoxime in human umbilical vein endothelial cells

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Indirubin is an active compound of *Polygonum tinctorium* Lour. (*P. tinctorium*). Recently, we synthesized the novel indirubin derivative, 5'-nitro-indirubinoxime (5'-NIO), and demonstrated that it has more potent anti-tumor activity *in vitro* and *in vivo* than any other reported indirubin derivatives. Although indirubin is also known to have anti-inflammatory activity, its mechanism of action is poorly understood. In this study, we evaluated the effect of 5'-NIO on the TNF-α induced inflammatory conditions of human umbilical vein endothelial cells (HUVECs) to determine if it would be useful as an anti-atherosclerotic agent. We found that 5'-NIO significantly inhibited the TNF-α-induced expression of MCP-1 and IL-8 in HUVECs. Furthermore, 5'-NIO strongly inhibited the adhesion of U937 cells onto the endothelial cells by decreasing expression of the adhesion molecules, ICAM-1 and VCAM-1. We also demonstrated that 5'-NIO plays its anti-inflammatory effect through the inhibition of NF-κB nuclear translocation and JNK activation. *In vivo* study using Sprague-Dawley rats showed that 5'-NIO treatment markedly attenuates the LPS-induced inflammatory cell infiltration, confirming the anti-inflammatory effect of 5'-NIO *in vivo*. Taken together, these findings indicate that 5'-NIO is a novel candidate with the potential for the treatment of atherosclerosis.

A2.36
Development and preclinical test of novel attenuated recombinant bacillus Calmette-Guerin vaccine rBCG-ABM01 for the intravesical bladder carcinoma immunotherapy

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Mycobacterium bovis bacillus Calmette Guerin (BCG)- immunotherapy has a well-documented and successful clinical history in the treatment of superficial bladder transitional-cell carcinoma (TCC). Nevertheless, regularly observed side effects, a certain degree of nonresponders and restriction to superficial cancers remain a major obstacle. Thus, alternative treatment strategies are intensively being explored. Here we reported the ability of multivalent vaccines of the recombinant BCG (rBCG-ABM01) in enhancing anti-cancer immunotherapy. Attenuated *M. bovis* BCG was engineered to secrete specific subunit antigens. The efficiency of protein was detected by flow cytometry and Wetern blot analysis. Antitumor effects were monitored by bioluminescence-imaging system (BLI), with measurement of cytokines and phenotyping of infiltrating lymphocytes in a murine orthotopic bladder-tumor model. Seven days after immunotherapy, mice treated with rBCG-ABM01 were found to have significantly higher Th1-polarized IFN-gamma concentrations than mice in the untreated controls. The frequencies of IL-2, IFN-gamma, and TNF-alpha producing cell were demonstrated higher than controls by cytometric beads array and quantitative PCR, respectively. A highly immunopotent rBCG-ABM01 was developed and it elicited immune responses with a high serum IFN-gamma level, inhibited tumor growth and prolonged survival in tumor bearing mice. Conceivably, the studies should provide clues leading to elucidate the role of the potential usefulness of recombinant BCG vaccine in human bladder carcinoma.

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A2.37
Interleukin-6, tumor necrosis factor-α polymorphisms and HBV infection status in two transplant patient groups

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Introduction: Single nucleotide polymorphisms (SNPs) in regulatory regions of IL-6 (-174 G/C) and TNFa (-308 A/G) have been shown to affect their expression and, consequently, interfering infections in humans. The aim of this study was to investigate the frequency of genotypes associated with the mentioned SNPs of IL-6, TNF-a and to determine their relation with hepatitis B virus (HBV) infection in two transplant patient groups.
Materials and methods: Hundred twenty-two patients have been enrolled in the study, 50 liver transplant (LT) and 72 kidney transplant (KT) recipients. HBV infection status have been investigated by serology and quantitative PCR. Cytokines SNPs have been identified by sequencing. Results have been statistically reasoned with Pearson's chi-square test.
Results: Sequencing and viral results showed that GG genotype at position -174 in IL-6 gene is predominant in LT patients with HBV infection markers, and GC genotype is more frequent in LT patients HBV free. Statistics revealed a significant correlation between G allele at IL-6 SNP and the absence of HBV infection in LT recipients. For KT patients HBV+, GG genotype at IL-6 SNP is more frequent. In both patient groups GG genotype at position -308 in TNF-a gene was predominant. For KT patients results showed a TNF-a genotype distribution, considering HBV infection status, near statistical significance.
Conclusions: G allele at position -174 in IL-6 gene seems to correlate with the absence of HBV infection in liver transplant recipients and A allele at position -308 in TNF-a gene is possible to be more present in kidney transplant recipients HBV negative.

A2.38
Inhibitory effect of phenolic components from Sargassum thunbergii on allergic inflammatory response in human basophilic KU812F cells

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In this study, we isolated three phenolic components, sargahydroquinoic acid and sargachromanol from Sargassum thunbergii by diverse chromatographic methods and investigated their anti-allergic effects. The anti-allergic effect of three phenolic compounds was explored by measuring cytokine production and

serum histamine level in A23187 stimulated human basophilic KU812F cells. Treatment with three compounds decreased histamine release and cytokine level such as interleukin-4 (IL-4), interleukin-13 (IL-13) and tumor necrosis factor-α (TNF-α) in A23187 stimulated KU812F cells. Furthermore, we examined their effects on FceRI expression, which is a high-affinity receptor for IgE on the cell surface. RT-PCR and Western blot analysis revealed that the phenolic components inhibited expression of FceRI receptor in both mRNA and protein levels. Data obtained from these results showed that three phenolic components from S. thumnergii exerted anti-allergic effect through negative-regulation of FceRI expression and decreased histamine release.

A2.39
Cell-free expression of intrinsically disordered subunits of multichain immune recognition receptors for NMR investigations

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Multichain immune recognition receptors (MIRRs) represent a class of surface receptors, expressed on cells of the immune system, responsible for the signalling cascade triggered by ligand binding to the extracellular domains. This signalling is achieved by intrinsically disordered cytosolic subunits of the receptors, containing immunoreceptor tyrosine-based activation motifs (ITAMs). Phosphorylation of these tyrosines serves as the first intracellular signalling step. The disordered cytosolic peptides of the T-cell receptor (Cd3ε, Cd3γ, CD3δ), the B-cell receptor (Igα, Igβ) and the IgE receptor on mastcells and basophils (FCεRIγ) have been included in this project. The different genes were cloned into the pEXP5-NT/TOPO vector and a S12 extract prepared from *Escherichia coli* was used as a basis for cell-free protein synthesis. In general, expression of peptide molecule suffers from low expression yields and a rapid proteolysis of the expressed peptides. All tested constructs were expressed in milligram quantities suitable for characterization with NMR. Both fully labelled 15N and 13C amino acids as well as selective labelling of selected amino acids, have been used to produce ITAM-containing peptides for NMR spectroscopy. Resonance assignments for two of the expressed subunits are currently underway.

A2.40
Leukapheresis filters-derived monocytes dedifferentiate into promonocytic cells with proliferation ability

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Background: Monocytes can differentiate into macrophage species and can be dedifferentiated and re-differentiated into several other cell types. We examined monocytes' ability to dedifferentiate into an ancestor form and the possibility of dedifferentiated cells cultivation and proliferation.
Methods: Monocytes were isolated from leukapheresis filters after transfusion. They were cultured for 7 days either in dedifferentiation medium containing IL-3, M-CSF and beta-mercaptoethanol (DD) or in control medium (C). Dedifferentiation was tested by flow cytometry using CD14, CD45, CD34, CD90, CD115, CD29 and CD105 markers, by fluorescent microscopy as well as by estimation of 3H-thymidine incorporation. After being dedifferentiated, cells were cultured in RPMI 1640 medium for 1 month.
Results: Monocytic lineage markers CD14 and CD45 levels remained unchangeable for DD cells and control group. Promonocyte markers CD34, CD90 and CD115 in DD cells were significantly higher as compared to the controls. Considering mesenchymal stem cell markers CD29 and CD105, no significant difference between the two groups was observed. Dedifferentiated cells were successfully cultured and multiplied and could be stored under liquid nitrogen without losing their characteristics.
Conclusions: Leukapheresis filters-derived monocytes are capable of dedifferentiating into promonocytic cells committed in monocytic lineage, but they do not present mesenchymal stem cells markers. Given that the dedifferentiated cells can be further cultured and proliferated, these cells may represent an abundant human cell source that can be used for investigating dedifferentiation and transdifferentiation mechanisms.

A2.41
Calcium influx induced by the Adenylate Cyclase Toxin from *Bordetella pertussis* triggers endocytic trafficking of integrins

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The adenylate cyclase toxin (ACT) of *Bordetella pertussis*, is a 1706 residue-long polypeptide that contains a N-terminal adenylate cyclase activity bearing domain and a pore forming domain that harbours ≈40 calcium-binding nonapeptide repeats characteristic of the RTX (Repeats in Toxin) family. ACT targets primarily $CD11b^+$ phagocytes and translocates across their cytoplasmic membrane the catalytic adenylate cyclase domain producing a rapid increase of intracellular cAMP that suppresses its bactericidal functions. Recently, we and others have reported that ACT induces intracellular calcium elevations that do not correlate with the toxin-induced cytotoxicity. The aim of the present work was to study the cellular consequences of the toxin-induced calcium influx. We show that the ACT-induced calcium rise is followed by CD11b/CD18 mobilization into raft-like microdomains. This movement is the result of the hydrolysis of a cytoskeletal protein, talin, likely through calpain activation. Migration of the β_2 integrin seems to be consequence of calcium influx and not the result of a direct interaction with ACT, as the cation rise also induces the migration of β_1 integrins, that are not specific receptors for the toxin. We also show that ACT and integrins, along with raft markers GM1 and flotillin-1, are rapidly internalised in a toxin-induced calcium rise-dependent process affecting an essential function of these immune cells i.e. adhesion. The removal from leukocyte plasma membrane of domains that contain key molecules such as integrins, may represent an advantageous strategy followed by pathogenic *Bordetella* to circumvent the host immune system.

A2.42
Structural studies of staphylococcal enterotoxin H in complex with T cell receptor and major histocompatibility complex class II

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Superantigens (SAgs) are bacterial toxins capable of cross-linking the immune receptors of the host, the T cell receptor (TCR) and major histocompatibility complex (MHC) class II, and thereby trigger a massive release of cytokines. This could lead to toxic shock syndrome, which can have a fatal outcome. Here, we present the crystal structure of the ternary complex between the superantigen, staphylococcal enterotoxin H (SEH), TCR and MHC, as well as the dimer complex, including only TCR and SEH. It is evident that SEH interacts with the variable α domain (Vα) of TCR, in sharp contrast to previously studied SAgs that interact with the Vβ domain. Due to the high structural conservation of amino acids in SEH that are crucial for the interaction, we propose that in addition to Vβ activation of T cells, there are SAgs, in addition to SEH, which are able to activate T cells through Vα as well. In addition to providing crucial information regarding the nature of TCR-mediated recognition of superantigens, the finding have central implications for future strategies aimed at preventing or modulating the often pathogenic response to superantigens.

A2.43

Abstract withdrawn

A2.44
Expression of rat NK cell receptor NKRP1B and its ligand Clrb in HEK293T cell line and their biophysical characterization

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Natural killer cells play a significant role in the immune response against tumor and infected cells. NK cells express a wide variety of surface receptors, including NKRP1, a C-type lectin-like family of both activating and inhibitory receptors. Recently, ligands have been found for some of these previously orphan molecules, some of them lying within the same family. This is also the case of rat Clrb as a cognitive ligand for rat NKRP1B. It has been shown that in rat, this inhibitory NKRP1B-Clrb mutual receptor system is subverted by rat cytomegalovirus protein RCTL, a viral version of Clrb, which serves as a decoy ligand for NK cells [1]. Here we present successful cloning and production of the above mentioned C-type lectin-like proteins based on transient transfection of HEK293T cell line in a suspension culture. This expression system allows us not only to obtain proteins of our interest with a satisfactory yield but also in their native conformation, removing the need for time consuming and often fruitless refolding procedures required in case of using the *E. coli* expression system. Moreover, the proteins are obtained in their glycosylated form. As glycosylation might participate on the lectin-lectin interaction this is considered an additional benefit of the system. Proteins were purified using IMAC followed by gel filtration, identified by mass spectrometry, characterized by analytical ultracentrifugation and Raman spectroscopy and crystallization trials were set up. This work is supported by the European Commission (Integrated project SPINE2-COMPLEXES, contract No. 031220).

Reference:

1. Voigt S et al, *Immunity* 2007

A2.45
Characterization of ZG16, a lectin-like protein found in the mucus layer of the intestine

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The mammalian intestine harbors complex societies of beneficial bacteria coexisting with the host. The first line of defense preventing these microorganisms to invade the underlying epithelia is a mucus layer with the heavily O-glycosylated MUC2 mucin as the main structural component. In order to obtain a greater understanding of the regulation, function and structure of this barrier we analyzed the mucus using proteomics to find additional components. One of the proteins found and further studied were the lectin-like protein ZG16, a small protein with 167 amino acids containing a signal sequence and a jacaline-like lectin domain. To address the protein properties, ZG16 was cloned, expressed in mammalian cell system and an antiserum was produced by the immunization of rabbits with the purified protein. Binding studies did not reveal any direct interaction with the MUC2 mucin or different glycoforms expressed on recombinant MUC1. Insted a strong binding to peptidoglycan, a bacterial cell wall element, was observed. As expected ZG16 also binds gram positive bacteria as they have peptidoglycan exposed to the surroundings. The viability of the bacteria after binding was unaffected in proliferation assays suggesting that ZG16 not have a direct bactericidal effect.

A2.46
Myelin basic protein peptide 46-62 ameliorates ongoing experimental allergic encephalomyelitis in DA rats

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The pathological role of antibodies raised against endogenous antigens in autoimmune diseases is widely accepted. Direct penetration of autoantibodies through the blood-brain barrier and their co-localization with neural tissue-specific autoantigens may explain their possible contribution in the neurodegeneration. Previously, we demonstrated that autoantibodies in multiple sclerosis (MS) reveal site-specific binding and cleavage activity toward myelin basic protein (MBP). Using recently created MBP-derived recombinant ''epitope library'' covering the entire protein sequence we determined epitopes of myelin basic protein specific for the autoantibodies isolated from MS patients. Further we administrated these peptides to DA rats with induced protracted relapsing experimental allergic encephalomyelitis (EAE) most closely related to MS. DA rats with EAE induced by syngenic spinal cord homogenate in complete Freund's adjuvant were treated by human MBP fragments 46–62, 81–102, 124–139, and 147–170 in native and liposome form. MBP 124–139 and 147–170 displayed only mild therapeutic effect but MBP 46–62 significantly reduced EAE, reflected by lower clinical scores and shorter EAE duration compared to controls. Treatment of DA rats by respected peptides entrapped in liposomes increase their therapeutic efficiency. Thus, obtained data may be used for effective and directed treatment of Multiple Sclerosis opening new paths in struggle against neurodegenerative diseases.

A2.47
Neurotrophin-3 adaptation marker to chronic and acute brain hypoxia

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Background: Chronic obstructive pulmonary disease (COPD) is a major cause of chronic hypoxia and cerebral ischemia involving a complex signaling cascade with an at least partial unraveled spatiotemporal pattern. In acute ischemia (ischemic stroke-IS), early excitotoxicity can lead to fast necrotic cell death, which produces the core of infarction. While brain cells are challenged by this deleterious mechanisms, they activate innate protective programs: synthesis of inflammatory cytokines and neuronal growth factors, members of neurotrophins family, suggesting that neuronal death is associated with an inflammatory reaction and a protective response. In chronic brain hypoxia, the inflammation intensity and the protective response efficiency are poorly characterized. The aim of this work was to begin the NT-3 comparative study between patients with COPD (chronic brain hypoxia) and patients with ischemic stroke (acute brain hypoxia), in order to establish a possible model of protective brain response to hypoxia.
Patients and methods: Forty-five patients were investigated after computed tomography-confirmed IS. We used ELISA to determine serum level NT-3 (as marker of the brain adaptation response). The results were compared with those obtained in 40 patients with confirmed COPD.
Results: significant increase of NT-3 serum levels was obtained in COPD compared with normal and ischemic stroke lots.
Conclusion: Increased neurotrophin-3 serum level could represent an important marker of neuroprotection, this neurotrophic factor being a potential therapeutic agent in acute or chronic brain hypoxia.

A2.48
Interleukin 8 – leader of the inflammatory cytokines in chronic obstructive pulmonary disease

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Background: Chronic obstructive pulmonary disease (COPD) pathophysiology is mainly characterized by inflammation throughout the central and peripheral airways, lung parenchyma, and pulmonary vasculature. Local defensive mechanisms are triggered by inflammatory stimuli, followed by signal transduction and activation of the transcription factor NF-kB, with synthesis of proinflammatory cytokines tumoral necrosis factor alpha (TNFα), interleukin 1-beta (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10), etc. Inflammatory cytokines IL-1β, TNFα, IL-6, IL-8 elicit defensive responses in lung parenchymal cells and bronchial cells, including activation of apoptosis. On the other hand, IL-10, the inhibitory cytokine, could also play an important antiapoptotic role, stimulating organ repair after injury. In addition, IL-8 promotes neutrophilia as well as neutrophils infiltration of bronchial tissue. We were interested in studying the cytokine signals which are involved in COPD development.
Patients and methods: Forty patients with confirmed COBP were investigated using blood samples. IL-1β, TNFα, IL-6, IL-8 and IL-10 were measured using ELISA kits.
Results: Even all cytokines serum levels were increased comparing with normal, only IL-1β, TNFα and IL-8 presented significant changes. The biggest concentration was obtained for IL-8.
Conclusion: Great serum level of IL-8 and its role of main chemoattractant factor for neutrophils strongly suggests that neutrophils activation and cytotoxicity are the main cause for the airways, lung parenchyma, and pulmonary vasculature damage in COPD.

A2.49
Helicobacter pylori neutrophil-activating protein (HP-NAP) activates the MAPK and PI3K/Akt pathway in human mast cells

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Helicobacter pylori (H. pylori), a Gram-negative bacterium, is thought to infect over half of the human population. *H. pylori* infection induces acute and chronic inflammation and plays a key role in gastric mucosal diseases. The neutrophil-activating protein of *Helicobacter pylori* (HP-NAP), one of its virulence factors, has been identified to activate neutrophils, monocytes, and mast cells. However, the mechanism of HP-NAP activates mast cells is not fully understood. In this study, we show that HP-NAP induces histamine release and interleukin-6 (IL-6) production in human mast cells. We also found that HP-NAP induces extracellular regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK), and Akt activation in human mast cells. The kinase inhibitor of MAPK/ERK, p38 MAPK and phosphotidylinositol 3-kinase (PI3K) suppress HP-NAP-induced IL-6 production. These results indicate that HP-NAP activates human mast cells through MAPK and PI3K/Akt pathway.

A2.50
Human lubricin expresses the sialyl Lewis x determinant and has L-selectin ligand activity

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Synovial fluid is responsible for lubricating the joint, which is the primary tissue for symptoms of osteoarthritis (OA) and rheumatoid arthritis (RA). Lubricin is an abundant mucin-like glycoprotein in synovial fluid. Except lubricating and protection of cartilage, lubricin also posses other biological functions such as chondroprotection and control of synovial cell growth. In this study, lubricin's O-linked oligosaccharides isolated from OA and RA' synovial fluid were investigated by LC-MS. O-glycan on lubricin were found to be mainly sialylated core 1, but small amount of sialylated core 2 structures, containing both sulphate and fucose, was found. The presence of sulphated and fucoslylated O-glycans on lubricin imply that its glycosylation may have specific function in addition to the lubricating property provided by simple core 1 structures. Further study showed L-selectin present on human lymphocytes was able to bind to lubricin. This indicates that lubricin may be involved in inflammatory cells recruitment influencing the L-selectin facilitated tethering.

A2.51
Promiscuity of binding/catalysis of antibodies – antidotes toward chemical weapons

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Immunoglobulins were shown to serve as an excellent template to generate de novo catalytic active centers. Both combinatorial and rational design approaches were used to improve their catalytic efficiency for numerous reactions. Organophosphorous poisons, (OP, nerve gases) may be regarded as one of the intriguing targets to develop antibody-based capturing machinery. Previously we obtained rationally designed antiidiotypic antibodies 9A8 against acetylcholinesterase which was shown to have pronounced specificity of its active center toward OP analogs. Alternatively using combinatorial selection of human phage display library by non-toxic phosphorus compounds we selected A17 ScFv antibody able to capture OP analogs. This antibody was expressed as full length antibody and Fab-fragment of it was crystallized and two 3D structures of non-modified and modified by phosphonate residues were solved with resolution 1.5 and 1.36 Å respectively. The 3D structures of 9A8 and both A17 antibodies were compared and mechanism of OP capturing was described. The microcalomitric studies allowed us to make quantitative conclusions concerning active sites of both antibodies. Rationally designed 9A8 has more dance active site, alternatively combinatorial selected A17 poses ''primitive'' rigid active center. 9A8 antibodies was interact with fluorescent analog of soman. A17 antibody hydrolyze paraoxon with low rate constant. These antibodies displayed certain specificity toward DNA. Rationally designed 9A8 was characterized as specific dsDNA binder and A17 was shown to catalyze dsDNA cleaving reaction. Thus both Abs has pronounced promiscuity of their catalytic centers.

A2.52
Molecular characterization of exhaled endogenous particles – a novel biological matrix non-invasively sampled from the respiratory tract

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We have recently developed a novel non-invasive method to sample non-volatile material in exhaled air [1]. The method is based on sampling exhaled endogenous particles using a cascade impactor. At present, little is known about molecular composition of the exhaled particles (PEx). It is expected that PEx composition is similar to the one of the respiratory tract lining fluid (RLTF). Here we present initial results on molecular characterization of PEx. The major component of RTLF is respiratory surfactant, consisting of phospholipids and proteins. Therefore these classes of molecules were our primary investigation targets. PEx were collected on silica plates using in-house built sampling device [1]. For lipid analysis, individual samples (30 l exhaled volume) were extracted and analyzed directly by MALDI TOF MS. For protein analysis, pooled samples from several individuals were collected (3000 l and 4400 l total exhaled volumes). Extracted proteins were separated by SDS-PAGE, subjected to in-gel trypsinolysis and analyzed by LC-MS for protein identification. Positive mode MALDI MS resulted in identification of several phosphatidylcholine (PC) species, with PC (16:0/16:0) being the most abundant as expected for pulmonary surfactant. Proteomic analysis of PEx resulted in identification of 235 proteins. A number of known pulmonary proteins, i.e. surfactant proteins, CC16, mucins were detected along with major plasma proteins. Identification of surfactant specific lipids and proteins in PEx confirms their origin to RTLF, making PEx a highly interesting biological matrix for monitoring biochemical changes in the distal airways.

Reference:

Almstrand, AC et al., (2009) *Anal.Chem.* 81, 662.

A2.53
Eukaryotic expression as an indispensable tool for preparation of native dimeric forms of NK cell C-type lectin-like receptors

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Natural killer cells are able to recognize and kill a variety of tumor and infected cells. The recognition is mediated by wide repertoire of cell surface receptors, both activation and inhibitory, belonging to two main structural families: immunoglobulin-like and C-type lectin-like. While ligands for the Ig-like receptors were shown to be MHC gp I proteins, ligands only for some of the lectin-like receptors are known up to date. Both families share relatively weak binding characteristics and in the case of lectin-like receptors, *in vitro* oligomerization clearly improves binding through increase in avidity. However, these lectin-like receptors were described mostly as dimeric *in vivo* and this minimal oligomer would be also the best for structural studies. Moreover, there is no crystal structure known of extracellular domain of any of these receptors in its full-length natural dimeric form; probably because prokaryotic expression of native dimers is rather difficult. Here we present successful design, cloning and production of dimeric forms of some mouse, rat and human NK cell lectin-like receptors belonging to NKRP1 and Clr families. Full-length extracellular receptor parts were expressed via transient transfection of HEK293 cell lines in suspension culture either alone or as a cleavable fusion with Fc fragment of human IgG1 to promote native dimerization. This fusion strategy resulted in cleaved pure covalent dimers of e.g. rat Clrb and purely dimeric Fc fusion of rat NKRP1B proteins and their structural characterization is currently under way. This work is supported by the European Commission (Integrated project SPINE2-COMPLEXES, contract No. 031220).

A2.54
New aspects of erlich ascites carcinoma development

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Erlich Ascites Carcinoma (EAC) cells growth kinetics *in vitro* and that correlation with infected mice surveillance are reported in present abstract. EAC cells were suspended in culture media

RPMI-1640, supplemented with 10% of bovine serum in penicillin vials. Incubation carried out in humidified chamber at 5% CO2 and 370C. The growth kinetics described with classical phases: lag-phase 0–3 days; exponential growth phase 3–12 days; stationary phase 12–16 days; decline phase 16–24 days of cultivation. Cell concentration droppings had been detected three times during 24 days long cultivation: at 7th, 14th, and 21st days. The experiment had been repeated several times, every of which confirmed reliability of fined out result. Observed EAC cell dropping phenomenon had not been described earlier for *in vitro* conditions, but is in sound with the *in vivo* observation described by Zamai A.S. (2007). EAC cells infected mice surveillance was also examined. High level of mortality at 10–14 days was observed. Analysis of correlation between cells growth *in vitro* and mice surveillance *in vivo* revealed those considerable inverse value (Spearman r: -0.89, p value 0.0020**) at 7–15 days of the experiment. These results let us to assume that observed EAC cells growth kinetics reflect the EAC fate in mice organism. This may be a substantial contribution to the tumor diagnosis and specification of period of malignant process. The observed phenomenon of EAC cells concentration dropping at their cultivation is an object of our current researches. We hope it will contribute to the understanding of details of cancer development and suggest more purposeful treatment.

A2.55
Effect of methylsulfonylmethane on LPS-IFN-gamma induced RAW 264.7 macrophage apoptosis

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Natural organosulfur compound methylsulfonylmethane (MSM) is a metabolite of dimethyl sulfoxide which occurs at low levels in various foods. It has been extensively used as a dietary supplement for its potential to improve human health and to reduce arthritic pain. When activated with LPS-IFN-gamma, macrophages undergo apoptosis time and dose dependently. Abnormal release of NO and cytotoxicity mediated by excess NO may lead to inflammation and tissue injury. Therefore, besides reduction in NO generation, therapeutic strategies aimed at inhibiting NO-dependent cell apoptosis may contribute to improving the outcome of sepsis. In this study we aimed to show the effect of MSM on LPS-IFN-gamma stimulated RAW 264.7 macrophage apoptosis.Cells were seeded to 6-well plates and co-incubated with MSM for 1 hour. Following co-incubation, cells were incubated in the absence or presence of LPS-IFN-gamma for 24 hours. Proliferation of cells was estimated by MTT test. According to MTT results, treatment with LPS-IFN-gamma significantly decreased cell proliferation and MSM increased proliferation of activated macrophages. Cells were then stained with Annexin V-FITC and observed and counted under a fluorescence microscope to identify if this enhanced proliferative effect of MSM was a result of protection against apoptosis. Our results have shown that LPS-IFN-gamma significantly induced macrophages to apoptosis and MSM protected macrophages from LPS-IFN-gamma induced apoptosis ($p < 0.05$). This proliferative effect may elicit from inhibition of NO production by MSM. This study shows that MSM exerts permissive antiapoptotic activity and can be used in disorders related with NO dependent apoptosis.

A3 – Metabolic Diseases

A3.01

Abstract withdrawn

A3.02

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A3.03

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A3.04
Silybin improves liver injury in experimental non alcoholic fatty liver disease

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Non Alcoholic Fatty Liver Disease (NAFLD) has a complex pathophysiology in which oxidative stress plays a pivot role. Silybin, a flavonolignan extracted from milk thistle, exerts a marked liver protecting action in a variety of experimental liver injuries. We aimed to clarify if silybin treatment may favourably impact on liver injury progression in an animal model of NAFLD. We explored the effects of a 4-week daily (20 mg/kg i.p.) administration of silybin in 6-week-old db/db mice feeding a methionine-choline deficient diet. We evaluated liver histology by the NAFLD activity score (NAS); mitochondria morphology by electron microscopy; liver 8-OHG, GSH and NFkB by ELISA; hepatic nitrite/nitrate by colorimetrical assay; expression of iNOS and 3-nitrotyrosine by western blot and immunofluorescence; mitochondrial respiratory chain activity (MRC) by spectrophotometrical analysis. In db/db mice, silybin treatment markedly improved liver injury as measured by NAS ($p < 0.01$); significantly preserved cristae morphology and reduced hepatic triglycerides content ($p < 0.001$). Silybin administration strongly decreased liver TBARS, 8-OHG and increased GSH ($p < 0.0001$ for all). Strikingly, silybin completely restored hepatic MRC ($p < 0.0001$ for all the five complexes) while inhibited NFkB p65 and p50 subunits binding activity ($p < 0.0001$ for both); consistently, it abolished liver iNOS expression ($p < 0.01$) and reduced nitrite/nitrates ($p < 0.05$) and 3-nitrotyrosine levels. In conclusion, silybin exerts potent antioxidant and antiinflammatory effects in murine NAFLD providing a strong rationale for the use of this compound in the management of patients with NAFLD.

A3.05

Abstract withdrawn

A3.06
Renal fibrosis and evidence for epithelial-mesenchymal transition in patients with nephrolithiasis

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We quantified fibrotic lesion in renal tissues of nephrolithiasis patients and evaluated its association with renal function. Presence of epithelial-mesenchymal transition (EMT) in nephrolithiatic renal tissues was investigated. Patients diagnosed with large kidney stone formation ($n = 50$) and age and sex-matched healthy controls ($n = 37$) were recruited. Fifty-eight renal sections from 38 patients were stained and analyzed. Fibrosis was assessed by Masson's trichrome staining. Co-expression of epithelial cytokeratins and mesenchymal markers (α-smooth muscle actin (αSMA) and vimentin) in renal tubular cells was detected by dual immunofluorescence staining. Expression of fibronectin, transforming growth factor-β1 (TGF-β1) and CD68 were investigated. Kidney function was significantly reduced in nephrolithiasis patients compared to healthy controls. Inflammation grading in nephrolithiasis renal tissues was correlated with percent fibrotic area. Renal fibrosis was inversely correlated with renal function. Cytokeratins co-expressed with αSMA and vimentin were observed in nephrolithiatic renal tubular cells, and these cells strongly expressed fibronectin and TGF-β1. Infiltration of CD68-positive cells was a common finding in inflamed renal sections. Conclusion, kidneys of nephrolithiasis patients displayed robust signs of inflammation and fibrosis. A close correlation of renal fibrosis with renal dysfunction was demonstrated. This is the first study to show evidence for renal tubular cells displaying signs of EMT in stone-containing kidneys. Plausibly, TGF-β1 triggers EMT which at least in part contributes to stone-induced renal fibrosis.

A3.07
Schedule dependency of insulin therapy, the four compartment model and mathematical modeling of blood glucose control

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Objective: To improve schedules for insulin (life's central metabolic hormone) treatment with less needed dose and less hypoglycemic attacks, during instillation of a special preparation into the external auditory channel.
Research Design and Methods: BS was hourly measured in 20 and less frequently in an additional number of diabetics to plot

an approximate curve of blood glucose level and the probable changes based on mathematical tools (interpolation, Autoregressive moving Average etc.). Data of twenty six patients were included in the design the mathematical model. Auditory instillation was done as reported before.

Results: A series of unexpected hyper and hypoglycemia were encountered that confirmed release of glucose from a special reservoir (compartment III) rather than attributable to the counter regularity hormones. Conventional treatments act as contributory. The plotted curve is a sine curve with noise. Based on mathematical model intermittent treatment every 47 minutes where the second derivative is zero (wane of BS lowering mechanisms) is most effective with the lowest needed insulin dose and hence no hypoglycemic attacks. Patients showed excellent control with the intermittent schedule with doses in the range of 50–70% of their conventional dose.

Conclusions: In diabetics (not normal persons) point of maximal effect and maximal need are not coincidental. Treatment based on the insulin need causes large fluctuations of BS levels and on the latter best BS control with less cumulative dose and less hypoglycemic attacks, respectively. This is the sign qua non that fixed intermittent treatment is more effective than event based schedule or continuous infusions.

A3.08
Continuous control of diabetes mellitus by infusion of Auditory Insulin (AI) preparation into the ear via a piezoelectric pump

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Objective: Based on previous study, we tried to use a tiny piezoelectric pump with a weight of 2 + 10gr or 2 + 70gr to instill the newly described insulin preparation into the auditory channel to find whether schedule dependency does exist in reality in patients with diabetes mellitus.

Research design and methods: We used a sophisticated mathematical model which declares intermittent therapy every 47 minutes (here 60 minutes) as the most effective schedule. This was compared with previous routine insulin dose. Twenty six patients with type I and II were enrolled to this trial for two to 14 weeks after normalization of fasting blood glucose level. Doses equivalent to 1.5–4.5 IU per hour in a 16/24 hour or 24 hour/24 hour were used.

Results: All patients showed a significant reduction of their FBS and random glucose levels to under 120 mg/dl and under 180 mg/dl respectively. Glycosylated Albumin normalized in all patients and Hemoglobin A1C levels were reduced significantly. The needed dose of Insulin was around 50–70% of the sc dose previously assigned to the patient. Attack of hypoglycemia never happened and the sense of well being and compliance were excellently preserved. Despite similar effectiveness the smaller pump and the 16 hour/24 hour rather than the 24hour/24hour were better tolerated.

Conclusions: As predicted, treatment via the auditory channel route is extremely effective in reducing blood glucose level to nearly normal levels without the risk of hypoglycemic attacks. A combined planned (programmed drive electronics) and interferred (by patients based on daily events) technique is possible and ideal.

A3.09
Treatment of 212 patients with Type I and II Diabetes Mellitus with a novel Insulin preparation which is avidly absorbed if instilled into the auditory channel: The Hamlet/Polonius effect

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Objective: To find new routes of insulin treatment, we have used one of the most effective preclinical preparations with no accompanying toxic agent to instill into the external auditory channel.

Research design and methods: Two hundred and twelve patients with type I and II diabetes mellitus were enrolled. After discontinuation of all antidiabetic medications based on blood glucose levels 6–60 IU equivalent insulin was instilled into the ear of resting patients and blood glucose level were evaluated at 15, 30, 45, 60, 90, 120, 150 and 180 minutes and serum insulin at 120 minutes. Patients were monitored for local and systemic side effects. Ten patients received a 99mTc tagged insulin preparation and were scanned for site of absorption.

Results: BS levels decreased substantially in 185 patients (87%) and less pronounced in another 10 (4.7%) with an overall response rate of 91.7%. Type I patients showed a reduction of BS with relative higher insulin levels. No local or systemic side effects were encountered. Scintigraphy demonstrated sites of absorption.

Conclusions: We conclude that this treatment is feasible and extremely safe. Auditory cerumen (earwax) might contribute to absorption. In contrast to other routes external auditory channel has a residual bioavailability of 100% and an immediate bioavailability of 30–50% and can be regarded as the ideal route for both bolus and continuous treatments. It not only enables us immediate reactions to daily Physical activities, meals, stress and sex, many treatment schedules mimicking natural insulin oscillations become possible. Faster insulin preparations are underway.

A3.10
Radix *Paeoniae rubra* is inhibiting PTP1B inhibitor through Pentagalloylglucose

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The protein tyrosine phosphatase 1B (PTP1B) is one of the main negative regulators of insulin signalling. PTP1B knockout mice not only show increased insulin sensitivity but also are resistant to weight gain when put on a high fat diet. Therefore, the inhibition of PTP1B is of substantial interest for the treatment of type 2 diabetes mellitus and the metabolic syndrome. We chose PTP1B as a target for bioassay-guided fractionation of root material of *Paeonia rubra*, a plant widely used in Traditional Chinese Medicine against the metabolic syndrome. After showing that the crude methanolic extract of radix *Paeoniae rubra* displays PTP1B inhibitory activity in an *in vitro* enzyme assay we fractionated the methanolic extract with Sephadex® LH-20 column chromatography. Fractions were tested for their pharmacological activity *in vitro* and further separation on Sephadex® LH-20 and by preparative HPLC lead to the isolation of one active pure compound: Pentagalloylglucose (PGG),

which is a tannin widely spread in plants. PGG inhibited PTP1B in the enzyme-based assay with an IC50 of 4.75 μM. In order to evaluate the activity in a more complex system, a cell-based assay with insulin-exposed human hepatoma cells was established. Insulin receptor phosphorylation was taken as a read-out to show that PGG was able to enhance phosphorylation at tyrosines Y1158, 1162, and 1163, the target sites of PTP1B. In summary, we show here that PGG is an inhibitor of PTP1B which could account for the known antidiabetic activity of radix *Paeoniae rubra*.

A3.11
Xanthinuria type I in two Czech families: Biochemical and molecular genetics analysis

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Xanthinuria type I (OMIM 278300) is a rare disorder of purine metabolism caused by xanthine dehydrogenase deficiency. The affected individuals are characterised by strongly diminished production of serum uric acid (UA) and high urinary excretion of hypoxanthine and xanthine. The patients may develop calculi in the urinary tract, acute renal failure and myopathy due to deposits of xanthine. We report a two probands without clinical symptomps. Female (38 year.): the low concentration of UA in serum (15 μmol/l, ref. 120–340) and urine (0.04 mmol/l, ref. 0.4–4.6) was detected, in five occasions UA was under the limit of detection. Excretion of xanthine in urine was 170 mmol/mol Cr (ref. < 25 mmol/molCr). Her two sons were investigated with normal biochemical findings. Second patient – male (25 year.): the concentration of UA in serum was not detected in two cases, in urine 0.03 mmol/l, excretion of xanthine 141 mmol/mol Cr. Both parents and his brother had normal biochemical findings. Allopurinol loading test indicated xanthinuria type I, the xanthine oxidase activities in patients were 0 and 0.4 pmoles/hour/ml of plasma. The activity from heterozygotes was within the control ranges. Sequence analysis cover promotor, 36 exons and intron-exon boundaries. We found three previously undescribed nonsence changes. Heterozygous deletion p.P214QfsX4 was found in both patients and three relatives. The second heterozygous sequence variant R881X was found in female and her son, R825X in male and his mother. The haplotype and statistical analysis of consanguinity is in process. Supported by grant MSM0021620806 and MZOVFN2005 Czech Republic.

A3.12

Abstract withdrawn

A3.13
Molecular mechanisms of plasma glucose-lowering effect of LVVYPW in streptozotozin-induced diabetic rats

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LVVYPW is a member of hemorphins family, an endogenous nonclassical opioid peptides derived from hemoglobin. Hemorphins were proposed to serve as homeostatic factors that switch on the compensatory systems in the organism during severe pathologies. Very recently we have revealed the plasma glucose-lowering effect of LVVYPW. Experiments were done on male Wistar rats aged 8–10 weeks (weight 200–220 g). Rats received single intraperitoneal (ip) injection of streptozotocin (STZ) at 60 mg/kg to induce diabetes. After ip injection of LVVYPW (1 mg/kg) into fasting STZ-induced diabetic rats, this peptide has been shown to decrease plasma glucose levels from 26.8 ± 0.61 mmol/l to 18.2 ± 1.88 mmol/l in 2 hour. Naloxone and naloxonazine, μ-opioid receptors (MORs) antagonists, abolished the plasma-lowering effect of LVVYPW. Thus, MORs are involved in anti-diabetic effect of LVVYPW. Earlier we have found out that Ca^{2+}/calmodulin(CaM)-dependent protein phosphatase calcineurin is involved in the molecular mechanisms of hemorphins action in physiological and pathophysiological conditions. LVVYPW recovered calcineurin activity, increased in STZ-induced diabetic rat plasma and brain. It is proposed that molecular mechanisms of hemorphins action in both physiology and pathology involve integration of Ca^{2+}/CaM/calcineurin pathway with MOR function.

A3.14
Aerobic metabolism in Rod outer segments: molecular basis for oxidative stress-related retinal pathologies

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Rods are associated with scotopic vision. Rod outer segment (OS), where phototransduction takes place [1], consists of a stack of flattened disks surrounded by the plasma membrane. Previous proteomic and biochemical analyses reported the functional expression of the respiratory chain complexes I-IV and F1Fo-ATP synthase in OS disks. Our present study confirmed the presence of an oxidative metabolism in rod OS, a more native sample than disks, isolated by a simpler procedure from bovine retinas [2]. Rod OS were characterized for purity. An oxygen consumption (stimulated by glucose and reverted by rotenone, antimycin A and KCN) and ATP synthesis (0.560 ± 0.084 μmol/min/mg), sensitive to the common ATP synthase inhibitors, was measured in rod OS. The presence of Cytochrome c oxydase (COX) and F1Fo-ATP synthase in the sample was verified by Semiquantitative Western-immunoblotting. Morever COX was also catalytically active. Co-localization of COX and F1Fo-ATP synthase with Rodopsin in rod OS by immunohystochemical analysis on mouse ocular sections confirm results. Data indicate that an oxidative phosphorylation occurs in rod OS, which do not contain mitochondria, thank to the presence of ectopically located mitochondrial proteins. This study may shed light on the pathogenesis

of many retinal degenerative diseases that correlate with energy availability or oxidative stress in the photoreceptors and on the efficacy of empirical treatments, such as hyperbaric or vitamin therapies, in these pathologies.

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A3.15
Systemic redox modifications in acute diabetic foot ulcers

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Peripheral neuropathy and atherosclerotic peripheral arterial disease are implicated in diabetic foot ulcers pathogenesis. Oxidative stress is associated with diabetes mellitus. The aim of this study is to investigate oxidative stress, protein glycation and inflammation in diabetic foot patients considering the pathogenic mechanisms of acute diabetic foot ulcers. Thirty eight hospitalized type 2 diabetic foot patients were divided in a subgroup defined by the presence of both neuropathy and arteriopathy ($n = 15$) and another subgroup with neuropathy only ($n = 23$). Twenty healthy subjects were involved. Increased erythrocyte enzymes activities for glutathione S transferase ($p < 0.05$), catalase ($p < 0.03$), higher plasma levels for fasting glucose ($p < 0.01$), dicarbonyls ($p < 0.01$), C reactive protein ($p < 0.04$), malonyldialdehyde ($p < 0.05$) but lower superoxide dismutase activity ($p < 0.02$) were determined in the diabetic foot subgroup with both complications versus subgroup with neuropathy only. There weren't any significant difference in the age, duration of diabetes, HbA1c and plasma lipids levels between subgroups. Studied parameters were modified in patients versus controls. In conclusion, the presence of atherosclerotic peripheral arterial disease in diabetic foot ulcers highlights important systemic redox modifications including: increased oxidative and carbonylic stress, increased levels of inflammatory markers and increased lipid peroxidation. Aggressive treatment focused on these pathogenic mechanisms should be instituted in order to prevent the development or evolution of coronary and renal arteries diseases in order to reduce cardiovascular mortality in diabetic foot patients.

A3.16
Evaluation of insulin-mimetic activity of new vanadyl-flavonoid complexes in alloxan-induced diabetes

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Aim: The aim of the study was the evaluation of the insulin-mimetic activity for new complexes of vanadyl with chrysin and quercetin.

Materials and methods: Diabetes was induced to male adult Wistar strain albino rats with intraperitoneally administered alloxan. The animals were divided into five groups, each of six animals: one control group received water orally (1 ml/100 g body weight), two groups received complexes of vanadyl with chrysin and quercetin (0.4 mmol/kg body weight) and two groups received the corresponding flavones (chrysin and quercetin, 0.8 mmol/kg body weight). The treatment was continued for 5 days. Blood was drawn from the animals and serum was separated for the assay of glucose (with standard glucose-oxidase method) and insulin (ELISA method).

Results: The vanadyl-quercetin complex determined a 42.89% decrease of the glucose level, even if the insulin level, compared to control, decreased with 7.01%. The vanadyl-chrysin complex leads to a 16.44% decrease of the glucose level, while the insulin level, compared to control, decreased with 5.26%. The insulin level increased with 56.14% under the effect of quercetin (correlated with a 10.53% decrease of glucose level), while chrysin determined a 1.79% decrease of the insulin level (correlated with a 24.24% decrease of the glucose level).

Conclusions: The mechanism of action of the new complexes tested in this study was extra-pancreatic, based on the fact that complexes of vanadyl with quercetin and chrysin determined the reduction of the glucose level which was not correlated with an increase of the insulin level.

A3.17

Abstract withdrawn

A3.18
Cytotoxicity of king cobra (*Ophiophagus hannah*) venom L-amino acid oxidase

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The cytotoxicity of purified king cobra (*Ophiophagus hannah*, Malaysia) venom L-amino acid oxidase (LAAO), against various cell lines and bacteria was examined. The enzyme exhibited strong cytotoxic activity against A549 (lung cancer) and MCF-7 (breast cancer) cells with IC_{50}s of 0.2 and 0.1 μg/ml, respectively, when examined by MTT method. Its activity against normal lung cell NL20 was, however, much weaker ($IC_{50} = 0.6$ μg/ml). DNA fragmentation patterns of the killed cancer cells were examined by TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick-end labeling (TUNEL) assay and agarose gel electrophoresis and the results suggested that king cobra LAAO caused cell death via induction of apoptosis. The LAAO also exhibited strong antibacterial activity against several strains of clinical isolated bacteria, including *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Klebsiella pneumoniae*, and *Escherichia coli* with minimum inhibitory concentration (MIC) value ranging from 0.78 to 50.0 μg/ml. Catalase significantly reversed the cytotoxic effect of LAAO. Thus, our results suggest that king cobra venom LAAO is a potent cytotoxic agent that acts via generation of hydrogen peroxide.

A3.19
Alteration of the enzymatic activity of SDH upon glycation in diabetic conditions

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Sorbitol dehydrogenase (SDH, EC 1.1.1.14) is a ubiquitous enzyme which catalyses the second step of polyol pathway. Here alteration of the enzymatic activity of SDH upon glycation in diabetic conditions has been investigated. After extraction and partial purification of SDH from sheep liver, the samples were incubated in defined concentrations of fructose and ribose and estimated for activity over time. The results indicate that the glycation can negatively affect the SDH functionality so that both the activity and the catalytic efficiency of this enzyme are reduced in comparison with the non-glycated control sample. These observations can explain the way of participation of the glycated SDH in the occurrence of diabetic complications.

A3.20
The impact of glycation on the kinetic parameters of sorbitol dehydrogenase

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Sorbitol dehydrogenase (SDH) is one of the potential targets of nonenzymatic glycation in diabetic conditions. This protein catalyzes second step in polyol pathway and is known to be involved in cataract and some other diabetic complications. In this study, SDH was extracted and partially purified from sheep liver. The purified enzyme was incubated in defined concentrations of ribose and fructose, glycation processes were followed up and equilibrium constants (Keq) for SDH reactions were determined. The product of glycation shows declined equilibrium constant in comparison with the non-glycated SDH as control in enzymatic transformation of sorbitol to fructose. Therefore, it can be deduced that, the forward reaction is drowsed due to effective glycation of SDH, during diabetic hyperglycemia. As a result, under such condition sorbitol concentration increases and osmotic pressure is elevated in turn as a potential causative mechanism of the diabetic cataract.

A3.21

Abstract withdrawn

A3.22
Screening of putative mutations in β-globin gene by DNA sequencing of β-thalassemia major patients from Izeh and Baghmalek cities of Iran

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Beta thalassemia is a heterogeneous inherited disorder of β-globin synthesis with over 200 different β-globin reported mutations worldwide. The highest prevalence of the disease occur in the Mediterranean region, parts of the North and West Africa, the Middle East, the Indian subcontinent and the Southern Far East and Southeastern Asia, all together composing the so-called thalassemia belt. To establish the molecular spectrum of the beta thalassemia in the Izeh and the Baghmalek cities in the Iran, 110 unrelated beta thalassemia major patients with high Bakhtiarian ethnic background were studied. Genomic DNA from blood was extracted and after amplification of β-globin gene by specific primers, full length sequencing of β-globin gene was done by DNA sequencing. The remarkable results revealed the relative high frequency of beta thalassemia mild (β+) causing mutations within the subjects. This mutations were -88(C > A), -28(A > C), −101(C > T) and 5UTR + 20 locating in the promoter or untranslated region of the beta globin gene. The individuals were homozygous or compound heterozygous for the mentioned nucleotide changes. The Iran encompasses a multiethnic population with vast spectrum of beta thalassemia mutations. The result described here could greatly facilitate screening for β-thalassemia and prenatal diagnosis and also will be of valuable help in the development of successful prevention programs for the population, at least for the regions of Iran.

A3.23
Investigation on the anticancer properties and subacute toxicity of cultivated tiger milk mushroom (*Lignosus rhinocerus*)

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The "Tiger-milk" mushroom (*Lignosus rhinocerus*) is an important medicinal mushroom used by the local tribal people in Malaysia. It is only found in a small geographic region, mainly Southeast Asia. This fungus was used by the locals to treat asthma, fever, cough, cancer and food poisoning. Recently, the mushroom has been successfuly cultivated. We investigated the anti-cancer activities of the crude extracts of a cultivar of tiger milk mushroom (cultivar CH2) using lung (A549) and breast (MCF-7) carcinoma cell lines as well as on normal human bronchus (NL20) cell line by MTT method. The IC_{50} was determined to be 450 μg/ml, 250 μg/ml and 3500 μg/ml respectively on A549, MCF-7 and NL20. DNA fragmentation assay showed a typical ladder pattern of DNA fragmentation, suggesting that the crude extract inhibits cancer cell growth via apoptosis. A 28-day sub-acute toxicity study using *Sprague-Dawley* rats was also carried out. The animals in control as well as in treated groups (50–1000 mg/kg) showed similar pattern of body weight gain, and the results of hematology and serum biochemistry also showed no statistically significant differences between the control and the treated groups.

A3.24
Grape-seed derived procyanidins inhibit dipeptidyl peptidase 4 (DPP4) activity

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The plasma dipeptidyl peptidase 4 (DPP4) activity is increased in subjects with chronic hyperglycemia and type 2 diabetes, and

inhibition of DPP4 activity is currently assayed as a mechanism to improve glycemic control. Procyanidins derived from grape seeds (GSPE) can ameliorate some hyperglycaemic situations, but their mechanisms of action are not clearly understood. At present we evaluate their effects on DPP4 activity. We measured DPP4 activity by colorimetric determination of product formation (H-Gly-Pro-pNA, 0.2 mM, Bachem) in different situations. First, we performed *in vitro* assays with human and rat (plasma, intestinal and from cell line Caco-2) DPP4 and found that GSPE inhibits DPP4 activity. Next, Caco-2 cells were used to evaluate chronic effects of GSPE on DPP4 activity. We found that 10 or 100 mg GSPE/L for 3 days decreased the DPP4 activity; by contrast, a higher dose (750 mg/l) for a shorter time (24 hours) had no effect. Finally, *in vivo* effects of chronic GSPE treatment on plasma DPP4 activity were analysed on two metabolically disrupted models: Wistar rats fed with a cafeteria diet and Zucker fa/fa rats. 35 mg GSPE/kg bw on fa/fa females for 9 weeks, did not revert the increase on DPP4 activity observed in the fatty animal versus the lean control. Instead, 50 mg GSPE/kg bw for 10 days inhibited DPP4 activity, which was partly decreased by a cafeteria diet. From all these result we can conclude that GSPE inhibits DPP4 activity acting directly on the enzymatic reaction, and also chronically reducing such activity. This study was supported by grant number AGL2008-01310/ALI the Spanish Government

A3.25
The evaluation of LCAT concentration in patients with DM1

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Introduction: Lecithin:cholesterol acyltransferase (LCAT) is one of the most important enzyme involved in HDL metabolism and promotes reverse cholesterol transport. Diabetes type 1 (DM1) is associated with lipoprotein abnormalities, therefore the aim of the study was to evaluate the LCAT concentration in DM1 patients.
Materials and methods: The studied group consisted of 81 DM1 (43.4 ± 6.3 years). It was divided into patients with ≤ 10 years (n = 54, subgroup1) and > 10 years (n = 27, subgroup 2) of DM1. 25 healthy people (31.2 ± 10.2 years) were a control group. Metabolic parameters were measured using standard laboratory procedures. IL-12 and LCAT concentrations were assayed by ELISA.
Results: DM1 patients had lower LCAT level than healthy group [5.5(4.1–6.3) versus 6.1(4.6–7.5) μg/ml]. In addition, the negative correlation between IL-12 and LCAT level has been shown in this group ($r = -0.307$, $p < 0.05$, $n = 81$). LCAT concentration was also decreased in patients of subgroup two in comparison to subgroup 1 [4.5(2.3–6.0) versus 5.6(4.5–6.4) μg/ml]. Moreover, higher ratio of LDL:HDL [1.7(0.7–3.7) versus 1.5(0.5–4.6)] and correlation between LCAT and IL-12 ($r = -0.511$, $p < 0.05$, $n = 27$) were found in subgroup 2.
Conclusion: Lower level of LCAT in DM1 patients could be one of the risks of atherosclerosis development. The LCAT concentration decreases upon DM1 duration, enhancing proatherosclerotic tendency in patients with long DM1 duration. The relation between the IL-12 and LCAT suggests, that low LCAT level inhibits the reverse cholesterol transport, what increases the chronic inflammatory process. *The study was supported by PhD grant (No402476337).*

A3.26
Glycation-induced changes in the molecule of glutathione S-transferase

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Glycation is a non-enzymatic process resulting in the impairment of protein functions during diabetes mellitus and age-related diseases. The reactive alpha-dicarbonyl compounds (e.g. methylglyoxal) and advanced glycation end products (AGEs) are formed. Alpha-dicarbonyls cause protein cross-linking and formation of AGEs mainly on intracellular proteins, which usually lose their biological activity and may persist in tissues. Glutathione S-transferases (GST, EC 2.5.1.18), group of intracellular enzymes involved in detoxification of xenobiotics, belong to the most abundant cytosolic proteins. We suppose that GST may be modified by glycation *in vivo*, which would provide a rationale of its use as a model protein for studying glycation reactions. Glycation of GST by methylglyoxal, fructose or glucose was studied (37°C, for up to 28 days). The course of protein glycation was evaluated using following criteria: enzyme activity, formation of fluorescent AGEs, amino group's content, protein conformation, cross linking and aggregation, carbonyl content, and changes in molecular charge of GST. The ongoing glycation by methylglyoxal 2 mM resulted in pronounced loss of GST enzymatic activity. It also led to the loss of 14 primary amino groups, which was accompanied by changes in protein mobility during native PAGE. Formation of cross-links with molecular weight of 65 and 135 kDa was observed. Obtained results can contribute to understanding of changes, which proceed in metabolism of xenobiotics during diabetes mellitus and ageing.
Acknowledgments: This study was realized with financial support from the Czech Science Foundation, Grant No. 524/09/P121.

A3.27
Adipocyte fatty acid binding protein (A-FABP) in Kupffer cell as a novel player in the pathogenesis of non-alcoholic fatty liver disease

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Obesity is a major risk factor for non-alcoholic fatty liver disease (NAFLD). Our recent study demonstrated that adipocyte fatty acid binding protein (A-FABP) is an early predictor for the development of obesity-related pathologies, including metabolic syndrome, type II diabetes and atherosclerosis. An increased level of A-FABP mRNA expression in patients with NAFLD has also been observed. This study aims to examine the role of A-FABP in the pathogenesis of NAFLD by using different mouse models. C57 male mice were intra-peritoneally injected with lipopolysaccharide (LPS) to induce acute liver injury or fed with high fat liquid diet to induce chronic liver injury. Depletion of Kupffer cell was performed by tail-vein injection of gadolinium chloride III to examine the hypothesis that AFABP is mainly expressed in hepatic macrophages. Hepatic A-FABP mRNA and protein levels were increased following acute injection of LPS or high-fat diet induction. It was accompanied by elevated levels of liver injury markers, alanine aminotransferase (ALT) and aspartate transminase (AST) and increased production of pro-inflammatory cytokines. Depletion of

Kupffer cells reduced the LPS-induced over-expression of A-FABP in both mouse models, indicating Kupffer cells were the major cell population for A-FABP expression in liver. The pharmacological inhibitor of A-FABP blocked LPS-induced production of pro-inflammatory cytokines in macrophages. Both endotoxin and high-fat induced liver injury were associated with elevated A-FABP expression in Kupffer cells, indicating an etiological role of A-FABP in the pathogenesis of NAFLD.

A3.28
Calciphylaxis – A highly regulated process due to deregulation of mesenchymal cell differentiation

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Calciphylaxis is a rare and life-threatening disease and occurs in patients with renal failure and diabetes. It is characterized by vascular and soft-tissue calcifications which results from an active cell-mediated process of calcification regulating proteins. We investigate several calcification inhibitors (Fetuin-A, Matrix-GLA-Protein (MGP), bone morphogenic protein7 (BMP7), osteoprotegerin (OPG) and osteopontin (OPN)) which play a role in the differentiation of chondrocytes and osteoblasts by automated immunohistochemistry. Further regulating proteins like BMP2 and 4, Tenascin-C (TNC), SOX9 and Indian Hedgehog (Ihh) are investigated. Ihh is involved in the differentiation from proliferated chondrocytes to prehypertrophic chondrocytes. During theses states of differentiation BMP7 is highly expressed. TNC is produced in association with the aggregating prechondrogenic mesenchymal cells in developing cartilages and it is involved in EMT. For this study formalin-fixed paraffin embedded (FFPE) subcutis tissue of 20 patient with calciphylaxis were compared with 22 normal controls. The vasculature, especially endothelial cells, intima, media and adventitia as well as the connective and fatty tissue were analysed. In calcified tissues BMP7 is over expressed in the vasculature as well as in the connective and fatty tissue. Furthermore Ihh and TNC was also higher expressed in calciphylaxis. MGP, OPG, OPN and BMP2 and 4 did not show any differences. Fetuin-A and Sox9 seems to be higher expressed. In contrast to other citations about the pathogenesis of calciphylaxis via osteoblastic differentiation we opine that vascular smooth muscle cells differentiate into chondrocyte-like cells.

A3.29
The levels of IL-6, IL2R and IGFBP-3 in patient with atherosclerosis

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Atherogenesis is highly complex and is modulated by numerous genetic and environmental risk factors. A large body of basic scientific and clinical research supports the conclusion that inflammation plays a significant role in atherogenesis along the entire continuum of its progression. Inflammation adversely impacts intravascular lipid handling and metabolism, resulting in the development of macrophage foam cells, fatty streaks and atheromatous plaque formation. Interleukin-6 (IL-6) may play a direct role in endothelial activation, or an indirect role when fibrinogen synthesis is stimulated. Interleukin-2 receptors (IL-2R) are expressed on activated T cells, B cells, and monocytes. Insulin-like growth factor binding protein-3 (IGFBP-3) inhibits cell growth, both directly by inhibition of DNA synthesis and indirectly by sequestration of IGFs, which stimulate cell growth. The aim of the present study was to examine the levels of IL-6, IL 2R and IGFBP-3 in atherosclerosis. Seventy patients with atherosclerosis (age 57.3 ± 13 years) and sixty control (age 60.9 ± 12 years) were included in our study. Blood samples were collected in the first 24 hours and at the end of three month. Serum IGFBP-3, IL6 and IL2R levels were determined by electrochemiluminescence method. We found increased level of IL-2R in the atherosclerosis group compared to control group (653.6 ± 322.4 U/ml versus 559.15 ± 297.3 U/ml, $p = 0.04$). There was no relationship between IGFBP-3 and IL-6 levels and atherosclerosis. Further investigations are required to disclose the mechanisms of elevation and the effects of increased serum levels of these factors in atherosclerosis.

A3.30
Effective delivery of encapsulated hydrophobic-type photosensitizers to the MCF-7 cancer cells

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The development of effective nanocarriers faces numerous challenges: reaching the target site preferably in a time controlled way, limiting the drug's adverse effects and ensuring biocompatibility. Present work is focused on effective delivery of hydrophobic cyanines to the cancer cells by poly(*n*-butyl cyanoacrylate) (PBCA) nanocapsules. We report on the synthesis and imaging of PBCA nanocarriers, their *in vitro* biological evaluation and the cellular localization of cyanine IR-768 delivered in the nanocapsules to MCF-7 cancer cells. Oil-cored PBCA nanocapsules were prepared as in [1] by interfacial polymerization in o/w microemulsions of gemini sugar surfactants (*N,N'*-bisdodecyl-*N,N'*-bis[(3-aldonylamido)propyl]ethylenediamines (bis(C12GA) or bis(C12LA)), *iso*-butanol, *iso*-octane and water. Nanoparticles with diameters in the range of 200 – 600 nm were visualized by atomic force microscopy (AFM) and scanning electron microscopy (SEM). *In vitro* studies on the erythrocyte hemolysis and the cell viability of breast cancer MCF-7 cells showed that the PBCA nanocapsules are safe carriers of cyanines in the circulation, having a very low hemolytic potential and being non-toxic to the studied cells. Fluorescence microscopy visualized the cyanine intracellular distribution and demonstrated that PBCA-nanocarriers effectively delivered the cyanine molecules to the MCF-7 doxorubicin-sensitive and -resistant cell lines.

Reference:

1. Pietkiewicz J, Zielinska K, Saczko J, Kulbacka J, Majkowski M, Wilk KA. *European J. Pharm. Sci.* 2010, **39,** 322.

A3.31
Acetic zinc influence on ethanol metabolism and basic membrane-bound enzymes activity in hepatic and brain cells under chronic alcoholic intoxication development

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Deficiency of zinc in a number of organs is observed under conditions of a chronic alcoholic intoxication. For the purpose of

correction of the specified dissonance use zinc salts among which low toxicity characterizes acetic zinc. White laboratory rats-males (weight 180–200 g) kept to standard vivarium diet were used in experiments. Animals were parted on three groups: 1 group – control animals; 2 group – rats with a chronic alcoholic intoxication which caused behind standard procedure; 3 group – rats with a chronic alcoholic intoxication which follow-up administration zinc acetate in a dose of 0.2 g on 100 g of animal mass one times into day. Ethanol metabolism systems functioning (alcohol dehydrogenase, aldehyde dehydrogenase activity, cytochrome P450 content) and functional condition of plasmatic membranes of rats liver and brain cells were investigated for 4, 6, 11, 16 and 21 days after the experiment beginning. It was shown, that under the chronic alcoholic intoxication there was dysfunction of the basic systems of ethanol metabolism through the decrease of ADG/AlDG system activity, activation of microsomal oxidations (increase of the cytochrome P450 content) and increase of basic membrane-bound enzymes activity (Na^+, K^+-ATPase, Ca^{2+}, Mg^{2+}-ATPase and 5'- nucleotidase) of rats liver and brain cells. The acetic zinc treatment resulted in the reduction of ethanol influence on investigated metabolism indicators (normalization of cytochrome P450 content, decrease of membrane-bound enzymes activities, increase of ADG and AlDG activities) in rats liver and brain cells in comparison with animals with a chronic alcoholic intoxication.

A3.32
Lebanese patients with Ornitine transcarbamoylase (OTC) deficiency: Genotype–phenotype

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OTC deficiency is the most common enzyme deficiency among Urea Cycle Defects. It is an X-linked nuclear-encoded mitochondrial enzyme, with variable clinical presentation and age of onset. Homozygous males may present at any age while heterozygous females are symptomatic in some cases. This study is the first report on the clinical presentation and genotype of Lebanese patients with OTC deficiency. Forty-nine subjects belonging to eight families were screened in our Metabolic Clinic. Patients presented at variable ages between 2 and 44 years. Clinical evaluation and metabolic work-up including ammonia level, plasma amino acids chromatography and urinary orotic acid quantification were done. Among late-onset cases, ten of the patients belonged to three consanguineous families from one village. Three of them passed away before diagnosis was made. The index patient was referred for incidental finding of moderate hyperammonemia (120 μm/l) during a work-up for epigastric pain. All symptomatic patients had hyperammonemia, low ornithine and citrulline levels, and increased orotic aciduria. They are successfully controlled with protein restricted diet, arginine supplementation and sodium benzoate in acute decompensations. Genomic DNA from patients and their families was isolated. The 10 exons of the OTC gene were amplified by PCR, separated and sequenced for mutation analysis and SNPs. Dot blot analysis was also used to screen for carriers. Sequencing analysis revealed a point mutation Arg40His and a polymorphism AAA- > AGA in Exon 2 in some of the patients. The remaining exons had normal sequences. Other genes will be screened in subjects with no identified mutations.

A3.33

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A3.34
Anti-inflammatory and liver protective action of bioactive complex rich in glycosaminoglycans obtained from small sea fish

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By an original patented technology we have obtained a bioactive complex from small sea fish rich in sulfated glycosamynoglycans (44–60%) essential amino acids (2–6.5%), essential fatty acids (1–2% ω-6) and mineral salts (Ca, Na, K, Fe, Mg, Se, Ni, Cu, Si). This bioactive complex, besides anti-hyaluronidasic, decreasing of elastase and collagenase activity favor *in vitro* collagen fibrils formation. Also, GAG bioactive complex can interrupt colagenolytic activity of degeneration processes of cartilage, recovering extracellular matrix and manifests a strong antioxidant activity. *In vivo* on Wistar rats with carragenan-induced paw edema, and with CCl4-induced liver toxicosis, we determinate an anti-inflammatory activity (81.4% in 24 hours) similar with diclofenac (83.7% in 24 hours) and liver protective effects by stimulation of hepatic catalase and inhibition of lipid peroxidation activity denoted by decreasing of MDA concentration. Taking into account the chemical composition of the GAG-bioactive complex obtained from small sea fish and the therapeutic effects highlighted by *in vitro* and *in vivo* experiments may be considerate this a very good source for medicinal products with valuable therapeutic properties and minimal side effects.

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A3.35

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A3.37
Three-dimensional quantitative structure-activity relationships (3D-QSAR) study of PPARγ partial agonists

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PPARγ is a member of the nuclear receptors superfamily that regulate the gene expression of proteins involved in glucose and lipid metabolism, regulation of adipocyte proliferation and differentiation and insulin sensitivity. Thiazolidinediones (TZDs) is one important class of synthetic agonist of PPARγ. TZDs are antidiabetic agents that target adipose tissue and improve insulin sensitivity, and are currently used in the treatment of type 2 diabetes. Despite the clinical benefit of these drugs, use of TZDs has been associated with adverse effects including weight gain, increased adipogenesis, renal fluid retention, and possible increased incidence of cardiovascular events. Therefore, new

PPARγ lligands with enhanced therapeutic efficacy and reduced adverse effects are needed. A promising new group of such ligands are selective PPARγ modulators (SPPARγMs). These compounds act as partial agonists of PPARγ and display different binding properties when compared with full agonists. For the first time, a set of PPARγ partial agonists were subjected to a 3D-QSAR study. The aim is to facilitate the rational design of more potent PPARγ partial agonists. To perform the 3D-QSAR study, six crystal structures whose ligands belong to a family of partial agonists were used as queries, and 60 indole PPARγ partial agonists with known EC50 values were used as a training and test set. The 3D-QSAR model obtained, showed a good correlation between the observed and predicted activities both in the training and test sets and thus may be useful in the design of new PPARγ partial agonists and predicting the EC50 values of possible PPARγ partial agonists.

A3.38
Antiplatelet activity of alpha-lipoic acid is mediated by PPARs

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Peroxisome proliferator-activated receptors (PPARs) including PPARα,β, and γ exist in human platelets and activation of PPARs inhibits platelet aggregation. Alpha-lipoic acid (ALA) occurring naturally in human diet exerts a protection against atherosclerosis and thrombosis. However, little is known about the effect of ALA on platelet activation. Thus, the study was to investigate whether the antiplatelet activity of ALA is mediated by PPARs. ALA (200–800 μM) dose-dependently inhibited rabbit platelet aggregation induced by arachidonic acid (AA) or collagen. Addition of GW6471 (PPARα antagonist) or GW9662 (PPARγ antagonist), only the antiaggregatory effect of ALA on AA-induced platelet aggregation was markedly reversed. However, GSK0660 (PPARβ antagonist) did not affect its antiplatelet activity. Similarly, the inhibitory effects of ALA on AA-induced thromboxane B2, O_2^- production, and Ca^{2+} mobilization were significantly reversed by simultaneous addition of GW6471 or GW6992. Moreover, addition of GW6471 or GW6992 markedly attenuated the increase of cyclic AMP formation by ALA. Interestingly, ALA also increased the interaction and immune-precipitation with PPARα,γand protein kinase Cα (PKCα) accompanied by a decrease of PKCα activity both in resting or AA-stimulated platelets. Similarly, addition of PPARα,γ antagonists markedly reversed the inhibition of PKCα activity by ALA in resting or activated platelets. In conclusion, we first show a direct inhibitory effect of ALA on platelet aggregation, and provide a novel mechanism for its action through involvement of an association with PPARs and PKCα in platelets, by which ALA may subsequently produce antiplatelet activity.

A3.39
Fibrillation of human insulin is accelerated upon interaction with PVP-coated nanoparticles under *in vitro* conditions

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Proteins can interact with different biological and non-biological surfaces. However, the role of surface, as promoters of pathological aggregation of amyloidogenic proteins, is rarely studied. Nanoparticles are materials at sub-micrometer scales, usually 1–100 nm, and provide excellent systems for modeling protein surfaces. In particular, they can be readily fabricated with dimensions comparable to biological macromolecules. Moreover, the large surface area, small size of nanoparticles and the ability of nanoparticles to influence protein folding and aggregation make them as an interesting tool for understanding molecular events behind the processes leading from native to fibrillar states of proteins. Here the fibrillation process of human insulin was investigated in the absence and presence of Polyvinylpyrrolidone (PVP)-coated nanoparticles. Formation of amyloid fibrils and effect of PVP-coated nanoparticles were verified through some of the specific methods of amyloid detection such as Thioflavin-T (ThT) fluorescence, Congo red binding, circular dichroism and atomic force microscopy. The process was found to be affected by the presence of the nanoparticles, which accelerate human insulin fibrillation. Kinetic fibrillation data, as monitored by ThT fluorescence, suggested that nanoparticles caused shorter lag (nucleation) phase; which is the rate-determining step during amyloid fibrillation, possibly by increasing the local concentration of protein molecules. The present studies may open interesting directions for work on fibrillation-related diseases and for the discovery of agents (e.g. nanoparticles), which may interfere with such processes.

A3.40

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A3.41
Rage activation by advanced glycation end products (age) enhances collagen IV accumulation in human embryonic kidney (hek 293) cells

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AGEs play major roles in the accumulation of extracellular matrix being involved in diabetic renal injuries. It seems that AGEs interaction with their receptor-RAGE leads to the activation of TGF-β and non-TGF-β pathways affecting the expression of several extracellular matrix proteins. Our aim was to evaluate the relationship between AGE, RAGE, TGF-βand collagen IV at the transcriptional and translational levels in HEK293 cells. The cells were incubated for 24 hours with AGE-BSA and BSA (control) at concentrations between 50–200 μg/ml in the presence or absence of 20 ng/ml anti-TGF-β1 or anti-RAGE antibodies. The mRNA expression of RAGE, TGF-β1 and procollagen α1(IV) was analyzed by quantitative real-time PCR. The protein levels of RAGE, type IV collagen and TGF-β1 were assessed by Western blot and ELISA. The concentration of 200 μg/ml AGE-BSA up-regulated the mRNA expression of RAGE, TGF-β1 and procollagen α1 (IV), the relative expression ratio (R) being 1.83 ± 0.2, 3.4 ± 0.09 respectively 4 ± 0.12. At 100 μg/ml AGE-BSA, R decreased to 1.41 ± 0.12, 1.23 ± 0.05 and 2.68 ± 0.17 for RAGE, TGF-β1, respectively procollagen α1 (IV), whereas at 50 μg/ml AGE-BSA, R diminished more in all cases. The co-treatment with anti-TGF-β1 or anti-RAGE anti-

bodies and 100μg/ml AGE-BSA down-regulated the mRNA expression of procollagen α1 (IV) to 0.49 ± 0.07 respectively 0.65 ± 0.09. The proteic expression was in good correlation with the mRNA one for RAGE, TGF-β1 and collagen IV. Our results suggest that the axis AGE-RAGE-TGF-β could be implicated in the development of tubule-interstitial fibrosis and glomerulosclerosis related to diabetic nephropathy.

A3.42
Assessment of mutated apolipoprotein A5

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Hypertriglyceridemia is an independent risk factor for coronary heart disease. According to the previous studies, triglyceride (TG) metabolic disorder was correlated with the apolipoprotein A5 (APOA5) gene polymorphisms. APOA5 can modulate the triglyceride hydrolase activity of lipoprotein lipase (LPL) through direct activation or indirect effects to reduce the plasma TG. However, the mechanism of APOA5 modulation is still unclear. We have identified a c.553G > T polymorphism, which was found in oriental populations only, causes a G185C substitution effect. Moreover, patients with c.553G > T polymorphism tend to suffer from hypertriglyceridemia. These phenomena inspired us that substitution of G185C might results in losing, or at least lowering the function of APOA5. The aim of this study is to assess the importance of glycine at 185th residue. Nineteen mutants of APOA5 protein were generated by site-direct mutagenesis. The mutant- and wild-type of APOA5 plasmid were transformed to *E. coli* BL21 and inducted at 37°C for 3 hour. Proteins produced were purified with nickel magnetic beads. A synthetic DMPC (1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine), which simulated VLDL structure, was constructed. Different APOA5 protein was added to the DMPC vesicle preparation and incubated at 24°C for 3 hour. Then LPL activity was measured. The results showed that the activation ability of LPL by all mutant-type of APOA5 was lower than that of wild type of APOA5 ($p = 0.0008$). This might suggest that the importance of 185GG, any mutation will decrease its activation ability.

A3.43
Oxidative stress alterations in peritoneal macrophages and polymorphonuclear leukocytes for type I autoimmune diabetic mice

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Diabetic hyperglycemia is associated with increased production of reactive oxygen species (ROS). The link between hyperglycemia and the complications of diabetes is unknown. Type I diabetic TCR-HA$^{+/-}$/Ins-HA$^{+/-}$ mice are obtained by mating BALB/c mice-expressing transgenes encoding a TCR recognizing an immunodominant epitope of influenza PR8 virus hemagglutinin (HA110-120) in association with I-Ed (TCR-HA$^{+/-}$), and B10.D2 mice expressing the PR8 influenza virus HA transgene (Ins-HA$^{+/-}$). Nitric oxide (NO) and O^{2-} levels in peritoneal macrophages and polymorphonuclear leukocytes (PMN) are quantified. Respiratory burst of PMN was evaluated by flow cytometry. Three stimuli are used: N-formyl Met Leu Phe (fMLP), phorbol-12- myristate-13-acetate (PMA) and opsonised *E. coli*. Lymphocyte populations CD45R/B220^{+}, CD4^{+} and NK1.1 cells were investigated by flowcytometry. In plasma we have determined the carbonyl content in oxidative modified proteins and global antioxidant activity. In diabetic mice, O^{2-} and NO production are higher compared to Balb/c mice. In peripheral blood and spleen of diabetic mice, a higher percentage of CD45R/B220^{+} and NK1.1 cells was detected by comparison with Balb/c mice. Plasma level of oxidized protein in diabetic mice was higher, while the antioxidant capacity was lower. In conclusion, all these characteristics indicate an altered oxidative stress in peritoneal macrophages and polymorphonuclear leukocytes associated with fulminant type I diabetes. This study was supported by PNII 41-074/2007.

A3.44
Identification and characterization of copy number changes: case study of familiar hemoglobinopathy

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Copy number variations (CNVs) are together with single nucleotide polymorphisms (SNPs) the main source of genetic variability in men. With regard to the extent of CNVs, they may posses significant phenotypic impact and are linked to group of pathological conditions and clinical syndromes. Using methods of DNA arrays, quantitative PCR, standard PCR and sequencing, we have identified and characterized large deletion in family with autosomal dominant familiar hemoglobinopathy. Hemoglobinopathies are very heterogenic group of disorders caused by the presence of abnormal structural variants of globin proteins or by underproduction of normal globin genes due to defects in α- or β- globin chain syntheses (thalassemias). We have studied family diagnosed by hemoglobinopathy with following clinical symptoms: preterm birth of severe anemic child with marked microcytosis and hepatopathy of various intensity, mild to severe anemia and marked microcytosis after several months of blood transfusions. We have performed mapping analysis using Affymetrix GeneChip Human Mapping 250K array and found heterozygous deletion 765 kb in length at chromosome 11: 5, 088, 500 – 5, 853, 400. This region contains β-globin cluster involving genes of β-globin chain (hemoglobine β, γ 1, γ2, ε). To refine break in deleted region, we have designed real-time PCR probes using Roche Universal Probe Library. Narowing of the interval allowed sequencing of delimited region. Finally, fast and simple assay for detection of deletion in other family members is set up. This study was supported by the grant from the Ministry of Education of the Czech Republic (MSM0021620806).

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A3.47
Polymorphisms within prothrombotic genes in overweight patients with coronary artery disease

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Background: Coronary artery disease (CAD) depends on a number of factors, genetic (including polymorphic variants encoding proteins involved in hemostatic mechanisms) and non-genetic (e.g. overweight or obesity). Methylenetetrahydrofolate reductase (MTHFR) catalyses remethylation of homocysteine (Hcys) to methionine. Increased levels of Hcys are related o decreased bioavailability of endothelial nitric oxide, well-known platelet aggregation inhibitor. Plasminogen activator inhibitor-1 (PAI-1) is a fast-acting inhibitor of tissue and urokinase type (t-PA and u-PA) plasminogen activators. Its increased plasma levels are associated with different thrombotic disorders. The aim of the study was to establish associations between the MTHFR 677C > T and PAI-1 –675_–674insG polymorphisms and CAD in overweight patients.
Methods: We analysed 114 angiographically documented CAD patients with overweight and 118 blood donors with normal body mass index (BMI). Both polymorphisms were genotyped using PCR-RFLP method.
Results: The MTHFR T allele and T allele carriers (CT + TT) were slightly more frequent in patients than in controls. We observed higher prevalence of PAI-1 5G allele as well as 5G5G homozygotes in overweight cases compared to controls with normal BMI. The results were on bound of significance (p = 0.082, OR = 1.38 and p = 0.067, OR = 2.02 respectively). Contemporaneous carrier-state of both analyzed polymorphic variants did not differentiate analyzed groups.
Conclusions: There are possible interactions between PAI-1 –675_–674insG polymorphism and overweight in determining the risk of coronary artery disease.
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A3.48
EPR evaluation of proteins engineered to carry Gd(III) contrast labels

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Apo A-I is the major protein constituent of high-density lipoprotein (HDL) and plays a central role in phospholipid and cholesterol metabolism. We have engineered apolipoprotein A-I (apoA-I) to contain a DOTA-chelated Gd(III) for the purpose of imaging reconstituted HDL (rHDL) biodistribution, metabolism, and regulation *in vivo*. Through the targeted substitution of cysteine in apo A-I, we have attached the thio-reactive MRI (magnetic resonance imaging) agent MTS-ADO3A to two distinct positions (55 and 76) in the protein. MRI imaging of infused mice shows apo A-I labeled with ADO3A migrates to both liver and kidney, the organs responsible for HDL catabolism, however the contrast properties of apo A-I are superior when the ADO3A moiety is located at position 55, compared to the protein labeled at position 76. Interestingly, conventional X-band EPR spectroscopy can detect differences in the Gd(III) signal when comparing the labeled protein in lipid-free to the rHDL state. Furthermore, both the highest MR contrast and narrowest Gd(III) EPR spectrum are achieved with using rHDL-associated apoA-I containing the ADO3A probe attached to position 55. While room temperature EPR requires > 1 mM Gd(III)-labeled protein to resolve the spectrum, the volume requirement is low (˜5 μl). Thus conventional X-band EPR spectroscopy provides a practical assessment for the suitability of imaging candidates containing the site-directed ADO3A contrast probe.

A3.49
The placenta and the gut: Regulation of barrier functions and inflammation by the maternal gut flora

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Pregnancy is characterized by an altered maternal metabolism, due to hormones produced by the placenta, and to the activation of the maternal immune system. The placenta plays a key role for maternal metabolism and immune responses, and regulates fetal nutrient supply, fetal growth and fetal programming. Impairment of placental functions, e.g in gestational diabetes mellitus (GDM) and preeclampsia (PE), will affect not only the mother, but also the fetus with both short- and long-term consequences for perinatal morbidities and chronic diseases in adult hood (e.g. cardiovascular diseases, obesity, DM). The commensal gut flora regulates a number of activities related to protective-, structural- and metabolic functions within and outside of gut. The aim of the present project was to examine the role of the maternal microbiota on placental functions with implications for gestational diseases (e.g. GDM, PE).
Methods and results: Placentas were obtained from Germ-free (GF) and normal (SPF) mice at day 19 of pregnancy. Isolated mRNA were analyzed with Illumina Sentrix® Beadchip Array MouseRef-8 V2 (25,600 probes) and demonstrated lower expression of components involved in tight junction signaling in GF compared to SPF mice. Transepithelial electrical resistance measurements showed that serum from conventionalized GF mice altered tight junctions in CaCo-2 cells. Furthermore, serum levels of IL-1β were elevated in pregnant GF mice compared to non-pregnant GF mice and to pregnant SPF mice.
Conclusions: The maternal gut flora affects properties of the placenta related to inflammatory responses and barrier functions with potential impact on feto-placental transport and metabolism.

A3.50
Discovery and mechanistic characterization of neolignans as PPAR-gamma agonists

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Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that play an important role in lipid and glucose metabolism. Among three subtypes of PPARs, PPAR-gamma is the best characterized and its agonists are useful for the treatment of insulin resistance and metabolic syndrome. Aim of our study was to identify new PPAR-gamma agonists by a pharmacophore' based virtual screening and characterize the activation profiles in the cellular level. This approach predicted several neolignans to bind to the PPAR-gamma ligand binding domain (LBD). To verify this prediction, the neolignans dieugenol, tetrahydrodieugenol, and magnolol were synthesized or isolated and PPAR-gamma binding studies were performed. Compared to the clinically used PPAR-gamma agonist pioglitazone, the neolignans exhibited a highly similar binding pattern and were binding to the PPAR-gamma LBD with EC_{50}s in the nanomolar range. The activity of these compounds was confirmed in a cell-based luciferase reporter model, showing a binding pattern suggesting partial agonism. In a coactivator recruitment assay, dieugenol, tetrahydrodieugenol, and magnolol also behaved as partial agonists and showed different recruitment patterns compared to pioglitazone. These neolignans induced 3T3-L1 preadipocyte differentiation in PPAR-gamma-dependent manner confirming effectiveness in a cell model with endogenous PPAR-gamma expression. In summary, this study identified several neolignans as novel PPAR-gamma agonists. Their interesting activation profiles suggest them as potential pharmaceutical leads or dietary supplements for the treatment of type 2 diabetes and the metabolic syndrome.

A3.51
Detection of advanced glycation end products in the serum of diabetic patients with chronic renal failure

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It is well known that advanced glycation end products (AGE) are correlated with an appearance of vascular complications in diabetic persons and have been identified as important modulators of the development and progression of diabetic nephropathy [1]. Most of the methods used for AGE determination in serum samples were able to identify a narrow spectrum of glycated molecules such as pentosidine, pyrraline, carboxymethyllysine-protein derivatives. The aim of the present study was using the new original antibodies which could identify the AGE in serum of diabetics being in five stage (eGFR < 15 ml/min/1.73 m^2) of chronic kidney disease (CKD). All patients were treated by insulin and had satisfactory glycemic control – HbA1c < 7.5%. Anti-AGE antibodies were obtained in our laboratory after rabbits immunization by selected fraction of glycated myoglobin [2]. The immunoblotting method allows to detect a wide array of AGE in 72% of serum samples, probably by recognizing the common epitopes presented in various glycating products. Control serum samples of healthy volunters contain some physiological level of glycated albumin. It is intriguing that the diabetic patients with a similar clinical phenotype exhibited the significant differentiation of high molecular weight AGE products in serum. We concluded that the new anti-AGE antibodies may be useful in detection of wide spectrum of AGE in diabetic serum samples. This report was supported by Grant N 401 3564 33 from Polish Ministry of Science and Higher Education and Grant No 1601 from Wroclaw Medical University.

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A3.52
Multifunctional protein enolase as a model system to investigate the protein glycation

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Hyperglycemia and oxidative stress generate the carbonyl shock in cells. Increase level of the reactive low molecular weight aldehydes, eg. methylglyoxal (MOG), 4-hydroxynonenal (4HNE), trans-2-nonenal (T2N) and acrolein (ACR) can modify protein during glycation. Enolase, such as one of most common intracellular proteins, is more susceptible for glycation than other glycolysis enzymes [1]. Probably, enolase protects others intracellular proteins against glycation by effective binding the reactive aldehydes [2]. The aim of this report is to study the ability of 4HNE, T2N, ACR and MOG to modify the human and mammalian enolase. The glycation was carried out at the conditions described previously for human muscle-specific enolase modified by MOG [3]. The incubation of human and pig muscle enolase with reactive aldehydes (1:300 molar ratio of enolase to glycation factor) caused the enzyme activity inhibition in the following order: 4HNE > MOG > ACR > T2N. The SDS/PAGE showed that the higher temperature of incubation (25°C, 37°C, 45°C) resulted in most complex mixture of the advanced glycation end products (AGE). Enolase modification by ACR and 4HNE produced more efficiently than MOG or T2N. SDS/PAGE analysis showed that colostrinine (peptides mixture from sheep colostrum) as well as carnosine and pyridoxamine has ability to decrease level of the high molecular weight AGE. These factors are able to protect muscle enolase against the decreasing of catalytic function during irreversible glycation in hyperglycemia.

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A3.53
Studies of human enolase interaction with plasminogen

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Enolase, as a multifunctional protein plays a receptor role, e.g. for plasminogen, on the surface of many cells [1]. Previously we localized an enolase on the myosarcoma cells (MESSA-1) surface and in the isolated outer membrane of the human breast carcinoma cells (MCF-7) [2,3]. In this report we prove that the enolase catalytic activity on the surface of intact MCF-7 cells is at

about 25% higher level than in of MESSA-1 cells, which suggest more intensive expression of the enolase protein on the breast cancer cell surface. The enolase was localized immunocytochemically in the cytoplasm and on the MCF-7 cells surface. Moreover, we determined the MCF-7 cell ability to superficial binding of human plasminogen. The enolase-plasminogen complex *in vitro* was detected in non-denaturing PAGE and by immunoblotting with rabbit anti-human alpha-enolase specific antibodies. The dissociation constant value (36 nM) for enolase-plasminogen complex was determined by using the surface plasmon resonance (SPR) technique and indicates the high binding ligand-receptor affinity. Our studies suggest that enolase as a plasminogen receptor on the cancer cell surface can be an essential factor of the metastasis mechanisms. This report was supported by Grant No 1602 from Wroclaw Medical University.

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A3.54
The changes in serum adiponectin levels by oral administration of surfactants in mouse

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Surfactants are amphiphilic and emulsify hydrophobic and hydrophilic compounds. They have been widely used for water-insoluble compounds in many food materials and pharmaceutical products. Some surfactants such as Cremophor EL have been considered to affect on biological and pharmacological activity. Bile acids, such as cholic acid, are biogenic amphiphilic molecules that play essential roles in the absorption of dietary lipids. Adiponectin, one of adipocytokine, is secreted from adipose tissue and is thought to play an important modulator of glucose and lipid metabolisms in insulin-sensitive tissues of both humans and animals. In this study, we investigated the influences of surfactants on mouse serum adiponectin levels. Mice are orally administered with ionic surfactants, such as cholic acid and 3-((3-Cholamidopropyl) dimetylammonio) propanesulfonate (CHAPS), and nonionic surfactants, such as Cremophor EL, Triton X-100, and Brij-35, separately. Serum adiponectin levels of mouse were measured by Western blotting with anti-adiponectin antibody. Cremophor EL, Triton X-100, and Brij-35 markedly increased adiponectin levels, while cholic acid reduced the level. Cremophor EL however failed to enhance adiponectin secretion from 3T3-L1 adipocytes. This paper will discuss the mechanisms of the function of surfactants.

A3.55
Changes in the energy status and glucose uptake rate of PC-12 cells induced by acute subcytotoxic or mildly cytotoxic hexavalent chromium insults

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In the 1920s, Otto Warburg reported that, contrary to most normal cells, cancer cells rely strongly on lactic fermentation for energy production, even in the presence of an adequate oxygen supply. Whether this metabolic reprogramming, known as the Warburg effect, is a causative effect in cancer or a mere epiphenomenon, has been a subject of intense debate, but, nowadays, it is increasingly viewed by cancer researchers as one of the fundamental alterations that occur during the process of malignant transformation. To investigate whether this metabolic reprogramming may underlie hexavalent chromium [Cr(VI)]-induced carcinogenesis, we have determined the effects of acute exposures to low levels of this well-established carcinogen on the rates of glucose uptake, lactate production and oxygen consumption in cultures of PC-12 cells. Moreover, we have also determined the effects of these exposures on the cells' adenylate energy charge, an important indicator of the cellular energy status. Slight, but statistically significant, changes in the glucose uptake ratios and adenylate energy charge of the cells were observed, pointing to a potential role for the Warburg effect in Cr(VI)-induced carcinogenesis. This work was supported by Centro de Investigação em Meio Ambiente, Genética e Oncobiologia (Grant CIMAGO 26/07).

A3.56
The effect of extracts from purple sweet potato leaves in adipocyte differentiation

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Obesity is characterized by an increase of adipose tissue as a result of positive imbalance between food intake and energy expenditure. Previous research has suggested that decreased proliferation and adipogenesis are mechanisms to reduce obesity. Purple sweet potato leaves (PSPL) contain a high content of polyphenolics which have been shown to exhibit a number of biological activities. In this study, we examined the effect of PSPL extracts on differentiation of 3T3-L1 preadipocytes and investigated how they work. 3T3-L1 preadipocytes were differentiated from fibroblasts to mature adipocytes and served as experimental materials. The cell line was stimulated by a mixture of insulin, dexamethasone, and methylisobutylxanthine (IDM). The effect of PSPL extracts varied with dose administration and with time course. The results obtained from MTT assay and BrdU incorporation showed that PSPL extracts could inhibit 3T3-L1 cells proliferation. PSPL extracts altered the protein amount of preadipocyte facror-1 (Pref-1) and CCAAT/enhancer binding protein α (C/EBPα). Our data suggested that PSPL extracts may modulate proliferation and differentiation of 3T3-L1 cells.

A3.57
Oral administration of GABA and phytosteryl ferulates prevent decreasing stressed mouse plasma adiponectin levels

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Stress perturbs physiological functions of mammals and is believed to exaggerate metabolic syndrome such as type 2 diabetes mellitus. Adiponectin, one of adipocytokines, binds to receptors in skeletal muscle and liver, increasing metabolisms of fatty acid and glucose and consequently improving insulin resistance. We previously demonstrated that oral administration of gamma

aminobutylic acid (GABA, derived from rice germ) and phytosteryl ferulates (PSFs, derived from rice bran) effectively increased plasma adiponectin levels in normal mice. In the present study, we have examined the effects of immobilization stress on the plasma adiponectin levels and the ameliorating effects of GABA and PSFs on the stress-induced hypoadiponectinemia. Mouse adiponectin was estimated through IR-fluorescent-Western blotting using anti-adiponectin antibody. Immobilization stress markedly reduced mouse plasma adiponectin levels. Oral administration of GABA increased adiponectin levels into normal levels. PSFs also recovered the levels. Simultaneous administration of GABA and PSFs drastically augmented the adiponectin levels. Encapsulated GABA and PSFs preparation newly developed successfully prevented the stress-induced hypoadiponectinemia. These results suggest that simultaneous intake of GABA and PSFs rich in rice products would prevent us from stress-induced metabolic syndrome.

A3.58
Robinia pseudo-acacia var. umbraculifera and Amorphastilbol enhance the glucose and lipid metabolism *in vivo* mice models for obesity and diabetes

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Introduction: Obesity has been demonstrated to be associated with lipid metabolic disorders, which commonly increase the risk of insulin resistance. Type 2 diabetes is also has been characterized by insulin resistance and hyperlipidemia.
Methods: Hypolipidemic effects of *R. pseudo-acacia var. umbraculifera* (RPA) and amorphastilbol (APH) were investigated in High-fat diet model (HFD). The anti-diabetic effects of RPA and APH were investigated in *db*/*db* mice model. After 8 weeks of administration, several biomarkers including body weight, food intake and blood glucose and mRNA levels for glucose and lipid metabolism were measured.
Results: Body, liver and adipose tissue weight were decreased in RPA and APH administrated HFD mice than control. The mRNA expression level of UCP2 was increased but G6Pase, decreased in liver and aP2, adiponectin and UCP3 mRNA were increased in adipose tissue. Blood glucose, triglyceride and free fatty acid levels were decreased while HDL-cholesterol was increased in *db*/*db* mice model. It was also improved glucose tolerance without any body weight changes. The mRNA expression levels of UCP2 and G6Pase showed same pattern in HFD model, and PPARγ and UCP3 were increased in adipose tissue.
Conclusions: We suggested that RPA and APH have a potential for treatment of type 2 diabetes and obesity by enhancing the glucose and lipid metabolism.

A3.59
Anti-inflammatory effects and mechanism of Hippocampus Kuda Bleeler derived compounds SE-1 and SE-2 on activated murine macrophages and microglial cells

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Inflammation has recently been implicated as a critical mechanism responsible for the neurodegenerative diseases. Macrophages and microglial cells are responsible for mediating inflammatory responses related to neurodegeneration. The two compounds isolated from sea horse Hippocampus Kuda Bleeler; 1-(5-bromo-2-hydroxy-4-methoxyphenyl) ethanone [SE-1] and 1-(2-hydroxy-4-methoxyphenyl) ethanone [SE-2] were studied as novel agents to suppress lipopolysaccharide (LPS) mediated inflammatory responses in cultured RAW264.7 and BV-2 cells. SE-1 and SE-2 significantly attenuated LPS induced release of inflammatory products such as nitric oxide, prostaglandin E2, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6). The compounds also down regulated the protein and gene levels of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), TNF-α, IL-1β and IL-6 in both cell lines. Molecular mechanism study shows that SE-1 and SE-2 inhibited the nuclear translocation of nuclear factor-κB (NF-κB) p65 and p50 subunits by attenuating the phosphorylation of IκBα. Also SE-1 and SE-2 suppressed the phosphorylation of MAPK molecules; JNK and p38. These results suggest the potential *in vitro* anti-inflammatory activity of SE-1 and SE-2, which inhibited the LPS stimulated inflammatory responses in RAW264.7 and BV-2 cells via suppressing MAPK and NF-κB signaling pathways.

A3.60
Anti-obesity effect of carboxymethyl chitin via AMPK and aquaporin-7 signaling pathway in 3T3-L1 adipocytes

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The aim of this study was to investigate the anti-obesity effect of carboxymethyl-chitin (CM-chitin), a water-soluble derivative of chitin, by measuring lipid accumulation and adipogenesis related factors in 3T3-L1 adipocytes. CM-chitin was synthesized by means of carboxymethylation reaction. Its inhibitory effect on lipid accumulation was investigated by measuring triglyceride content and glycerol release level. The gene and protein levels associated with adipogenesis were determined using RT-PCR and western blot analysis. Treatment with CM-chitin reduced triglyceride content and enhanced glycerol secretion in a dose-dependent manner. CM-chitin induced the down-regulation of adipogenesis related transcriptional factors and adipocyte specific gene promoters. Moreover, the specific mechanism by CM-chitin was confirmed by transcriptional activations of the phosphorylated adenosine monophosphate-activated protein kinase (AMPK) and aquaporin-7. These results suggest that CM-chitin exerts anti-adipogenic effect on lipid accumulation through modulations of AMPK and aquaporin-7 signal pathways.

A3.61
Glucosamine derivatives promote osteoblastic differentiation in osteoblastic MG-63 cells

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In this study, the effects of glucosamin derivatives (glucosamine-6-phosphate: PGlc and glucosamine-6-sulfate: SGlc) on osteoblastic cell differentiation were evaluated in osteoblastic MG-63 cells. Our results provided evidences that glucosamin derivatives

(PGlc and SGlc) have the ability to increase the alkaline phosphatase (ALP) activity, the osteocalcin (OC) level and the collagen level as phenotypic markers during osteoblastic differentiation. Moreover, the effect on BMP-2 activity, which is an important factor for bone formation, remodeling and mineralization, was examined by using ELISA, RT-PCR and western blot analysis. Treatment of osteoblastic cells with glucosamin derivatives (PGlc and SGlc) enhanced BMP-2 activity. The present study may provide new insights to the role of glucosamin derivatives in the osteoblastic differentiation and possibility for its application as a supplement in bone health.

A3.62

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A3.63
Structural properties of amyloidogenic apoA-I mutant L178H

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Apolipoprotein A-I (apoA-I) is the main protein constituent of high-density lipoprotein (HDL), commonly referred to as ''the good cholesterol''. HDL and apoA-I have numerous atheroprotective functions including anti-oxidative and vasodilatory effects, and transport of cholesterol from peripheral tissues to the liver. For these processes to occur apoA-I must exhibit significant structural plasticity. However, mutant variants of apoA-I have been shown to be amyloidogenic. Here we focus on a natural apoA-I mutant that contains a leucine to histidine substitution at position 178 in the C-terminal region. It has previously been shown that this apoA-I mutant aggregates into amyloid fibrils together with the transport protein transthyretin and forms deposits in the heart, larynx and skin. Aiming to understand the process of amyloid formation, we here present data on time-dependent structural changes as measured by circular dichroism spectroscopy, ANS binding and limited proteolysis. In contrast to most amyloidogenic proteins which exhibit increases in beta-strand structure, we show a large increase (> 30%) in the alpha-helical content of apoA-I L178H upon incubation at 37°C, conditions at which the wild type structure appears unperturbed. Time studies of dimyristoyl phosphatidylcholine (DMPC) binding indicate reduced lipid binding ability for the L178H mutant compared to wild type. Also, stability studies performed using the denaturing agents guanidine and urea show a greater involvement of hydrophobic interactions within the L178H mutant than in wild type apoA-I.

A3.64

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A3.65
Chronic high-fat-diet feeding effects on liver insulin resistance in male and female rats

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Numerous studies point to oxidative stress as initial key event triggering obesity-induced insulin resistance in several tissues, but the etiology of insulin resistance in liver remains unclear. Taking into account the gender differences in the incidence of obesity-associated disorders, the aim of the present study was to investigate the effect of obesity on insulin signaling pathway in liver of male and female rats, as well as its relationship with gender differences in hepatic lipid accumulation. Ten week-old Wistar rats of both genders were fed a pelleted standard diet (3% w/w fat) or a palatable high-fat-diet (HFD) (24% w/w fat) for 26 weeks. Glucose, insulin and resistin levels were measured in serum. Lipid content as well as IRβ, IRS-2, AKT and JNK protein levels were measured in liver. Serum markers of insulin resistance were markedly lower in control female rats than in males and liver IRβ, IRS-2 and AKT protein levels showed the opposite profile. In response to the HFD feeding, female rats exhibited a greater increase of both body and fat depot weights than males; in contrast, hepatic lipid accumulation was higher in males. With the dietary treatment serum markers of insulin sensitivity were impaired in female rats although without reaching the levels of insulin resistance shown by males. Moreover, IRβ and AKT activation decreased markedly in female rats while IRS-2 inhibitory phosphorilation increased in this gender. In this context, the increased insulin resistance shown by HFD female rats, together with their greater adipose tissue expandability, could be adaptive mechanisms that prevent liver from lipotoxicity.

A3.66
HSD11B1 gene polymorphisms in Bosnian patients with type 2 diabetes and metabolic syndrome

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Conversion of inactive cortisone to active cortisol by the enzyme 11beta-hydroxysteroid dehydrogenase type 1 (11beta-HSD1) enhances glucocorticoid receptor activation in target tissues. Increased expression of 11beta-HSD1 in adipose tissue is associated with obesity, glucose intolerance, type 2 diabetes and metabolic syndrome. In this study, we investigated association of two *HSD11B1* gene polymorphisms with metabolic syndrome and type 2 diabetes. DNA samples from 80 diabetic patients with metabolic syndrome were genotyped for two polymorphisms in *HSD11B1* gene: rs846910: G > A and rs45487298: insA by high resolution melting curve analysis. Influence of genotypes on clinical and metabolic parameters was assessed (fasting glucose, insulin, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, HbA1c, ALT, GGT, creatinine and urea levels). The mutated allele frequency was 0.06 and 0.24 for rs846910: G > A and rs45487298: insA polymorphism, respectively. The rs846910: G > A polymorphism was significantly related to higher total cholesterol and LDL cholesterol levels. The rs45487298: insA polymorphism was associated with lower plasma glucose levels. Our results show that common rs45487298: insA polymorphism in *HSD11B1* gene may be associated with better glycemic control in diabetic patients with metabolic syndrome.

A3.67
Differential expression and localization of insulin receptor in renal tubule cells of diabetic rats

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Insulin is essential for energy management in the body; however, its role in kidney metabolism has not been extensively studied. Its binding to the insulin receptor leads to a cascade of intracellular signalling events, which lead to a regulation of multiple biological processes such glucose metabolism. The insulin receptor (InsR) is a member of the receptor tyrosine kinase superfamily, and an integral glycoprotein of the plasma membrane in most mammalian cells. Here, we studied the expression, protein levels and localization of InsR in a long-term type 1 diabetes rat model induced by streptozotocin. Comparative qRT-PCR analysis demonstrated a significant increase in the expression of the InsR gene in renal tubule cells (RTC) of diabetic rats. In contrast, western blot analysis showed a significant reduction of the InsR subunit β level in these same cells. Furthermore, InsR immunoreactivity was localized in basolateral and apical plasma membrane of the RTC in renal cortex in control rats, while this pattern changed to a cytoplasmatic localization in the long term diabetic rats. This differential subcellular distribution and expression suggests the involvement of the InsR in the metabolic changes of the RTC of diabetic rats and its implication in the diabetic nephropathy (Foundation M. Botin; DID-UACh: 2006–19).

A3.68
Hedera helix L. against prostate cancer metastasis

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Hedera helix L., is a member of Araliaceae family, has cosmetic usage. The plant has such properties as antiproliferative, cytotoxic, antimicrobial, antifungal, antiprotozoal, antiinflamatory effects. The aim of this study is to apply the extracts of leaf and unripened fruits of *H. helix* prepared with ethanol, on highly metastatic Mat-LyLu and weakly metastatic AT-2 cells, which are rat prostate cancer cells of the Dunning Model *in vitro* and to determine the effects of these extracts on the cell proliferation and migration of these cell lines of different metastatic abilities. Cell proliferation was detected spectrophotometrically by MTT (methyl thiazolyl diphenyl tetrazolium) assay. Cell migration, however, was investigated via wound healing assay. Our results showed that the ethanolic leaf extract of *H. helix* was inhibited both proliferation and migration depending on dosage and time in highly metastatic Mat-LyLu and weakly metastatic AT-2 cells. Ethanolic fruit extract of *H. helix*, however, supressed the migration of highly metastatic Mat-LyLu cells where it didn't produce a significant change on the proliferation of this cell line. Although AT-2 cells migration did not effected by the ethanolic fruit extract, their proliferation was inhibited. In conclusion, the ethanolic fruit extract of *H. helix* may suppress metastasis unaffected by proliferation. However effect of the ethanolic leaf extract of *H. helix* did not clear on metastatic ability of the cancer cells due to inhibition of cell proliferation.

A3.69
Lipid trafficking alterations in acid ceramidase-deficient human fibroblasts

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Farber disease (FD) is a fatal sphingolipidosis caused by the deficient activity of acid ceramidase (AC). This hydrolase is responsible for the degradation of ceramide (Cer) into sphingosine and free fatty acid in lysosomes. Although some advances have been made in the knowledge of the genetic and biochemical bases of AC-deficiency, little is known about the disease molecular pathogenesis. One pathogenic event common to several sphingolipidoses could reside in blocking of intracellular transport to or from lysosomes at several related cellular points with subsequent endosomal/lysosomal jam. The present study investigated whether intracellular trafficking of specific lipid molecules is blocked in FD. Increased filipin staining, indicative of high levels of intracellular unesterified cholesterol, was observed in AC-deficient cells and found to co-localize with a lysosomal marker. A similar observation was made for the ganglioside GM2. By fluorescence microscopy BODIPY-LacCer was found to be endocytosed from the plasma membrane to endosomes/lysosomes, in contrast to normal control cells where the lipid analog was transported to the Golgi complex. For BODIPY-C5-Cer, an increased level at the Golgi complex was seen in FD cells. Overexpression of AC corrected lipid traffic imbalances observed in AC-deficient fibroblasts. These data provide evidence for the importance of a steady-state level of lysosomal Cer for lipid homeostasis that, when disrupted, may lead to an unbalanced level of sphingolipids and cholesterol and subsequently, to alterations in cell signalling specific pathways with impact in intracellular lipid trafficking.

A3.70

Abstract withdrawn

A3.71
No evidence for the involvement of carnitine palmitoyltransferase 1 transmembrane domains in the formation of an acylcarnitine pore

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Carnitine palmitoyltransferase 1 (CPT1) is recognized as the enzyme catalyzing the rate limiting reaction in the β-oxidation of long-chain fatty acids, converting long-chain acyl-CoAs into the respective carnitine ester. It remains controversial how these acylcarnitine intermediates have access to the intermembrane space to be further transported by carnitine acylcarnitine translocase and reconverted back to long-chain acyl-CoAs by carnitine palmitoyltransferase 2. It has been previously shown that rat

CPT1A has a hexameric quaternary structure arrangement, suggesting that this oligomeric complex could function as a pore channelling acylcarnitines through the outer mitochondrial membrane (OMM). We aimed to investigate if oligomerization of CPT1 to form such pore would indeed be the only pathway by which acylcarnitines enter into the intermembrane space. Oxidation of C16-carnitine was measured in control and CPT1 deficient intact human fibroblasts in order to disclose if this process is independent of CPT1 transferase activity but dependent on the hypothesized pore formation. We found that using C16-carnitine as substrate total β-oxidation in CPT1 deficient cell lines (carrying a nonsense mutation) is not affected. Thus, although both CPT1 function and structure are severely impaired in the deficient cell lines, C16-carnitine is still able to access the mitochondrial matrix to be further metabolized. Facing this, it seems that either the pore formed by oligomerization of CPT1 does not function as a channel for the produced acylcarnitines or this is not the exclusive route (and certainly not the major one) involved in acylcarnitines channelling through the OMM.

A3.72

Abstract withdrawn

A3.73
Molecular pathogenesis of a new glycogenosis caused by a mutation in glycogenin-1

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We recently described a new disease in a patient with cardiomyopathy and muscle weakness. Histochemical staining revealed a striking glycogen deficiency in skeletal muscle and an abnormal carbohydrate accumulation in the heart. Sequencing of the glycogenin-1 gene, *GYG1*, revealed a nonsense mutation in one allele and a missense mutation, Thr83Met, in the other. The allele with the missense mutation, the only allele expressed, resulted in inactivation of the autoglucosylation of glycogenin-1. Glycogenin-1 is autoglucosylated with approximately 10 glucose residues at tyrosine 195 and thereby initiates the glycogen-synthesis. To further analyze the pathogenesis of the Thr83Met mutation, human glycogenin-1 was expressed in CHO cells. Wild type glycogenin-1 was autoglucosylated whereas the Thr83Met mutated form was not. Similar results were obtained when apo-glycogenin-1 was expressed in a cell-free system and autoglucosylated *in vitro*. To test if threonine in position 83 is functionally important for autoglucosylation we replaced it with other amino acids in the cell-free *in vitro* system. Serine in position 83 was functional whereas tyrosine and phenylalanine were not. The main difference between the functional and the non-functional amino acids, that we tested, is size suggesting that the amino acid in position 83 is of sterical importance. Two different catalytic activities are carried out by glycogenin-1: The linkage of glucose to tyrosine 195 and the elongation of α-1,4 linked glucose molecules. By mass spectrometry we demonstrated that Thr83Met mutated glycogenin-1 was unable to catalyze the tyrosine-O-glucose linkage explaining the inactivated autoglucosylation.

A3.74
Lipin 1 gene polymorphisms in Bosnian population with type 2 diabetes and metabolic syndrome

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Metabolic syndrome is a cluster of risk factors for atherosclerotic disease and type 2 diabetes mellitus comprising obesity, insulin resistance, hypertension, and dyslipidemia. The individual components of metabolic syndrome are strongly influenced by genetic factors. Lipin 1, a novel molecular protein expressed by adipocytes, has marked effects on adipose tissue mass, insulin sensitivity, and glucose homeostasis. Thus, we hypothesized that genetic variants within lipin 1 gene *(LPIN1)* are associated with traits of the metabolic syndrome. The study was done on 70 patients diagnosed with metabolic syndrome and type 2 diabetes. All subjects involved in the study were patients of General Hospital Tesanj (Bosnia and Hercegovina). DNA from those patients was isolated from the whole blood using the QIAGEN method. A total of two single nucleotide polymorphisms (SNPs) in *LPIN1* gene, rs11693809 and rs2716610, were analyzed by real-time PCR with hydrolyzing probes. Correlations between biochemical parameters (glucose, HbA1c, insulin levels, HDL and LDL cholesterol, triglycerides, serum proteins, liver enzymes, etc) and rs11693809 and rs2716610 polymorphisms in *LPIN1* gene were tested. Our analysis revealed correlation between AST (aspartate aminotranspherase) activity and the rs11693809 polymorphism in diabetic patients with metabolic syndrome.

A3.75
Assessment of oxidative stress and cytokines in saliva and plasma of patients with oral aphthous ulcer lesions

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Recurrent aphthous ulcerations (RAU) are common oral inflammatory lesions. Predisposing factors such as trauma, microbial factors, food, drug reactions, immune disorders, hormonal imbalance and smoking, all of which trigger free radical formation and upset the oxidant/ antioxidant balance of an organism, as well as familial tendency, are believed to be involved in the etiology of RAU. With its contents of antioxidant molecules and enzymes, saliva and serum are the first step of the defensive mechanism against free radicals. The aim of the present study was to determine the salivary and serum malondialdehyde (MDA) level, glutathione (GSH) level, tumor necrosis factor- alfa (TNF-α) and interleukin (IL-2 and -10) levels in patients with RAS. Salivary and serum MDA and GSH levels were determined by using high performance liquid chromatography (HPLC) and also cytokines levels were evaluated by ELISA. Salivary and serum MDA and GSH levels were significantly elevated ($p < 0.01$) when compared to with control group. Although interleukin -2 and TNF- α levels were found high levels, IL-10 level was low with RAU in patients. The antioxidant system and levels of cytokines have been affected in the process of RAU.

A3.76
Effects of high-fat-diet feeding on skeletal muscle mitochondrial biogenesis in male and female rats

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High-fat-diet (HFD) feeding leads to overweight, insulin resistance and oxidative stress and has been associated to a decrease of mitochondrial mass and function. Since gender differences in mitochondrial function and oxidative stress have been reported, the aim of the present study was to investigate whether there is a gender dimorphism in the consequences of a HFD feeding on rat skeletal muscle mitochondrial biogenesis. Male and female Wistar rats of 2 months of age were fed a hypercaloric and hyperlipidic diet or a control pelleted diet for 26 weeks. Soleus and gastrocnemius muscle protein levels of PGC-1alpha, TFAM, COXII, UCP3, and antioxidant defences, as well as markers of oxidative damage were measured. In both genders, HFD feeding increased PGC-1alpha and TFAM levels in soleus and gastrocnemius muscles and COXII in the later. HFD fed male rats showed higher oxidative damage than their female counterparts in both muscles, and lower Mn-SOD and Cu-SOD levels in gastrocnemius. Soleus UCP3 levels were higher in control female rats than in males and increased with HFD feeding only in males, whereas HFD feeding increased UCP3 levels in gastrocnemius muscle of both genders. In conclusion, oxidative damage induced by HDF feeding is more marked in male rats, probably due to their lower antioxidant protection compared to female rats. In response to HDF feeding, male rats increased mitochondrial differentiation in a higher degree than females, suggesting gender differences in mitochondrial biogenesis in order to counteract the consequences of the increased availability of metabolic fuels.

A3.77
Determineted to bile salts deconjugation ability of free and immobilized these strains and cholesterol removal by some Bifidobacterium sp

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The aim of this study is primarily to evaluate the deconjugation of sodium taurocholate by four strains of Bifidobacterium sp. selected according to cholesterol removal capacity. Influence of different bile concentrations on cholesterol removal has been studied. Secondly, it has been targeted to determine the utility of immobilization effects on the deconjugation of sodium taurocholate of strains which have the highest deconjugation property. Bifidobacterium breve (A28, S4 and S9) and Bifidobacterium bifidum (A10) strains were used in this study. It has been confirmed that strains which have cholesterol removal capacity (A28, S4) were able to deconjugation of sodium taurocholate than those low cholesterol removal capacity (A10, S9) and the highest cholesterol removal (18%) was observed by strain A28 in 3 mg/ml bile concentration. Viability and deconjugation of sodium taurocholate of immobilized strains were determined. Deconjugation of sodium taurocholate and cholesterol removal by immobilized and free cells was studied and it has been determined that immobilized cells were more effective. According to these results, immobilized forms of B. breve A28 and S4 can be planned to be used in probiotic industry because of having higher cholesterol removing, viability and deconjugation ability.

A3.78
Gender differences in adipose tissue fatty acid composition and lipid management in response to changes in dietary fat

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High-fat diet (HFD) feeding usually leads to hyperphagia and body weight gain, but macronutrient proportions in the diet can modulate energy intake and fat deposition. The mechanisms of fat accumulation and mobilization may differ significantly between depots, and gender can also influence these differences. The aim of the study was to determine gender differences in fatty acid (FA) composition of visceral and subcutaneous adipose tissues in response to changes in diet composition. We further analyzed the influence of both dietary FA and levels of proteins involved in tissue FA uptake and release on fat deposition. Wistar rats of both genders were fed a HFD (30% w/w fat) or a control diet (3% w/w fat) for 14 weeks. FA composition was analyzed by gas-chromatography and levels of LPL, HSL, α2-AR, β3-AR and PKA were determined by Western blot. HFD feeding increased adiposity without changes in body weight and induced a fat accumulation in adipose tissues, which was gender- and depot-dependent. FA content of HFD was reflected in the tissue FA composition, with an increase in the proportion of MUFAs and a decrease in that of PUFAs in both fat depots. The sexual dimorphism in adiposity index could be associated to changes in the levels of proteins involved in tissue lipid management, and also related with a differential response to dietary FA content. Thus, the lower tissue fat accumulation of HFD female rats may be linked to lower LPL levels and higher levels of intermediates in the lipolytic pathway.

A3.79
Effect of ovariectomy and 17beta-estradiol on mitochondrial function and insulin response in rat skeletal muscle

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Sexual dimorphism in mitochondrial function has been evidenced in several tissues, including skeletal muscle. Sex hormones, especially estrogens, could play a key role in mitochondrial energy metabolism regulation, which is intimately linked to insulin response. In this context, estrogens seem to preserve mitochondrial structure and function by reducing oxidative stress and inhibiting apoptosis process. The aim of the present study was to evaluate the effects of ovariectomy and a subsequent estrogens supplementation on mitochondrial function, as well as its relationship with insulin sensitivity in skeletal muscle (soleus and gastrocnemius). 13-weeks old Wistar rats were used and divided into four experimental groups: controls both male and female, ovariectomized and ovariectomized supplemented with 17beta-estradiol. Soleus (prevalently oxidative) and gastrocnemius (prevalently glycolytic) muscles were homogenized and protein levels related with insulin response (GLUT-4, IRS, IRbeta) and mitochondrial function (ATPase, COXIV, TFAM) were measured by Western-blot. The results indicated that ovariectomy induced an insulin sensitivity decrease in soleus muscle, which was partially reversed by estradiol administration. On the other hand, the levels of marker proteins of mitochondrial function were diminished in gastrocnemius

muscle of castrated animals, and estrogens replacement recovered the effect of ovariectomy. As a whole, results show an effect of hormonal environment on functionality of skeletal muscles, and they could also suggest a different response from muscle tissues to ovariectomy, depending on their prevalent metabolism.

A3.80
Synthesis of novel 1,3-Diarylpyrrazol derivatives as potent inhibitors of COX-2 and thromboxane synthase

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It is well known that inhibition of the enzyme cyclooxygenase (COX) is the principal mechanism for the efficacy of NSAIDs. Among all the COX isomers selective inhibition of COX-2 which is being induced during inflammation is off interest since the resulting drugs lack the gastric side effects associated with classical NSAIDs. However, their prolonged use causes cardiovascular side effects which limit their clinical use. Researchers suggested that COX-2 inhibitors may increase the risk of thrombotic effects by reducing the production of prostacyclin (PGI2), while exerting a negligible effect on Thromboxane A2 (TXA2), level. PGI2 formation is associated with vasodilatation and reduction in platelet aggragation. In contrast, TXA2 promotes vasoconstriction and platelet aggregation. While traditional NSAIDs were known to inhibit both PGI2 and TXA2 formation, the more focused inhibition of COX-2 by selective inhibitors leaves TXA2 unaffected tipping the balance toward a prothrombotic effect. Based on these results, dual inhibition of COX-2 and thromboxane synthase emerged as a new strategy to provide effective and safer NSAIDs lacking the drug associated GI and cardiovascular side effects. Thus, we have designated and synthesized novel compounds which bear a central pyrrazole ring whose 1,3,4-positions are substituted with pheny, 3-methyl-2(3H)-benzoxazolinone moieties and substituted cinnamic acid residues, respectively. The results of inhibitory potency against COX-2 were evaluated in an enzymatic assay using human whole blood. Platelet aggregation was measured using an aggregometer according to the turbidimetry method of Born and Cross.

A3.81
Characterization of the molecular basis of acute intermittent porphyria

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Acute intermittent porphyria (AIP) is an inherited disease caused by reduced activity in the third enzyme of the heme biosynthetic pathway, hydroxymethylbilane synthase (HMBS). The reasons for this reduction and the consequences of the mutations are not completely understood. However, valuable information about the phenotype-genotype correlations can be obtained by functional studies on both the wild-type (wt) enzyme and certain disease causing mutations found in specific regions of HMBS. For these studies we have initially chosen R116W, K132N, R167W, R173W and V215E mutations, representing: i) mutations around the active site and/or interfering with proper binding of the co-factor dipyrromethane, ii) mutations far from the active site and predicted to affect overall folding and flexibility, and iii) mutations affecting efficiency of transcription or translation. Presently, we have expressed recombinant human wt-HMBS and the 5 selected mutations in *E. coli* using pGEX expression vectors. The enzymes have been purified on Glutathione-Sepharose 4B and we have obtained high yields and purity for wt and most mutants. We are at present characterizing the steady-state kinetic parameters of the mutants comparative to the wt as well as their thermostability by differential scanning calorimetry, circular dichroism and fluorescence spectroscopy. Wt-HMBS shows a high thermostability (T_m = 79.5°C), and some of the mutants reveal a decreased thermostability, in addition to kinetic defects. Strategies aiming to stabilize the mutants are being investigated. The results obtained so far aid to understand the pathogenic mechanisms in AIP and will be presented at the conference.

A3.82
Research of antihypoxic drugs effect with myocardial disturbances

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Cardiovascular diseases accompanied the phenomena a hypoxia and ischemia depending on development of pathological state. In pathology of all these states matters, permeability of membranes, violation of energy and plastic metabolism, of vessels endothelia, intensification of lipid oxidation processes, increasing formation of super oxide anions and decline of activity of the free radical scavenging system. Therefore for prevention of harmful influence of the hypoxia states at ischemia, in case of occurring of extreme situations it is necessary to apply medications, which simultaneously influence on the indexes of energy metabolism, execute the function of free radicals scavengers, inhibit processes of lipids oxidation. In an experiment on rats, mise designed by hypoxia of various aethiology at previous introduction of acetylcystein, taurine, arginine, corargin, glutargin. Investigated influence of these preparations on the indexes of energy metabolism (content of adenylic nucleotides, creatine phosphate, activity of creatine phosphokinase), indexes of lipid oxidation, system of free radical scavenging (activity of catalase, super oxide dismutase) in tissues of myocardium, and also activity of inducible NOS (iNOS II) in the platelets of animals. Under hypoxia of various aethiology the probed preparations are different prevented the changes of energy metabolism indexes, lipid oxidation, system of free radical scavenging. As a result of high activity of iNOS the nitric oxide (NO), which executes a cytotoxical function under the pathology, is produced. For the correction of these changes applied donors of NO: arginine, corargin, glutargin.

A3.83

Abstract withdrawn

A3.84

Abstract withdrawn

A4 – Neurobiology

A4.01
Capsaicin is a key molecule in neuropathic pain induced by diabetes

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Diabetic peripheral neuropathy is a common chronic complication of diabetes mellitus. Transient receptor potential (TRP) ion channels are involved in sensing physical and chemical stimuli. TRPV1 is a Ca^{2+}permeant non-selective cation channel expressed predominantly by unmyelinated C-fibers and thinly myelinated A-δ fibers and plays a major role in inflammatory thermal sensation. Capsaicin is the active component of chili peppers (which are plants belonging to the genus Capsicum) and is the main compound responsible for TRPV1 activation. In this study, we have used a transgene-mediated diabetes model to study the role of capsaicin in diabetic peripheral neuropathy. We have used sensory neurons from dorsal root ganglia prelevated from TCR-$HA^{+/-}$/Ins-$HA^{+/-}$ mice and Balb/c. Whole cell patch-clamp recording and immunofluorescence microscopy have been employed. The larger amplitude and reduced desensitization of TRPV1 currents induced by capsaicin, obtained in TCR-$HA^{+/-}$/Ins-$HA^{+/-}$ mice, closely resemble changes identified in streptozotocin-induced diabetes in rats. Expression of TRPV1 was significantly higher in the double-transgenic diabetic mice. Capsaicin treatment reduced the TRPV1 expression. In diabetes, the link between abnormal pain sensitivity and hyperglycemia resulting from insulin deficiency is not clear, but our study proves that capsaicin plays a major role in regulating TRPV1 function and expression in diabetes. This study was financed by the research grant PNCDI2 41-074/2007.

A4.02
The use of spect scintigraphy with I-123 ioflupane in differentiation of patients with parkinsonian syndromes and essential tremor

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Introduction: Parkinson's Disease is a progressive neurodegenerative disease. It is important to diagnose the disease in its early stages to rule out symptomatic Parkinsonism or Parkinsonian Syndromes and start specific treatment. Dopamine transporter imaging such as 123I-Ioflupane has been developed to directly measure degeneration of dopamine presynaptic terminal.
Aim: The study of patients with clinically uncertain Parkinsonian Syndromes, in order to help differentiate Essential Tremor from Parkinsonian Syndromes related to idiopathic Parkinson's disease, with scintigraphic imaging with 123I-Ioflupane.
Patients & Method: Sixteen consecutive patients nine male and seven female (58 ± 9 years old) with movement disorders, were included in the study. The administered dose was 110MBq123I-Ioflupane. Lugol's solution were given to each patient to minimize uptake of radioactive iodine by the thyroid gland. In each patient, SPECT scans were acquired on a gamma-camera at 3 hours post-injection. The acquired images were interpreted visually with consensus between two nuclear medicine physicians.
Results: Normal images are characterized by two symmetrical crescent-shaped areas of equal intensity. Abnormal images are either symmetric or asymmetric with unequal intensity and/or loss of crescent. Abnormal images with reduced DAT density in the striatum, were seen in nine patients and these patients had neurodegenerative Parkinsonism. The other seven patients showed homogeneous and symmetrical uptake in the striatum and in these patients Parkinson's disease was ruled out. (images shown)
Conclusions: SPECT imaging with 123I-Ioflupane is a very useful tool to differentiate benign essential tremor from Parkinson's disease.

A4.03

Abstract withdrawn

A4.04
Protective effects of KHG25857 against glutamate-induced excitotoxicity in rat glial cultures

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We have screened new drugs with a view to developing effective drugs against glutamate-induced excitotoxicity. In the present work, we show effects of a new drug, KHG25857 against glutamate-induced excitotoxicity in primary rat glial cultures. Pretreatment of glial cells with KHG25857 for 2 hour significantly protected glial cells against glutamate-induced excitotoxicity in a time- and dose-dependent manner with an optimum concentration of 100 μM. The drug significantly reduced production of proinflammatory cytokines, tumor necrosis factor-α, and interlukin-1β in glutamate-induced excitotoxicity. The drug also prevented glutamate-induced intracellular Ca^{2+} influx and reduced the subsequent overproduction of nitric oxide and reactive oxygen species. Furthermore, the drug preserved the mitochondrial potential and inhibited the overproduction of cytochrome c. In addition, the drug effectively attenuated the protein level changes of β-catenin and glycogen synthase kinase-3β. These results suggest that KHG25857 effectively protected primary cultures of rat glial cells against glutamate-induced excitotoxicity.

A4.05
Antioxidant status in Schizophrenia

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Although the biochemical mechanisms involved in schizophrenia are not yet completely understood, there are mounting evidences

that oxidative stress-mediated cell damage plays a role in the path physiology of schizophrenia. In the present research we have chosen to study the biochemical blood values of reduced G-SH, GR, GPx, TAS, SOD, creatine and creatinine. For this reason we have selected one group of 21 male schizophrenic subjects with medication and a control group of 18 healthy subjects. For all the schizophrenic subjects, the creatinic index [IC = creatine/(creatine + creatinine)] was considerably lower than the normal level of 0.5. It was also observed an important decrease below the normal range of the reduced G-SH for all the patients in the schizophrenic patients group compared with the normal reduced G-SH levels seen in the control group. For GR, TAS and GPx we have seen in the case of the schizophrenic patients lower decreases than in the case of G-SH, but statistically significant when compared with the values seen for the control group. The SOD was found increased, possibly as an adaptive response to free radical overload. We may conclude that in schizophrenia there exists very probably some imbalances in the antioxidant defenses of the organism and some possible perturbations of the energy metabolism and of the processes of detoxification of ammonia that seems to be indicated by the imbalances of the creatinine/creatine ratio manifested in the reduction of the creatinic index.

A4.06
Minerval induces ER stress and apoptosis in astrocitoma 1321N1 cells but not in MRC-5 fibroblast cells

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Minerval (2-hidroxi-9-cis octadecenoic acid) impairs lung cancer (A549) cell proliferation upon modulation of the plasma membrane lipid structure and subsequent regulation of protein kinase C localization and activity. However, this mechanism does not fully explain the regression of tumors induced by this drug in animal models of cancer. We have investigated the effect of palmitate and Minerval, on endoplasmic reticulum (ER) stress pathways and induction of apoptosis in 1321N1 astrocytoma-cells and MRC-5 fetal fibroblast cells. For this purpose the cells were incubated in the presence or absence of Minerval and/or palmitate for 12, 24 and 48 hour under low-serum conditions. In this context, 1321N1 cells underwent apoptosis, as determined by caspase-8 activation and PARP degradation. Concomitantly, the levels of ER stress markers, phosphorylated eIF2, Ire1-alfa, XBP-1, and CHOP, were increased compared with control (untreated) cells. In MRC-5-cells, Minerval did not induce ER-stress whereas palmitate induced the expression of the above ER stress markers. In summary, Minerval induces ER stress and apoptosis in 1321N1 human astrocytoma cells, but not in MRC-5 human fibroblast cells. These data provide relevant data about the mechanism of action of Minerval and its high efficacy in the absence of toxic effects.

A4.07
Some biochemical changes in the brain of young rats subjected to Chlorpyrifos neurotoxicity

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Chlorpyrifos (CPF) is one of the most commonly used organophosphorus (OP) pesticides in the world. Like the other OP, CPF (O, O-diethyl-3, 5, 6-trichloro-2-pyridylphosphorothioate) inhibits the enzyme acetylcholinesterase (AChE), which destroys acetylcholine, the neurotransmitter that activates cholinergic neurons. But number of scientist repeatedly demonstrated that CPF toxicity is not limited to cholinesterase inhibition alone but can act by other mechanisms. The aging brain shows selective neurochemical changes involving several neural cell populations. Increased brain metal levels have been associated with normal aging and a variety of neurodegenerative disorders. Copper is an important modulator of NMDA-receptor activity, zinc – of glutaminergic transmission. It is thus important to elucidate the mechanisms by which metal homeostasis of brain is maintained and how metals function in cellular processes, including neurotoxic damages. In present work we have studied levels of copper, zinc and iron in hippocampus, cerebellum and cerebral cortex of growing rats subjected to low doses of Chlorpyrifos. Simultaneously we have studied changes in activity of the antioxidant enzymes such as catalase, glutathione peroxidase and superoxide dismutase. Our data demonstrates age-dependent changes of investigated metal levels in different brain regions. We observed also some age-dependent and brain region-dependent changes in antioxidant enzymes activity. A whole series of biochemical analysis of different parts of rat brain and our previous experiments with Morris water maze confirm the CPF neurotoxicity and at the same time impel for investigations on cellular and molecular level.

A4.08
Energetic metabolism of myelinated axons: a new correlation among demyelination and axonal degeneration in Multiple sclerosis

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Multiple sclerosis (MS) is a demyelinating disease in which the myelin sheaths around the axons are damaged by immune system. During MS, the loss of myelin doesn't simply cause a lowering of speed of conduction but also an axonal necrosis. Although, this is the major determinant of irreversible neurological disability, the mechanisms of axonal loss are poorly understood. Among the proposed hypotheses for degeneration of demyelinated axons, it was suggested that axonal degeneration depends on energy depletion [1]. Our recent study has demonstrated that the whole respiration chain is present in isolated myelin; moreover, these proteins are able to produce ATP, consuming oxygen [2]. This suggest that myelin sheath, not only surrounds the axon, but supplies it, through energy production. So we hypothesize that the demyelinization would imply a dramatic ATP depletion and consequent loss of functionality, axon survival and cytoarchitecture, suggesting a new neuro- trophic role for the sheath. By both biochemical and imaging techniques, we have observed that the presence and the functionality of the complete electron transport chain, normally present in myelin sheath, decrease drastically in the MS plaque. Moreover, this loss appears more serious in the active plaques respect the very early active plaques. These results could give a new input to the understanding of the correlation between the myelin loss and the axonal degeneration, clarifying the neuro-trophic role of myelin.

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A4.09
Involvement of proteases in oxidative stress-induced necrotic cell death

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At present necrosis is considered as perfectly determinate process which can be delayed or switched to apoptosis at some defined stages. Formerly the involvement of proteolytic enzymes such as calpains and cathepsins in cells death processes was revealed in a number of investigations. The aim of our study is to elucidate the contribution of different protease types in oxidative stress (OS)-induced necrosis of cultured neuronal cells. An effect of three protease inhibitors – benzamidine (tripsin-like serine protease inhibitor), leupeptine (inhibitor of serine and cysteine proteases) and pepstatine A (inhibitor of aspartate proteases) on rat pheochromocytoma PC12 cells survival after OS was investigated. OS was performed by 30-min incubation of PC12 cells in medium with 1 mM H_2O_2. Necrotic cells were detected by staining with trypan blue dye 4 hour after OS induction. It was shown that benzamidine, leupeptine and pepstatine A suppress OS-induced necrosis of PC12 cells. At concentrations of 100 (benzamidine), 0.1 (leupeptine) and 50 mkM (pepstatine A) we observed the highest effect expressed in reducing the number of necrotic cells – 45, 25 and 43%, respectively. Using different schemes of inhibitors application it was established that benzamidine and pepstatine A were more effective when applied before and during H_2O_2-incubation. On the contrary cytoprotective effect of leupeptine was independent of application period. The data obtained indicate that benzamidine- and pepstatin A-sensitive proteases are involved in early stages of necrosis, whereas leupeptine-sensitive – in later stages. This study was supported by the Russian Foundation for Basic Research (Grant 08-04-01760).

A4.10
L-carnitine regulator – mildronate exerts acute anticonvulsant and antihypnotic effects

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A structural analogue of L-carnitine and an inhibitor of its biosynthesis, mildronate is used in clinics as an anti-ischemic drug for neurological applications. The effects of mildronate on the nervous system have primarily been evaluated after long-term treatment, which significantly decreases the tissue concentrations of L-carnitine. However, the effects of acute administration of mildronate have not been systematically examined before. Mildronate (200 mg/kg, acute intraperitoneal administration) exerted dose-dependent anticonvulsant activity in a chemoconvulsant pentylenetetrazole (PTZ)-induced clonic and tonic seizure test but did not change the effects of a convulsion-inducing dose of (+)-bicuculline, a γ-aminobutyric acid receptor antagonist. Mildronate also showed a dose-dependent antihypnotic effect on ethanol-induced loss of righting reflex, and it partially inhibited the reduction in cortical cerebral blood flow observed after ethanol administration. However, in a PTZ-induced seizure test, mildronate significantly stimulated the anticonvulsant activity of ethanol. The anticonvulsant activity of mildronate was completely blocked after pre-treatment with α2-adrenergic receptor antagonist yohimbine (2 mg/kg) and nitric oxide (NO) synthase inhibitor NG-nitro-L-arginine (10 mg/kg). These results demonstrate that acute administration of L-carnitine regulator mildronate exerts anticonvulsant and anti-hypnotic effects. The mildronate activity involves the α2-adrenergic receptor and NO-dependent mechanisms. These findings indicate that acute administration of mildronate could be beneficial for the treatment of seizures and alcohol intoxication.

A4.11
Dimensional alterations of planar Purkinje cell dendrites in reeler mice

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Reeler mice provide an animal model to understand mammalian brain development and brain pathologies. Particularly interesting are structural problems at the cellular level: the atrophy of the cerebellum with disrupted cortical cytoarchitecture is a typical pathology in such a model, also observed in many neurological disorders. However, the important alteration patterns of Purkinje cell dendrites remain largely unknown. Here we provide direct evidence that the alignment of Purkinje cells in normal mice becomes disrupted in reeler mice. We observed this important alteration pattern by combining synchrotron X-ray microscopy with Golgi staining, visualizing the 3-dimensional (3-D) neuronal micro-architecture of ultrathick specimens (up to 1 mm). We specifically found dimensional alterations of planar Purkinje cell dendrites, i.e. abnormal 3-D arborization in reeler mice. The quantitative analysis of the branching patterns in a 3-D geometry revealed that the branching angle did not significantly change from 77 ± 8 (mean ± SEM) in spite of the dimensional alteration, whereas the length of branch segments strongly increased with a large variation with respect to normal specimens. The fractal dimension of the Purkinje cells, estimated in 3-D coordinates, decreases from 1.723 to 1.254, revealing a significant reduction of dendritic complexity. These dimensional alterations, in addition to abnormal Purkinje cell migration during cerebellar development, could be a factor in neurological disorders including motor malfunction in reeler mice.

A4.12
The N-terminus of amyloid-beta plays a crucial role in its aggregation and toxicity

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The aggregation of Amyloid Beta (Aβ) peptide into insoluble amyloid fibrils that deposit in the brain is one of the primary pathogenic events in Alzheimer's disease. We have previously shown, using a Drosophila model of Aβ toxicity, that the N terminus of the Aβ peptide, despite being unstructured in the mature Aβ fibril, nonetheless affects Aβ induced neurodegeneration *in vivo*. In order to understand the contribution of the N terminus of Aβ to its aggregation behaviour, we have investigated a number of rationally designed N-terminal mutants *in vitro*. We find that single amino acid mutations in this region affect significantly the kinetics of Aβ aggregation *in vitro* as measured by a range of spectroscopic techniques. Furthermore, we observe striking differences in the morphology of the aggregated species formed by these different Aβ mutants when imaged with TEM

or AFM and also in the β-sheet content of their mature fibrils. Interestingly, mutants with an increased net charge or lower hydrophobicity tend to show slower aggregation kinetics, and to form more ordered aggregates whereas mutations that reduce net charge or increase hydrophobicity favour faster aggregation kinetics and poorly structured aggregates. In addition, the exposed hydrophobicity of aggregates formed in the early stages of aggregation is correlated to their toxicity. These findings demonstrate not only that the N-terminus of the Aβ peptide plays a crucial role in its aggregation and toxicity but also suggest that this region of Aβ may modulate *in vivo* toxicity by altering the conformations of aggregates that it forms.

A4.13
Selective PMCAs silencing modulates calmodulin genes expression in cAMP-differentiated PC12 cells

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Transient Ca^{2+}waves or prolonged fluctuations in its concentration are well-established phenomenons observed during neuronal cell differentiation. Ca^{2+}action in the regulation of signaling pathways may be realized in both direct or indirect ways. However, to sustain proper signal transduction, Ca^{2+}concentration must be tightly controlled. Among others, the most sensitive mechanism is a plasma membrance Ca^{2+}-ATPase (PMCA), existing in four functional isoforms. While PMCA1 and PMCA4 are widely distributed, PMCA2 and PMCA3 are unique for excitable cells. The activity of PMCA is regulated by Ca^{2+}/Calmodulin (Ca^{2+}/Calm) complex, the most important naturally existing activator. Using PC12 cell lines with suppressed PMCA2 or PMCA3 isoforms, we explored the role of PMCAs in dibutyryl-cAMP induced differentiation as well as cAMP effect on Calm genes expression. Calm is encoded by three independent genes: *calmI*, *calmII* and *calmIII*, all together producing an identical protein. Using real-time PCR technique, we demonstrated that expression of *calmI* and *calmII* was increased in both transfected lines, although to a different degree. Signals from cAMP pathways may be also mediated via CREB, a transcription factor that is able to activate numerous downstream genes. In our study, CREB phosphorylation correlated with activation of ERK and JNK, showing possible ways of CREB acivation. These data indicate on the unique connection between PMCAs composition and expression profile of CaM genes during PC12 cell differentiation. Supported by: 502-16-809, 502-16-810 and 503-6086-2 from the Medical University of Lodz, Poland.

A4.14
Beta amyloid fibril destabilizing activities of some pyrazoline derivatives

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Alzheimer's Disease (AD) is a common progressive neurodegenerative disease characterized by intraneuronal neurofibrillary tangles and the extracellular deposition of the amyloid β-peptide. Aβ peptide,is shown to be a proteolytic product of amyloid precursor protein (APP), and polymerization of Aβ (1–40) and Aβ (1–42) peptides,lead to the formation of insoluble β-amyloid fibris (fAβ) in AD brains. Destabilization of preformed fAβ is suggested to be an attractive therapeutic target fort he tratment of AD. Increased brain and platelet monoamine ozidase-B (MAO-B) activities in AD patients suggested that ozidants formed by MAO catalysis may cause the neurodegeneration, and MAO inhibitors have some neuroprotective effects. Recent studies indicating that MAO-B inhibitors precent the Aβ-fibril aggregation and senie plaque formation in AD are promising. On the basis of our preliminary studies showing that some N-substituted thiocarbamoyl derivatives had potent monoamine oxidase inhibitory activities, in the present study we synthesized 1-N-substituted thiocarbamoyl-3-phenyl-5-(pyrrol-2-yl)-4,5-dihydro-(1H)-pyrazole derivatives as MAO inhibitors and evaluated their β-amyloid fibril destabilizing activities using spectroscopy, fluorescence spectrocopy, electron microscopy and MALDI Mass Spectroscopy. Our results showed that 1-N- substituted thiocarbamoyl-3-phenyl-5-(pyrrol-2-yl)-4,5-dihydro-(1H)-pyrazole derivatives directly inhibited the deposition of fAβ *in vitro*. Although exact mechanism of the anti-amyloidogenic activity of these derivatives is unclear, pyrazolines appeared as the future promising molecules fort he development of new anti-Alzheimer drugs. This work was supported by the Scientific and Technical Research Council of Türkiye (TUBITAK), with a Project number of 107T783.

A4.15
Organophosphorus pesticides used in Turkey make a covalent bond with butyrylcholinesterase detected by mass spectrometry

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Organophosphorus pesticides used most commonly in Turkey include methamidophos, dichlorvos, O-methoate, and diazinon. These toxic chemicals or their metabolites make a covalent bond with the active site serine of butyrylcholinesterase (BChE). Our goal was to identify the adducts that result from the reaction of human BChE with these pesticides. Highly purified human BChE was treated with a 20-fold molar excess of pesticide. The protein was denatured by boiling and digested with trypsin. MS and MSMS spectra of HPLC-purified peptides were acquired on a MALDI-TOF-TOF 4800 mass spectrometer. It was found that methamidophos added a mass of +93, consistent with addition of methoxy aminophosphate. A minor amount of adduct with an added mass of +109 was also found. Dichlorvos and O-methoate both made dimethoxyphosphate (+108) and monomethoxyphosphate adducts (+94). Diazinon gave a novel adduct with an added mass of +152 consistent with diethoxythiophosphate. Inhibition of enzyme activity in the presence of diazinon developed slowly (15 hour), concomitant with isomerization of diazinon via a thiono-thiolo rearrangement. The isomer of diazinon yielded diethoxyphosphate and monoethoxyphosphate adducts with added masses of +136 and +108. MSMS spectra confirmed that each of the pesticides studied made a covalent bond with serine 198 of BChE. These results can be used to identify the class of pesticides to which a patient was exposed.
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A4.16
Epigallocatechin-3-gallate (EGCG) binds to TTR and modulates its amyloidogenicity

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Transthyretin (TTR) is an amyloidogenic protein being known more than one hundred TTR variants associated with hereditary amyloidosis. Although the details of the TTR amyloid fibril formation process are not completely known, the pathway involves TTR tetramer dissociation into monomers with altered conformation that will re-associate in an aggregated form, polymerize and form amyloid fibrils. Approaches for TTR amyloidosis that interfere with any step of this cascade of events have therapeutic potential. In this study we tested (-)-epigallocatechin-3-gallate (EGCG), the most abundant catechin of green tea, as inhibitor of TTR amyloid formation. We demonstrate, through biochemical and biophysical methods that EGCG binds strongly to isolated TTR, *in vitro*, and binds to TTR in human plasma, ex vivo. We also show, by stability assays through isoelectric focusing in partial denaturing conditions that EGCG acts as TTR stabilizer, either for wild type TTR or TTR V30M variant. In addition, we demonstrate by transmission electron microscopy (TEM) analysis that EGCG inhibits TTR aggregation *in vitro* and in a cell culture system. We also found that EGCG acts as amyloid fibril disruptor when tested *in vitro* for wild type TTR, TTR Y78F and TTR L55P amyloid fibrils. The dual effect of EGCG as TTR stabilizer and fibril disrupter makes it a good candidate for therapy or as model for other molecules to be used as therapeutic agents in TTR amyloidosis. Based in these results, *in vivo* experiments in mice transgenic for human TTR V30M in a null background were performed and suggestive for a significant reduction of tissue TTR deposition.

A4.17
The role of Phe329 in the inhibition of human butyrylcholinesterase by malachite green and methyl green

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Earlier kinetic studies in our laboratory have shown that cationic triarylmethane dyes are highly effective as inhibitors of cholinesterases with Ki in the micromolar range. In the present study, aiming to identify the binding sites of the dyes and their functional contacts with the active site, we have explored the role of Phe 329 in the inhibition of human butyrylcholinesterase (BChE) by malachite green (MG) and methyl green (MeG). The mutation Phe329Ala was introduced into human BChE by PCR-mediated site-directed mutagenesis with Pfu polymerase. Wild type and mutant BChE were stably expressed in human embryonic kidney cells and purified by anion exchange and procainamide-Sepharose affinity chromatography. Studied at 25°C, in 50 mM MOPS buffer (pH 8), with butyrylcholine as substrate, MG acted as single-occupancy, linear mixed type inhibitor of wild type BChE with Ki = 0.071 ± 0.004 μM. MeG caused competitive inhibition of wild type BChE with Ki = 0.17 ± 0.01 μM. Under the same experimental conditions, the two ligands caused complex, nonlinear inhibition of the Phe329Ala mutant and the kinetic pattern of inhibition indicated cooperative I binding at two sites. Intrinsic K' values ($=[I]^2_{0.5}$ extrapolated to [S] = 0) for MG and MeG were 2.0 ± 0.60 and 5.3 ± 1.1 μM, respectively. The differences in kinetic pattern and Ki values pointed to Phe 329 as a critical residue in determining enzyme conformation as well as ligand binding.

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A4.18
The dynamic of some plasma oxidative stress markers in stroke patients undergoing rehabilitation

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Introduction: Both *in vitro* and *in vivo* studies indicate oxidative stress is a key element in stroke etiology. Cerebral ischemic insult leads to overproduction of reactive oxygen and nitrogen species with deleterious consequences both for nervous tissue and other organs. Most of the studies investigated oxidative stress in acute stroke patients. Little attention was paid to post-acute stroke patients.
Objective: We investigated the dynamic of some oxidative and nitrosative plasma markers in stroke patients.
Methods and patients: The study group has 20 patients (8 males/12 females, 69.452.49 years old) who presented to our rehabilitation department from June 2009 till January 2010. Blood samples were taken at the beginning (moment 0) and at the end (moment 1) of the hospitalization time (131 days). We assessed the concentrations of total thiols, glutathione, advanced oxidation protein products (AOPP), protein carbonyls, and 3-nitro-tyrosine. We also assessed the concentrations of total plasma proteins, albumin, and the activity of -glutamyl transpeptidase (GGT). A control group of 20 subjects (5 males/15 females, 62.783.15 years old) was created. Informed consent was obtained from all the participants in study or from their belongings.
Results and discussions: The concentration of all parameters decreased from moment 0 to moment 1 with the exception of total proteins and total thiols. We found statistical significant differences only for protein carbonyls and 3-nitro-tyrosine concentrations and GGT activity. This study will be completed with other oxidative and nitrosative stress markers. ± ± γ ±

A4.19
Plasma status of homocysteine and lipoprotein (a) during chronic phase of stroke

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Introduction: Homocysteine (Hcy) and lipoproteine (a) [Lp(a)] are two factors responsible for endothelium aggression. Both

parameters are well known stroke risk factors. While the mechanisms of Hcy toxicity are somewhat understood, the exact mechanism relating Lp(a) with stroke is not well understood.

Objective: We evaluated the level and dynamic of Hcy and Lp(a) concentration in ischemic stroke patients.

Methods and patients: The study group has 10 patients (4 males/6 females, 73.33.6 years old) who presented to our rehabilitation department. Blood samples were taken at the hospitalization moment (moment 0) and at the discharge moment (moment 1). The hospitalization periode of time was 131 days. We assessed plasma homocysteine and Lp(a) concentrations using commercially available kits. We also assessed routine biochemical analysis (blood glucose, triglycerides, total cholesterol, HDLcholesterol, LDLcholesterol). The control group consists of 10 subjects (3 males/7 females, 62.83.7 years old). Informed consent was obtained from all the participants in our study or from their belongings.

Results and discussion: We found that during the hospitalization periode of time Hcy concentration increased, while Lp(a) concentration decreased. We found statistical significant difference only for Hcy. Also, we found a negative correlation between these two parameters. In order to obtain a better image about the dynamic of these parameters we need further investigation using larger study population and other parameters relevant for endothelium status after ischemic stroke.

A4.20
Manipulation of lipidic environment of prion protein-enriched domains isolated from rat cerebellar granule cells

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The Prion protein is a GPI-anchored protein primarily concentrated in neuronal cells. Under certain conditions its cellular form, PrPC, can convert into the lethal isoform, PrPSc. This conversion occurs in particular regions of the membrane, called lipid rafts. These microdomains enriched with cholesterol and glycosphingolipids are thought to play a crucial role both in physiological functions and in alternative folding of the PrP. The aim of our project is to establish if the alteration of cell lipid composition can modify the membrane distribution of PrPC within raft or non-raft regions and to promote the activity of disintegrins (ADAM10/17) upon PrPC. Due to this reason, granule cells obtained from the cerebella of 8-days-old SD rats were incubated after 8 days in culture with 2×10^{-6} M GM1 or GD1a at 4°C or 37°C for 4 hours. Detergent resistant fractions (DRM) preparation was accomplished according to Palestini et al., 2000 and proteins in all gradient fractions were separated and analysed by EF/WB. Lipids were separated by HPTLC and analysed by radiochromatoscanner. The treatment with GM1 at 4°C led to a remarkable enrichment of this ganglioside in DRM and slightly increased the content of PrPC and ADAM10/17 in this region. At 37°C GM1 was enriched in DRM and a moderate ganglioside incorporation was detectable in non-rafts membrane regions corresponding to gradient fractions from 8 to 12. In DRM PrPC and ADAM10/17 amounts decreased. Further experiments with GD1a are in progress; through the use of these ganglioside we mean to verify whether their major encumbrance can change into a bigger extent proteins distribution within raft and non-raft membrane regions.

A4.21
Evidence for a glial protective role in Mn^{2+}-induced neurotoxicity

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Manganese is a vital element and cofactor of key enzymes in mammals. However, high levels of this ion in the body give rise to Mn^{2+} deposits in brain, inducing manganism, a Parkinson-like disease. Little is currently known about the mechanisms involved in this neurotoxicity, and potential treatments or transporters involved in Mn^{2+} detoxification. In this work we have used mouse primary cultures to explore Mn^{2+} effect on neural and glial cells. MTT assays showed that the presence of Mn^{2+} decreased the survival of both cell types, being glial cells more resistant to Mn^{2+} toxicity than neurons. Immunofluorescences showed that Mn^{2+} specifically affected cellular integrity and produced fragmentation of Golgi membranes, containing the secretory pathway Ca^{2+}/Mn^{2+}-ATPase isoform 1 (SPCA1) as a functional candidate for Mn^{2+} detoxification. Besides, the cellular effects induced by Mn^{2+} were less pronounced in glia, which also prevented neurons against degeneration. On the other hand, the removal of Mn^{2+} from cultures or the addition of EDTA ceased the toxic effect of Mn^{2+} and allowed a partial reassembly of the Golgi complexes, mainly in glia. These findings lead to an understanding of the neurotoxicity linked to chronic-Mn^{2+} exposure in order to look for new therapeutic approaches. This work was supported by Grants from MICINN (BFU2008-00182) and Fundación Marcelino Botín, Spain.

A4.22

Abstract withdrawn

A4.23
Human cartilage glycoprotein gp-39 (YKL-40) is a putative oncogene

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Gliomas are the most frequent malignant primary brain tumors. They are highly aggressive, invasive, and neurologically destructive. Earlier it was found that HC gp-39 (human cartilage 39-kDa glycoprotein) gene was expressed at markedly higher level in glioblastomas compared to normal brain cells. Described results in accordance with previously published data indicate that YKL-40 may be used as a potential molecular marker for brain glial tumors. To determine whether YKL-40 possessed oncogenic properties we established human embryonic kidney HEK293 cell line that stably expressed YKL-40. Presence of YKL-40 in cells transfected by hybrid plasmid was tested by Western-blot and immunocytochemistry. The YKL-40-expressing clones grew as colonies in soft agar in contrast to the parental HEK293 cells or HEK293 cells transfected with the empty vector. To determine whether YKL-40 could influence tumor initiation and development *in vivo*, HEK293 cells that stably expressed YKL-40 and HEK293 cells transfected with the empty vector were used for stereotaxic intracerebral implantation to female wistar rats. On the 21th day after implantation tumor appeared in three rats with implanted HEK293 cells which stably expressed YKL-40, two rats had smaller tumor after 1 month. The volume of tumor was

443.9 mm^3. No tumor growth was observed for at least 6 weeks in five rats which were injected with the empty vector-transfected HEK293 cells as a negative control. Histochemical analysis of intracerebral tumor HEK293_YKL40 showed arrangement of YKL-40 on the periphery of the tumor. Thus expression of YKL-40 in HEK293 cells was sufficient to induce *in vivo* tumorigenicity.

A4.24
Identification of new interactions of tyrosine kinase Ack1 in mouse brain

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Ack is a tyrosine kinase having several protein-protein interaction domains: an SH3 domain, a region able to interact with Cdc42 (Cbd domains: Cdc42 binding domain) and several proline riche regions. In previous work we have shown the pattern of expression of Ack1 in adult and fetal tissues. Since this molecule has several protein-protein interaction doamins, we have used two strategies in order to identify some of the putative molecules able to interact with Ack. In the first approach we have used immunoprecipitation followed by Mass- Spectrometry to identify the associated proteins; in the second strategy we have purified by affinity chromatography molecules interacting with GST-Ack1 proline-rich regions and we have identified them by Mass spectrometry. With these techniques we have described several new molecules, among others lymbic associated protein, beta and gamma actin, keratin-6 alpha and several unnamed proteins. This data together with other molecules already described to interact with Ack such as Grb2, Nck, beta-1 integrin and the nucleotide exchanger Dbl makes Ack1 to be a molecules with multiple possible partners. In addition we show how overexpression of Ack1 in cultured neurons from hippocampus or cerebellum increases the number of dendrites, and branching.

A4.25
Characterization of site specific O-glycan structures within the mucin-like domain of α-dystroglycan from human skeletal muscle

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The glycosylation of the extracellular protein α-dystroglycan is important for its ligand binding activity and altered or blocked glycosylation are associated with several forms of congenital muscular dystrophies. By immunoprecipitation and sialic acid capture-and-release enrichment strategies we isolated tryptic glycopeptides of α-dystroglycan from human skeletal muscle. Nano-LC/MS/MS was used to identify both glycopeptides and peptides corresponding to the mucin-like and C-terminal domain of α-dystroglycan. The O-glycans found had either Hex-O-Thr or HexNAc-O-Ser/Thr anchored structures, which were often elongated and frequently, but not always, terminated with sialic acid. The HexNAc-O-Ser/Thr but not Hex-O-Thr glycopeptides, displayed heterogeneity regarding glycan core structures and level of glycosylation site occupancy. We demonstrate for the first time glycan attachment sites of the NeuAcHexHexNAcHex-O structure, corresponding to the anticipated Neu5Acα3Galβ4GlcNAcβ2Man-O glycan (sLacNAc-Man), within the mucin-like domain of human α-dystroglycan from human skeletal muscle. In summary, 24 glycopeptides were characterized from human α-dystroglycan, which provide insight to the complex *in vivo* O-glycosylation of α-dystroglycan.

A4.26
Excess normal prion protein (PrPC) in rat central nervous system is responsible for the myelin damage associated with vitamin B12 deficiency

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It has been widely accepted that the myelinolytic lesions associated with vitamin B12 (cobalamin, Cbl) deficiency in rats are caused by increased synthesis of tumour necrosis factor(TNF)-α in the central nervous system (CNS). However, TNF-α also regulates the PrPC synthesis in a cultured cell line and it has been suggested that the octarepeat region (OR) of PrPC has myelinotrophic action. We therefore investigated whether the cascade of events responsible for the CNS myelinolytic lesions associated with Cbl deficiency may include abnormal PrPC levels in the CNS of totally gastrectomised (TGX) (i.e. Cbl-deficient) rats 2 months(mo) after total gastrectomy (TG) (when the myelin lesions are ultrastructurally most severe). We repeatedly injected anti-OR antibodies (Abs, bought from the University of Zürich), heat-inactivated anti-OR-Abs or anti-TNF-α Abs intracerebroventricularly (i.c.v.) for 2 months after TG, and injected recombinant PrPC i.c.v. into normal adult rats for 2 months. PrPC levels in spinal cord (SC) and cerebrospinal fluid (CSF) of TGX rats treated with saline alone were significantly increased in comparison with laparatomised controls. The SC myelin ultrastructure was normal in the anti-OR Ab-treated TGX rats, whereas SC myelinolytic lesions were still present in the TGX rats treated with heat-inactivated anti-OR Abs and in the PrPC-treated rats. The TGX rats treated with anti-TNF-α Abs showed a normal SC myelin ultrastructure and no increase in SC or CSF PrPC levels. Taken together, these data demonstrate that the SC myelinolytic lesions observed in Cbl-deficient rats are due to increased local PrPC levels caused by increased TNF-α synthesis.

A4.27
Imaged by LA-ICP-MS: metal accumulations after cerebral infarct in course of time

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In recent years accumulations of metals were increasingly described in context with brain diseases. Cerebral infarcts in particular cortical infarcts lead to secondary degeneration of neurons in thalamic areas corresponding to the damaged cortical part. In rats infarcts can be directed stereotactically to defined cortical sections by injection of the photo reactive substance rose bengal and adjustment of a light source to the skull. At different time points after infarct induction we prepared cryo sections of the rat brain. The slices were analyzed in respect of metal accumulation

by using quantitative Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS) and correlated with neurodegeneration. The neurodegeneration was detected by cis-[18F]-D-proline, a new radiopharmacon used for Positron Emission Tomography (PET). Thereby, we plotted a map of metal distribution in the rat brain and metal accumulation in the infarct area as well as in the area of secondary neurodegeneration. The metal accumulation relating to time after infarct induction was analyzed. The results can improve the understanding of the involvement of metals in brain damage particularly with regard to metal triggered oxidative stress described as cause of neurodegeneration. Furthermore LA-ICP-MS can deliver new information for the improvement or advancement of bioimaging by PET and MRI.

A4.28

Abstract withdrawn

A4.29
Influence of Nanobodies on the aggregation properties of Alpha-synuclein

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The fibrillation of α-synuclein (αS) and its deposition in Lewy Bodies is one of the key pathological hallmarks of Parkinson's disease (PD). However, much remains to be understood about the mechanism of αS aggregation and its relationship to PD pathogenesis. The present work is focused on *in vitro* αS aggregation, using a number of novel conformation specific antibody fragments (Nanobodies) that have been determined by NMR to target different epitopes in monomeric αS. The binding of each Nanobody to αS has been characterised in atomic level detail and its effect on the kinetics of the αS aggregation pathway has been investigated closely. In the future, this approach will help to study the physical and structural properties of αS aggregates and understand how to prevent their formation.

A4.30
D-methionine in noise induced hearing loss

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Oxidative stress in the cochlea is considered to play an important role in noise induced hearing loss. The purpose of this study is to understand the mechanism underlying the noise induced increase in reactive oxygen species (ROS) in the inner ear. The changes in superoxide dismutase (SOD), catalase, lipid peroxidation (LPO) and the auditory brainstem response (ABR) were measured in the cochlea 1, 7 and 14 after noise exposure (4 kHz octave band at the intensity of 118 dB SPL for 8 hours) in C57BL/6 mice. In addition we also studied the action of an antioxidant D-methionine (D-met) to investigate its role in preventing this noise induced oxidative stress and hearing loss. D-met was able to scavenge the free radicals resulting in a significant decrease in LPO levels from noise exposure controls. In summary, the findings of this study indicate that the time dependent alterations in scavenging enzymes facilitate the production of free radicals and D-met is effective in attenuating the noise-induced oxidative stress and associated functional loss in mice cochlea.

A4.31
Increased ERG amplitudes in a model of type 2 diabetes

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Purpose: To examine how retinal functional changes correlate with blood glucose levels.
Methods: Zucker diabetic fatty (ZDF) rats (8–22 weeks old) were compared with lean control rats using full field electroretinography (ERG). A group of ZDF rats received a long-acting insulin analog once daily from 17 weeks of age to reduce glucose levels to <10 mM. ERGs were performed in the three groups until 22 weeks. The expression of specific retinal cell markers was examined at 23 weeks of age.
Results: ZDF rats showed significantly higher scotopic a- and b-wave amplitudes ($>10\%$) from 12 weeks of age compared to lean controls. A delay in the implicit times of all oscillatory potential (OP) wavelets of about 10% was also observed in ZDF rats at 14 weeks of age and onwards. Amplitudes were normalized within 3 hours after insulin administration, with no effect on OP latencies. Over time, insulin treatment produced a small shortening of OP latencies and a further decrease in a- and b-wave amplitudes, which became lower than observed in non-diabetic animals of the same age. Glial cell markers were up-regulated in diabetic rats, but not in insulin-treated animals.
Conclusions: Hyperglycemia led to higher ERG amplitudes, probably reflecting the ability of the outer retina to utilize the additional glucose as substrate. This is corroborated by the observation that insulin treatment reduced the retinal responses. The fact that insulin treatment produced sub-normal amplitudes may correlate with the initial worsening of retinopathy seen in some patients. The activation of glial cells noted in the untreated ZDF animals may, directly and/or indirectly, contribute to the effects observed.

A4.32

Abstract withdrawn

A4.33
Loss of megalin expression in the outer retina in models of photoreceptor degeneration

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Purpose: Megalin, a member of the low-density lipoprotein receptor (LDLR) family, binds and takes up a range of ligands, including the neuroprotectants metallothionein I and II (MT-I + II). The purpose of the present study was to examine the distribution of megalin in rodent models of *Retinitis Pigmentosa* (RP) in relation to that of MT-I + II.
Methods: Megalin and MT-I + II expression were analyzed in normal mouse and rat retinas and in retinas of *rd1* and *rds* mice and of RCS (Royal College of Surgeon) rats of various ages. The identity of labelled cells was verified by co-staining with various specific cell markers. Sites of possible interaction between megalin and MT-I + II were identified with an *in situ* proximity ligation assay (PLA).
Results: Strong up-regulation of MT-I + II was observed in Müller cells in degenerating retinas, which coincided with the onset

of glial fibrillary acidic protein expression (GFAP) in *rd1* and *rds* mice. In normal animals, megalin was expressed in the nerve fiber layer, in the region of the inner and outer segments, and in Müller cell processes in older animals. *In situ* PLA revealed that interactions between megalin and MT-I+II might occur at the inner and outer margins in the normal retina. In degenerating retinas, however, megalin expression was lost in the outer retina early in the degenerative process in all three models.

Conclusions: Expression of MT-I+II appears to be controlled by different events in the three pathologies. A transfer of MT-I+II from e.g. Müller cells into photoreceptors appears less likely as megalin expression was progressively lost, suggesting that the potentially neuroprotective actions of metallothionein may be limited.

A4.34
Diosgenin-induced neuronal differentiation of rat neural stem cells requires ERK1/2

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Diosgenin, a steroidal sapogenin is extracted from the root of wild yam (Dioscorea villosa). It has been reported to have various biological activites, including antioxidant and anti-inflammatory effects. In this study, we examined the effect of diosgenin on neuronal differentiation of neural stem cells (NSCs). NSCs obtained from 14-day-old rat embryos were propagated as neurospheres and culture under differential conditions with or without diosgenin for 5 days. Diosgenin significantly increased the percentage of microtubulin associated protein 2 (MAP2) in positive neurons compard with the control cells. In addition, these results show that diosgenin promote the differentiation of NSCs into a neuronal phenotype characterized by neurite outgrowth, the expression of neuronal marker protein Tuj1 and voltage-operated $Ca2^{+}$channel. Furthermore, diosgenin induced an increase in the phosphorylation of the extracellar signal regulated kinase 1/2 (ERK1/2). U0126, a selective ERK1/2 kinase inhibitor blocked both the phosphorylation of ERK1/2 and the ability of diosgenin to promote differentiation of NSCs into neuron. The structure of diosgenin is similar to that of estrogen. To investigate the possible role of the estrogen receptor (ER) in the course of a diosgenin-induced neurogenic effect, we used the highly specific ER antagonist ICI182,780 to blocked ER-related pathway. The addition of ICI182,780 did not alter diosgenin-induce neural differentiation. This data indicated that diosgenin does not require ER activation in neural differentiation. Taken together, our results suggest that diosgenin can induced NSCs differentiation through ERK1/2 activation in a ER-independent pathway.

A4.35

Abstract withdrawn

A4.36
The chaperone-like protein HYPK acts together with NatA in cotranslational N-terminal acetylation and prevention of Huntingtin aggregation

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The human NatA protein N-α-terminal-acetyltransferase complex is responsible for cotranslational N-terminal acetylation of proteins with Ser-, Ala-, Thr-, Gly-, and Val-N-termini. The NatA complex is composed of the catalytic subunit hNaa10p (hArd1) and the auxiliary subunit hNaa15p (hNat1/NATH). Using immunoprecipitation coupled with mass spectrometry we identified endogenous HYPK, a Huntingtin (Htt) interacting protein, as a novel stable interactor of NatA. HYPK has chaperone-like properties preventing Htt aggregation. HYPK, hNaa10p and hNaa15p were associated with polysome fractions indicating a function of HYPK when associated with the NatA complex during protein translation. Knockdown of both hNAA10 and hNAA15 decreased HYPK protein levels possibly indicating that NatA is required for the stability of HYPK. The biological importance of HYPK was evident from HYPK-knockdown HeLa cells displaying apoptosis and cell cycle arrest in the G0/G1 phase. Knockdown of HYPK or hNAA10 resulted in increased aggregation of an Htt-EGFP fusion with expanded polyglutamine stretches, suggesting that both HYPK and NatA prevent Htt aggregation. Furthermore, we demonstrated that HYPK is required for N-terminal acetylation of the known *in vivo* NatA substrate protein PCNP. Taken together, the physical interaction between HYPK and NatA seems to be of functional importance both for Htt aggregation and N-terminal acetylation.

A4.37
$GABA_B$ receptors are heteromultimers with a family of auxiliary subunits

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Metabotropic $GABA_B$ receptors mediate the action of GABA (γ-aminobutyric acid) the main inhibitory neurotransmitter in the brain. They regulate synaptic transmission and signal propagation by controlling the activity of calcium and potassium channels. The $GABA_B$ receptor is a G-protein coupled receptor composed of two homologous proteins: $GABA_{B1}$ and $GABA_{B2}$. However, the heterologously expressed $GABA_{B(1,2)}$ receptor heteromer failed to reproduce the functional diversity observed with native $GABA_B$ receptors. To test the hypothesis that functional diversity results from additional yet unknown partners we have used a proteomic analysis by combining affinity purifications of membrane protein complexes from rat brain with quantitative

mass spectrometry. The analysis identified $GABA_B$ receptors as complexes of $GABA_{B1}$, $GABA_{B2}$ and a subset of the family of "potassium channel tetramerization domain-containing" (KCTD) proteins. Further biochemical, immunhistochemical and electrophysiological studies showed that KCTD isoforms 8, 12, 12b and 16 are tightly associated with the C-terminus of $GABA_{B2}$. They exhibit a distinct expression profile in the brain and localize to the plasma membrane of pre- and postsynaptic compartments in the hippocampal CA1 region. The receptor properties of the $GABA_{B(1,2)}$ heteromer were changed through coassembly with the KCTD proteins: the KCTD proteins increase agonist potency, alter the G-protein signaling of the receptors by inducing rapid onset and pronounced desensitization. Together, our results establish the KCTD proteins as auxiliary subunits of $GABA_B$ receptors that determine the pharmacology and kinetics of the receptor response.

A4.38
Retinoic acid effect on β-amyloid$_{(25-35)}$ toxicity in PC12 cells

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Beta-amyloid is a peptide known for constituting plaques in brains of Alzheimer disease patients. Formation of amyloid plaques results in overproduction of reactive oxygen species and is accompanied by a disturbed calcium homeostasis. There is an increasing amount of data concerning potential retinoids protection against Alzheimer disease. Retinoic acid attenuates accumulation of amyloid plaques and can support regeneration of cells by stimulation of neurodifferentiation. In this study we focused on the effect of one of the most toxic β-amyloid fragments (25-35) on cell viability. Moreover, we examined if retinoic acid can protect the cells against $\beta A_{(25-35)}$ cytotoxicity. Our models were pseudoneuronal PC12 control cells, and PC12 line with suppressed expression of PMCA3, a neuron-specific isoform of calcium pump. Viability of the cells was measured spectrophotometrically with WST-1 reagent. The results show that retinoic acid, in a concentration dependent manner, can protect control PC12 cells against toxic effect of $\beta A_{(25-35)}$. We did not observe this effect in PMCA3-reduced cells. It may suggest that the defensive effect of retinoic acid could depend on calcium homeostasis. This work was supported by The European Social Fund and the national budget within the framework of Measure 2.6 of the Integrated Regional Operational Programme ("Stypendia wspierajace innowacyjne badania naukowe doktorantow" Project) and from Medical University of Lodz grant 503-6086-2.

A4.39
Multiparametric fluorescence microscopy imaging of amyloid beta allows for direct observations of protein misfolding *in vitro* and in cells

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Misfolding and aggregation of amyloid beta peptides into oligomers and subsequently amyloid fibrils have detrimental effects on synapse function and cell viability, accounting in part for the memory loss and neuronal degeneration associated with Alzheimer's disease. Whilst amyloid beta aggregation can be readily studied *in vitro* by established biophysical techniques, it has to date, been very difficult to directly monitor aggregation or identify the aggregation state of misfolded species *in vivo* with both spatial and temporal resolution. Multiparametric fluorescence microscopy allows for pixel-by-pixel acquisition of not only fluorescence intensities, but also of more responsive parameters such as fluorophore excited state lifetime and steady-state anisotropy and we have recently shown how these parameters sensitively report on the aggregation state of an engineered á-synuclein fusion protein with a c-terminal yellow fluorescent protein tag *in vitro*, in cells and *in vivo*. Here we have studied misfolding of amyloid beta (Ab40 and Ab42) *in vitro* and in cultures of neuronal cells. Cross-validation of multiparametric imaging data obtained *in vitro* with fluorescence spectroscopy, transmission electron microscopy and biochemical assays has allowed us to correlate certain fluorescence lifetime signatures and fluorescence anisotropy characteristics with the specific appearance of monomeric, oligomeric and fibrillar amyloid beta in our samples. Using this information we have then been able to identify the same species in a cellular context by examining the extracellular appearance, internalization and in-cell compartmentalization of Ab40 and Ab42.

A4.40
Heat inactivation of tissue samples to stabilize proteins, peptides and their modifications

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Removal of a sample from its natural surrounding leads to major disturbance of tissue homeostasis. The action of proteases and other enzymes can rapidly change the composition of the proteome and post-translational-modifications (PTMs). Subsequent analytical results reflect a mix of *in vivo* proteome and degradation products. Here, a novel stabilization system was used to treat fresh and frozen tissue samples to stop degradation and preserve the *in vivo* proteome. The system utilizes rapid heat inactivation to eliminate enzymatic activity in tissue and thereby enable detection of endogenous peptides and monitoring of important PTMs, such as phosphorylations. Stabilization was assessed by nano-LC-MS, MALDI, and western blotting on samples from brain, muscle, and liver. Inadequate sample handling cause an increase in degradation fragments which has been shown in these experiments. However, after immediate sample stabilization, no protein degradation fragments were detectable. The mass spectrometrically identified peptide peaks in stabilized samples consisted of several known neuropeptides, endogenous peptides, and novel, potentially biologically active, peptides. Most peaks detected in the untreated group originate from proteins such as hemoglobin, cyclophilin, NADH dehydrogenase, synuclein and other highly expressed proteins. Accordingly, the assayed functions of proteases, phosphatases and cytochrome C oxidase showed clear inactivation after stabilization. The levels of phosphorylated forms of CREB, GSK and MAPK remained unchanged after 2 hours in room temperature after stabilization as the levels of the same proteins in untreated samples decreased in only 10 minutes.

A4.41
Aggregation and toxic mechanisms of the poly-Q containing protein ataxin-3 in an intracellular environment

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Poly-glutamine (poly-Q) diseases are neurodegenerative inherited disorders caused by the expansion over a pathological threshold of a poly-Q tract in the involved protein. In our work we have studied the poly-Q protein ataxin-3 (AT-3), the causative agent of spinocerebellar ataxia type 3. AT-3 consists of a globular N-term domain and a flexible tail containing the poly-Q tract. In particular, since the pathogenic mechanisms are still poorly understood, we investigated the relationship between AT-3 toxicity and protein aggregation in an intracellular environment. We used in our studies four human AT-3 variants expressed in *E. coli* to explore the correlation between proteins aggregation pathway and toxicity by biochemical and biophysical techniques. Growth curves analysis and structural investigations onto soluble and insoluble AT-3 protein fractions evidenced a multi-domain aggregation mechanism and a toxic effect dependent on soluble protein concentration. Considering that AT-3 deleterious effects on a bacterial host underlines a cytotoxic effect independent from organism complexity, we investigated general mechanisms by which toxicity can act. Even if no significant differences in ROS levels were observed, we have identified alterations in membrane integrity by SDS susceptibility test and propidium iodide staining. Furthermore, we employed our *E. coli* system as a model for evaluating the intracellular effects of redox status on aggregation and toxicity of AT-3, which contains six cysteins not involved in disulfide bridges. Our preliminary results demonstrated a possible role of disulfide bridges in aggregation process and oligomers generation.

A4.42
Modified analogues of ACTH fragments as new candidates for effective drugs

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The molecular mechanism underlying regulatory peptides biological action keeps unclear for the time being. In spite of the question, medical products based on peptides found wide application in clinics as effective drugs and prophylactics, for example neuropeptide Semax. As shown before Semax exposing to P2 membranes of different brain structure cells undergoes fast and aggressive proteolysis by membrane enzymes. Some of peptide fragments appeared holds their own biological activity, individual specificity and directional influence. In this work we tried to clear two key processes underlying neuropeptide biological action molecular mechanism: proteolysis and receptor specific binding on brain cells plasmatic membranes. Four original modified analogues of ACTH fragments containing in structure PGP C-terminal fragment been tested: ACTH (4-7)-Pro-Gly-Pro, ACTH (6-9)-Pro-Gly-Pro, ACTH (7-10)-Pro-Gly-Pro, ACTH (15-18)-Pro-Gly-Pro. All molecules shown different stability after exposition to plasmatic membranes of brain cells or nasal mucosa. It was shown that in time necessary for penetration sufficient for physiological action peptide quantities into the brain by intranasal way, the most part of molecules keeps stable. Exposed to P2 membranes of different brain structure cells, each of synthetic ACTH analogues undergoes individual proteolysis. The ACTH analogues influence on specific binding of well-known selective ligands (CP-55,940, WIN 55,212-2, Dihydrocapsaicin and other) was shown. This new artificial neuropeptides, we suppose, could give the best fit as a new drugs for clinical and prophylactic application.

A4.43
Proteomic study of proteins tightly associated with tau polypeptide in neurofibrillary tangles in brains with Alzheimer's disease

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Alzheimer's disease (AD) is the commonest cause of dementia in the elderly and is characterized by the presence of neurofibrillary tangles (NFTs) and neuritic plaques. Both structures represent dense accumulations of abnormal insoluble filaments. NFTs are formed by paired helical filaments (PHFs) composed by assembled tau polypeptides. The integral constituents of PHFs remain unknown mainly due to the high insolubility of NFTs. In this study we performed a proteomic analysis of NFTs to identify those potential polypeptides that could be closely bound to tau and that could have a possible role in the tau pathological assembly. Paraformaldehyde-fixed-brain tissue sections from three Mexican AD cases were immunolabeled with the conformational phosphor-dependent AT100 monoclonal antibody (Ser 199, Ser202, Thr205, Thr 212, Ser 214). We isolated 2300 NFTs from each case using a PALM system and pooled them for further studies. Proteins were extracted from pooled NFTs using several solubilization methods and were digested with trypsin. Generated peptides were separated by two-dimensional HPLC and analyzed by electro spray ionization and tandem mass spectrometry. Mass spectra were used to identify the polypeptides using different databases. We identified 57 polypeptides, which included some proteins related to metabolism, cytoskeleton, antioxidant proteins, protein kinases and ubiquitin. To verify the identification by proteomic analysis, we performed immunofluorescence and laser confocal microscopy for some polypeptides identified. This study demonstrates the advantages to combine techniques for high quality isolation of polypeptides and proteomics in fixed brain tissues.

A4.44
Hyperphosphorylation of tau polypeptide and appearance of fibrillar amyloid-β deposits correlate with aging in a triple transgenic mouse model of Alzheimers disease

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Hyperphosphorylated neurofibrillary tangles (NFTs) and fibrillar deposits of amyloid-β (Aβ) are the neuropathological hallmarks of Alzheimers disease (AD). Molecular mechanisms involved in

formation of such structures are not fully understood. However, the existence of animal models, such as the 3xTg-AD transgenic mouse, is very important to address this important issue. 3xTg-AD mouse carries gene mutations in the β-amyloid protein precursor (βAPP_{swe}), presenilin-1 ($PS1_{M146V}$) and tau_{P301L} and its brain characteristically presents Aβ deposits and tau pathology, being cortex and hippocampus the most affected regions. Here we analyzed the presence of a variety of phosphor-dependent tau epitopes and tau C- and N-terminus markers at different ages of the 3xTg-AD mouse, and the temporal appearance of such epitopes and Aβ deposits using the 499, 46.1, Alz-50, 499, S199, S231, S396, S400, S404, and AD2 antibodies. Double immunolabeled tissues were counterstained with thiazin red and observed through a laser confocal microscope. Our results showed that appearance and distribution of hyperphosphorylated tau correlated with age. Tau aggregates were first observed at hippocampus in a 3 months old mouse. Interestingly, the appearance of tau aggregates in the cortex was observed at 7 months and they were very evident by the age of 18 months. By contrast, Aβ deposits were first observed at seven months and they were clearly identified at 18 months. This study revealed that the appearance of phosphor-tau epitopes preceded at least four months to Aβ deposits. This observation confirms the usefulness of the 3xTg-AD mouse in the study of AD neuropathology.

A4.45
Effect of *Bacopa monniera* extract on the expression of neuropeptide genes in SH-SY5Y neuroblastoma cells

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The memory formation requires the action of several proteins and the alterations of protein expression have been attributed to memory functions. Neuropeptide Y (NPY) acts as a peptide neurotransmitter and neuroendocrine hormone in the nervous system. Several studies indicate that NPY and cAMP responsive element binding protein (CREB) played important roles in the formation of memory. Therefore, expression levels of both peptides also related with memory. Furthermore, it had been found that several herbs affect the memory; one is *Bacopa monniera* (brahmi). Thus far, there are little published scientific studies of the effects of brahmi on neuropeptide expression in neuronal cells. Therefore, the aim of this study was to examine the effect of brahmi on the expression of neuropeptide genes related to memory in SH-SY5Y neuroblastoma cells. SH-SY5Y neuroblastoma cells were cultured, then differentiated by 10 μM retinoic acid and treated with 100 μl/ml of brahmi extract for 2, 3 and 6 day to observe the duration of treatment that affects the mRNA expression. After mRNA extraction, cDNA was generated and the NPY and CREB genes were amplified by RT-PCR. Density of each band was evaluated by SynGene GeneTools® software with paired samples *t*-Test statistics. Our data showed that the expression at gene level of NPY remained unchanged. On the contrary, our results showed that CREB mRNA level significantly increased ($p < 0.05$) by more than 2-folds after brahmi treatment for just 2 days. Alteration of CREB expression affected by brahmi could be one pathway in memory improvement, and this should be further analyzed.

A4.46
Comparison of blood toxic and plasma essential elements of the autistic Turkish infants

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The whole blood total mercury and lead levels;plasma manganese, copper, zinc and selenium levels of autistic infants and their healthy siblings and casually selected healthy infants who are not autistic were measured and compared. Whole blood total mercury levels of autistic and control-1 are (11.97 ± 3.75 μg/l), (5,18 ± 2.24 μg/l), ($p < 0.001$),respectively, autistic and control-2 are (11.97 ± 3.75 μg/l), (6.06 ± 2.46 μg/l), ($p < 0.001$)respectively and significantly higher in autistics. Whole blood lead levels of autistic and control-1 are (16.14 ± 5.25 μg/l), (7.69 ± 2.17 μg/l), ($p < 0.001$) respectively, autistic and control-2 are(16.14 ± 5.25 μg/l), (9.62 ± 2.92 μg/l), ($p < 0.001$)respectively and significantly higher in autistics. Plasma manganese levels of autistic and control-1 are(6.41 ± 2.45 μg/l), (3.64 ± 0.92 μg/l), ($p < 0.001$) respectively, autistic and control-2 are (6.41 ± 2.45 μg/l), (4.11 ± 0.94 μg/l), ($p < 0.001$) respectively and significantly higher in autistics. Plasma copper levels of autistic and control -1 are (1557.48 ± 298.84 μg/l), (1274.09 ± 177.21 μg/l), ($p = 0.004$) respectively, autistic and control - 2 are (1557.48 ± 298.84 μg/l), (1212.93 ± 271.41 μg/l), ($p < 0.001$ respectively and significantly higher in autistics. Plasma zinc levels of autistic and control-1 are (1469.80 ± 173.81 μg/l), (1611.01 ± 148.72 μg/l), ($p = 0.048$) respectively, autistic and control-2 are (1469.80 ± 173.81 μg/l), (1624.28 ± 201.76 μg/l), ($p = 0.020$) respectively and significantly lower in autistics. Plasma selenium levels of autistic and control-1 are (34.83 ± 7.14 μg/l), (77.03 ± 18.84 μg/l), ($p < 0.001$) respectively, autistic and control-2 are (34.83 ± 7.14 μg/l), (76.71 ± 19.22 μg/l), ($p < 0.001$) respectively and significantly lower in autistics.

A4.47
The blood-brain barrier permeability during convulsions in the rat pups born from folic acid supplemented mothers during their pregnancy

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Folic acid (FA) is used to prevent neural tube defects and takes part in important biochemical reactions in the body. In recent years it became a popular molecule to prevent cardiovascular and cerebrovascular diseases. In our study we investigated the effects of FA on the blood-brain barrier (BBB) permeability, brain water content (BWC), Zonula Occludens-1 (ZO-1) and Glial Fibrillary Acidic Protein (GFAP) immunoreactivity during convulsions in rat pups. We used 5 months old rat pups born from mothers supplemented 5 mg/kg/day FA intraperitoneally during their pregnancy. Evans blue method used for assesing BBB permeability. Pentylenetetrazole (PTZ) induced convulsions caused a remarkable increase on the BBB permeability ($p < 0.01$). But in PTZ + FA group the BBB permeability significantly decreased when compared to PTZ group ($p < 0.05$).

BWC significantly increased in PTZ group when compared to controls ($p < 0.05$), it is not changed in PTZ+FA group. Although ZO-1 coloring power was found to be similar to control group, in PTZ+FA group ZO-1 immune reactivity was found to be increased. This result is original because it represents that FA might have an effect on the expression of ZO-1 protein during BBB development. GFAP immun reactivity increased in PTZ group when compared to control group. In PTZ+FA group GFAP immune reactivity was detected to be decreased when compared to PTZ group. The results of this study suggest that FA has protective effects on the BBB permeability decreasing paracellular passage of blood born molecules during convulsions in rat pups born from mothers supplemented FA during their pregnancy.

A4.48
Purification and mass spectrometric characterization of tau in human cerebrospinal fluid

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One of the typical brain lesions of Alzheimer's disease (AD) is neurofibrillary tangles consisting of hyperphosphorylated forms of the microtubule-stabilizing protein tau. While significantly elevated concentrations, both of total tau and of phosphorylated tau, is found in the cerebrospinal fluid (CSF) of AD patients, it is still unknown which isoforms are present and which phosphorylation sites are utilized. Molecular characterization of tau in CSF presents an analytical challenge because of the high heterogeneity of the protein and its low concentration in CSF. We present a purification protocol based on immunoprecipitation that enabled, for the first time, mass spectrometric characterization of tau in human CSF. The tau purification method was optimized with regard to several experimental parameters including selection of antibody, antibody immobilization and CSF pre-treatment. ELISA was used to monitor tau recovery in each step. The IP samples were analyzed by Liquid chromatography (LC) coupled to either matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) or electrospray (ESI) hybrid linear ion trap Fourier transform ion cyclotron resonance mass spectrometry (QIT-FTICR MS). Nineteen tryptic fragments of tau were detected, identifying three tau isoforms. The ability to detect tau by MS in a 2-ml CSF sample paves the way for clinical CSF studies on the tauopathies. This study was supported by grants from the Swedish Research Council (projects 2006-6227 [HZ] and 2006-2740 [KB]), the Sahlgrenska University Hospital, the Göteborg Medical Society, Swedish Brain Power, Stiftelsen för Gamla Tjänarinnor, and the Alzheimer Foundation, Sweden.

A4.49
Excretion by urine of toxic and essential elements of the autistic Turkish infants

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Urine total mercury, lead, manganese, copper, zinc and selenium levels of the autistic infants and their siblings were measured and compared. The binary comparison of age value results of autistic ($n = 16$), control-1 ($n = 6$) (their siblings) groups whose urine analyses were done are; autistic and control-1 are(5.93 ± 1.98), (8.37 ± 4.28), ($p = 0.052$) respectively and there is no significance. Urine total mercury levels of autistic and control-1 are (10.34 ± 5.81 μg/day), (18.41 ± 7.3 μg/day), ($p = 0.019$) respectively and significantly lower in autistics. Urine lead levels of autistic and control-1 are (10.34 ± 5.81 μg/day), (15.00 ± 8.11 μg/day), ($p = 0.048$) respectively and significantly lower in autistics. Urine manganese levels of autistic and control-1 are (2.34 ± 2.20 μg/day), (3.83 ± 2.96 μg/day), ($p = 0.118$) respectively and no significance.Urine copper levels autistic and control-1 are (18.90 ± 6.42 μg/day), (18.9 ± 7.67 μg/day), ($p = 0.999$) respectively and no significance. Urine zinc levels of autistic and control-1 are (1190.40 ± 613.10 μg/day), (1284.89 ± 501.19 μg/day), ($p = 0.637$) respectively and no significance. Urine selenium levels of autistic and control-1 are (47.22 ± 21.6 μg/day), (42.58 ± 25.9 μg/day), ($p = 0.587$) respectively and no significance. When the obtained results are evaluated, it gives rise to think that autistics are exposed to more neurotoxicity with toxic metals influence as especially mercury and lead and additionally copper and manganese levels are found to be higher in autistics however zinc and selenium levels were lower. Also, the lesser excretion of especially mercury and lead with urine when compared with healthy infants recurs to the mind that these metals accumulate in the body.

A4.50
TNF correlated with reelin in postmortem brain in depression patients

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One of the hypothesis about the inflammatory factor involved in major depression converge to many proinflammatory substances such as TNF-α, IL-6. TNF-α which is balanced with other cytokines in brain takes part in synaptic plasticity, but it may be excitotoxic effect to brain when it excess. We search the correlation marker by using the Stanley Neuropathology Consortium Integrative Database(SNCID) which is a web based method to integrate the Stanley Medical research Institute data sets. We found that TNF-α is significantly increased in major depression ($n = 15$) compared with normal controls($n = 15$), not in schizophrenia, bipolar affective disorder in frontal lobe. Sperarman's test showed that TNF-α level in frontal lobe is significantly correlated with dopamine D2 receptor mRNA in prefrontal lobe ($p < 0.01$), myelin oligodendrocyte glycoprotein mRNA in frontal lobe($p < 0.01$), glial fibrillary acidic protein mRNA in frontal lobe($p < 0.01$). Also, IL-6 in frontal lobe($p < 0.01$), reelin($p < 0.01$) in frontal lobe is correlated with TNF-α. It is novel finding that TNF-α is correlated with reelin in human postmortem brain. Reelin helps migration of neuroblasts in developing brain as well as adult brain. It also participates in synaptic plasticity via modulate longterm potentiation(LTP). In summary, we suggest that mentioned correlated markers involve in synaptic plasticity, especially, correlation with reelin is novel finding.

A4.51
Hyperosmotic stress and UV radiation stimulate tau phosphorylation

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Tau is a microtubule associated protein which has an important role in the development of Alzheimer's Diseases (AD) and other

diseases known as Tauopathies. When Tau is hyperphosphorylated, becomes in an insoluble form, the NFT. Although many protein kinases phosphorylate Tau *in vitro*, its phosphorylation *in vivo* is not well known yet. In this work we studied Tau phosphorylation by ERKs and SAPKs induced by osmotic stress (sorbitol) and Ultra-Violet (UV) exposure in HEK 293 cells transfected with human Tau (pCMV5-hTau40). The phosphorylation and activation of ERKs, p38s and JNKs by sorbitol and UV were analyzed by Western-blot using antibodies against the phosphorylated forms of these kinases and their substrates. Sorbitol and UV both induced the phosphorylation and activation of p38 and ERK 1/2 and both turned off in a time-dependent manner. JNKs showed a different behaviour remaining activated in the time scale used in this study, 2 hours. Sorbitol stress induced Tau phosphorylation at Thr-50 and Ser-235 in a time course very similar to the observed in ERKs and p38s activation. The use of inhibitors (PD98059 and PD184352) suggested that Tau phosphorylation at Thr-50 is ERKs dependent. UV stress induced Tau phosphorylation at Thr-50 and Ser-235 in a p38s and JNKs dependent manner, as revealed the use of SB203580 and SP600125.

A5 – Infectious Diseases

A5.01

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A5.02

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A5.03
Successful treatment of vaginitis with a pegylated vanadium compound that shows extreme activity against a series of bacteria, fungi and protozoan species: a sign of very ancestral molecular target

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Objectives: To combat the growing problem of sexually transmitted diseases and simplifying the treatment of vaginitis.
Materials and Methods: In a phase I trial based on preclinical and toxicology studies; we treated patients with refractory vaginal discharge of different etiologies with the most active of twelve pegylated Vanadium compound. The 3 concentrations were based on V2O5 weight from which the material derived as it is pegylated and releases Vanadium with time. With strict eligibility criteria, patients with refractory vaginal discharge (6 month duration, no response to many local and systemic therapies) were treated. Thorough pre-treatment and post treatment studies including cultures were performed. Patients received a single session of local vaginal washing with the solution. Re-examination and culture after one week was performed.
Results: Ninety two patients responded completely after the first, 25 after the second and one patient after the third session with a complete response rate of nearly 100%. Compliance was excellent and nobody showed major side effects. Symptoms due to PID waned and cervical ulcers healed in almost all patients in question. Bacterial vaginitis responded best while fungal and Trichomonas vaginitis responded later but completely as well.
Conclusions: Concerning the current explosion of STD in recent decades this single session treatment shows much promise and study for viral and other germs inactivation is underway. Activity against a series of bacteria, fungi, parasites (*in vitro* on intestinal metazoans and worms) arises the question whether a very ancient and ancestral molecule is targeted by this pegylated Vanadium compound.

A5.04
Molecular methodology used in detection of influenza A H1N1 outbreak

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H1N1 subtype of influenza A type, as a cause of novel influenza A outbreak is a challenge for using different molecular methodology. It appears to be of swine origin and contains a unique combination of gene segments that has not been identified in the past. We compared the different methodology used in typing and subtyping of Influenza A H1N1 virus. Typing of influenza virus isolated from nasopharyngeal swabs was performed using conventional (qualitative) RT PCR and NASBA influenza A/B commercial kit. Real time RT PCR methods recommended by CDC was used for H1N1 subtyping using four sets of primers and probes for human Influenza A-M (matrix gene), swine Influenza A, swine H1 and human RNaseP (as a internal control for RNA quality) gene segment. First 20 suspected patients for H1N1 were analysed using the all three methodology. Twelve of them were confirmed as Influenza A positive using Influenza conventional PCR reaction. Using NASBA Nuclisens Easy Q influenza A/B method only two were Influenza A positive. CDC recommended real time RT PCR protocol shows fourteen influenza A positives. Two of them were seasonal Influenza A-H3N2 (the same one detected with NASBA) and the rest were H1N1 subtypes. The rest of samples were tested using only the CDC recommended methodology. Conventional and NASBA methods failed to detect the novel H1N1 positive samples giving negative results for influenza A type, maybe due to a possible rearrangements on the novel virus and the less sensitivity of the methods. Recommended molecular method is the Real time RT PCR able for simultaneously carry out the detection, typing, and subtyping of influenza viruses.

A5.05
Klebsiella pneumoniae with fucose synthesis gene mutation displays reduced virulence in mice

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Human liver abscess caused by *Klebsiella pneumoniae* (KP) is a recently emerging disease. One such strain from clinical isolate, Kp5, induced high fatality in infected mice. A characteristic of KP is its mucoid phenotype due to large capsule. The capsule of Kp5 was found to possess high amount of fucose that was not found in non-virulent strain causing urinary tract infection. The Kp5 rarely interacted with mice peritoneal macrophages which were induced by thioglycolate stimulation, indicating the fucose-containing capsule could be used by bacteria to evade host immune reaction. Mutant of Kp5 (Kp5m) was constructed by inserting an apramycin resistant gene cassette to the *gmd*, one of the fucose synthesis genes, and incorporated into Kp5 genome by conjugation. The Kp5m displayed very low virulence to mice and compared to the wild type Kp5, the Kp5m showed lower growth rate, higher interaction with mouse peritoneal macrophages, higher ability to form biofilm and lower toxicity to cultured HepG2 cells. The bacterial morphology also showed some abnormal filamentous phenotype which was not seen in Kp5. The results suggested that the mutation reduced bacterial virulence in mice, in addition also influenced bacterial growth and physiology.

A5.06
The implication of mtDNA mutations in HPV-induced cervical carcinogenesis

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High risk human papillomaviruses (HPV) are known to be the etiological agent of cervical cancer disease. On the other hand, other

cofactors are considered to be important in cervix carcinogenesis. Mutations in mitochondrial DNA (mtDNA) as well as alterations in mtDNA content have been reported in numerous cancers examined to date. The D-loop region has been shown to be a mutational hot spot in human cancer. In order to evaluate the role of mtDNA mutations in cervical lesions progression, cervical smears (collected in liquid cytology medium from 50 women, 29–55 years old) were investigated. Total genomic DNA (High Pure PCR Template, Roche Diagnostics) was isolated from scrape specimens from patients with different citology (normal cervical epithelium, ASCUS, LSIL, HSIL and cervical cancer) and tested for HPV DNA presence (InnoLipa Genotyping kit- Innogenetics). To elucidate a causative role of mtDNA in cervical lesions, mtDNA mutations were investigated using Mutector mtDNA kit. In patients with normal cytology mtDNA mutations were detected in less than 15% of the cases, as compared with 50% of LSIL and HSIL cases. mtDNA mutations were detected in 60% cases of cervical cancer and only in 10% ASCUS. These studies provide strong evidence that instability in the D-loop region of mtDNA may be involved in cervical dysplasia. We suggested that mtDNA mutations may play a role in cervical precursor lesions and cancer but the mechanism of carcinogenesis remains to be solved.

A5.07
HIV V3 structures as novel HIV immunogens

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A major objective in HIV-1 vaccine development is identifying immunogens that can induce antibody response against epitopes that are shared among all or many virus strains. An important HIV-gp120 epitope is the third variable (V3) region which binds to chemokine receptors CCR5 (''R5 viruses'') and CXCR4 (''X4 viruses''). Based on our previous NMR structure determination we discovered that V3 peptides from both X4 and R5 strains adopt a β-hairpin conformation. In the R5 conformation, two oppositely charged residues were juxtaposed, enabling a favorable electrostatic interaction that stabilized this conformation. In the X4 conformation, electrostatic repulsion between these residues forced a one register shift in the N-terminal strand of the V3. Despite this difference, both strains share a β-hairpin conformation. Peptide immunogens, mimicking these V3 structures were designed, synthesized and administered to rabbits. These immunogens induced high titer antibody responses against gp120 and demonstrated neutralizing activity against different HIV strains. Our promising results suggest that our novel approach using V3 peptides constrained to, and thereby mimicking, the conformations created in V3 results in more effective vaccine candidates than flexible V3 peptides. Our novel use of structural data is an important step forward in the design of HIV-1 vaccines.

A5.08

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A5.09
Redox and antioxidants status plant extract by oxidative stress related diseases

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Natural antioxidants are extensively studies for their capacity to protect organisms and cell from damage induced by oxidative stress, the latter being considered a cause of infectious diseases. The major aim of the present study was to investigate the role of the plant extract (PE) as natural antioxidant in modeling oxidative stress (hemolysis of erythrocytes). We obtained changes by the measuring of redox potential and value of pH for extracts with different degree of ethanol. However calculation the value of rH pointed that all PE can be as restoring factor by free radical oxidation processes. It is spoken about fact, that our PE can be use as natural inhibitors in during oxidative damage in organism. Using electron spin resonance (ESR) technique we investigated *in vitro* free radical production by erythrocyte suspension with PE. We also studied the influence ethanol extracts on hemolysis kinetics induced by HCl solution in different situation. Antioxidant status of plant extracts was determination in erythrocyte suspensions by important products of lipid peroxidation. So, we try to develop novel approaches to correlate the level of lipid peroxidation and membrane damage for erythrocyte system. Our results shown that choose a method of extraction (time, temperature, using solution, etc.) can be determinate that pharmaceutical effect, which necessary for concrete case of organism damage. The method of peroxide hemolysis we suggest for using as specific test for detection of the presence different disease in an organism carried out by the activation of the lipid peroxidation processes. This work was supported ANSEF Grant 07-NS-biochem-1440.

A5.10
Lipid chlorohydrins are biologicaly active markers of inflammatory relevant oxidant – hypochlorite

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Hypochlorite, a known pathologically relevant oxidant, gives the wide spectrum of his chlorination and oxidation products. Our previous experiments proved that chlorohydrins arise as major products upon the reaction of hypochlorite with unsaturated phosphatidylcholines. Having in mind their specifity, they are regarded to be a good marker of hypochlorite, but their possible biological role is still to be studied. Thus, we decided to check the effects of the presence of chlorohydrins in the cell membrane on the basic cell function. Fusion of liposomes labeled with NBD-PE, cell cycle, mitochondrial potential quantified with JC-1 and the level of caspase-3 stained stained with FITC-conjugated mAb was analysed with flow cytometry. Cell viability and proliferation ability was evaluated with MTT test and on the basis of the DNA content, respectively. Flow cytometry demonstrated fast fusion of chlorohydrin liposomes labeled with NBD-PE with cells in both cell suspension and monolayer. Incorporation of chlorohydrins into cells was followed by reducion of cell viability and proliferation ability depending on the order of chlorohydrin. Cell growth arrest was observed in G0/G1 phase with simultaneous reduction of cell population in S and G2/M phase. Contemporaneosly, increase of the level of active form of caspase-3 and drop of mitochondrial potential was found in cells cultured in the presence of chlorohydrin liposomes. Phosphatidylcholine chlorohydrins, except having the quality to be a good hypochlorite marker, appear to be biologically actve and the intensity of the effects were correlated with the number of HOCl-molecules substituted to the fatty acid chain.

A5.11
Sodium nitrite treatment reduce hepatic ischemic-reperfusion injury

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Recent studies suggest that nitric oxide synthase (NOS)-independent NO sources mediates protecting effect towards ischemic injury on hypoxic or ischemic conditions. The purpose of this study is to investigate the cytoprotective effect of NO^2- administration in various concentrations and different time periods, to hepatic ischemic/reperfusion injury. For the control group, hepatic I/R protocol (45 minutes I and 5 hour R) was performed on albino mice whereas for the study group, sodium nitrite ($NaNO^2$, i.p.) was administreated in four different concentrations (1.65, 16.5, 165, 1650 μg/kg), 12 and 24 hours before the I/R protocol. Following reperfusion period, alanin aminotransferase (ALT) and aspartat aminotransferase (AST) levels in plasma and antioxidant enzyme activities in liver homogenates were determined and tissues were evaluated histologically in control and study groups. Plasma ALT and AST levels found to be increased after I/R protocol in control group whereas decreased in NO^2- study group. Malondialdehyde levels were found to be decreased in NO^2- study group compared to control group inversely proportional to $NaNO^2$ concentration. Reduced glutathion (GSH) levels and GSH/Oxidized glutathion (GSSG) ratios were found to be significantly increased while GSSG content were found to be decreased in NO^2- study group compared to control group. Mophologic findings of hepatic protection by NO^2- were evaluated histologically in various proportions. Our results suggest that NO^2- administration 12 and 24 hours before I/R can reduce the oxidative injury by enhancing reduced glutathion content and antioxidant enzyme levels; thus this can play a part in the protection towards I/R injury.

A5.12

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A5.13
Polyamine protective functions under antibiotic-mediated bacterial DNA damage

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Background: DNA is known to be the main target of reactive oxygen species that can be produced in response to a variety of antibiotics.
Aim: The investigation of polyamine defense functions being accomplished by their antioxidant activity and DNA protection in response to the impacts of two different antibiotic families.
Methods: Determination of hydroxyl radicals with 3′-(hydroxyphenyl)fluorescein cell staining followed by fluorescent detection on microplate rider. Registration of DNA damages by agarose gel electrophoresis of pDNA samples extracted out of *E. coli* cells exposed to antibiotics. Cell estimation by serial plating on LB media.
Results: (1) Fluoroquinolone (levofloxacin) and aminoglycoside (amikacin) sublethal impacts on *E. coli* cells produced concentration effects on hydroxyl radical formation.
(2) Antibiotics investigated here resulted in cell DNA fragmentation.
(3) *E. coli* polyamines putrescine and spermidine were found to scavenge hydroxyl radicals produced by fluoroquinolone and aminoglycoside.
(4) Likewise, polyamines reduced the DNA fragmentation caused by fluoroquinolone and aminoglycoside.
(5) Furthermore, polyamines notably raised cell viability in response to all of investigated antibiotics.
Conclusions: Antibiotics referring to two investigated families, regardless of their specific intracellular targets produced cell hydroxyl radical formation and DNA damages. *E. coli* polyamines exerted their antioxidant and DNA-protective effects in response to fluoroquinolone and aminoglycoside. Polyamine positive effects on cell viability were exhibited in response to all of antibiotics investigated here.

A5.14
Prolyl oligopeptidase of *Trypanosoma brucei* hydrolyzes native collagen, peptide hormones and is active in the plasma of infected mice

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Proteases play important roles in many biological processes of parasites, including their host interactions. In sleeping sickness, *Trypanosoma brucei* proteases released into the host bloodstream could hydrolyze host factors, such as hormones, contributing to the development of the disease's symptoms. In this study, we present the identification of the *T. brucei* prolyl oligopeptidase gene (poptb) and the characterization of its corresponding enzyme, POP Tb. Secondary structure predictions of POP Tb show a structural composition highly similar to other POPs. Recombinant POP Tb produced in *E. coli* was active and highly sensitive to inhibitors of *T. cruzi* POP Tc80. These inhibitors, which prevent *T. cruzi* entry into non-phagocytic cells, arrested growth of the *T. brucei* bloodstream form in a dose-dependent manner. POP Tb hydrolyzes hormone peptides containing Pro or Ala at the P1 position at a slightly alkaline pH, and also cleaves type I collagen *in vitro* and native collagen present in rat mesentery. Furthermore, POP Tb is released into the bloodstream of *T. brucei* infected mice where it remains active. These data suggest that POP Tb might contribute to the pathogenesis of sleeping sickness.

A5.15

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A5.16
The HPLC (High Performance Liquid Chromatography) analysis of aflatoxins in maize from the main production areas in Iran

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Fifty-one maize samples, intended for animal feed and human consumption, were collected from the four main maize production provinces in Iran and analyzed by high-performance liquid chromatography (HPLC) for contamination by four naturally-

occurring aflatoxin analogues (AFB1, AFB2, AFG1, and AFG2). Most of the maize samples obtained from Kermanshah and Mazandaran provinces were contaminated with aflatoxins, especially AFB1 and AFB2. The maximum AFB1 (276.3 μg/kg) and highest level of total aflatoxins (AFT) (316.9 μg/kg), were detected in a maize sample collected from Kermanshah province. The mean aflatoxin level from contaminated samples (52.60 μg/kg) from Kermanshah was significantly higher ($p < 0.0001$) than those in maize from the other three provinces, and exceeded all the maximum tolerated levels (MTLs) set for AFT in maize. The level of AFB1 in 15.8% of the total samples was above the MTL (5 μg/kg) for AFB1 in maize. The mean contamination level of AFT (23.86 μg/kg) was higher than MTL for maize in Iran (20 μg/kg). The levels of AFB1, AFB2, AFG1, and AFG2 ranged between not detected (< 0.1 μg/kg) and 276.3, 30.4, 9.1, and 1.1 μg/kg in maize grains, respectively. The relatively high level of this carcinogenic mycotoxin indicated that the Iranain people are potentially endangered to hepatocarcinoma.

A5.17
Novel allosteric inhibitors of HIV-1 integrase on the base of multimodified oligonucleotides

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The integration of HIV-1 DNA into the infected cell genome is one of the key steps in the viral replication cycle. The viral integrase is thus a key potential target for new antiviral drugs. Short single-stranded oligonucleotides conjugated with hydrophobic molecules inhibit integrase efficiently, inducing dissociation of the integrase–viral DNA complex. The dependence of the conjugate inhibitory activity on the oligonucleotide length and structure as well as on the structure of hydrophobic molecules has been studied. The nature of hydrophobic moiety plays the main role in the modulation of the conjugate inhibitory activity. They efficiently inhibit the catalytic activity of integrases with mutations in the catalytic core isolated from HIV-1 strains resistant to known integrase inhihbitors, styrylquinoline and raltegravir. Intracellular delivery of the inhibitor containing eosin has been investigated by using two different cell-penetrating peptides. One of them promotes an efficient cellular uptake of the inhibitor in cell nuclei. When bound to the peptide, the oligonucleotide conjugate exhibits a potent antiviral activity and inhibits HIV-1 replication in MT-4 cells with EC50 about 0.4 nM. This study was supported by the CNRS and Russian Foundation for Basic Research, projects 08-04-01293 and 09-04-93108-CNRSL and Grant of President of Russian Federation MK-2627.2009.4.

A5.18
The saliva proteomes of the amazonian triatomine vectors of Chagas' disease *Rhodnius brethesi* and *Rhodnius robustus*

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The triatomine bugs that act as vectors of Chagas' disease are haematophagous organisms in all of their evolvement stages. Their feeding success is greatly related to their salivary proteins that allow these insects to access their food by counteracting host haemostatic mechanisms, such as platelet aggregation, clotting and vasoconstriction, as well as host inflammatory reactions. Since these components are involved in the *Trypanosoma cruzi* transmission process, the salivas of epidemiologically significant Amazonian triatomine bugs, *Rhodnius brethesi* and *Rhodnius robustus*, were submitted to proteomic studies. Initially, the experimental conditions for wide-pH-ranged separation by two—dimensional gel electrophoresis (2DE) were optimized. The average number of spots of these two saliva samples was around 130. Comparative analysis of the 2DE maps revealed very few divergences between the two species. The identification of the spots was made by peptide mass fingerprinting, peptide fragmentation and *de novo* sequencing using MALDI-TOF/TOF technology. The data revealed largely anti-haemostatic components, mainly lipocalins. Our results show that the *R. brethesi* and *R. robustus* saliva proteomes are rich in platelet aggregation inhibitors which promote the blood feeding of these insects. Support: PRONEX-FAPDF, CNPq, CAPES, FINATEC and FINEP.

A5.19
Bacterial proteases cleaves the MUC2 and may cause ulcerative colitis

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The distal colon is covered with protective mucus layers, an outer loose and inner firm layer in mouse. The loose layer is heavily colonized by bacteria. Little is known about the role of bacterial proteases and break down of the mucus. MUC2 is major component of this mucus layer which is polymerized by dimerisation via C-termini and trimerization via N-termini producing a large network like structure. Our results shows that Bacteriodes fragilis and Porphyromonas gingivalis are found to cleave the N and C terminal of the MUC2 at specific cleavage sites without disrupting the intra cystine disulphide bond which stabilizes the mucus network. Other cleavages are predicted to dissolve the mucus gel. These findings suggest that the bacteria can degrade the mucus layer which causes the inflammation by contacting the epithelial cells directly may result in ulcerative colitis in human.

A5.20
Enzymes of the lysine biosynthetic pathway as targets for antifungals?

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Systemic infections caused by human pathogenic fungi in immunocompromized patients continue to be one of the important clinical problems. Limited availability of safe and efficacious antifungal chemotherapeutics and emerging resistance to existing drugs stimulates search for novel molecular targets for antifungals. The α-aminoadipate pathway (AAP) of L-lysine biosynthesis is unique in fungi and thus has been so far considered one of the potential targets for the rational design of antifungal drugs. In the present communication we report results of our studies on characterization of four enzymes catalyzing initial steps of AAP from the opportunistic fungal pathogen *Candida albicans*: homo-

citrate synthase (HCS), homoaconitase (HA), homoisocitrate dehydrogenase (HICD) and α-aminoadipate aminotransferase (AadAT). The appropriate genes were cloned from the *C. albicans* genomic DNA, expressed in *E. coli*, their products were isolated as oligoHis-tagged proteins and characterized in terms of steady-state kinetic parameters, substrate specificity and cofactor requirements. Differences in the enzymatic properties of two isoforms of HCS encoded by the *LYS21* and *LYS22* genes were noted. Several potential inhibitors of the AAP enzymes were designed, synthesized and tested for their enzyme inhibitory potential. Finally, virulence of the constructed lys21Δ/lys22Δ mutant of *C. albicans* in the disseminated murine candidiasis model was compared to that of the wild-type strain. Conclusions questioning a possibility of exploitation of AAP enzymes as targets in chemotherapy of systemic fungal infections have been presented.

A5.21
Isolation, cloning and sequence analysis of lactate dehydrogenase gene from *Theileria annulata*

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Theileriosis is a serious animal disease that transmitted by ticks. It is caused by obligate intracellular parasitic protozoa of the genus *Theileria*. Infection by *Theileria* spp. parasites results in production losses and high mortality in susceptible animals. Currently available antiparasitic drugs are effective in animals but animals may remain carriers and treatment is most effective in the early stages of the disease. Isolation, cloning and analysis of lactate dehydrogenase from *T. annulata* which causes tropical theileriosis has been targetted in the present study with the ultimate aim of designing new antiparasitic drugs that will hopefully have wider mode of action in animals. The target sequence was isolated from the genomic DNA by PCR and cloned into the vector pGEM-T easy from a *Theileria* species for the first time, to our knowledge. Sequencing of the whole gene from both directions indicated that open reading frame was interrupted by two introns. There are also several single nucleotide exchanges in the sequence. Most remarkable is a five residue insertion in the active site loop region that might be an attractive target for inhibitors of the enzyme.

A5.22
Association of first response antioxidant enzymes activities and course of blood infection

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Reacting free radicals act an essential part in pathogenesis of systemic inflammatory response syndrome, sepsis, severe sepsis and septic shock. Vitamins and trace elements as further enzyme cofactors redistribution is typical for sepsis which could be immediately concerned with relative deficit of circulating antioxidants. Deficiency of antioxidant enzyme glutathione peroxidase cofactor, selenium is commonly found in critically ill patients, particularly those suffering from severe infections mentioned and polytrauma. Glutathione peroxidase prevent organic substrate peroxidation by nonspecific reduction of peroxides following glutathione depletion. We attempt to improve survivance of critically ill patients by selenium supplementation. Oxidative stress become irreversible approximately in 6–24 hours from tissue hypoxia that's way when shock arises, selenium intravenous injection supplementation has to be started as soon as possible. Optimal timing and dosage was 750 μg/day between 1 and 3 weeks depending on severity of the disease. Preliminary results showed increase in glutathione peroxidase activity in a group of selenium treated in comparison to placebo group treated by physiological solution from 0.19 μkat/l to 0.45 μkat/l. Inverse activity of glutathione reductase from 1.23 μkat/l to 0.65 μkat/l was estimated. According to improvement of patients clinical state evaluated by APACHE II score as well as 10% mortality decrease in treated group in comparison to placebo group we consider activation of another selenium containing antioxidant. Selenium supplementation could cause thioredoxin reductase activation which uses the same reducing agent to reduce oxidated thiols.

A5.23
Structural analysis of Toll-like receptor 2-activating lipoprotein from *Vibrio vulnificus*

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IlpA, a surface protein of the human pathogen *Vibrio vulnificus*, is the first lipoprotein to be characterized in *Vibrio* spp. as a major immunostimulant. Previously, it was characterized that IlpA was subject to lipidation at its N-terminal cysteine residue. The resulting IlpA then activates Toll-like receptor 2 in human cells, and induces overproduction of proinflammatory cytokines closely associated with septic shock in infected individuals. To identify structural features of IlpA, we determined the crystal structure of IlpA at 2.6 Å resolution. Specifically, IlpA consists of two homologous domains, each with α/β topology, similar to the structure of solute-binding protein which is a component of ATP-binding cassette transporter. In fact, binding of L-methionine was observed in the pocket between the two domains, suggesting that IlpA is an L-methionine-binding protein. The structural features of IlpA in this study, along with the immunological properties of IlpA identified previously and other solute-binding proteins, suggest that solute-binding lipoproteins of ATP-binding cassette transporter present at the bacterial cell surface could serve as pathogen-associated molecular patterns to Toll-like receptor 2, causing host immune responses against infection.

A5.24
Comparative analysis of the Tn1546 element from newly isolated and identified vancomycin resistant *staphylococcus aureus* strain isolated from burn suffering human patients hospitalized at intensified care unit Sulaimani Central Hospital, Iraq

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Vancomycin-resistant Staphylococci was not analyzed extensively in Iraq yet. *Staphylococcus aureus*, a major cause of potentially

life-threatening infections acquired in health care settings and has developed resistance to most classes of antimicrobial agents soon after their introduction into clinical use. In many health care institutions around the world, glycopeptides such as vancomycin provide effective therapy against most strains of multi-drug-resistant *S. aureus*, including MRSA.
Materials and Methods: With PCR and DNA sequence analysis, the Tn1546 elements, a mobile genetic element that encodes vancomycin resistance proteins in enterococci and staphylococci was amplified and the sequences of which was compared to the prototype Tn1546 element from enterococci, and also to the previously sequenced Tn1546 of VRSA recorded in gene bank/ NCBI.
Results: In 2008, the first ten clinical isolates of vancomycin-resistant Staphylococcus aureus (VRSA) containing vanA gene with variable vancomycin MICs ranged from 32 to >128 μg/ml were recovered in Sulaimani Emergency hospital for war victims/ Sulaimani/Kurdistan region of Iraq. They possessed differences in its Tn1546 elements, which explain various expression level of VanA gene displayed by vancomycin MICs. The "Sulaimani-VRSA-Tn1546" element was different by distinct modifications in nucleotide sequences and showed partial homology with an enterococcal transposone Tn1546-like element.
Conclusions: *S. aureus* isolate has been shown to be vancomycin-resistant, due to a Tn1546 transposon analogue and proved to be effective as an alternative model approach for pathogenic bacterial characterization.

A5.25
Novel identification of different categories of regional species of diarrheagenic *escherichia coli* from human stool samples by using multiplex pcr technique

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Enteropathogenic *Escherichia coli* (EPEC) are one of the oldest diarrheogenic *E. coli* and were recognized as an infantile diarrhea cause. EPEC strains are often the most frequently isolated bacterial diarrheal pathogens. EPEC can be isolated from stools up to 2 weeks after cessation of symptoms. Presence of the eae gene responsible for A/E lesions and the bfpA gene that located on a plasmid, called the EPEC adherence factor (EAF), have been used to classify this group of bacteria into typical and atypical strains.
Material and Methods: DNA was extracted from Growing bacterial colonies. Amplification primers for (eaeEp-f, eaeEp -rltEt-f, ltEt-r, stEt-f, stEt-r StxEH-f, xEH-r, CVD-EA-f, MWG, Germany), were used to amplify *E. Coli* specific sequences that where separated on a 1X TBE Agarose gel. After electrophoresis, UV transilluminator was used to detect DNA binding ethidium bromide which appears as light fluorescent bands. Mann–Whitney *U* test for independent samples was used to analyze these data. $p < 0.05$ was considered as statistically significant. At least for three times experiments where repeated.
Results: PCR tested isolates from 111 EPEC patients only 45 (40.5%) were positive for the local eaeA gene and considered as true EPEC while, more categories of *E coli*, 5/19 (26.3%) showed st and/or lt genes of ETEC, and 19 (21%) showed PCVD gene of EAEC.
Conclusions: *E. coli* characterization by Multiplex PCR is important because a considerable number of pathogenic *E. coli* remains non determinate by conventional diagnostic techniques during infection course.

A5.26
Bacterial biofilms characterisation in human Iraqi patients suffering from urinary tracts infection

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Bacterial biofilm help clinicians explain some challenging areas in pathogenesis, diagnosis, and urinary tract infections (UTI) treatment. Biofilm formation needs two properties: cells adherence to surface & accumulation to form multilayered cell clusters Genetic and molecular biofilm formation basis in human pathogenic bacterial strains is multifaceted Microscopic observation confirmed that many types of UTIs (e.g. catheter-associated infections, struvite urolithiasis).
Methods: Causative agents Isolation and identification of pre, per and post operative bacteriuria, biofilm formation on microtiter plates and urinary catheter, biofilm formation inhibition via chemical surfactants and biofilm antibiotic susceptibility to antimicrobial agents. Ionic surfactant formation analysis (SDS) and anionic surfactant (Tween 80) at 0.2% in TSB in addition to biofilm antibiotic susceptibility assay & bacterial isolates molecular analysis via Pulse Field Gel Electrophoresis (PFGE) & parallel biochemical & serologic samples analysis was done.
Results: SDS significantly inhibited biofilm formation on microtiter plate wells. Most isolated uropathogens were, *E. coli*, *S. aureus* and *K. pneumoniae*. Bacteriuria incidence in controls was 28%, *E. coli* were 10%, *S. aureus* 8%, *P. aeruginosa* 2% and 1% *S. faecalis*. Catheterization is most important risk factor for bacteriuria pre & post operatively. Antibiotics application did not eradicate bacterial biofilm on catheter surface.
Conclusions: The there was a domination of *E. coli*, *S. aureus* and *K. pneumoniae* isolated uropathogens. PFGE is suitable for pathogenic bacteria characterization analysis.

A5.27
Microbiological detection of catheter-associated urinary tract infection in Iraqi patients of different sexes and age groups

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Urinary catheters are inserted in millions patients in acute-care hospitals. Catheter-associated urinary tract infection (CAUTI) is the most common nosocomial infection in hospitals, comprising >40% of all institutionally acquired infections & the 2nd nosocomial bloodstream infection cause. We investigate the catheter role in pre and post operative bacteriuria in patients undergone cystoscopy.

Methods: 154 patients admitted to Medical City Hospital in Baghdad for Diagnostic cystoscopy, Transurethral resection of bladder tumor (TURT) and Transurethral Resection of Prostate (TURP) were included. Patient's age, sex and surgery duration were studied as bacteriuria risk factors. 462 urine samples, 158 cystoscope swabs, 65 irrigation samples and 112 antiseptic samples were investigated to determine bacteriuria incidence, causative agents.
Results: In non catheterized patients 26.3% of the patients had bacteriuria, 90% of catheterized patients showed bacteriuia pre operatively ($p < 0.05$). Patients with pre operative catheterization developed bacteriuria post operatively, while 49% of patients with no previous catheterization history developed post operative bacteriuria ($p < 0.05$). Post operative bacteriuria in patients undergone surgery <45 minutes was 49% which was significantly less than those undergone surgery more than 45 minutes 80% ($p < 0.05$). *Escherichia coli* was the most common organism isolated in pre, per and post operative samples in TURT and TURP group.
Conclusions: Catheterization and surgery duration >45 minutes were found to be the most important risk factors for bacteriuria post operatively.

A5.28
Detection of a new local endemic *V. cholerae* type as a result of a comparative molecular analysis screening approach of different natural infectious sources in Iraq

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In the time period between (2000 and 2009) serious cholera outbreaks occurred in different parts of Iraq. Here, pulse field gel electrophoresis (PFGE) was used as a DNA fingerprinting tool for first time in Iraq in order to detect possible infection sources and to compare between environmental & clinical isolates associated to cholera outbreaks.
Methods: Two sample groups, namely one of 100 acute diarrhea patients stool samples and another of 100 water samples from nine sites across Iraq were collected 2004–2006. Pathogen isolation & identification was according to standard methods. Gene Navigator System was used for PFGE, the running condition was; 100 mA/200 Volt / switching time 50 sec/20 hours using Not I restriction enzyme. Bacterial isolates molecular analysis was by Pulse Field Gel Electrophoresis (PFGE). Parallel biochemical and serologic samples identification was done.
Results: *V. cholerae* isolation from stool of nine patients with profuse watery diarrhea, eight were O1 (six ogawa and two inaba, El Tor Biotype) & one isolate was NAG. Water samples were positive to Vibrio cholerae in Wasit (four isolates) in Baghdad (one isolates). PFGE analysis showed two clinical isolates (Inaba) genetically identical therefore epidemiologically related while, NAG isolates one clinical & one environmental isolates were genetically identical therefore epidemiologically unrelated representing a novel local strain and indicated that water is the infection source.
Conclusions: Clonal relationship between clinical and environmental isolates exists and PFGE is a useful tool for epidemiologic surveillance of *V. cholerae*. Also two new local endimic *V. cholerae* types was detected and characterized.

A5.29
Accounting for loop flexibility of SARS-CoV papain-like protease in computational drug discovery

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The papain-like cysteine protease (PLpro) encoded by the SARS coronavirus (SARS-CoV) replicase gene represents a potential target for antiviral drug development. The flexible loop around the PLpro active site shows substantial conformational adaptation between open and closed forms upon ligand binding, major challenge in computational drug discovery/design efforts. We used molecular dynamics to extensively sample the loop conformations and the phi-psi dihedral torsion change of Tyr269-Gln270 (loop residues) on inhibitor binding. Snapshots from molecular dynamics simulations of apo (open) and inhibitor bound (closed) forms (20ns each), of the loop were used to develop a two-pass pharmacophore model using computational solvent mapping, to eliminate false positives and account for protein flexibility in virtual screening. The dynamic model was trained on 101 PubChem compounds with 78% accuracy and tested on a test set of 59 in-house compounds with 78% accuracy. Hits retrieved from pharmacophore screening were docked into the two bound crystal structures of PLpro. We apply an adjusted scoring function (validated against the test set with an accuracy of 70%) to re-rank the docked compounds; top scoring compounds from both the docking runs were clustered based on 2D fingerprints and chosen for experimental testing. The results are quite promising, especially compared to traditional flexible docking approaches where a poor rank correlation of 16% was achieved between the experimental and predicted values. This model will be used for future database screening for discovery of novel leads against the SARS PLpro. Supported in part by NIH grant AI060915.

A5.30
Syphilis-kodecytes – novel function-spacer-lipid (FSL) modified red cells capable of sensitive and specific detection of syphilis antibodies

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The ability to detect infectious agents is a prerequisite to the treatment and containment of transmittable diseases. Many diagnostics assays are based upon the detection of antibodies in serum against the infectious agent and often rely on ELISA or agglutination based assays to determine the infectious status. Using function-spacer-lipid (FSL) constructs bearing peptides (1) representative of an infectious agent attached to human red blood cells, offers a potential alternative diagnostic approach to the detection of antibody-defined infections. Using the syphilis spirochaete as a model, and a peptide selection algorithm, a series of FSL constructs were created and attached to human red cells creating syphilis-kodecytes. These kodecytes were then tested in routine blood typing platforms against serum from patients and blood donors of known syphilis status. This approach has

already been successfully used to detect ethnic specific blood group antibodies (1). It was found that syphilis-kodecytes used in blood grouping platforms are capable of the same sensitivity and specificity as ELISA and Particle Agglutination assays. The ability to engineer the FSL construct and replace the functional peptide for other infectious markers allows for a highly flexible and rapid-development technique for the detection of antibody-defined infectious agent markers, and may be an important low-technology tool for use in epidemics and pandemics, particularly in third world environments.

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Reference:

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A5.31
Elevated advanced oxidation protein products levels in chronic liver disease. Relation to severity of complication in liver cirrhosis

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Background/Aims: Advanced oxidation protein products (AOPPs) and Nε-(carboxymethyl) lysine-modified advanced glycation end products (CML-modified AGEs) may contribute to progressive liver disease in the cirrhotic patients and influence the potency of oxidative stress. Little is known, however, regarding the relationship between the oxidative stress-related biomarkers and development of cirrhotic complications. The aim of this study was to evaluate the association between serum AOPPs and CML-modified AGEs levels and the severity of complications in liver cirrhosis.

Methods: Data of inpatients with cirrhotic complications were collected retrospectively. The serum AOPPs and CML-modified AGEs or levels as well as severity of complications of 45 inpatients was analyzed.

Results: The serum AOPPs level was strongly associated with the severity of liver function impairment as assessed by Child-Pugh and MELD scores ($p < 0.001$). Even a low (below median values) AOPPs level (19.8 μmol/l) was associated with severe complications. High (above median values) AOPPs or CML-modified AGEs levels (19.8 μmol/l and 1.4 μg/ml, respectively) indicated the existence of higher incidence of vascular disease ($p < 0.001$), and diabetes ($p < 0.01$) as well as infection ($p < 0.05$).

Conclusions: High (above median values) AOPPs level may indicate the existence of severe complications associated with liver cirrhosis.

A5.32
Inhibition of dengue virus type-2 (DENV2) replication by flavanoid-derived compounds

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Dengue viruses, mosquito-borne members of the Flaviviridae family, are the causative agents of dengue fever and its associated complications. More than 2.5 billion people in over 100 countries are at risk of infection. There is currently no treatment or vaccine available for dengue infection. This study employed whole-cell organism *in vitro* methods to study the inhibitory property of the flavanoid-derived compounds against DENV2 activity. Results showed that at concentration not exceeding the maximum non-toxic dose (MNTD), these compounds completely prevented DENV2 infection in HepG2 cells as indicated by the absence of cytophatic effects. The antiviral activity assessed by virus inhibition assay showed strong inhibition in a dose dependent manner. All five compounds exhibited inhibitory activity with a range of potency strengths of 72–100%. The plaque forming unit per ml (pfu/ml) was reduced prominently with a maximum reduction of 98%. Amplification of DENV2 protease (NS2B/NS3) RNA transcript harvested from *in vitro* inhibition assay showed decreased in the band intensity indicating reduced viral load. Quantitative RT-PCR results showed reduced copies of DENV2 protease/ng RNA relative to increased concentration of inhibitors. Thus, it was evident that synthesized compounds YK 51, YK 73 and YK 73x implicated their antiviral properties by inhibiting the DENV2 replication through reduction of the protease gene. The mode of actions is also strongly attributed to the functional groups of saturated piperidine ring and the carbamates or BOC. These highly potent inhibitors have great potential to become lead antiviral agent for dengue.

A5.33
Inflammatory and cytotoxic systemic effects induced by intratracheal instillation of pm10 and pm1

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The effects of particulate matter (PM) include asthma, cancer, cardiovascular and lung diseases. PM10, in the lungs, induces alveolar and systemic inflammation. Moreover, PM smaller than 100 nanometers can pass through cell membranes and migrate into other organs, could produce systemic inflammation and increase risk of thrombosis. Therefore, we evaluated the different inflammatory and cytotoxic potential of PM10 collected in an urban area in Milano in two seasons (summer'08 and winter'08) on the lungs, and the different systemic effects of PM1 collected in the same area and seasons. BALB/c mice were intratracheally aerosolized with suspensions of PM10 from different seasons (100 ug/100ml); after 3 hours, 24 hours, mice were sacrificed and BAL was collected, cytologically and biochemically characterized (total and differential cell counts, ALP, LDH, TNF-a, Hsp70, sPLA2), while lungs were excised for further analyses (HO-1, Casp8, NFKB). PM1 from different seasons (100 ug/100ml) was instead aerosolized once every 2 days for a total of three instillations; 24 hours after the last exposure, mice were sacrificed and BAL was collected, cytologically and biochemically characterized (total and differential cell counts, ALP, LDH, TNF-a, Hsp70, sPLA2). Lungs were excised for further analyses (HO-1, Casp8, NFKB, MDA), as brain (CD68, GFAP, TNF-a, MDA) and heart (HO-1, TNF-a, MPE, MDA). Blood samples were collected for coagulation markers analyses (Fibrinogen, sP-selectin, PAI-1). This strategy permits us to discriminate between stagionality pulmonary and systemic effects of PM10 and PM1, collected in the same Milano urban area. Supported by Fondazione Cariplo to P.Palestini.

A5.34
Interleukin 6 (IL-6) and Neurotrophin 3 (NT-3) dynamics in children with meningitis and encephalitis

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The systemic inflammatory response induced by brain neuroinfections is an important target for the actual research. The activation of microglial cells represents the key element of the inflammatory response to viral aggression in acute viral meningitis (M_VIR) and encephalitis (E_VIR) by multiple etiologies. The inflammatory reactions and the efficacy of the protective mechanisms are two factors with control the course of neurological infections. The present work investigated the possible correlation of NT-3 with IL-6 from children with (M_VIR) and (E_VIR). NT-3 and IL-6 serum and CSF level were determined using ELISA kits. The patients were divided into two groups: -group I including children with M_VIR, HSV-, treated or not with dexamethasone (DEXA+ /DEXA-), n = 18 (20.3 ± 3.72 years) - group II including children with E_VIR, HSV+, DEXA+, n = 7 (20 ± 2.86 years). An inverse correlation was found between NT-3 and IL-6 serum levels in group I DEXA+ and in those of the group II DEXA- with a moderate course, while a direct correlation between the two serum parameters was detected in patients of group II DEXA- who died. In addition, an inverse correlation between the CSF NT-3 and IL-6 levels was found in survivor patients of the group I and II. The inverse correlation between NT-3 and IL-6 suggests that NT-3 may exert an immuno-modulatory action on IL-6 responses in survivor children after the viral neurological infections. The absence of this correlation corresponded to imminence of death. DEXA therapy did not influence the serum and CSF NT-3 dynamics.

A5.35
SNPs in MUC2 are expected to cause a nonfunctional protein and maybe an explanation for Ulcerative Colitis

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Ulcerative colitis is an inflammatory bowel disease of unknown cause. MUC2 mucin is the major component of the intestinal mucus barrier. Nearly 180 SNPs were found in the MUC2 coding region of which 95 SNPs are non-synonymous. Five frame shift mutations in the N-terminal region of MUC2 caused by insertions or deletions disrupt the reading frame which leads to termination resulting in truncated protein. Mutations in the MUC2 gene are predicted to be linked to a dysfunctional MUC2 which could provide a genetic explanation for ulcerative colitis. Analysis of the SNPs which are evolutionarily stable makes them easier to use when the disease traits is followed in population studies. SNPs in MUC2 could probably be of help to determine the likelihood of an individual predisposed to ulcerative colitis disease.

A5.36
Transcriptional and protease activity analysis as tools for increasing rotavirus protein production in *Saccharomyces cerevisiae*

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Rotavirus is the major etiological agent of diarrhea in children and young animals. Actual vaccines based on attenuated or inactivated virus could cause a disease transmission by reversing the attenuation or inefficient inactivation. Virus-like particles (VLP) have emerged as excellent vaccine candidates, as they are not infectious and their size and structure result in excellent immunostimulating properties. Rotavirus VLP assemble in the absence of other non-structural proteins have the same topological and functional characteristics as native viral particles. Therefore, the structural proteins possess the information required for particle assembly. A challenge that must be overcome in rotavirus VLP expression in yeast is increasing the heterologous protein production to promote self-assembly into VLP. The present work uses transcriptional and protease activity analysis for yeasts capable of expressing scaffold rotavirus protein for VLP formation. Various Saccharomyces cerevisiae strains variants expressing VP6 rotavirus protein using constitutive promoters were used. Microarray analysis was used to access the overall state of the cell, linking phenotype to specific transcription factors, metabolites and overall biological shifts in the cell. In this regard, using DNA microarrays and protease activity tests, the yeast's biological response to foreign protein production could be measured.

A5.37
Three-year prevalence survey of nosocomial infection in the same hospital

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Objective: To investigate the prevalence of nosocomial infection (NI) in the same hospital in the past 3 years.
Methods: The survey form of personal case was filled by adopting the method of combining clinical investigation and consulting inpatients medical records.
Results: The average rate of nosocomial infection (NI) was 4.8%; rate of NI was different in the different; there were not significant difference among age, hospitalization, invasive operation, infection sites, antibiotics using in the past three years, but, the rate of NI were changing in the different department in the 3 years.
Conclusions: The rate of NI were changing in the different hospitals and departments; The the survey and control of NI should be strengthened in the department and the season with high rate of nosocomial infection.

A5.38
A analysis on early diagnosis of nosocomial infection caused by pulmonary tuberculosis in patients with diabetes mellitus

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Objective: To reduce the nosocomial infection caused by Mycobacterium Tubereulosis in patients with diabetes mellitus, some characteristics for early diagnosis of nosocomial infection was studied.
Methods: The clinical data of a total of 316 diabetes mellitus patients with pulmonary tuberculosis were made a retrospectively investigation from January 1998 to December 2007.
Results: (1) The age was 55.01 ± 12.77 and 61.92 ± 11.18 years old in the patients with active and inactive pulmonary tuberculosis respectively; (2) The pulmonary tuberculosis history was more frequent in the patients with inactive pulmonary tuberculosis than with active pulmonary tuberculosis; (3) The frequency of cough with profuse sputum and the sputum positive rate with Mycobacterium Tubereulosis were 48.4%, 80.0%, 25.2%, 10%; The GHBA1c, C peptide and the control of blood glucose level were 10.74 ± 2.95%, 0.56 ± 0.38 nmol/l, 13.11 ± 5.63 mmol/l, 8.68 ± 2.08%, 0.66 ± 0.48 nmol/l, 10.33 ± 9.41 mmol/l in the patients with active and inactive pulmonary tuberculosis respectively, there were significant difference for these markers between the two groups ($p < 0.05$).
Conclusion: These clinical manifestations with frequent cough with profuse sputum, the sputum positive with *Mycobacterium Tuberculosis* and surveillance of blood glucose level are helpful to early diagnosis of pulmonary tuberculosis in the patients with diabetes mellitus. It is very important to screen the information for reducing the nosocomial infection of *Mycobacterium Tuberculosis*.

A5.39
A analysis on the clinical features of A/H1N1 influenza infection

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Objective: To reduce stuffs and patients being infected in hospitals, the clinical features of A/H1N1 influenza infection were studied.
Methods: The clinical data of 17 patients with A/H1N1 influenza were made a retrospectively investigation in June 2009, the data included the change of body temperature, blood routine and chest X- rays check, 18 patients with ordinary influenza were studied as the control group.
Results: There were not significant difference for the change of body temperature, blood routine ($p > 0.05$); The chest X- rays check were normal in the two groups; All patients were confirmed positive for A/H1N1 RNA when they had fever, and were negative for A/H1N1 RNA when they had no fever in the A/H1N1 influenza group, those patients with ordinary influenza were all negative for A/H1N1 RNA when they had or not fever; These patients with A/H1N1 influenza or ordinary influenza had no complications, and all had good prognosis.
Conclusion: A/H1N1 influenza did not have any special clinical manifestations compared with ordinary influenza.

A5.40
A study on hospital infection in two parts of the same hospital

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Objective: To investigate the nosocomial infection characteristics in the two parts of the same hospital under the same adminstration mode.
Methods: The clinical data of patients with microbiogically documented nosocomial infection from January 2007 to December 2007 were retrospectively analyzed. Pathogens were identified by bacterial biochemical identification panel, antimicrobial susceptibility tests were done by disc agar diffusion test.
Results: The prevalence rate of nosocomial infection was 4.9% and 4.8% in the two parts of the same hospital; There were not significant difference in the distribution of infection site and bacilli, but there was significant difference in fungi among pathogens isolated from nosocomial infection cases(16.0% versus 8.0%), and the rate of nosocomial infection were changing in the different departments in the two parts of the same hospital.
Conclusion: There are different nosocomial infection characteristics even if in the same hospital, The nosocomial infection should be strengthened in the departments with more in-patients, according to the developping program of hospital.

A5.41
A clinical analysis of nosocomial fungal infection in same hospital for 4 years

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Objective: To investigate the nosocomial fungal infection in same a hospital. The pathogens, the infection routes, the high-risk factors, the drug-resistance of nosocomial fungal infection and its relationship with the use of antibiotics in same a hospital from January 2005 to December 2008.
Methods: The clinical data of patients with microbiogically documented nosocomial fungal infection were retrospectively analyzed. Pathogens were identified by fungal biochemical identification panel, antimicrobial susceptibility tests were done by disc agar diffusion test.
Results: The mean incidence rate of nosocomial fungal infections in a hospital from 2005 to 2008 was 8.04%. Most lower respiratory tract infection, accounting for 54.5%, followed by urinary tract and skin infection. Among high-risk factors, antibiotics use accounts for 42%, followed by ventilator use, accounting for 17.9%. Candida albicans is the major type of pathogens, accounting for 93.7%. 64 cases of drug sensitivity tests were carried out (30.9%), in which the strains were most sensitive to 5-FC, showing strongest resistance to Itraconazole.
Conclusions: Lower respiratory tract was the most frequent infection site; The main pathogens of nosocomial fungal infection were Candida albicans (93.7%); The management including rational use of antibiotics, microbiogical culture and susceptibility test should be strengthened to reduce the nosocomial fungal infection.

A5.42
A analysis on the effect of the distribution of pathogens and their antimicrobial resistance by administrative intervention

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Objective: To evaluate the effect of the distribution of pathogens and their antimicrobial resistance by controlling the use of the third generation cephalosporin.
Methods: Pathogens were identified by bacterial biochemical identification panel, antimicrobial susceptibility tests were done by disc agar diffusion test.

Results: (1) Six thousand five hundred ninety-one strains were isolated from clinical samples in the past 3 years, the most common pathogens were Enterobacter (24.7%) *Klebsiella pneumoniae* (20.8%) *Pseudomonas aeruginosa* (12.0%) *S. taphylococcus aureus* (11.4%) *Staphylococcus epidermidis* (6.8%). (2) The antimicrobial resistant rate of Gram-negative bacilli was decreasing after the controlling the use of the third generation cephalosporin.
Conclusion: It can reduce the drug resistant rate of Gram-negative bacilli to control the use of the third generation cephalosporin.

A5.43
Hepatitis c virus infection induces pro- and antiapoptotic cell response: who is the winner?

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Introduction: Apoptosis plays a central role in chronic hepatitis C virus (HCV) infection. Although the activation of cell death signals has been reported, HCV infection persists in most patients suggesting a pro-survival adaptation, eventually developing hepatocellular carcinoma. This study focused on the role of mitochondria in the activation of pro- and antiapoptotic response in cells expressing HCV proteins.
Materials and Methods: Human Osteosarcoma U2-OS cells inducibly expressing the HCV polyprotein; huh7.5 hepatoma cells transfected with full length HCV genome.
Results: Long term induction of viral proteins in U2-OS cells induced a cyclosporine A-sensitive cytochrome c partial release from mitochondria, revealed by immunofluorescence, western blot and spectral analysis. In HCV-transfected Huh7.5 cells, release of the apoptosis inducing factor (AIF) with no apparent nuclear translocation was also observed. HCV positive cells displayed an HIF-dependent enhanced glycolysis, charachterized by up-regulation of the mitochondria-bound Hexokinase II (HKII); preliminary data on signal transduction pathway revealed the i-perphosphorylation of Glycogen synthase kinase 3β(GSK3β).
Conclusion: HCV causes a cell stress activating an early apoptotic response, the entity of which likely depends on the cell type. Nevertheless a wide series of cell survival mechanisms are also triggered resulting in a metabolic adaptation possibly favouring carcinogenesis. Based on our results, we propose a pro-survival mechanism linking HCV infection to inhibition of GSK-3β, stabilization of HIF1α and up-regulation of HKII, the last events causing a glycolytic shift and protecting from apoptosis.

A5.44
Glyceollins, a novel class of soybean phytoalexins, inhibit tumor angiogenesis through destabilization of HIF-1α

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Pathological angiogenesis is triggered by various cytokines and growth factors secreted in tissue microenvironment and essential for the progression of angiogenic diseases. Anti-angiogenesis therapy recently has been found to be one of the most promising therapeutic strategies for angiogenic diseases, such as cancer and rheumatoid arthritis. Glyceollins are a novel class of soybean phytoalexins with potential cancer-preventive anti-estrogenic effects. However, little is known about their anti-angiogenic/-vasculogenic activity and the underlying mechanisms. In this study, we found that glyceollins inhibited vascular endothelial growth factor (VEGF)- or basic fibroblast growth factor (bFGF)-induced cell proliferation in a dose-dependent manner in human umbilical vein endothelial cells (HUVECs). Glyceollins inhibited VEGF- or bFGF-induced migration, tube formation and release of matrix metalloproteinase (MMP)-2 in HUVECs. Glyceollins significantly suppressed *in vivo* microvessel development in chick choriallantoic membrane (CAM) and in flk-1:GFP transgenic zebrafish. Glyceollins significantly inhibited tumor progression in Lewis lung carcinoma inoculated in mice through the suppression of microvessel density detected by CD31 antibody. Glyceollins inhibited phosphorylation of extracellular regulated kinase 1/2, c-Jun N-terminal kinase as well as p38 MAPK and focal adhesion kinase (FAK) induced by VEGF or bFGF. Hypoxia-inducible factor (HIF)-1α expression was decreased via increase of ubiquitination by glyceollins. In conclusion, glyceollins act as a novel inhibitor for angiogenesis which might be used for preventing and treating hypervascularized diseases such as cancer.

A5.45

Abstract withdrawn

A5.46
Mutation of *ipaB* in *Shigella flexneri* 2a attributes to gradual decrease in shigellosis patients in a Taiwan township from 1996 to 2000

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A shigellosis outbreak of *Shigella flexneri* 2a occurred in a Taiwan township during August, 1996 and extended to 2000, with 39, 46, 42, 33, and 13 closely-related isolates recovered yearly from 1996 to 2000. Fourteen isolates from 1996 were initially tested, and seven isolates were found to contain the virulent antigen IpaB and seven did not. The relative multiplicity and cytotoxicity in human macrophage-like cells and the relative multiplicity in human colon adenocarcinoma Caco-2 cells of the IpaB-containing isolates were determined to be 352.94–51.24%, 117.60–48.57%, and 177.48–50.7%, respectively, whereas those of the non-IpaB-containing isolates to be 7.71–2.41%, 35.44–6.18%, and 1.64–1.25%, respectively. These 14 isolates were subcultured for 10 consecutive days and, except for isolate SH3160, IpaB-minus colonies were obtained from the 10th-day cultures of all of the isolates. The relative multiplicity and cytotoxicity in macrophage-like cells and multiplicity in Caco-2 of these thirteen 10th-day IpaB-minus isolates were 23.79–1.18%, 31.81–9.87%, and 7.66–0.38%, respectively, whereas those of a randomly-chosen isolate from the 10th-day subculture of SH3160, which was IpaB-plus, were 68.27%, 101.6%, and 88.28%, respectively. Of the isolates recovered in 1996, 1997, 1998, 1999, and 2000, 84.6%, 67.3%, 61%, 69.7% and 92.31%, respectively, had their first-day cultures containing IpaB, and 23.08%, 28.26%, 7.14%, 0%, and 0%, respectively, had their tenth-day cultures containing IpaB. Thus, mutation in *ipaB* was likely the reason for gradual decrease in numbers of *S. flexneri* infection in the Taiwan township.

A5.47

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A5.48

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A5.49
Diagnosis of trichomonas vaginalis infection: the sensitivities and specificities of PCR, microscopy and culture methods

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Trichomoniasis is a prevalent venereal protozoan infection which has been associated with human immunodeficiency virus acquisition and preterm birth. Currently, culture is considered the standard technique for the detection of *Trichomonas vaginalis*. The aim of this study was to compare wet-mount, culture and Polymerase Chain Reaction (PCR) methods to diagnose *T. vaginalis* in vaginal swab and urine specimens. For this, the vaginal and urine samples of 51 infected women were evaluated. The sensitivity and specificity of PCR using vaginal samples were 98 and 100%, respectively. Out of 51 infected women, 16 (31.37%) had vaginal and urine wet-preparation positive for *T. vaginalis* and 39 (76.47%) were positive by culture. The sensitivity and specificity of PCR using urine specimens were 58.82 and 100%, respectively. Our results showed that the use of urine-based detection of *T. vaginalis* is not suitable in women. But, the PCR-based detection of *T. vaginalis* using vaginal specimens would be an appropriate alternative to culture. The correct and precise diagnosis of trichomoniasis will cause to increase efficiency therapy and better control of this disease which reduce complications of infection.

A5.50
Application of real-time TaqMan multiplex PCR in epidemiologic study of *B. burgdorferi* and *A. phagocytophilum* in tick samples

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For the detection of *B. burgdorferi* and *A. phagocytohilum*, etiologic agents of Lyme disease and human granulocytic ehrlichiosis respectively, large amount of methods has been described, which include antibody and antigen detection assays (immunofluorescence assay, enzyme-linked immunosorbent assay, Western blotting, culture isolation, and PCR-based (polymerase chain reaction) assays). The present study describes the validation of real-time TaqMan multiplex PCR assay for the detection of simultaneously infection of *B. burgdorferi* and *A. phagocytohilum* in field-collected tick samples from Latvia. DNA samples of 472 individual field-collected ticks were examined by real-time TaqMan multiplex PCR assays, that amplify the 23S ribosomal RNA gene from *B. burgdorferi* and msp2 gene from *A. phagocytohilum*. Results of the study showed that the prevalence of *B. burgdorferi* in ticks was 44.5%, the prevalence of *A. phagocytophilum* – 1.0% and mixed infection was observed in 0.8% cases. In contrary to nested PCR, TaqMan PCR shows hihger sensitivity and result in significant savings in time, reagents, thermal cycler usage and overall cost. The results confirm that TaqMan PCR assay is suitable for epidemiologic studies of B. *burgdorferi* and *A. phagocytophilum* in the tick samples. In this study the simultaneously infection of ticks with *B. burgdorferi* and *A. phagocytophilum* was observed. Further studies of other human tick-borne pathogens including TBE virus (tick-borne encephalitis) will be necessary in order to get the full picture of coinfection in regional ticks to assess the risk they pose to the human population.

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A5.51
Mucoid phenotype switch in *Burkholderia multivorans* clinical isolates

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Burkholderia cepacia complex (Bcc) is a group of 17 closely related bacterial species from the *Burkholderia* genus that are important opportunistic pathogens in cystic fibrosis and chronic granulomatous disease patients. Bcc bacteria can lead to a rapid decline in lung function and septicemia, causing the cepacia syndrome. Exopolysaccharides are one of the hypothesized virulence traits and are produced by strains from all Bcc species (1, 2). Phase variation from mucoid to nonmucoid phenotype may occur during the course of infection and is hypothesized that the mucoid phenotype is correlated to persistence in the lung, whilst the nonmucoid phenotype is associated to increased disease severity (2). In order to understand which factors can trigger the mucoid phenotype switch, two isogenic mucoid and nonmucoid sequential clinical isolates from a cystic fibrosis patient's lung (*Burkholderia multivorans* D2095 and *Burkholderia multivorans* D2214) were grown under stress conditions: low iron availability, microaerophilia, oxidative stress and antibiotics. Switch from mucoid to nonmucoid was obtained under microaerophilic conditions. The strains were shown to be isogenic although their phenotypes differ in exopolysaccharide production, motility, antibiotic resistance and virulence in *Galleria mellonella*. A transcriptomic approach was performed in order to identify regulatory mechanisms underlying phenotype switch in Bcc bacteria. Gene expression profiling revealed altered expression of genes encoding cell envelope components, proteins involved in central metabolism, chemotaxis, motility and iron uptake. Further work to understand phenotype switch is being performed.

A5.52
Vascular endothelital growth factor (VEGF) and lactate dehydrogenase (LDH) in the pleural effusions caused by different etiology

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Objective: To explore the clinical validity of vascular endothelial growth factor (VEGF) in the pleural effusions caused by different etiology.

Methods: VEGF in the pleural effusions caused by different etiology were measured by ELISA. LDH measurements were performed on a selective, discrete, multichannel analyzer using standard methodology.

Results: (1) VEGF levels in the pleural effusions were 240.29 ± 11.52, 217.72 ± 49.51, 68.03 ± 50.70 pg/ml in the patients with parapneumonic effusions(PPE), tuberculosis effusions(TBE) and transudative pleural effusions respectively. There were significantly higher VEGF in the patients with PPE, TBE than those with transudative pleural effusion ($p < 0.05$). But, no significantly difference between the patients with PPE and TBE. (2) LDH levels in the sera were 156.75 ± 35.26, 142.94 ± 42.17, 128.57 ± 81.38 U/l with PPE, TBE and those with transudative pleural effusion respectively, there were not significantly difference among them($p > 0.05$). (3) LDH levels in the pleural effusions were 1135.25 ± 747.85, 328.5 ± 178.89, 126.29 ± 60.16 U/l with PPE, TBE and those with transudative pleural effusion respectively, there were significantly difference between PPE, TBE and transudative pleural effusion ($p < 0.05$), and significantly difference between PPE and TBE ($p < 0.05$).The ratio of LDH in the pleural effusion and serum significantly difference between PPE and TBE ($p < 0.05$), but no difference in the transudative pleural effusion.
Conclusion: The detection of VEGF and LDH has diagnostic values in differentiating exudative and transudative pleural effusions, and PPE has more serious infection response than TBE.

A5.53
A analysis on the diagnosis value of thoracoscopy and closed pleural biopsy in the tuberculous pleuritis

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Objective: To evaluate the diagnosis value of thoracoscopy and percutaneous pleural needle biopsy in the pleural fluid patients with tuberculous pleuritis.
Methods: One forty-nine patients with tuberculous pleuritis and pleural fluid were analyzed from January 2003 to March 2007.They were allocated to thoracoscope group (n = 19), concluding operational biopsy (n = 2) and percutaneous pleural needle biopsy group (n = 130).Two examinations were evaluated and compared for the diagnosis of tubeculous pleuritis.
Results: Among the 149 patients of tuberculous pleuritis and pleural fluid, 16 patients were found granulation tissue, chronic inflammation and so on in their pleural tissue in the thoracoscope group (n = 19),which confirmed to the histological changes of tuberculous pleuritis and the detection rate was 84.21%.There were 67 patients whose histological changes conformed to tuberculous pleuritis in the percutaneous pleural needle biospy group and the detection rate was 51.54%.The difference of detection rate between the thoracoscope group and the percutaneous pleural needle biospy group was significant ($p < 0.05$).
Conclusion: As examinatons for tuberculous pleuritis, the detection rate of thoracoscope and operational biopsy was larger than percutaneous pleural needle biospy, which were ralated to field of vision, thus needs further investigation.

A5.54

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A5.55
Contributions of nucleotide excision repair, DNA polymerase η, and homologous recombination to replication of UV-irradiated herpes simplex virus type 1

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The effects of UV irradiation on herpes simplex virus type 1 (HSV-1) gene expression and DNA replication were examined in cell lines containing mutations inactivating the XPA gene product required for nucleotide excision repair, the DNA polymerase η responsible for translesion synthesis, or the Cockayne syndrome A and B (CSA and CSB) gene products required for transcription-coupled nucleotide excision repair. In the absence of XPA, CSA and CSB gene products, virus replication was reduced 10^6-, 400-, and 100-fold, respectively. In DNA polymerase η mutant cells, HSV-1 plaque efficiency was reduced 10^4-fold. Furthermore, DNA polymerase η was strictly required for virus replicaton at low multiplicities of infection but dispensable at high multiplicities of infection. Knock down of Rad51, Rad52, and Rad54 levels by RNA interference reduced replication of UV-irradiated HSV-1 150-, 100-, and 50-fold, respectively. We find that transcription-coupled repair efficiently supports expression of immediate early and early genes from UV-irradiated HSV-1 DNA. In contrast, the progression of the replication fork appears to be impaired, causing a severe reduction of late gene expression. Since the HSV-1 replisome does not make use of PCNA, we attribute the replication defect to an inability to perform PCNA-dependent translesion synthesis by polymerase switching at the fork. Instead, DNA polymerase η may act during postreplication gap-filling. Homologous recombination, finally, might restore the physical and genetic integrity of the virus chromosome.

A5.56
Impact of different parameters upon the expression of certain virulence factors in escherichia coli strains isolated from marine water

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Objective: To investigate the expression of 10 virulence factors (VF) in 100 *E. coli* strains isolated from Black Sea Coast, in different cultivation conditions simulating different environmental stress factors. Material and methods. Cell-associated (i.e. adhesion to HeLa cells) and soluble (i.e. lecithinase, lipase, gelatinase, caseinase, amylase, aesculin hydrolysis, DN-ase, sheep blood haemolysis, CAMP-like factor) VFs was investigated in variable incubation temperatures; NaCl and glucose concentration; pH; aerobic and anaerobic incubation. Results. The tested strains developed ability to growth at 22°C, 37°C, 44°C, irrespective to the salinity, pH and glucose concentration, in aerobic and anaerobic incubation conditions. The VF were better expressed at 37°C, followed by 22°C, especially adherence ability, siderophores, amylase and caseinase production. At 0% NaCl only amylase and siderophores production was expressed, at 5–6% NaCl sheep blood haemolysis and CAMP-like factors, while caseinase was expressed at 6% and 10% NaCl. The adherence to

HeLa cells was decreased by higher salinities. The amylase and caseinase were better expressed at pH 9.6 and siderophores at pH 5. The higher glucose concentrations (3%) inhibited the expression of siderophores and caseinase. THE CAMP-like factor was better expressed in the presence of *S. aureus*, rather than Rhodococcus equi. In conclusion, the present results demonstrated the high adaptation ability of enterobacterial strains of water origin to different environmental stress conditions and the possibility of virulence potential expression even in limiting environmental conditions.

A5.57
Profiling of *Mycobacterium tuberculosis* gene expression during infection in genetically different mouse models

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Changes in gene expression in response to host defense mechanisms are necessary for the survival and functional activity of pathogenic bacteria. Analysis of these changes is crucial for a better understanding of the pathogenesis of infectious disease and development of effective treatment approaches. We performed the comparative investigation of transcriptomes of *M. tuberculosis* (H37Rv strain) *in vivo*. The transcriptomes were compared at different stages of infection in genetically susceptible (uneffective immune response) and genetically resistant (effective immune response) mice. Co-denaturation and co-renaturation of excess bacterial genomic DNA with the cDNA prepared on total RNA of the infected tissue allowed us to select the bacterial fraction of the cDNA sample. We performed quantitative and qualitative characterization of transcriptomes by high-throughput massive parallel sequencing of samples. *M. tuberculosis* genes transcribing in lung tissues of genetically resistant and genetically susceptible (4 and 6 weeks of infection) mice were identified. Comparative analysis allowed defining a number of genes, which expression do not depend on host resistance to tuberculosis. Among them there are 9 genes of lipid metabolism and 12 genes involved into cell wall processes. The corresponding proteins are the known or predicted virulence factors and could be further considered as potential therapeutical targets. This work was supported by the RFBR grant #08-04-01053 and the Russian Academy of Sciences grant (Physico-chemical biology. Structural, functional and evolutional analysis of genomic cis-regulatory systems).

A5.58
Targeting the nucleotide metabolism of human pathogen *T. brucei*

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Trypanosome brucei is a pathogen that infects humans (African sleeping sickness) and cattle (Nagana). There are no vaccines available for this disease. The present drugs are toxic, expensive or not active against all variants of the parasite. When we have grown trypanosomes in the presence of 1 mM deoxyadenosine they died within a few hours. Nucleotide pools analyses showed intracellular accumulation of dATP and depletion of ATP pools in the trypanosomes. We have shown that *T. brucei* adenosine kinase is crucial for the accumulation of dATP and depletion of ATP1. However, when we used deoxyadenosine at lower concentrations than 1 mM, it was instead degraded by the trypanosomes. We found that *T. brucei* methylthioadenosine phosphorylase was responsible for this degradation and it was only when deoxyadenosine was used at a concentration high enough to saturate this enzyme it had a strong effect on parasite survival. These results suggest that deoxyadenosine analogs which are MTP-ase cleavage resistant can be used as antitrypanosomal drugs. Our preliminary experiments on trypanosomes infected mice with adenine arabinoside (Ara-A) showed that non cleavable deoxyadenosine analogs can be developed into new drugs against trypanosomes.

Reference:

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A5.59
The levels of serum total antioxidant status (TAS) and cytokines in hepatitis B virus (HBV) infection

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Inflammatory-immune damage occurs in liver pertaining to Hepatitis B virus. Reactive oxygen species(ROS) are also considered to be the cause of this damage. However, antioxidant protection systems are formed against the deteriorating effects of ROS in organisms. It was aimed to detect and compare total antioxidant and cytokines(IL-2, TNF-α) levels of chronic and occasionally diagnosed inactive HBsAg carriers. In this study, 25isolated anti-HBc total(+), 25HbsAg and anti-HBc total(+) patients' sera, 20chronic HBV patients' sera and 10 healthy people's sera (control group) were used. The mean values of TAS were found 1.29 ± 0.20 mmol in chronic HBV patients, 1.24 ± 0.15 mmol in inactive HBsAg carriers, 1.16 ± 0.5 mmol in isolated anti-HBc(+) patients and 0.93 ± 0.10 mmol in control group as Trolox equivalent/l. The results revealed that there was no statistically significant difference among the patients' group as a statistically significant difference was found between the control and the patients' groups ($p < 0.05$). Moreover, TAS values of isolated anti-HBc(+) and inactive HBsAg(+) carrier patients were found to be higher than control group as it was seen in HBV infected patients similarly. The result pointed out that the disease was progressing chronically. Besides, there was a significant increase in the levels of TNF-α and IL-2 in chronic HBV. It was concluded that the detection of serum TAS values and cytokines levels could be considered very important for the anticipation of the progression and ongoing to chronic hepatitis of HBV infected patients.

A5.60
The supramolecular organization of glycosphingolipid-receptors in *lipid rafts* affects the biological action of Shiga toxins

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Shiga toxin-producing *Escherichia coli* (STEC) are responsible for courses of disease from asymptomatic carriage to uncomplicated

diarrhea, bloody diarrhea, and the life-threatening hemolytic-uremic syndrome (HUS). Shiga toxins (Stxs) bind to the glycosphingolipid (GSL)-receptor globotriaosylceramide (Gb3Cer/CD77) and cause microvascular endothelial injury in specific target tissues. GSLs are located primarily in supramolecular cholesterol-enriched membrane microdomains, *lipid rafts*, being the prerequisite for efficient receptor-mediated endocytosis and retrograde transportation resulting in Stx-caused cellular injury. We aimed to characterize the microdomain-associated GSLs of endothelial cells from different vascular beds. In specific buoyant microdomain fractions of the plasma membrane, obtained by sucrose density gradient ultracentrifugation, Stx receptors were detected by thin-layer chromatography (TLC) overlay assays using specific anti-Gb3Cer antibodies or Stx in conjunction with anti-Stx antibodies. By means of immunofluorescence microscopy we could demonstrate that Stx receptor GSLs colocalize with different types of microdomains in micro- and macrovascular endothelial cells. To demonstrate the impact of the molecular assembly of microdomains on Stx receptor function, we disrupted the *lipid rafts* followed by cell viability assays. Importantly, the more sensitive microvascular endothelial cells showed a higher content of microdomains being resistant to cholesterol depletion, whereas the microdomains of less sensitive cells could be easily disrupted.

A5.61
Whole genome sequencing of a multiresistant bacterium isolated from a wastewater treatment plant receiving effluent from antibiotic drug manufactureing

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Antibiotics save millions of lives every year and are crucial for fighting disease worldwide. However, the heavy usage also leads to antibiotic resistance - one of the most important challenges for the health care sector. As bacteria can move between environments, and resistance genes often are transferred horizontally between species, it is important to protect also the environmental bacteria from excess antibiotic exposure. We have sequenced the genome of a multiresistant strain of *Ochrobactrum intermedium*, a common environmental bacterium and opportunistic pathogen. This particular strain was isolated inside a treatment plant in India receiving waste water from 90 bulk drug manufactures, and was found to be resistant to 36 of 39 tested antibiotics. Massively parallel pyrosequencing (454 sequencing) of its genome resulted in an average sixteen-fold coverage. Comparative genomics were used to analyze the genome of the resistant strain in relation to the already sequenced reference strain. The analysis revealed structural differences between the strains including both larger deletions and insertions. Several point mutations were also detected in coding sequences as well as in ribosomal RNA. We identified 12 additional resistance genes in the isolate, compared to the reference strain. The quinolone target enzymes DNA gyrase and topoisomerase IV showed nine amino acid changes in the protein sequences for the isolate, two of which are known to cause quinolone resistance in *Escherichia coli*. The results presented here demonstrate the power of second generation sequencing technology as well as the need for sustainable management of antibiotic waste.

A5.62
Antibiotic-contaminated effluent promotes mobile resistance in environmental bacteria

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The high use of antibiotics has caused an accelerating development of antibiotic resistance - one of the largest threats to human health. Bacteria defend themselves against antibiotics by acquiring resistance genes, i.e. mobile genetic elements that can be shared between species. The collection of resistance genes is particularly large in environmental bacteria and they may therefore constitute recruitment pools of resistance genes for pathogens. Recently, we have documented high environmental releases of antibiotics, but the effects on the bacterial communities and their resistance genes have not yet been studied. We sampled river sediments up and downstream from a treatment plant releasing high levels of fluoroquinolone antibiotics. The metagenomes were sequenced using massively parallel pyrosequencing generating 500,000 reads compsing 250 million bases. Functional analysis revealed high levels of resistance genes providing resistance to several classes of antibiotics, including sulfonamides, aminoglycosides and fluoroquinlones. Based on structural similarities, we also found several putative novel resistance genes previously not reported. The metagenomes also contained a high abundance of integrons and transposons facilitating the horizontal transfer of resistance genes. In addition, we found several high copy number resistance plasmids, two of which have previously not been described. We conclude that releases of high levels of antibiotics released into the environment strongly promote both resistance genes and elements supporting horizontal gene transfer. These results therefore stress the importance of a sustainable management of antibiotic waste world-wide.

A5.63
Glyco-bioinformatic and statistical analysis of inflammatory response in different tissue types

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Glycosylation is one of the most important post-translational modifications of proteins, known to be involved in pathogen recognition, the innate immune response and protection of epithelial membranes. However, when compared to the tools available for the processing of high-throughput proteomic data, the glycomic domain is severely lacking. We are involved in development of glycomic solutions at many levels; the interpretation and assignment of oligosaccharide structure from LC-MS and MS/MS data; the development of methods for high-throughput comparison of samples; public-domain storage and presentation of LC/MS and MS/MS data for published oligosaccharide structures and the application of existing statistical tools to the analysis of oligosaccharide data-sets. Here we present our prototype database for public domain storage of published oligosaccharide structures. This is being developed to store our oligosaccharide LC-MS and MS/MS data. The peak lists are available for download and can be imported into a suitable program for visualisation. The database can be searched by parameters including

parent mass, structure, organism or tissue type. Stemming from this work the availability of data sets of samples from diverse studies leads to the need for global glycosylation analysis using existing statistical tools. We used various data sets of control and disease state from different tissue types and created cluster plots which identified important components in recognising inflammatory related responses. This data shows the value of statistic analysis of large glycomic data-sets in building a comprehensive picture of the role of glycosylation in inflammatory response.

A5.64
ShotgunFunctionalizeR – an R-package for functional comparison of metagenomes

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Microorganisms are ubiquitous in nature and constitute intrinsic parts of almost every ecosystem. A culture-independent and powerful way to study microbial communities is metagenomics. In such studies, functional analysis is performed on fragmented genetic material from multiple species in the community. The recent advances in high-throughput sequencing have greatly increased the amount of data in metagenomic projects. At present, there is an urgent need for efficient statistical tools to analyze these data. We have created ShotgunFunctionalizeR, an R-package for functional comparison of metagenomes. The package contains tools for importing, annotating and visualizing metagenomic data produced by shotgun high-throughput sequencing. ShotgunFunctionalizeR contains several statistical procedures for assessing functional differences between samples, both for individual genes and for entire pathways. In addition to standard and previously published methods, we have developed and implemented a novel approach based on a Poisson model. This procedure is highly flexible and thus applicable to a wide range of different experimental designs. We demonstrate the potential of ShotgunFunctionalizeR by performing a regression analysis on metagenomes sampled at multiple depths in the Pacific Ocean.

A5.65
Exploring the microbial resistome in river sediments exposed to extraordinary high levels of antibiotics

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The rapid development and propagation of antibiotic resistance in pathogenic and opportunistic bacteria is a major threat to public health worldwide. The phenomenon has been widely studied in the clinical setting, but comparatively little is known about the prevalence and diversity of antibiotic resistance in communities of environmental bacteria, often referred to as the environmental resistome. As the external environment may function as a reservoir of resistance genes to human pathogens, we are interested in how environmental bacteria are affected by antibiotic pollution. We have previously isolated microbial DNA from river sediments taken up- and downstream from a water treatment plant that processes waste water from several pharmaceutical plants producing antibiotics. In a previous study, we used deep sequencing to identify unprecedented frequencies of known resistance genes to several classes of antibiotics in these samples. In this study, we aim to functionally characterize the resistome in a more open and exploratory way by screening genomic DNA libraries transformed into sensitive hosts. To generate the libraries, several experimental strategies were explored, including mechanical shearing and enzymatic digestion of the isolated DNA followed by blunt- or sticky end cloning into different plasmids, subsequently transformed into sensitive *E. coli*. Pros and cons of the different strategies will be discussed along with preliminary results of the screening against selected antibiotics.

A5.66
Killing of Escherichia coli by Shigella flexneri

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An outbreak of *Shigella flexneri* occurred in a Taiwan township in 1996 and extended to 2000. Of the 180 isolates recovered, 74 isolates (the epidemic strain isolates) had PFGE pattern N1X1, whereas the remaining 106 isolates (the non-epidemic strain isolates) had 51 PFGE patterns closely-related to N1X1. The non-epidemic strains likely resulted from deletions of the epidemic or other non-epidemic strains. From 1996 to 2000, 77%, 82%, 100%, 100%, and 100% of the epidemic strain isolates and 15%, 66.6%, 55%, 33%, and 0% of the non-epidemic strain isolates were found to be capable of killing *E. coli* strains recovered from rectal swabs of healthy persons. Thus, for the epidemic strain, isolates with the killing activity survived better yearly than those without the killing activity. But for the non-epidemic strains, deletions occurred continuously and isolates with killing activity disappeared yearly. A fosmid clone of an epidemic strain isolate (SH3160) was identified for the killing activity, and the *col* Ib gene was further identified. The *col* Ib gene located in a plasmid in SH3160 which could be transferred from *S. flexneri* to *E. coli* and vice versa by conjugation. Upon treatment of *E. coli* that carried plasmids containing *col* Ib and the downstream immunity gene with mytomycin C, a 65-kd protein was induced. This protein could also be detected in cultural medium of *E. coli* that carried plasmids containing *col* Ib under the control of *lac* promoter. A (His)6-tagged colicin Ib protein was purified and its killing activity was demonstrated *in vitro*.

A5.67
The *Entamoeba histolytica* EhTBP and EhTRF1 transcription factors are GAAC-box binding proteins *in vitro*

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Entamoeba histolytica is the protozoan parasite which causes human amoebiasis. In this organism, few transcription factors

have been characterized. The *Entamoeba histolytica* TATA-box binding protein (EhTBP) is the first basal transcription factor that has been studied. Recently, we described the cloning and characterization of the *Ehtrf1* encoding gene, another member of the TBP family. The *E. histolytica* core promoters have three conserved elements that control the transcription start site: the TATA-box, Inr and GAAC-box. It has been described that the GAAC element controls the transcription start site and the expression levels. It also directs a new transcription start site, which is independent of TATA or Inr elements. There has also been determined that the GAAC-box is recognized by an unknown nuclear protein named GAAC-binding protein (GBP). In this work we describe the ability of EhTRF1 and EhTBP to bind to the GAAC element. Recombinant EhTRF1 and EhTBP polypeptides were expressed in bacteria as 26 kDa and 30 kDa, respectively, and were purified by IMAC using native conditions. To determine the GAAC-binding capacity of rEhTRF1 and rEhTBP, we performed electrophoretic mobility shift assays that revealed the formation of EhTRF1- and rEhTBP-GAAC complexes, which were competed with 200-fold molar excess of unlabeled GAAC or TATTTAAA oligonucleotides, but not with an excess of muted GAAC oligonucleotide or an unspecific competitor. We determined the dissociation constants of rEhTRF1 and rEhTBP for the GAAC-element. Our results indicated that EhTBP and EhTRF1 polypeptides are GAAC-binding proteins *in vitro*.

A5.68
Porphyromonas gingivalis HmuY protein as a potential marker of chronic periodontitis

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Porphyromonas gingivalis as a member of the red complex bacteria is considered one of the main etiological factors of chronic periodontitis. The bacterium has developed sophisticated mechanisms to communicate and evade host epithelial cells which are the first barrier preventing successful inflammation. The bacterium-host cell cross talk is a complex process which involves different types of interactions. Low-iron accessibility is one of the first indicators that bacterium has reached host environment. As a response, several proteins are recruited to acquire iron or heme from host proteins. HmuY, an outer membrane lipoprotein, is able to sequester heme directly from hemoglobin and deliver it to a *P. gingivalis* outer-membrane heme receptor HmuR. High expression levels under iron-depleted conditions, a unique tertiary structure and low sequence homology to other bacterial proteins make HmuY protein a promising molecular marker of chronic periodontitis. Here we examined the presence of the *hmuY* gene and its expression levels in dental plaque samples of chronic periodontitis patients. Sequences of the *hmuY* gene found in different patient samples were compared. In order to determine the role of HmuY in the development of inflammation, we used an *in vitro* model consisting of epithelial cell line infected with *P. gingivalis*. We examined expression levels of the HmuY protein in bacterial cells interacting with epithelial cells and expression levels of several Toll-like receptor proteins in infected epithelial cells. We showed that HmuY protein is a specific marker of *P. gingivalis* that influence the bacterial-epithelial cell cross talk.

A5.69
The *Entamoeba histolytica* EhTBP and EhTRF1 transcription factors bind to different TATA variants *in vitro*

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In eukaryotes, the TATA-box binding protein (TBP) forms the SL1, TFIID and TFIIIB complexes, which are specific for the RNA polymerases (RNAP) I, II, and III, respectively. TBP in TFIID is the first factor that binds to promoter to recruit TFIIA, TFIIB, RNAP II-TFIIF, TFIIE and TFIIH transcription factors to ensemble into the pre-initiation complex and initiate gene transcription. Mammalian TBPs can bind to a large number of diverse TATA elements. An exhaustive statistical genomic survey documented that the TATA-box is an A/T-rich eight bp segment, often flanked by G/C-rich sequences. In the case of *Entamoeba histolytica*, few basal transcription factors have been characterized. Previously, we described the *in vitro* EhTBP binding affinity for different TATA sequences found in *E. histolytica* gene promoters and others designed by us producing mutations in the TATA-box. Recently, we reported the *Ehtrf1* gene cloning and demonstrated the ability of EhTRF1 to bind to TATTTAAA-box. In the present work, we describe the capacity of both EhTRF1 and EhTBP to bind to other TATA variants *in vitro* by electrophoretic mobility shift assays and determine their dissociation constants. Recombinant EhTRF1 and EhTBP proteins were expressed in bacteria as 28 kDa and 30 kDa 6His-tagged polypeptides, respectively, and purified by IMAC through Ni^{2+}-NTA-agarose columns. Purified rEhTRF1 or rEhTBP polypeptides formed DNA-protein complexes with TATA-box variants containing C's or C's in the first, third, fifth and sixth positions, because these positions have been reported as important for DNA-binding activity for human and yeast TBPs.

A5.70
TT virus infection among blood donors in south of Iran

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Introduction and Objective: Recently, a new infective agent of humans, TT virus (TTV), was isolated from the serum of a patient with posttransfusion hepatitis of unknown etiology, in Japan. The prevalence of TTV in different population groups, including apparently healthy individuals, is very high. The aim of this study was to determine the prevalence TTV in healthy blood donors.
Methods: Sera were collected from 499 healthy blood donors. None of them had clinical signs of acute or chronic hepatitis and serological markers for hepatitis A, hepatitis B, and hepatitis C viruses were negative. TTV DNA was amplified and detected using semi-nested PCR, followed by gel electrophoresis.
Results: Sixty-six of 499 (13.40%) samples had TTV DNA detected by PCR. From 66 TTV DNA positive, 63 were male

and three of them were female (p = 0.874). The mean age of people with TTV DNA was 34.46 ± 9.25 years old.
Conclusions: The prevalence of TTV DNA in south of Iran was 13.40%. As our cases were apparently healthy individuals, we propose that the role of TTV in the pathogenesis of acute and chronic liver diseases must be considered for further investigation.

A5.71
Serum alanine-aminotransferase and aspartate-aminotransferase levels in blood donors

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Increased activity of serum aminotransferases such as alanine-aminotransferase (ALT) and aspartate-aminotransferase (AST), in blood donors may identify infection with non-A, non-B hepatitis, and is being used as a surrogate test for preventing post-transfusion viral hepatitis. We have studied ALT and AST levels in 499 consecutive blood donors to determine their association with gender, and hepatitis virus infection markers. Sera were collected from 499 healthy blood donors, tested for viral infection by ELISA and then ALT and AST levels were determined. Four hundred sixty-seven men and 32 women were studied; ALT activity was 26.80 ± 27.75 IU/l (mean ± SD) and ALT range was 10–142 IU/l. AST activity was 25.58 ± 24.12 IU/l (mean ± SD) and AST range was 10–124 IU/l. The upper normal value for men was 40 IU/l and 34 IU/l for women. From 499 cases, 71 people had an ALT level than normal value, and 68 (95.8%) of them were men and 3 (4.2%) of them were women (p = 0.417). From 499 cases, 67 people had an AST level than normal value, and 65 (97%) of them were men and 2 (3%) of them were women (p = 0.218). None of them had clinical signs of acute or chronic hepatitis and serological markers for hepatitis A, hepatitis B, and hepatitis C viruses were negative. It seems clear that different cutoff values should be considered for men and women, and ALT & AST elevation is influenced by many factors.

A5.72
In-vitro inhibitory effect of BLIP derived peptides on TEM-1 beta-lactamase

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Bacterial resistance against beta-lactam antibiotics has been a global health problem mainly caused by the presence of beta-lactamases. The combination of the beta-lactam with beta-lactamase inhibitors has proven to be a successful strategy to combat the escalating problem of beta-lactam resistance mediated by class A beta-lactamases. Most of the research on inhibitor binding to beta-lactamase has focused on TEM-1 class A beta-lactamase due to its prevalence. Beta-lactamase inhibitory protein (BLIP) has been shown to be a potent inhibitor of *E. coli* RTEM-1 beta-lactamase. The interaction between beta-lactamase and BLIP leads to the design of candidate peptide based drugs for the inhibition of beta-lactamase. In this study, this approach has been used to investigate RTEM-1 inhibition by the BLIP derived peptide (residues 45-53) and its variants. *E. coli* cells harboring the pUC18 plasmid that carries the bla gene of RTEM-1 beta-lactamase, was used for periplasmic beta-lactamase synthesis. This enzyme was purified by osmotic shock method and followed by a combination of anion exchange (DEAE-cellulose) and gel filtration (Sephadex G-100) chromatographies. *In vitro* beta-lactamase inhibition by BLIP derived peptides was assayed on both pure RTEM-1 and partially purified RTEM-1 beta-lactamase using CENTA as the substrate. This study has been supported by TUBITAK project 108M644.

A5.73
Production of recombinant varicella zoster gE in CHO-K1 cells for improved serological diagnostics of VZV CNS infections

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Herpes simplex virus 1 and 2 (HSV-1, HSV-2) and varicella zoster virus (VZV) can all cause CNS disease. Discrimination between these infections is needed to optimize antiviral treatment. Initially, PCR detection of viral DNA in the cerebrospinal fluid (CSF) is the diagnostic method of choice. However, one week after onset the analysis usually has to rely on serological grounds. The present technique used – IgG analysis of paired CSF and serum samples by ELISA – is often based on crude preparations of viral antigens. It is characterised by low specificity and discrimination of the infections can not be accomplished due to serological cross reactivity. The VZV gE protein is an immunodominant and specific B-cell antigen for VZV and an ELISA candidate. Attempts using bacterial or baculovirus expressed VZV gE did not improve the diagnostic ability of the test. Instead, a plasmid carrying the His6-tagged extracellular part of VZVgE was transfected into mammalian CHO-K1 cells. Stable clones were generated and cultivated in a perfusion lab scale bioreactor (1.5 l) at pH 6.9, 37°C, and a dO2 of 40% under serum free conditions. Spent medium was concentrated and the recombinant protein was purified on a his-tag column. The product was used for serological diagnostics of IgG against VZV gE in ELISA. Preliminary results showed a high sensitivity and no cross reactivity between sera with high titres of IgG against only HSV-1 or only VZV. In paired serum-CSF samples the novel antigen could discriminate between VZV and HSV-1 encephalitis, which may solve a long-lasting diagnostic problem.

A5.74
New details of inhibition of HCV helicase by lead compounds derived from benzotriazole

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Hepatitis C virus, an enveloped, highly mutable RNA virus is responsible for major cases of acute hepatitis and liver cancer. There is intense need for developing efficient treatments. Viral protein NS3 helicase/NTPase seems to be very attractive and still hardly examined molecular target, because blocking of its helicase activity prevents HCV replication in the hepatoma cell.

Nucleoside base - like compounds have been reported to act as HCV NS3 helicase inhibitors. 4,5,6,7-tetrabromobenzotriazole (TBBT) and 5,6-dichloro-1-(β-D-ribofuranosyl)benzotriazole (DCBT) inhibit unwinding of DNA substrate with IC50 within μM range without any effect on the rate of ATP hydrolysis, catalysed by the enzyme. Several attempts were made to elucidate the mechanism of inhibition of HCV helicase activity by benzotriazole derivatives. Recently we investigated binding of this two lead compounds to isolated domain 1 of HCV helicase using NMR spectroscopy. The results throw some light on the question of mode of action. From chemical shift mapping experiments we concluded, that DCBT binds to domain 1 in similar way as ATP. By contrary, TBBT binds in slightly different manner. Both compounds interacts with so called ''spring helix'', which was recently shown to participate in coupling between ATP hydrolysis and DNA binding/unwinding. Basing on the rachet mechanism of DNA unwinding by NS3 helicase, the investigated benzotriazole derivatives can be expected to interfere with the transition states of the NS3 – DNA – ATP complex and freeze the helicase in a particular conformation.

A5.75
Fragmentation, ubiquitination and mosaicism of HIV/SIV genetic components in the context of thermo-stable adenoviral (Ad) vectors as novel vaccine approaches against HIV-1/AIDS

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Broadening the immune response through development, optimisation and evaluation of HIV-1/SIV genetic components is a rational strategy towards HIV-1/SIV vaccination studies. We originally aimed to induce immunity to HIV-1 infection by developing a cohort of vectors in which HIV-1 genetic components have been engineered to stimulate broader CTL responses. Full-size gag (but also pol/env) genes including an across clade conserved/mosaic epitope were engineered, fused to mono- or tetra-ubiquitin (Ub) sequences and further tested on DC and non- DC cell lines by using plasmid, *in vitro* synthesised mRNA, or adenoviral vectors. We subsequently constructed rAd vectors carrying genetically fragmented ubiquitinated fusions for either HIV (>10) or SIV (>7) gag genes in an attempt to reduce antigenic competition and alter epitopic dominance. To this context, sugar mixtures were identified which optimally preserved and thermostabilised adenoviral-based vaccines infectivity whilst maintaining the strength and rigidity in a micro-needle array, aiming towards elimination of cold-chain distribution of a vaccine to Sub-Saharan African countries. Vectors retained substantial infectivity (i.e. >55% eGFP+ cells) for up to 3 months trough a temperature range tested (-20°C to +40°C). Transcutaneous injections of dessicated Ad OVA-GFP vectors (4E109 vg) in C57BL6 mice or footpad-mediated migration of Ad5-CMV-eGFP to lymph nodes demonstrated functionality of Ad-sugar formulations. We are currently extending our studies using either *in vitro* CTL epitope-mapping or *in vivo* plasmid prime/Ad boost strategies. SIV constructs will be forward tested in non-human primate models (cynomolgus macaques).

A5.76
Analysis of SPS-sensor dependent gene expression in *Candida albicans*

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Candida albicans is a commensal organism that lives as a benign member of the microflora of healthy individuals. Changes in the host environment can induce *C. albicans* to mount virulent infections. The majority of women experience at least one episode of acute vulvovaginal candidiasis (VVC), and 5% suffer recurrent chronic infections (>4 episodes annually). Importantly, chronic VVC affects nominally healthy women without measurable immunodeficiencies; the factors contributing to chronic infections remain elusive. *C. albicans* use the SPS-sensor to regulate the activity of two latent transcription factors Stp1 and Stp2. Activated Stp1 induces the expression of genes required for the catabolic utilization of host proteins, including the established virulence factor *SAP2*, a secreted aspartyl protease (SAP), and several oligopeptide transporters (OPTs). Activated Stp2 induces the expression of permeases that catalyze transport of amino acids into cells. Quantitative methods to monitor SPS-sensor dependent gene expression reveal that the induction of the SPS-sensor, or the introduction of a dominant activated *STP1** allele greatly enhances the expression of *SAP2* (>100 fold), *OPT3* (12 fold), *OPT4* (7-fold) and *OPT5* (24 fold). Fluorescence reporter constructs that monitor Stp1 (P*SAP2-RFP*) and Stp2 (P*CAN1-GFP*) regulated genes have been developed, enabling the integration of nutrient-based signals to be visualized in real-time in single cells. We are applying these tools to understand how fungal cells sense and respond to the vaginal environment, i.e. the nutrient content of cervical mucus, and the high content of amino acids and protein present in serum and semen.

A5.77

Abstract withdrawn

A5.78
Optimization of PCR protocol for the detection of genus *Cronobacter*

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A new bacterial genus *Cronobacter* of the family *Enterobacteriaceae* was established in 2008. *Cronobacter spp.* are Gram-negative, facultative-anaerobic, rod-shaped bacteria, which are recognised as causative agents of neonatal bacteraemia, meningitis and necrotising enterocolitis. According to the Commission Regulation No 1444/2007, the food law of European Union insists on the absence of *Cronobacter spp.* (not detectable in 10 g of food product) in infant formulae. Although the gold standard for the detection of *Cronobacter spp.* in food is the conventional cultivation method, there is trend to develop rapid and user-friendly techniques. Here, we present data of the development of the PCR protocol with different sets of primers for the detection of *Cronobacter spp.* The reliability of protocols was evaluated with the collection of 60 bacteria, including *Cronobacter* strains and other food relevant microorganisms, the detection limits were determined. The characteristics (specificity and sensitivity) of developed procedures are discussed.

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B1 – Metabolic Networks

B1.01
Reporting and capturing uniform enzyme function data

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The multi-disciplinary approach of systems biology combines a variety of modern experimental techniques and provides endless opportunities to generate huge amounts of enzyme structure and activity data. However, all these data in both the literature and in databases suffer from the fact that they are incomparable due to incomplete and fragmented descriptions of materials and methods, and therefore are, to some extent, unreliable. Moreover, experimental results have been collected under quite disparate conditions so that researchers often are faced with the problem of the range of method-specific enzyme data. This causes problems when data move between researchers whose data are supplied by laboratories that use different methods, and can, in the worst cases, lead to misinterpretation of laboratory findings. After a general agreement within the scientific community had been achieved, that there is an urgent need for the standardization of data reporting, the STRENDA Commission, founded and supported by the Beilstein-Institut, proposed guidelines for reporting minimum information on both experiments and results. These guidelines are embedded in the international checklists development project MIBBI and have been adopted by the major biochemical journals. Additionally, in cooperation with journals, database producers and the community, an electronic data-submission form was developed to enable prospective data submission by the authors in accord with the standards for the reporting of functional enzyme data. The guidelines and the submission form will be presented in detail along with further aims of the STRENDA Commission.

B1.02
Adiponectin level in Iranian non obese with acute myocardial infarction

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Backgrounds: Adiponectin is an adipose tissue-derived mediator with significant antiatherogenic properties. A few studies were done in acute phase of myocardial infarction especially in non obese patients. We design a study to investigate the association between adiponectin concentration and acute phase of myocardial infarction in non obese patients.
Methods: This case-control study was done in Paymaneah Hospital (Jahrom, Iran) from February 2007 to May 2008. Plasma adiponectin levels were measured in 43 patients with AMI (mean age: 62.7 ± 13.3 years, male: 67.4%) at the first 24 hours of admission and 43 normal controls (mean age: 62.1 ± 12.3 years, male: 55.8%) matched for age, sex and other CAD risk factors.
Results: Adiponectin levels in patients with AMI (3.36 ìg/ml) were significantly lower than that of the control group (5.03 ìg/ml) ($p < 0.0001$). Lower adiponectin were independently associated with higher risk of AMI (odds ratio = 8.97; 95% CIs: 2.3–34.5; $p = 0.001$). Adiponectin levels negatively correlated with triglyceride (r: -0.46, $p = 0.002$) and total cholesterol ($r = -0.32$, $p = 0.03$) in the case group and with body mass index (BMI) in control subjects.
Conclusion: The present study showed that adiponectin was associated with AMI in non obese patients but it is not related to sex, age and other CAD risk factors.

B1.03
Adiponectin, triglyceride and cholesterol in acute myocardial infarction

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Adiponectin which is secreted by adipose tissues has been reported to have direct antiatherosclerotic effects also plasma adiponectin levels have been shown to be decreased in patients with cardiovascular diseases and hypertension. To determine the relationship between plasma adiponectin levels and serum triglyceride and cholesterol concentrations in patients with acute myocardial infarction and in healthy control group, this case-control study was carried out on 43 patients with acute myocardial infarction (AMI) and 43 age, sex, and other classic cardiovascular risk factors matched healthy persons with normal coronary artery angiography as control group. Blood samples were taken from the patients at first 24 hours of admission. Plasma adiponectin levels were measured by ELISA method in the patient and the control groups. Plasma adiponectin levels in the patients and the control group were 3.328 ± 1.716 ìg/ml and 5.014 ± 1.456 ìg/ml, respectively ($p < 0.001$). Serum triglyceride concentrations in the patients and the control group were 146.05 ± 99.595 and 144.23 ± 99.824, respectively ($p < 0.933$). Serum cholesterol concentrations in the patients and the control group were 146.30 ± 45.209 and 171.84 ± 33.765 respectively ($p < 0.046$). There was a significant reverse relationship between plasma adiponectin levels and serum triglyceride ($r = -0.056$, $p = 0.002$) and cholesterol ($r = -0.323$, $p = 0.035$) concentrations in the patients, but this relationship was not found in the control group. The results suggest that low plasma adiponectin level may be an acute myocardial infarction risk factor. Further comprehensive studies are needed to explore effect of serum triglyceride and cholesterol concentrations on plasma adiponectin level.

B1.04
Serum adiponectin level in patients with benign prostatic hyperplasia

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Introduction and Objectives: Adiponectin is known as an inhibitory factor for cell replication. Its level decline in malignan-

cies and obese patients. We evaluated its level in patients with LUTS due to BPH.
Methods: Seventy five patients with BPH enrolled in this study and divided to three groups according to AUA symptom score. Serum adiponectin level checked and compared.
Results: In group I (Mild LUTS) 21 patients, Mean age was 66.8 years and mean adiponectin level 12.4 ug/ml, group II (Moderate LUTS) 30 patients, mean age70.1 years, mean adiponectin 5.58 and group III (Severe LUTS) 24 patients, mean age 71.8 years and mean adiponectin 1.5, respectively.
Conclusion: Serum adiponectin level decline in patients with BPH, however it is a benign tumor and its level correlate with the symptom score (More severe LUTS has lower level).

B1.05
Plasma homocysteine levels in woman with polycystic ovary syndrome

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Polycystic ovary syndrome (PCOSy) is a condition characterized by oligomenorrhea and androgen excess, affects 6–10% of the women in reproductive period.Although the pathogenesis is still uncertain, many studies suggest that PCOSy may increase risk for insulin resistance, type 2 diabetes, dyslipidemia, cardiovascular risk. Woman with PCOSy would be expected to be at significantly increased risk for atherosclerotic disease. Evidence suggests that homocysteine(Hcy) may promote atherosclerosis by damaging the inner lining of arteries and promoting blood clots as a result of inflammation. Hyperhomocysteinemia has been shown as independent predictor of cardiovascular morbidity in patients with atherosclerosis. The aim of our study was to determinate levels of Hcy in woman with polycystic ovary syndrome. Thirty patients (age, 23, 5 $\pm$ 5.5) with PCOSy and twenty four (age, 25.5 $\pm$ 4.3) healthy, were involved in the study.Total Hcy was measured using fluorescent immunoassay. Statistically significant differences in serum concentration of Hcy were observed between groups. Mean Hcy level we found as (10.2 $\pm$ 2.9 versus 7.0 $\pm$ 1.5) in PCOSy and normal group respectively ($p < 0.05$). For Macedonian population we found statistically significant increased Hcy levels in woman with PCOS. Because an increased concentration of total Hcy has been shown as and independent risk factor for cardiovascular alterations, it is essential in this group of woman are taken measures for early prevention. Routine quantification of total Hcy in woman with proven PCOSy would be simple and safe method for early prevention of cardiovascular diseases

B1.06
Cloning and characterization of a novel Nudix hydrolase encoded by nudC gene from plant pathogen *Pseudomonas syringae pv. tomato str DC 3000*

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Nudix hydrolases are widely distributed pyrophosphatases with a conserved amino acids motif GX5EX5[UA]XREX2EEXGU (where U is a hydrophobic residue). They hydrolyze a variety of substrates, predominantly nucleoside diphosphate derivatives. The proposed role of Nudix enzymes is to maintain cellular homeostasis. However, it can not be excluded that some of these proteins from pathogenic organisms may play a role in pathogen invasion processes. The aim of this work was to characterize the first Nudix hydrolase from plant pathogenic bacteria *Pseudomonas syringae pv. tomato str DC 3000*. PCR reaction was used to amplify gene NP_792526 encoding the 278 aa NudC protein *P. syringae* genomic DNA. PCR product was cloned into expression vector. The recombinant NudC hydrolase was expressed in *E. coli* and purified by affinity chromatography. The enzymatic activity was tested with the purified recombinant NudC protein and a number of different substrates. At reaction conditions typical for Nudix hydrolases, at pH 8.0 and in the presence of $Mg2^{+}$ ions, the preferred substrate of this enzyme was NADH. An attempt to elucidate the biological role of the NudC protein was undertaken by creating mutants with disruption of the gene encoding the NudC protein.

B1.07
Plasma membrane associated enzymes for sphingolipid structural changes and neuronal development

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Sphingolipids are ubiquitous components of mammal cell membranes, but are particularly abundant in the nervous system, and within the nervous system, in neurons. Since sphingolipids are concentrated at the subcellular level in the plasma membranes, where they reside asymmetrically in the extracellular leaflet. Several evidences suggest that changes of the cell sphingolipid content and composition are necessary for the differentiation and functions of neurons in culture. These changes could be obtained very rapidly with changes of plasma membrane organization by modulating plasma membrane-associated enzymes. Expression and activity of these enzymes have been characterized in different cellular models. In cultured neurons it has been demonstrated the presence and the pH depending of sialidase NEU3 and of a sialyltransferase, whereas in human fibroblasts of β-galactocerebrosidase, β-glucocerebrosidase GBA1, β-glucosilceramidase GBA2, sialidase NEU3, sphingomyelin syntase and sphingomyelinase. In future studies we would like to translate the information from fibroblasts to cultured neurons.

B1.08
Two homologous genes in arthrobacter phenanthrenivorans encode 1-hydroxy-2-naphthoic acid dioxygenases with similar catalytic properties

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A new species named Arthrobacter phenanthrenivorans sp. nov. Sphe3 isolated from creosote-contaminated soil in Greece, can grow on phenanthrene, a toxic PAH of three aromatic rings, as the sole source of carbon and energy (Kallimanis A., Kavakiotis K., Perisynakis A., Spröer C., Pukall R., Drainas C. and Koukkou A. I. Int. J. Syst. Evol. Microbiol. (2009);**59**(2):275–279). In bacterial pathways for degradation of phenanthrene via o-phthalate, the 1-hydroxy-2-naphthoic acid is ring cleaved by a 1-H2NA dioxygenase producing 2′-carboxybenzalpyruvate. Ring cleaving dioxygenases play a central role to the decomposition of doubly hydroxylated aromatic compounds. 1-H2NA dioxygenase is unique among such dioxygenases, because it can cleave a singly hydroxylated aromatic ring. We have identified two genes in the *A. phenanthrenivorans* genome, encoding 1-H2NA dioxygenases

with 90% homology at the nucleotide level, coding for two polypeptides of 387 amino acids, respectively. One of them, diox1, was located on a mega plasmid and the other, diox2, on the chromosome. Both genes were subcloned in the pET29c vector (Novagen), overexpressed in E. coli BL21(DE3) and purified to electrophoretic homogeneity through Ni+-NTA chromatography. Both purified protein fractions Diox1 and Diox2 exhibited 1-H2NA dioxygenase activity. The existence of two homologues encoding for 1-H2NA with similar catalytic properties indicates a gene duplication or transposition within the genome of Arthrobacter phenanthrenivorans Sphe3.

B1.09
Relationship of polyP metabolism and bioinsecticide biosynthesis in *Bacillus thuringiensis*

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Polyphosphate (polyP) which is a linear polymer consisting of tens to hundreds of phosphate molecules joined together by high-energy anhydride bonds. PolyP is synthesized by polyP kinase (PPK) using the terminal phosphate of ATP as the substrate. It is degraded to phosphate by exopolyphosphatase (PPX) enzyme. Although polyP polymer has very important functions (energy storage, resistance to stress factors, regulation of gene expression etc.) in the microorganisms studied, to our knowledge there is only one study about the role of this polymer in *Bacillus*. In 2004 Shi et.al., deleted the genes (*ppk*, *ppx*, *pap*) responsible for the polyP metabolism and showed that polyP is necessary for the motility, biofilm formation and sporulation in this microorganism. *Bacillus thuringiensis*, which produce δ-endotoxin, used for biological control of insects. Some mineral elements especially phosphate, which were added into the culture media, has been shown to play very important stimulatory effect on toxin production (Banerjee-Bhatnagar, 1999; Kurt et.al., 2005). In this study, it is aimed to obtain hypertoxic mutant strains by changing the phosphate metabolism of *B. thuringiensis israelensis*. For this purpose; *ppk* and *ppx* genes of *B. thuringiensis* were cloned separately and together and the effect of overexpression of these genes on endotoxin production were determined. Optimum conditions (pH, temperature, carbon source) for toxin production by mutant strains and also resistance of these strains to different stress factors (pH, temperature, oxidative stress, heavy metals) were tested. This study will be the first example which relate polyP metabolism with bioinsecticide biosynthesis.

B1.10
The effects of dexketoprofen on paraoxanase and nitric oxyde synthase activities in rat liver tissues

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Dexketoprofen-trometamol is a water-soluble salt of the dextrorotatory enantiomer of nonsteroidal anti-inflamatory drug ketoprofen. In this study we investigate the relationship between low-high dose of Dexketoprofen and enzyme activities such as Paraoxanase (PON) and Nitric Oxyde Synthase (NOS). Twenty-six wistar albino rats in this study were randomly divided into 3 groups: Group-I = Control group (n = 10), Group-II = Low dose group (treated i.p 10 mg/kg/day Dexketoprofen for 1 week; n = 10), Group-III = High dose group (treated i.p 20 mg/kg/day Dexketoprofen for 1 week; n = 6). At the end of the study, rats were sacrified and their livers were removed. In the liver tissues, NOS and PON activities were measured. Statistical analysis were performed using Mann Whitney-*U* test. The PON activity were significantly elevated in high dose group (6.78 ± 0.77 IU/mg protein) compared to low dose (4.34 ± 0.64 IU/mg protein) and control (3.80 ± 0.46 IU/mg protein) groups $p < 0.05$, $p < 0.025$ respectively. There was no significant difference between PON activities of the control and low dose groups. NOS activity were significantly incresed both high dose and low dose groups (7.45 ± 0.84 and 5.61 ± 0.42 IU/mg protein) compared to control group (2.70 ± 0.36 IU/mg protein), $p < 0.025$ and $p < 0.01$ respectively. Also, NOS activity was found significantly elevated in high dose group versus low dose group $p < 0.05$. In conclusion, we suggested that high dose of dexketoprofen induced the NO production by activating the NOS enzyme expression. On the other hand, elevated PON activity, as an antioxidant enzyme, could protect lipoproteins from oxidative stress.

B1.11
Characterisation of two new enzymes involved in lysosomal phospholipid degradation

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The incomplete degradation of phospholipids plays an important role in the pathogenesis of several lysosomal storage disorders. However, the array of lysosomal enzymes involved in this degradation is incompletely known. Here we present the purification and characterisation of two new enzymes involved in this process. Lysosomal phospholipase A1 acting on phosphatidylcholine was originally reported by Mellors and Tappel in 1967 and Fowler and deDuve in 1969; but never fully characterised biochemically or genetically. The enzyme was purified to homogeneity from bovine kidney as a 75 kDa glycoprotein compromised of two peptides of 25 and 50 kDa joined by noncovalent forces. Remarkably this enzyme was absent from bovine brain suggesting a tissue specific expression. In transfected COS-cells the enzyme was transported to the lysosomes via the mannose-6-phosphate receptor (MPR). Lysosomal lysosphingomyelinase is an acid sphingomyelinase like protein that lacks the N-terminal saposin-like domain, resulting in no intrinsic activity towards sphingomyelin. The enzyme was purified from bovine kidney and brain as a 55 kDa glycoprotein. One of the glycans linked to the brain form was a phosphorylated high-mannose type, suggesting that lysosomal lysosphingomyelinase is sorted to the lysosomes via MPR. Although the lack of any of these two enzymes would be expected to cause a lysosomal lipid disease, no lysosomal storage disease caused by their absence has yet been reported.

B1.12
The effects of acetaldehyde on steady-state metabolism of ethanol in perfused rat liver as treated with cyanamide assessed by kinetic mechanism-based rate equations of the alcohol dehydrogenase

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Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) are the principal enzymes responsible for ethanol metabolism in mammalian liver. To mimic ALDH2 deficiency which is found in 40—45% of East Asians, rat liver ALDH was inhibited by treatment with cyanamide. At doses of 0.2 mg/kg and 1.5 mg/kg cyanamide, rat liver ALDH activities were found to be inhibited by ˜50% and ˜75%, respectively. Steady-state metabolism of ethanol was determined in single-pass perfused rat liver inhibited by cyanamide. Concentrations of ethanol, acetaldehyde and acetate in the effluent perfusates were determined using GC or HPLC. The acetaldehyde concentrations were elevated from ˜10 to ˜70 and ˜110 μM by 0.2 mg/kg and 1.5 mg/kg cyanamide, respectively, when perfused with 2 mM ethanol; and from ˜20 to ˜120 and ˜200 μM when perfused with 10 mM ethanol. We compared the experimental data to those from computer simulations using the steady-state kinetic mechanism-based complete rate equations of rat ADH1. The results indicate that the elimination rates of ethanol are decreased due to inhibition of ALDH activity and this can be quantitatively explained by build-up acetaldehyde with the computer simulations.

B1.13
The effect of polyphosphate kinase deficiency on antibiotic production in *Streptomyces coelicolor*

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Polyphosphate (polyP), which is found in every living cell, is a linear polymer consisting of tens to hundreds of phosphate molecules joined together by high-energy anhydride bonds. Polyphosphate, which is synthesized by the enzyme polyP kinase (PPK), is an important energy store and has been shown to have a crucial role in the regulation of adaptive responses of cells to physical and chemical stresses. Streptomycetes are biotechnologically important bacteria and they are well known for their ability to produce a wide variety of bioactive compounds. Chouayekh and Virolle (2002) have reported that ppk gene encoding polyP kinase has a negative effect on antibiotic biosynthesis in Streptomyces lividans. In this work, ppk gene of Streptomyces coelicolor has been disrupted and the effect of this mutation on actinorhodin and undecylprodigiosin biosynthesis was investigated. The results of this study showed that ppk gene has the same repressing effect on antibiotic biosynthesis in *S. coelicolor*. Although undecylprodigiosin production by *S. coelicolor* ppk strain is same, actinorhodin production increased about five times at 120 hour compared to wild type. Mutant strain grow only on antibiotic containing rich medium and not sporulate effectively. Although wild type strain don't show oxidative stress sensitivity, mutant strain was sensitive to H_2O_2 in the conditions tested.

B1.14
Dequalinium inhibits autophagy in the human leukemic cell line NB4: apoptosis resistance mechanism or cell death inducer?

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Autophagy is the process in which cellular compounds are degraded by the lysosome and then recycled.Although it can serve as a protective mechanism against apoptosis and starvation, excessive autophagy results in a non-apoptotic programmed cell death. However, there is evidence of a crosstalk between autophagy and apoptosis. The tumoral supressor p53 seems to inhibit autophagosome formation, preventing the mitochondrion recovery process and inducing apoptosis. Our group has demonstrated that the antitumoral agent dequalinium (DQA) induces apoptosis in the human acute promyelocytic leukemia cell line NB4.Previous results showed a partial inhibition of the DQA effects on apoptosis and on the intracellular levels of reactive oxygen species at a low dose of DQA in combination with the proteasome inhibitor MG-132,which induces apoptosis per se. Since MG-132 inhibits programmed protein degradation,it is possible that the proteins that accumulate in the cells then undergo a different type of degradation.Therefore, we studied apoptotic and autophagic events in NB4 cells treated with MG-132 and/or DQA and the possible implication of p53. Our results indicate that DQA induces apoptosis and inhibits autophagy,and that it triggers p53 nuclear translocation in NB4 cells. We suggest that MG-132 could promote autophagy,therefore resulting the combination with DQA in a competition with apoptosis.This might explain the reduction in apoptosis that occurs in co-treatment with DQA (10 μM, 48 hours).Moreover, we suggest that p53 could be,at least in part, responsible for the reversion of this effect at higher doses of DQA. This work was supported by UAH (GC2009-001).We thank JCCM & UAH for fellowships.

B1.15
Variations in the metabolic network of *Saccharomyces cerevisiae* during adaptive evolution on galactose

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Exploring the changes in the metabolic network that improve fitness in a new niche is an essential step for evolutionary study. Galactose metabolism in *S. cerevisiae* is an interesting target for finding new metabolic network combinations because all GAL genes are well elucidated and their expression is strongly induced by galactose. We evolved wild-type *S. cerevisiae* using serial dilution on galactose minimal media. We subjected this yeast to adaptive evolution in three lineages, starting from a common ancestor, to increase the specific growth rate. After about 400 generations, the growth rate increased similarly in all the lineages, but the fermentative capacity of the evolved mutants from the lineages varied substantially. Glucose-1-phospate consuming reactions were commonly induced in the mutants from the three lineages while the MAP kinase pathway was repressed, as revealed by transcriptome analysis. The intracellular concentration of glucose-1-phoshate significantly reduced while amount of glycogen and trehalose were considerably increased in all the mutants. We also sequenced the genomes of the three evolved mutants from each lineage. We will present an integrated analysis of the changes that are common to the three lineages and those that are specific to each lineage. The two most striking features of this study are that (i) the mutants evolved to possess very diverse phenotype, despite having the same growth rate and (ii) there were no transcriptional changes in the genes of the galactose pathway. The implications of the result on metabolic network analysis and impact of systems biology on evolutionary study are discussed.

B1.16
Effect of the antitumor agent dequalinium upon human prostate cancer cells

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Carcinomas are the most common cause of death due to cancer in humans, with prostate carcinoma as one of the most prevalent cancers. PC3 cell lineestablished from human prostatic adenocarcinoma metastasis to bone, posses characteristics of highly malign neoplasm and therefore is an appropriate model to investigate carcinoma responses. Dequalinium (DQA), a member of delocalized lipophilic cations family, is accumulated and retained in mitochondria of carcinoma cells due to a higher negative mitochondrial transmembrane potential of these cells in comparison to normal cells. Mitochondrion, which makes an integral contribution on the regulation of main cellular events, is an adequate target for tumor cell eradication. Previous results have shown that DQA displays a different cytotoxic activity depending on the cell type. However, the exact mechanisms underlying DQA activity are not yet fully understood. Therefore, the aim of this study was to investigate the effect of this agent on the PC3 cell line in order to acquire a better knowledge about the DQA action mechanism in different cell lines.
Preliminary results show a cytotoxic effect of DQA on PC3 cell line inducing the cell death by apoptosis or necrosis depending on the DQA concentration and the time of the treatment employed. Alterations of mitochondrial function have been evidenced by a loss of mitochondrial transmembrane potential, reactive oxygen species accumulation and other events. The current study improves the knowledge on DQA as a novel anticancer agent with a potential application in human prostate carcinoma therapy.

B1.17
Structural correlations in bacterial metabolic networks

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Evolution of metabolism occurs through the acquisition and loss of genes whose products acts as enzymes in metabolic reactions, and from a presumably simple primordial metabolism the organisms living today have evolved complex and highly variable metabolisms. We have studied this phenomenon by comparing the metabolic networks of 134 bacterial species with known phylogenetic relationships. We consider the "super-network" consisting of the union of all networks where each reaction-node is tagged with the number of organisms it belongs to (organism degree, OD). Network analysis shows that common reactions are found at the centre of the network and that the average OD decreases as we move to the periphery. Nodes of the same OD are much more likely to be connected to each other compared to a random null-model, and this trend persists up to a distance of five reactions, which is similar to the average pathway length of 4.4 reactions, as defined in MetaCyc. Further, we propose a neutral model of metabolic evolution driven by horizontal gene transfer (HGT), which is only directed by biochemical constraints. The model is intialised with a single reaction-node, and by adding new nodes one at time metabolic networks are formed. Although these networks are unrelated and their only similarity stems from biochemical constraints, their "super-network" still exhibits the same structure as the bacterial counter-part. This suggests that bacterial metabolic network evolve through HGT and to a large extent are formed by biochemical constraints.

B1.18
Characterization of cellular composition of evolved strains of *Lactococcus lactis*

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The lactic acid bacteria *Lactococcus lactis* has a well-characterized metabolism in which sugars are converted to mainly lactic acid. While *L. lactis* grows homofermentatively under anaerobic conditions with high concentrations of glucose, the bacteria switches to more efficient mixed fermentation in the presence of other sugars such as maltose and low concentrations of glucose. Inefficient and incomplete metabolism when a more efficient alternative appears available is a widespread phenomenon. In order to understand the evolutionary advantage of the inefficient use of substrates, *L. lactis* MG1363 has been evolved in four parallel chemostats in chemically defined medium, CDMPC, developed for long term cultivation. Since the evolutionary path is followed in four parallel experiments adaptive evolutionary paths can be compared to the final outcome. The evolved strains were extensively characterized with respect to gene reorganization, gene deletion/duplication as well as changes at the mRNA and protein level. Evolved strains were found to have an increased lag phase when revived in batch culture as well as having an increased stability during stationary phase. Also, strains have an increased secretion capacity when compared to the original strain. In addition to insights into overflow metabolism, constraints governing long-term mutation and adaptation in *L. lactis* can be analyzed and so contribute to our understanding of strain stability.

B1.19
Genome scale metabolic models of three bacteria in the human gut

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Microbial intestinal metabolism is thought to affect many human disease states such as diabetes, obesity and inflammatory bowel diseases. Vast amounts of resources are spent on generating genotype data of the gut microbiota but the understanding of the link to phenotype is still limited. To fully understand the interplay between the microbes and the human host and to successfully hypothesize interventions, a more integrated approach is needed. Genome-scale metabolic models (GSMM) have successfully been used to link genotype and phenotype by predicting essential genes and describing the metabolism of several organisms. The formulation is mathematically tractable, scalable and requires a minimum of measured parameters. The construction of GSMM includes data base mining to annotate genes to reactions. Our automated GSMM framework will be used to infer reactions not yet annotated. We plan to construct GSMM for some of the most important species in the gut, namely *B. thetaiotaomicron, E. rectale* and *M. smithii*. By flux balance analysis, it is possible to identify essential genes and interspecies metabolites. Integrated analysis of transcriptome data on GSMM highlights

otherwise unseen effects by identification of reporter metabolites. It will also be possible to simulate optimal conditions for butyrate production, important in inflammatory bowels diseases. Furthermore, the energy harvesting efficiency by the gut microbiome can be quantified with different microbiome compositions and substrates which are related to obesity development. Simulations can generate likely hypotheses for clinical interventions such as probiotics, prebiotics, therapeutics and altered diet.

B1.20
Aspects of sex-specific LRP-2 expression in kidney epithelial cells and the effect of modified LDL particles on receptor regulation

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Diseases including inflammation, diabetes, and obesity can lead to the generation of lipoproteins modified by oxidation, glycation, alkylation, and nitration. Uptake of modified LDL, without efficient degradation, leads to formation of foam cells, causing pathologies including atherosclerosis and glomerulosclerosis. Dyslipidemia accelerates renal damage. In our study we focus on the low-density lipoprotein receptor-related protein 2 (LRP2), which is highly expressed in the renal proximal tubule. We want to elucidate its function in the uptake of molecules involved in lipid metabolism in the kidney. Furthermore, we want to identify the underlying mechanisms of steroid hormone regulation of LRP-2 expression and test if estrogen has a protective effect. Two human kidney cell lines were used. Native HDL and LDL were isolated and modified LDL forms were generated. After incubation with lipoproteins, LRP2 expression was determined. Modified LDL particles caused an increase in LRP2 transcript levels. Furthermore, we were able to show that administration of estrogen enhances transcript expression. Now we address the question, if the incubation with estrogen alters the effect of lipoprotein particles. The inhibition of LRP2 by the receptor-associated protein (RAP), which prevents binding of other ligands, will elucidate receptor dependent regulations. The regulatory mechanisms of LRP2 and its roles in diseases leading to renal damage are not well established. Further studies of sex-related differences in kidney structure and functions will enable us to understand the development of various renal diseases, leading to a better knowledge of pathogenesis and prevention.

B1.21

Abstract withdrawn

B1.22
Reconstruction of a comprehensive *Saccharomyces cerevisiae* genome-scale metabolic and regulatory network

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The yeast *Saccharomyces cerevisiae* is a well studied eukaryotic organism used in many model experiments. Several genome-scale models of the yeast metabolic network exist and these can be used for performing in silico simulations of the system and as a scaffold for omics data integration. All the previous reconstructions of the yeast metabolic network differ in scope and the way they were constructed and difference in metabolite nomenclature makes mapping between the different models difficult. Therefore, there is a need for a new, consensus model that describes as many cellular mechanisms as possible, and also suits good for simulations. We have constructed a new, more comprehensive, genome-scale metabolic model for yeast. The model was based on the consensus network, and a previous model, iIN800 which includes useful information about lipid metabolism. Known transcription factors and gene regulations were also included in order to improve the prediction power of the model. In order to achieve an even more comprehensive description of the cellular mechanisms going on in yeast a stoichiometric model including the genes and reactions involved in the protein synthesis were constructed. This includes many processes, e.g. transcription, translation, splicing, mRNA degradation, post-translational modifications etc. The reconstruction of this network was made by database searches and literature mining. The model was validated by comparing simulations to experimental data from chemostat fermentations under different conditions, e.g. C-limited and N-limited growth. The result is a model that can simulate the system and also serve as a good platform for omics data integration.

B1.23
Energetic metabolism involved in the regulation of porcine spermatozoa motility

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There are two pathways for ATP production in mammalian spermatozoa, glycolysis and mitochondrial respiration. Their role in the control of one of the main spermatozoa functions, motility, remains under investigation. Our previous results showed that porcine spermatozoa motility is regulated by glycogen synthase kinase-3α (GSK3α). Semen from 8 Duroc boars was used and spermatozoa motility was analyzed by computer assisted ISAS® program. GSK3 phosphorylation was evaluated by western blotting. Treatment with the glycolysis inhibitor, the glucose analogue 2-deoxy-D-glucose (DOG) led to a slight reduction in porcine spermatozoa motility (decreases in % motile and % rapid spermatozoa) together with a clearly more progressive movement (significant increases in LIN and STR coefficients and in the % of progressive spermatozoa). However, treatment with mitochondria activity inhibitors rotenone and cyanide caused almost a complete inhibition in porcine spermatozoa motility parameters. In parallel to a blockade in spermatozoa motility, mitochondria activity inhibitors led to a reduction in the phosphorylation levels of the GSK3α isoform in these male germ cells with a non appreciable effect in GSK3β isoform. However, treatment of spermatozoa with DOG had no appreciable effect in GSK3α/β phosphorylation. In summary, our results suggest that the porcine spermatozoa motility is clearly dependent on the mitochondrial activity. However, the glycolysis seems to be involved in the regulation of the porcine spermatozoa progressive movement. Supported: JUEX-PRI07A100 (Spain).

B1.24
Proteome analysis of moderately halophilic bacteria response to oxygen limitation

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Halophilic microorganisms,which have ability to live in saline environments, provide a variety of applications in different fields

of biotechnology. The technical applications of compatible solutes,biopolymers and enzymes that are active and stable at high salt contents,are important for industry. The biodegradation of some organic pollutants and the production of alternative energy are other fields of applications of these microorganisms. Moderately halophilic bacteria is on interest because of its ability to grow in a wide range of salt concentration from 3% to 15%. Proteomics is the characterization of all proteins in a biological system. This study aims to investigate response mechanism of moderately halophilic bacteria, Halomonas sp.ADD12, to oxygen limitation using proteomic tools. Strain ADD12 was isolated from Çamalti Saltern,Turkiye,and has an ability to grow between 5% and 25% NaCl concentrations and it can degrade phenol. 16s rDNA analyses showed that isolate ADD12 was belong to Halomonas genus. To examine oxygen limitation response,Strain ADD12 was grown on aerobic and semi-aerobic conditions. Growth curves were determined at these conditions and microorganisms were harvested at the end of exponential phase. For both conditions two dimensional gel electrophoresis were conducted and proteome maps were visualized. It was seen from the maps, there was an inhibition of whole protein and enzyme production systems.The differently expressed proteins were analyzed by Proteome-Analyzer 4700.Proteins involved in oxygen limitation response were determined. This work was supported by Marmara University,Research Fund Projects FEN-C-DRP-181208-0286.

B1.25
Phytosteryl ferulates affect cross-talk between mTOR and NF-kappaB cascade

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Hydroxycinnamic acid derivatives, observed ubiquitously in plants, have some physiological functions. We have recently reported that phytosteryl ferulates inihibited DNA-binding of NF-κB. mTOR (mammalian target of rapamycin) was shown to be a key kinase acting downstream of the activation of the phosphatidylinositol 3 kinase (PI3K). Cumulative evidence supports the hypothesis that mTOR acts as a master switch of cellular catabolism and anabolism, signalling cells to expand, grow and proliferate. In this study, we investigated rapamycin effects upon NF-κB, because rapamycin is a specific inhibitor of mTOR kinase, downstream of PI-3K/AKT cascade. It was observed that rapamycin plus phytosteryl ferulates synergically inhibited DNA-binding of NF-κB. Phytosteryl ferulates might thus counteract mTOR in inflammatory diseases by modulating key redox sensitive gene transcriptions via PI-3 kinase-AKT-mTOR signaling pathways in the inactivation of NF-κB. Hence, phytosteryl ferulates via the PI3K/AKT/mTOR axis appears to be an important biological product for therapeutic intervention.

B1.26
Metabolic trade-offs in competition for resources between *Saccharomyces cerevisiae* and *Kluyveromyces lactis*

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One of the postulates of the theory of evolution is the competition for limited resources. Previous studies have shown that there is a trade-off between yield and rate in resource utilization and ATP production pathways, implying that different metabolic strategies will result in different competition outcomes. Two main metabolic strategies used by micro-organisms are respiration and fermentation. Respiration maximizes the efficiency of ATP production, resulting in increased yields at the expense of the rate. The whole population benefits from this cooperative use of resources, but individual fitness is reduced. In contrast, fermentation is an inefficient ATP production strategy, but it runs at a higher rate. This is a ''cheat'' strategy because the fitness of fermenting individuals is increased at the expense of the average population fitness. Our goal was to study competition between the two and find conditions, such as resource availability, population and spatial structure, that favor dominance of those strategies. We used *Saccharomyces cerevisiae* and *Kluyveromyces lactis* tagged with different fluorescent proteins as models for fermentation and respiration, respectively. We experimentally determined physiological parameters by individual cultivations and then used those parameters in dynamic spatial and non-spatial models to simulate competition experiments. To validate our models, we studied population dynamics in mixed batch, chemostat and spatially structured cultures. The results will show population and resource utilization dynamics in different conditions and we will discuss implications of those results in the evolution of metabolic strategies.

B1.27
Levosimendan protect aganist ischemia-reperfusion injury in the isolated perfused rat heart model

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Positive inotropic agents play an important role in treating acute decompensation of patients with heart failure due to left ventricular systolic dysfunction. Levosimendan, calcium-sensitizing agent, increases contractility by enhancing the sensitivity of myofilaments to calcium by binding to the C cardiac troponin in cardiac muscle in a calcium-dependent way. The aim of this study was to investigate whether levosimendan has protective effects on myocardial ischemia-reperfusion injury. After anesthesia heart were placed in cold Krebs-Heinseleit and mounted on Langendorff apparatus. Hearts were assigned into three groups: first, after 30 minute ischemia in order to induce, ischemic postconditioning 3 times 10 second ischemia separated by 10 second reperfusion protocol was used; second, non-postconditioned group was used as control; and third, 10-8 mol levosimendan was added to the reperfusion fluid after 30 minute ischemia in order to induce chemical postconditioning. In all these groups, 30 minute global ischemia was followed by 120 minute reperfusion. cTnT and cTnI expression were made with Western Blot and apoptotic differences were matched with TUNEL method. 10–8 mol levosimendan and ischemic post-conditioning significantly increased cTnT and cTnI expression compared with control hearts. Also, ischemic post-conditioning significantly reduced levosimendan cTnT expression compared with Levosimendan. Levosimendan significantly reduced apoptosis compared with control and ischemic post-conditioning groups. Levosimendan has a cardioprotective effect when administered after ischemia against ischemia-reperfusion injury. Protective effect is associated with via decreased apoptosis.

B1.28
Genome sequencing, annotation and analysis of *Saccharomyces cerevisiae*: from genotype to phenotype for industrial biotechnology applications

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Saccharomyces cerevisiae is the most well characterized eukaryote and the preferred microbial cell factory for the industrial biotechnology applications. Genome sequencing of *S. cerevisiae* strain S288C, the first eukaryote genome sequence reported, provided a framework for gene annotation through functional genomics. Of particular relevance for metabolic engineering and industrial biotechnology, an annotated genome sequence was a prerequisite for genome-scale metabolic network reconstructions. Since the genome sequence of *S. cerevisiae* was released, the technologies have advanced and costs associated with whole genome sequencing have decreased substantially. Therefore this has opened an opportunity for the use of genome sequencing as a commonplace tool for advancing functional genomics and strain engineering. In this work we propose that high-throughput genome sequencing, annotation and analysis of *S. cerevisiae* strain CEN.PK 113-7D as commonly used in laboratory. We present the developed genome browser and single nucleotide polymorphism (SNP) database of *S. cerevisiae* strain CEN.PK 113-7D. To directly link genotype to phenotype information, then cellular behavior was characterized in well-controlled batch fermentations on glucose and galactose, complimented with transcriptome analysis. We demonstrate that S288C, the strain utilized for the publically available *S. cerevisiae* genome sequence, exhibits a typical *S. cerevisiae* behavior related to central carbon metabolism as compared to CEN.PK113-7D, a common laboratory strain for industrial biotechnology applications.

B1.29
Are Lewis rats less efficient than Sprague-Dawley rats to cope with heat stress?

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Heat is an environmental factor threatening health and even life. The time during which an organism is able to cope efficiently with heat stress (heat tolerance) is highly variable among individuals. Glucocorticoid signalling is probably involved as hypothalamic-pituitary-adrenocortical (HPA) axis impairment is associated with decreased heat tolerance. Energy metabolism is one of the possible relay between HPA axis activation and heat tolerance. Sprague-Dawley (SD) and Lewis (L) rats, differing by their HPA axis regulation, were instrumented for body temperature (T_{abd}) recording, then exposed during 24 hours in warm ($T_a = 33°C$) or neutral ($T_a = 22°C$) environment. At the end, frontal cortex and peripheral metabolisms were analyzed. L rats had a lesser T_{abd} throughout heat exposure. Blood corticosterone was similar between strains in each condition. At neutrality, L rats had higher blood glycerol level than SD rats ($p < 0.05$). In the heat, L rats had lower glycemia ($p < 0.001$) and lactatemia ($p < 0.001$) levels but higher blood glycerol ($p < 0.10$) and free fatty acids ($p < 0.001$) levels than SD rats. At neutrality, no difference was observed between strains in brain content of 5-AMP-activated protein kinase (AMPKα) and phosphorylated protein (p-AMPKα), acetyl-CoA carboxylase protein (ACC) and phosphorylated protein (p-ACC). In the heat, L rats exhibited higher AMPKα ($p < 0.001$), lower p-AMPKα/AMPKα level ($p < 0.10$), but similar ACC and p-ACC/ACC levels than SD rats. Altogether, it suggests that L rats favoured glycolic pathway, whereas SD rats had shift to lipidic pathway, a more adapted metabolism. These metabolic changes are not associated with substantial difference in brain energy status.

B1.30
The effect of prenatal/postnatal nicotine administration on oxidant/antioxidant status in rats

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Cigarette smoking is common in societies worldwide and has been identified as injurious to human health. One difficulty lies in the fact that cigarette smoke is composed of lots of toxic chemicals including nicotine that causes addiction. Until now, rare studies have examined the effect of nicotine on oxidative stress in infants. However the results are sophisticated. For this reason, we aimed to determine the effect of long term nicotine exposure on oxidant and antioxidant system during prenatal and postnatal period. In our study, Swiss Albino rats were divided into three groups as control ($n = 10$), low dose nicotine ($n = 10$) and high dose nicotine ($n = 9$). Nicotine hydrogen tartrate was given to rats in nicotine groups per oral in tap water freshly prepared everyday for 12 months in a dosage of 2.22 mg/kg body weight (BW) and 33.3 mg/kg BW, respectively. No significant difference in plasma level of total antioxidant status, 8-hydroxy-deoxyguanosine, total sulfhydryl groups and erythrocyte lysate Malondialdehyde(MDA), Catalase and superoxide dismutase (SOD) levels in comparison of controls and nicotine groups. Only plasma cotinine, metabolite of nicotine levels ($p < 0.01$) and liver MDA levels were higher ($p < 0.05$) and kidney SOD activity was lower ($p < 0.05$) in nicotine groups in correlation with nicotine dosage. Since, 12% of the pregnant population continue to smoke cigarettes, low dose nicotine replacement therapy during pregnancy may be better than smoking.

B1.31
Physiological characterization of *Aspergillus oryzae*

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The filamentous fungus *Aspergillus oryzae* is widely used as a microbial cell factory for large scale heterologous protein production. *A. oryzae* is also known as a natural organic acid producer. However, the metabolisms and regulations of organic acid production in *A. oryzae* are poorly characterized. Furthermore, the media composition has a major impact on the performance of this organism and the economic feasibility of an industrial fermentation process. We therefore evaluated the utilization of different nitrogen and carbon sources by *A. oryzae*. In this study, we aim for a deep investigation of the cellular mechanisms of the utilization of different nitrogen and carbon sources. Firstly, we performed batch cultivation on different carbon sources (e.g. glucose and glycerol) and nitrogen sources (e.g. peptone and di-ammonium phosphate). Further, genome-

wide expression analysis was performed to analyze the transcriptome under these different conditions and to identify key-players in the metabolism and regulation. An updated genome-scale metabolic model of *A. oryzae* was used as a scaffold for simulations under different conditions. The embedding of the omics data into the *in silico* work was found to accelerate the optimization process of future industrial applications of this filamentous fungi. The derived results will be used for the construction of a strain that is performing as well on the cheaper nitrogen source.

B1.32
Cloning and expression studies of putative *Rhizopus oryzae* hexokinase genes in *Saccharomyces cerevisiae*

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Rhizopus oryzae is a filamentous fungus which can ferment sugar to ethanol, and lactic acid. Increasing demand of the world for use of renewable carbon sources has put this and similar organisms into a position where the biotechnology industries are more and more interested with these organisms. *R. oryzae* can grow on some renewable carbon sources which makes it a good candidate for production of ethanol as well as lactic acid. In the light of studies done for *Saccharomyces cerevisiae*, a well studied industrial and model organism, we are trying to investigate the regulation of glycolytic pathway of *R. oryzae*. In this study, we have used data from annotated genome database of *R. oryzae* to clone hexokinase genes of this organism. By doing blast search we obtained ten probable genes having two conserved domains ''hexokinase 1'' and ''hexokinase 2''. These domains are present in all of the known hexokinase sequences cloned and characterized from other organisms including *S. cerevisiae*, *Aspergillus niger* and *Hansenula polymorpha*. We obtained PCR products of the five of the probable genes with the primers designed for them and these were also replicated by using P*fx* polymerase. At the end of the sequencing of two of them, it was proved that the PCR product has no introns and these are expressed gene in *R. oryzae* genome. Complementation study was done for one of them in triple hexokinaseless mutant of *S. cerevisiae* and growth was observed on selective medium. Same experiments will be carried out for the rest of the probable genes.

B1.33
Changes at transcriptome level during host-parasite interaction using *Perkinsus olseni* as a model

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Bivalves represent one of the most important marine resources worldwide. Parasitic infections like the one caused by *Perkinsus* sp., a member of the Alveolata group, which also comprises parasites highly relevant for medical and veterinary purposes like Plasmodium and Toxoplasma, significantly decrease shellfish production inducing severe economical/ecological damages. Here we report the trancriptomic response of Perkinsus sp. to its natural host the clam *Ruditapes decussatus*, revealing at the same time the complexity of the pathways present in this unicellular eukaryote. Illumina and 454 sequencing were used simultaneously to cover up an important part of Perkinsus transcriptomic. Two datasets, comprising *P. olseni* transcripts collected under normal culture conditions and when exposed to clam hemolymph, were sequenced using an illumina. Four hundred and fifty-four sequencing of a normalized cDNA library was used to fill up gaps between illumina contigs using hybrid sequencing assembling. This first attempt to characterize the mechanisms involved in host-parasite interaction using high-throughput sequencing unveiled the complexity behind these events, suggesting that this species might be less dependent on its host for its metabolism than its relatives and allowed the identification of some important chemotherapeutic target at metabolic level. The combination of this data, together with the annotated genome of *P. marinus*, will provide a set of genomic tools to study host-parasite interactions in this marine model system, for which there is a growing interest worldwide.

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B1.34
Combined effects of melatonin with epirubicin and doxorubicin in MCF-7 breast cancer cell lines

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Side effects of chemotherapeutic agents cause serious problems in cancer treatment. Thus new combined treatment strategies have been widely studied. Melatonin has been shown to inhibit cell proliferation in MCF-7 cell lines. The purpose of this study was to investigate antiproliferative effects of melatonin combined with epirubicin and doxorubicin in time and dose dependent manner on MCF-7. We have also aimed to determine the characteristics of combined effects of melatonin with epirubicin and doxorubicin. For these purposes MCF-7 cells were treated with different doses of melatonin (0.5–1500 nM), epirubicin (10–50 μM) and doxorubicin (10–50 μM) for 24-48–72 hours. MTT assays were carried out to investigate the effects of treatments on cell viability. MTT assays demonstrated that cell death was occurred in a dose and time dependent manner in epirubicin and doxorubicin treatments. Our results show that 30 μM concentrations of doxorubicin and epirubicin caused 41% and 50% inhibition of cell viability at 48 hours respectively. Combined effect of 1000 nM melatonin with 30 μM epirubicin or 30 μM doxorubicin enhanced cell death the ratio of 10–15% at 48 hours. Western blot data of caspase 3 also confirmed these results. Our data suggests that all treatments induce cell death in MCF-7 and combined effect of 1000 nM melatonin with 30 μM epirubicin or 30 μM doxorubicin enhance cell death. Although further studies needed we suggest that melatonin can be a candidate as an additive agent in chemotherapeutics and these results may be a basis for the researches to determine anticancer use of melatonin in breast cancer.

B1.35
The antitumour derivative, C-1748, affects CYP3A4: crosstalk between drug metabolism, CYP3A4 expression and enzymatic activity

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Individual variety in drug metabolism is one of the major causes of treatment failures during cancer therapy. Understanding of the relations between changes in the level of gene expression and catalytic activity of enzymes involved in metabolism of the drugs might lead to the development of novel strategies for regulation of these process. Therefore, much attention has focused on the induction or inhibition of CYP3A4 cytochrome P450 isoenzyme, which is involved in the biotransformation of more than 50% of all drugs clinically used. It is known that expression of CYP3A4 is affected by different factors from genetic polymorphism to xenobiotic exposure. We have previously demonstrated that C-1748 was transformed in HepG2 cells and the number and concentration of metabolites were associated with CYP3A4 protein level. To confirm this findings, we determined the effect of C-1748 on the expression and enzymatic activity of CYP3A4 in HepG2 cells. Using a combination of reverse transcriptase RT-PCR and HPLC analysis, we found that the studied compound significantly decreased the expression of CYP3A4 mRNA and also enzymatic activity of this enzyme at EC50 concentration, after a short period of time. We demonstrated that this effect of C-1748 was reflected in the protein levels. However, cell incubation for a longer time with C-1748 resulted in an increase of CYP3A4 mRNA expression levels and its enzymatic activity relative to control. The presented studies showed for the first time that C-1748 affected the protein level, gene expression and enzymatic activity of CYP3A4 in HepG2 cell line in the different way that observed for other antitumour agents.

B2 – Signal Transduction

B2.01
Insulin and heparin bioactivation of 3D NWPF discs by water/O2 plasma for l929 fibroblast cell cultivation

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The goal of this study was to obtain COOH functionalities on the surface of 3D, non woven polyester fabric (NWPF) discs by using low pressure water/O_2 plasma assisted treatment. The plasma treatment was performed in a cylindrical, capacitively coupled RF-plasma-reactor in three steps: H_2O/O_2-plasma treatment; in situ (oxalyl chloride vapors) gas/solid reaction to convert –OH functionalities into –COCl groups; and hydrolysis for final–COOH functionalities. DoE (Design of Experiment) software program was used for the optimization of plasma modification. COOH and OH functionalities on modified surfaces were detected quantitatively by using fluorescent labeling technique and an UVX 300G sensor. Plasma treated and oxalyl chloride functionalized samples were biologically activated with insulin or heparin molecules by using spacer PEO (polyoxyethylene bis amine). Success of immobilization process was checked qualitatively with ESCA analysis. The average amount of immobilized insulin and heparin onto NWPF surfaces were determined as 146.09 and 4.81 nmol/cm^2, respectively. Our results showed that water/O_2 plasma assisted treatment worked very well for functionalization (COOH) and bioactivation of 3D NWPF discs comparing with wet-chemistry methods. Cell culture experiments indicated that functionalization of NWPF discs and/or nanotopographies on the disc surfaces were effective on L929 cell adhesion and proliferation.

B2.02
Insulin like growth factor-1 stimulated erythropoietin production is mediated by ERK kinase pathway

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Purpose: Erythropoietin (Epo) is a known angiogenic factor. It is postulated that it may play a role in proliferative diabetic retinopathy (PDR). Since insulin like growth factor-1 (IGF-1) has also been implicated in the development of proliferative diabetic retinopathy, we have investigated the effect of IGF-1 on intracellular Epo production.
Methods: hRPE cell cultures were established from human eyes. Cell viability and proliferation were determined by the trypan blue exclusion method (T) and by thymidine incorporation (3H-thy). Localization of Epo in hRPE cells was examined by immunohistostochemistry. Endogenous 14C-methionine-Epo (14C-Epo) production was quantitated by immunoprecipitation. hRPE cells were also treated with PD98059 (PD), a selective inhibitor of ERK kinase. Data were analyzed by Student *t* test.
Results: hRPE cell proliferation was stimulated by IGF-1 in a dose dependent manner as determined by 3H-thy and T. PD significantly inhibited IGF-1 stimulated 3H-thy incorporation in these cultures: (990.6 ± 187.46 versus 1296.4 ± 209.75, CPM ± SEM, $p < 0.05$,N = 4). IGF-1 also stimulated immunoprecipitated 14C-Epo in a dose dependant manner and this stimulation was also inhibited by PD (1481.93 ± 385.20 versus 5636.94 ± 2212.04, CPM ± SEM, $p < 0.05$, N = 5). Immunohistochemical studies demonstrated more densely stained Epo-positive cells in presence IGF-1 than in control and IGF-1 + PD.
Conclusions: These data suggest that IGF-1 is mitogic in hRPE cells and the ERK kinase may be involved in mitogenic effect and in Epo synthesis. Development of an ERK Kinase inhibitor may be of therapeutic value in the treatment of proliferative ocular complications.
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B2.03
Assigning the property of electricity conduction to the DNA molecule can explain many problems with signal transduction and transcription factors (TF)

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Objective: To solve the problem of sequence recognition of TF by trial and error which is not economic we tried to find novel mechanical and electrical properties within the DNA molecules.
Materials and Methods: Based on data of Shirakawa et.al, we plotted new molecular conformations with different angles and axes rotations of DNA to find two molecular landmarks for electricity conduction namely alternating single and double bounds of the Sp2 hybridization nature and intermolecular channels that can harbor free electrons. We questioned whether tautomery of the bases can open the double stranded DNA long before topoisomerase enzymes come into effect. Besides, the distance between double and single bounds were compared between different electricity conducting polymers (polypyrole, polythiophene etc.) and DNA.
Results: DNA can cautiously be regarded an electricity conducting polymer which is doped with the histones. The wave of electricity causes a slower mechanical wave that melts (exposes) DNA at special sites to facilitates recognition by TFs and hence attract the transcription machinery. The time lag between electrical and mechanical waves is of utmost importance in this theory.
Conclusions: (1) DNA is probably an electricity conducting biopolymer which increases speed of response and facilitates recognition of targets by TFs.
(2) Entrance of specific TF through nuclear pores is the first signal for transcription in form of electrical excitation and gene exposure.
(3) This mechanism reduces the number of needed TF and makes general TFs more cell-specific; a few TFs can erase a series of gene inductions in different cells.

B2.04

Abstract withdrawn

B2.05

The eIF2α kinase PKR modulates the hypoxic response by Stat3 -dependent transcriptional suppression of HIF-1α expression

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Within the tumor microenvironment, hypoxic conditions trigger important cellular responses which promote angiogenesis, metabolic reprogramming and tumor progression. Hypoxia-inducible Factor-1α is a key transcription factor that is activated by hypoxic conditions to induce the expression of genes that contribute to tumor growth. In addition, cells respond to hypoxic stress not only by upregulating HIF-1α but also by inhibiting global protein synthesis through several mechanisms including the phosphorylation of the alpha subunit of eIF2 at serine (S) 51. In mammalian cells, phosphorylation of eIF2α is mediated by a family of kinases which respond to distinct forms of stress. We investigated whether these kinases are involved in the regulation of HIF-1α. Our results demonstrate for the first time that the double-stranded (ds) RNA-dependent protein kinase PKR plays a significant role in suppressing HIF-1α expression specifically at the transcriptional level. Transcriptional repression of the *HIF1A* gene by PKR is sufficient to impair the upregulation of HIF-1α and its downstream genes under hypoxic conditions. Inhibition of *HIF1A* gene transcription by PKR is independent of eIF2α phosphorylation but requires inhibition of the activity of the signal transducer and activator of transcription 3 (Stat3). We also provide evidence that transcriptional repression of *HIF-1A* requires the T cell protein tyrosine phosphatase (TC-PTP), which acts downstream of PKR to suppress Stat3. Our data reveal a novel tumor suppressor function of PKR through the inhibition of Stat3 activity and HIF-1α expression, which takes place independently of eIF2α phosphorylation.

B2.06

Screening a kinase inhibitor library for multiple target inhibitors of VEGFR2 and PDGFRb

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Background: VEGFR2 and PDGFRbeta play an important role in tumor angiogenesis, in tumor growth and metastasis. Targeting these two signaling pathways may prevent the tumor progression. Clinical results show that anti-angiogenic therapy can normalize the tumour vasculature and improve the delivery of therapeutics.
Objects: The aim of this study was to discover potential new VEGFR2 and PDGFRb inhibitors with the application of biochemical and cellular assays.
Methods: The compounds to be tested were selected from Vichem's NCL, library of kinase inhibitors. These molecules are ATP binding site inhibitors and allosteric inhibitors and represent a diverse set of potentially active chemical structures. For the *in vitro* study we have chosen recombinant VEGFR2 and PDGFRbeta kinase proteins and EA.hy926 and Kaposi's sarcoma cell lines. We used Transcreener Kinase Assay with Direct Immunodetection of ADP for the biochemical studies. For the cellular assays we used CellTiter-Glo Luminescent Cell Viability Assay based on quantitation of the ATP present, which signals the presence of metabolically active cells. sWe have determined the apoptotic effect and the ADME parameters of the active compounds.
Results: We have optimized the biochemical assay and executed the high throughput screening of molecules on VEGFR2 and PDGFRb kinases. We have identified lead compounds which inhibit both kinases with nanomolar IC50 values and demonstrated their antiproliferative and apoptotic effects on cell lines.
Conclusions: The selected lead compounds have shown promising results. The anti-angiogenic potentials of these compounds will be further evaluated with an angiogenesis tube formation assay.

B2.07

Biochemical characterization of novel EGFR and c-Met single and dual inhibitors

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Non-small cell lung Cancer (NSCLC) is one of the most frequent cancer type with high mortality worldwide. NSCLC is less sensitive to conventional chemotherapy. Therefore, specially developed and targeted tyrosine kinase inhibitors (TKIs) against its central oncogene EGFR (epidermal growth factor receptor), might be promising alternatives. Patients with somatic activating mutation in EGFR (e.g. L858R) are principally responsive to current TKI therapies. However, most of these patients become resistant to treatment after prolonged administration of these drugs, because of a secondary T790M mutation in EGFR and/or Met oncogne amplification. Our scientific aim was to search for novel inhibitors for the most important kinases, regarding the EGFR-related signaling pathways, in the small molecular Nested Chemical LibraryTM. The activity of wild type and mutant EGFRs, c-Met and c-Src recombinant kinases were measured using an ADP-detecting fluorescence-based assay platform (Transcreener®). Based on the results of our biochemical assay system, we found molecules around two novel, patentable core structures inhibiting EGFR L858R. Furthermore, we also found promising, reversible EGFR L858R/T790M inhibitors. Finally, a compound family proved to be EGFR L858R and c-Met dual inhibitor. The anti-tumor potentials of these compounds will be evaluated applying NSCLC cell lines and further ADME-Tox examinations are also planned. In summary, we optimized a cost efficient, recombinant enzyme based HTS (high throughput screening) assay platform and selected efficient drug-like lead compounds inhibiting mutant EGFRs and c-Met.
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B2.08
The antiproliferative effect of known and novel PLK1 inhibitors on different cancer cell lines

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Polo like kinase 1 (PLK1) is essential in the regulation of mitotic entry, spindle formation, chromosome segregation and cytokinesis during mitosis. High expression and activity of this kinase occurs in many human tumor types. The inhibition of PLK1 activity results in their mitotic arrest and apoptosis thus the regression of tumors. We screened known and novel PLK1 inhibitors on 18 different cell lines to characterize their antitumor effect. Among these cancer cell lines were two (A431 and A549) with mutation in the PLK gene and one of them (Jurkat) showed high level of PLK protein. The viability of the cells was measured using a luciferase-coupled ATP quantification assay (CellTiter-Glo; Promega, Madison,WI). In this assay, luminescent signal is proportional to the amount of ATP and thus to the number of metabolically competent cells. The cells were treated with 10 μM of each of the PLK1 inhibitors, and the luminescent signal was recorded after 72 hour incubation. The compounds were further tested to define their EC50 values on all cell lines. These molecules have shown promising results in the inhibition of cell proliferation on each cell line with known genomic imprinting. There were no significant differences in the EC50 values in case of cells with or without PLK mutations. Our results indicate the effectiveness of the novel and known molecules and provide useful new data of their inhibition potential on 18 cancer cell lines.

B2.09
Biochemical characterization of novel PKD1 inhibitors using kinase assays and cellular studies

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Background: Protein kinase D1 (PKD1) is the member of protein kinase D, a novel family of serine/threonine kinases targeted by for example the second messenger diacylglycerol. PKD1 is present in VEGF signaling pathway and has a role in inflammation by the production of proinflammatory cytokines in endothelial cells or angiogenesis through the phosphorilation and nuclear exclusion of HDAC5 and HDAC7.
Objects: The aim of this study was to discover potential new PKD1 inhibitors with the application of biochemical and cellular assays.
Results: We have optimized the IMAP (Immobilized Metal Assay for Phosphochemicals) assay and performed the high throughput screening of molecules on PKD1. The compounds to be tested were selected from Vichem's Nested Chemical Library, a library of kinase inhibitors. These molecules are ATP binding site inhibitors and allosteric inhibitors and represent a diverse set of potentially active kinase inhibitor structures. For the the biochemical study we used recombinant PKD1 protein and for the cellular assay EA.hy926 endothelial cell line. We have identified hit molecules which inhibit PKD1 with nanomolar IC50 values and determined their antiproliferative, apoptotic effects and ADME parameters.

B2.10
The antipsychotic drug haloperidol inhibits cholesterol biosynthesis, disrupts lipid rafts and impairs insulin signalling in SH-SY5Y cells

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Conventional antipsychotics, such as haloperidol, are drugs used in schizophrenia, though they are not free of side effects. Cholesterol is concentrated in lipid rafts which are seen as major platforms for signal transduction events such as insulin receptor.
Objective: To examine the effects of haloperidol on cholesterol synthesis, lipid rafts composition and insulin signalling in a neuroblastoma cell line.
Methods: Sterols were analyzed by reversed-phase HPLC. Lipid rafts were isolated by sucrose gradient fractionation and analyzed by western blot. The cells were incubated with DiI-LDL and immunodetected by fluorescence microscopy.
Results: We found that haloperidol inhibited cholesterol synthesis affecting Δ7-reductase > Δ8, 7-isomerase > Δ14-reductase activities, in that order, which causes a change in the sterol composition of lipid raft and non-raft membrane domains. These effects were accompanied by profound alteration of lipid rafts functionality and impaired insulin signalling. Free cholesterol abrogated the effect of haloperidol on insulin signalling. In contrast, LDL was much less effective in preventing the effects of haloperidol, which is attributed to the drug's inhibition of intracellular vesicular trafficking.
Conclusions: The disruption of lipid rafts and subsequent alteration of raft-dependent insulin signalling, as a consequence of both the inhibition of cholesterol synthesis and interference in intracellular cholesterol transport, should be considered as additional actions possibly involved in the undesirable effects of haloperidol.
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B2.11
Functional analysis of P2Y12 randomized libraries in yeast *Saccharomyces cerevisiae*

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G protein coupled receptors (GPCR) are targets of 40% of therapeutics currently used in clinical practice. Purinergic receptor 12 (P2Y12) belongs to GPCR and is a crucial factor for blood coagulation. Several antiplatelet agents: prasugrel, ticlopidine, clopidogrel that are widely used for preventing of stent thrombosis take their action via P2Y12. Functional characterization of P2Y12 regions that are involved in ligand binding is very important in development of novel therapeutics on P2Y12. In this study several amino acid positions of P2Y12 that are involved in ligand binding were characterised. First randomised libraries of P2Y12 were created in PCR using random primers. Obtained

randomised libraries of P2Y12 were cloned into yeast expression vector and transformed into corresponding *Saccharomyces cerevisiae* strain containing genetic modification of the mating-signal transduction pathway that enables agonist dependent growth on special selective medium. Variants of randomised P2Y12 were isolated, sequenced and tested for functional activity by natural agonist ADP. Analysis of randomised libraries showed that heterogeneity of obtained variants highly depends on primer heterogeneity that may affect occurrence of some P2Y12 variants more often than others. In summery some of analysed amino acids have important role in P2Y12 action and must be considered in design of new therapeutics on P2Y12.

B2.12

Abstract withdrawn

B2.13
The *Drosophila* EcR DNA-binding domain (EcRDBD) with its C-terminal extension possesses a hormone-dependent NLS activity

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The insect ecdysteroid receptor is a transcription factor complex comprised of two nuclear receptors, the 20-hydroxyecdysone receptor (EcR) and the Ultraspiracle protein (Usp). EcR and its ligands are attractive targets for development of gene switches in humans and in agricultural insect control. Our previous study suggested that N-terminal region of EcR has the significant impact on subcellular distribution of all EcR isoforms. In order to define nuclear localization signals (NLSs), series of EcR mutants tagged with yellow and cyan fluorescent proteins (YFP and CFP) were prepared and examined in different cell lines. Our data revealed the presence of the novel NLS in the N-terminal region of EcR. Moreover, we determined a complex of NLS spreading over the DNA-binding domain of EcR (EcRDBD) and its N- and C-terminal extensions. The YFP-EcR protein with mutated NLS located in the N-terminal extension of EcRDBD was distributed equally within the analysed cells or localized predominantly in the cytoplasm. However, in the presence of 10^{-5} M Muristerone A, the protein was localized almost exclusively in the nucleus. Thus, the complex NLS located within EcRDBD and its C-terminal extension exhibits the hormone-dependent NLS activity. This work has been supported by the Polish Ministry of Science and Higher Education; (Grant number: 2827/P01/2007/32) and by Wroclaw University of Technology.

B2.14
Role of MAP-kinase signaling in the apoptosis induced by acyclic nucleoside phosphonates PMEG and PMEDAP

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The mitogen-activated protein kinases (MAPK) comprise three Ser/Thr kinase cascades. Extracellular-signal-related kinases (ERKs) respond to growth factors or other mitogenic signals by promoting cell proliferation. The other two pathways - p38 MAPK and the c-Jun N-terminal kinase (JNK) pathways promote stress response such as inflammation or programmed cell death. In this study, we investigated the involvement of MAPK in the cytotoxicity of acyclic nucleoside phosphonates (ANPs) PMEG and PMEDAP in CCRF-CEM and HL-60 cells. Gene expression profiling in PMEG-treated CCRF-CEM cells revealed up-regulation of p38β, γ and δ mRNA, which was however not detected at the protein level. Neither of the two ANPs induced p38 kinase phosphorylation in CCRF-CEM cells whereas increased p38 phosphorylation was observed in HL-60 cells. ERK kinase pathway was also activated by the ANPs while JNK kinase remained unaffected. Pretreatment of the cells with selective p38 inhibitor SB203580 resulted in a marked diminution of the ANPs-induced apoptosis as indicated by a decrease in caspase 3 cleavage. On the other hand, its effect on PARP cleavage was negligible suggesting that ANPs-induced PARP cleavage is not entirely dependent of caspase 3 and/or p38 kinase activation. Inhibition of ERK and JNK pathways did not prevent apoptosis induced by the ANPs indicating that they are not relevant for the apoptotic effects of these compounds.
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B2.15
Calyculin-A induces phosphorylation and translocation of myosin phosphatase target subunit 1 in THP-1 leukemic cells

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Myosin phosphatase holoenzyme (MP) is composed of a PP1 catalytic subunit (PP1c) and the myosin phosphatase target subunit (MYPT1). MYPT1 is generally distributed between the cytoplasm and the nucleus of cells, and its nuclear import is dependent on the presence of nuclear localization signal sequence located close to the N-terminus. In THP-1 leukemic cells MYPT1 is localized almost exclusively in the nucleus as revealed by confocal microscopy of the cells and by Western blots of subcellular fractions. The level of phosphorylation of MYPT1 at Thr695 and Thr850, which are implicated in the inhibition of MP activity, is low. Treatment of the cells with the phosphatase inhibitor calyculin-A (CL-A) induces translocation of MYPT1 from the nucleus to the cytoplasm followed by an increased phosphorylation level at the inhibitory sites and a decreased mobility of MYPT1. This supports the occurrence of additional phosphorylation events, since phosphorylation of MYPT1 at the two inhibitory sites only does not alter its mobility on SDS-PAGE. Inhibition of Rho-kinase did not influence the localization pattern, suggesting that Thr695 and Thr850 residues might not be involved in mediation nuclear transport. Contrarily, the distribution of PP1c isoforms between the subcellular fractions remains approximately the same upon CL-A treatment, suggesting that shuttling of MYPT1 between the nucleus and the cytoplasm requires its dissociation from PP1c. Further experiments are required to establish whether phosphorylation site(s) in MYPT1 close to the nuclear localization signal sequence might be involved in the regulation of the nuclear transport of MYPT1 and its association with PP1c.

B2.16
Diacylglycerol Kinase alpha mediates HGF-induced Rac activation and membrane ruffling by regulating atypical PKC and RhoGDI

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Diacylglycerol kinases (DGKs) convert diacylglycerol into phosphatidic acid (PA). We showed that DGKa is required for growth factor-induced cell migration and ruffling, by promoting Rac plasma membrane targeting and activation. Thus we investigated the signalling pathway through which DGKa controls Rac activation at ruffling sites. The dynamics of Rac targeting to the plasma membrane are regulated by RhoGDI, which complexes with Rac and allows its localized activation. Moreover, atypical PKCz, which is directly regulated by PA, has been recently demonstrated to regulate Rac activation through the release from RhoGDI. We show inhere that PKCz/i associates with RhoGDI and is required for both HGF- and constitutively-active myr-DGKa-induced ruffle formation and Rac/RhoGDI recruitment to ruffling sites. In this context, we show that: (i) DGKa inhibition prevents HGF-induced PKCz/i recruitment to ruffling sites; (ii) myr-DGKa, as well as cell treatment with PA, is sufficient to induce PKCz/i activation and translocation to ruffling sites; (iii) DGKa is dispensable for ruffle formation and RhoGDI recruitment to ruffling sites induced by constitutively-active myr-PKCz; (iv) DGKa is required for HGF-induced RhoGDI recruitment and for Rac/RhoGDI complex dissociation; (v) myr-DGKa is sufficient to induce Rac and RhoGDI recruitment at ruffling sites in a PKCz/i-dependent manner. Overall, our data demonstrate that DGKa, by producing PA, provides a key signal that, through atypical PKCz/i activation and plasma membrane recruitment, in complex with RhoGDI and Rac, allows Rac activation leading to formation of membrane ruffles, which constitute essential requirements for cell migration.

B2.17
Diacylglycerol kinase alpha regulates SDF1α-induced cell invasion by regulating atypical PKC and matrix metallo proteinases 9

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Diacylglycerol kinase enzymes convert diacylglycerol into phosphatidic acid. We demonstrated that that HGF and VEGF activate diacylglycerol kinase alpha (DGKα) in a Src-dependent manner and its activity is required for epithelial and endothelial cell migration, invasion, proliferation and angiogenesis. Recently we unveiled a new signalling pathway linking HGF receptor to Rac activation and formation of membrane protrusions, through DGKα-mediated regulation of atypical PKCs (aPKC) and RhoGDI. SDF1α, member of the chemokines CXC subfamily, and its receptor CXCR4, a G protein coupled receptor, mediate survival, proliferation, and invasion of breast carcinomas. Here we show that in MDA231, breast cancer cells, upon SDF1α stimulation, DGKα is activated and recruited to the plasma membrane in a pertussis toxin-sensitive manner. Both DGKα silencing and inhibition of its activity impair SDF1α-triggered invasion and chemotaxis. In particular, DGKα activity is required for SDF1α-induced matrix metallo proteinases 9 activation (MMP9), elongation of cell protrusions through extra cellular matrix, recruitment of aPKC at the plasma membrane and targeting of both β1 integrin and MMP9 at the tip of cell protrusions. Finally the expression of a constitutively membrane-bound active DGKα mutant is sufficient for formation of protrusions as well as targeting of both β1-integrin and MMP9 at the protrusion tips and activation of MMP9 gelatinolytic activity in an aPKC-dependent manner. Altogether, these data indicate that DGKα activation stimulates SDF1α-induced breast cancer cell invasion, by regulating aPKC-mediated protrusions in ECM and MMP9 targeting and activation.

B2.18
Generation and characterization of cell lines with stable suppression of key enzymes of poly(ADP-ribose) metabolism

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Poly(ADP-ribosyl)ation, catalysed by poly(ADP-ribose) polymerases (PARP-s), is a reversible post-translational modification of proteins. The ADP-ribose polymer is formed by sequential attachment of ADP-ribose moieties from NAD+ to acceptor proteins. The major enzyme catalyzing poly(ADP-ribose) catabolism is poly(ADP-ribose) glycohydrolase (PARG). Poly(ADP-ribosyl)ation is involved in the maintenance of genome integrity, DNA repair and regulation of gene expression. However, the exact mechanism of these regulations and the possible role of PARG in these processes is fairly unknown. Reports based on the use of PARP-1 knock out mice have demonstrated that inhibition or genetic ablation of PARP-1 provides remarkable protection from oxidative stress-related conditions. Little is known, however, about the role of poly(ADP-ribose) polymers and PARG protein in these cellular events. Here, we employed a lentiviral-mediated, gene-specific shRNA system to establish A549 cell lines with stable suppression of the PARG and PARP-1 genes. Our data demonstrate that shPARG and shPARP-1 cell lines display efficient reduction of targeted mRNAs and the corresponding proteins. Oxidative stress induces transient nuclear synthesis of poly(ADP-ribose) polymers. In the shPARP-1 cell line, however, poly(ADP-ribose) synthesis was strongly suppressed. Poly(APD-ribose) synthesis was apparently unchanged in shPARG cells; however, degradation of the polymer was significantly delayed. The efficient suppression of PARP-1 and PARG that has been achieved in A549 cells is a suitable tool for investigation of the role of poly(ADP-ribose) metabolism in oxidative stress in special regard to cell death.

B2.19
Expression of TGF-beta/Smads signalling in human cervical cancers

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High risk human papillomaviruses (hrHPV) are considered etiologic agents of cervical cancer. TGF-b (transforming growth fac-

tor-beta) is well known for its anti-proliferative effects and the neoplastic cells often lose their sensitivity to TGF-b. A characteristic alteration associated with malignant progression is the loss of responsiveness to transforming growth factor-b1 (TGF-b1)-induced cell growth inhibition. The aim of this investigation was to define the molecular basis of TGF-b1 function in cervical cancer. 30 cervix biopsies harvested from women (30–57 years old) who underwent surgical treatment for squamous cervical cancer (SSC), were included into the study. All samples were tested for hrHPV DNA presence (Linear Array, Roche). 5 biopsies from HPV DNA negative women (42–53 years old) who were subjected to hysterectomia for other causes were included as control group. In order, to explored TGF-b1 signaling pathway the expression of TGF-b1, TGF-b1 receptors and Smad 2 were estimated in Real-Time RT-PCR (ABI) and in Western- blot analysis. The study revealed an abnormal overexpression of TGF-b1 in 36.66% (12/30) of SSC cases as compared with control group and an abnormal reduction of type I TGF-b1 receptor in 23.33% (7/30) of cervical cancer tissues. In addition, 16.66% (5/30) of the tumour samples presented a reduction in Smad2 expression (as confirmed by Real-Time and Western-blot investigations). Our results demonstrated that in human cervical cancer the disruption of TGF-b/Smad signalling pathway that might contribute to the malignant progression of cervical dysplasia.

B2.20
Immunocytochemical analysis of subcellular localization and content of S6 kinase during cell cycle progression

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The ribosomal protein S6 kinases are important components of PI3K signal transduction pathway. S6Ks play a key role in the regulation of cell growth by stimulating protein synthesis in response to growth factors and nutrients. In the mammalian cells two forms of S6Ks are expressed, namely S6K1 and S6K2 that play both redundant and distinct functional role. It was shown, that selective inhibition of S6Ks led to proliferation delay. The goal of our work was to study the subcellular localization and content of S6Ks during cell cycle progression. Human breast cancer cells MCF-7 were cultured on the cover glass slides. Anti-S6K1 rabbit antibodies/anti-Ki-67 mouse antibodies and anti-S6K2 rabbit antibodies/anti-Ki-67 mouse antibodies mixtures were used to detect S6K1 and S6K2 content in proliferating cells. The patterns were examined using fluorescent microscopy. Our data shows, that S6K1 and S6K2 are localized in the cytoplasm during interphase, however weak positive reaction was revealed in cell nuclei as well. The population of studied cells demonstrated the same level of intracellular content of S6Ks. Immunocytochemical and immunofluorescent analysis revealed that the content of S6K1 and S6K2 increased during mitosis. In metaphase, anaphase and telophase the immunofluorescent reaction become much more bright and prominent. The statistical analysis confirmed the corelation between increased S6K1, S6K2 content and activation of proliferation process.

B2.21

Abstract withdrawn

B2.22
Glucocorticoid induced upregulation of apoptotic cell phagocytosis in human dendritic cells

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We have recently observed that transglutaminase 2 (TG2), a crosslinking enzyme, is involved in the regulation of the apoptophagocytic system in mouse macrophages; its deletion leads to impaired apoptotic cell phagocytosis and to the development of autoimmune disorders. Glucocorticoids represent powerful anti-inflammatory compounds due to their capacity to inhibit inflammatory cell recruitment and down-regulate production and responsiveness to pro-inflammatory cytokines. Long-term exposure of human monocytes to the synthetic glucocorticoid dexamethasone (Dex) reprograms their differentiation toward a pro-resolution phenotype, and this includes increased capacity for phagocytosis of apoptotic cells. In our study we have investigated the role of TG2 in phagocytosis of apoptotic cell by Dex treated immature dendritic cells (iDC). Dex increases the phagocytosis of apoptotic neutrophils by iDC. A Taqman Low Density Array with 95 apopto-phagocytic genes showed that Dex downregulated the expression of TG2 among other genes, but upregulates various phagocytosis receptors like MERTK, CD14, cell surface molecules like adenosine A3 receptor, bridging molecules like C1QA and receptors that take part in signalling such as ALOX5 or effectors (e.g. DNASE2). Although TG2 is strongly downregulated by Dex, immature dendritic cells have increased phagocytic capacity, which is due to the use of alternative pathways upregulated by dexamethasone.

B2.23
TSC2/PP5 interaction: novel phosphatase for tumor suppressor?

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Understanding the relationship between growth and proliferation in multicellular organisms requires identification of the key regulators of growth control and understanding of how they regulate growth and how growth is linked to cell proliferation. Recent progress in understanding the mechanisms of growth control indicates that the tumour-suppressor tuberous sclerosis complex TSC1/2 plays a major role in negative regulation of cell growth. TSC1/2 complex made of two proteins TSC1 and TSC2. Mutations in either TSC1 or TSC2 genes are genetically linked to TSC syndrome, connected with the development of hamartomas in numerous organ systems. Both protein products of TSC1 and TSC2 genes form an intracellular complex exerting GAP activity towards a small GTP binding protein Rheb. The activity of TSC1/2 complex is regulated by multiple phosphorylations of TSC2 mediated by several kinases, such as PKB, AMPK, etc. So far, very little is known about the molecular mechanisms of TSC2 dephosphorylation. In our previous research using the Y2H-screening we have identified a number of potential TSC2 binding partners including protein phosphatase 5 (PP5). The aim of this study was to characterize interactions between TSC2 and PP5. Data presented in this

work indicate that TSC2 interacts with PP5 *in vivo* and efficiency of such interaction depends on physiological status of cells. In addition, PP5 is capable of dephosphorylating TSC2 *in vitro* at AMPK potential sites. Our data suggest that the physiological relevance of TSC2/PP5 interaction should be in reversing the stimulatory signaling mediated by AMPK on TSC2 activity.

B2.24
Short and long-term regulation of the RhoA specific GEF isoforms Net1/Net1A during TGF-β mediated regulation of epithelial polarity and EMT

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In the present study we analyzed the role of TGF-β in the regulation of the RhoA specific guanine exchange factor Net1. The two GEF isoforms Net1 and Net1A exist mainly in the nucleus and partly in the cytoplasm. Cytoplasmic Net1A localization is slightly higher than Net1 leading to an increased ability of Net1A to activate RhoA. We report here that TGF-β transcriptionaly upregulated the Net1A but not the Net1 isoform in HaCaT keratinocytes. This was correlated with an upregulation of the cytoplasmic protein levels of Net1A that potently activated RhoA. Both Smad2 and Smad3 proteins are involved in the induction of Net1A by TGF-β. This was evident by overexpression experiments using adenoviral mediated gene expression of wtSmad2 and wtSmad3 as well as by silencing experiments using siRNAs targeting Smad2 or Smad3. The induction of Net1A by TGF-β was also completely abolished using the MEK1 inhibitor UO126, implying the involvement of the Erk pathway in this process. We also report that both isoforms were differentially downregulated upon long term TGF-β treatment. Indeed, Net1 was downregulated at the mRNA level while Net1A was targeted for degradation at the protein level, as indicated by using the MG132 proteasome inhibitor. Finally, siRNA mediated silencing of both Net1 and Net1A resulted in disruption of E-Cadherin mediated adherens junctions in HaCaT keratinocytes. Our results emphasize the role of Net1A in TGF-β mediated regulation of epithelial cell polarity and epithelial to mesenchymal transition.

B2.25
Identification of the cellular targets and mechanism of action of the glycerophosphoinositols

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The glycerophosphoinositols (GPIs) are ubiquitous, bioactive metabolites produced by the phospholipase A2IVα activity on membrane phosphoinositides. With their mechanism of action including modulation of adenylyl cyclase and Rho-GTPase activities or increase of intracellular calcium levels, the glycerophosphoinositols have multiple effects: induction of cell proliferation in thyroid cells, stimulation of the chemotactic response in lymphocytes, modulation of actin cytoskeleton organisation in fibroblasts and reduction of the invasive potential of metastatic cells. In order to define the GPIs mechanism of action, we set out to identify their protein targets. Therefore, a proteomic approach based on high-throughput-differential-LC-MS/MS analysis was employed using modified GPIs, with a biotin moiety bound to their glycerol backbone (biotinylated-GPIs). The targets identified so far include proteins known to be involved in cell signalling, cytoskeleton organisation, protein folding and metabolic processes. We have focussed our attention on the Src-homology-phosphatase-1 (Shp-1), a well-known regulator of Src activation, since it could be related to the reported signalling pathway leading to the glycerophosphoinositol-mediated modulation of the actin cytoskeleton, that involves Src. We have first verified the binding between biotinylated-GPIs and recombinant Shp-1, in pull-down assays using streptavidin-coated beads and confirmed their direct interaction. Preliminary experiments using in-vitro phosphatase assays show that GPIs affect the Shp-1 enzymatic activity. With this approach we have defined the cascade involved in the GPIs-mediated control of the actin cytoskeleton.

B2.26
Decrease of thyrocyte functional activity *in vitro* is accompanied by subcellular redistribution of S6K1 and S6K2 in primary monolayer culture

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Thyroid function is closely related to its follicular structure. Loss of this specific organization in primary culture leads to the dedifferentiation of thyrocytes. TSH and insulin are the main regulators of thyroid function and realise their effect through PI3K signalling pathway. The ribosomal protein S6 kinase is an important member of this pathway. Depending on physiological status of tissue, S6K could be found in the cytoplasm or in the nucleus as well. The aim of this work was to determine subcellular localization of S6K1 and S6K2 forms of rpS6 kinase in the primary monolayer culture of thyrocytes obtained from undamaged follicles. Rat thyroid follicles were placed at low density into dishes coated with gelatine. At the 3rd, 6th and 10th day of cultivation the distribution of to S6K1 and S6K2 in cell compartments was subjected to immunocytochemical and Western blot analysis. Upon cultivation follicles completely lost their organization and transformed into uniformed monolayer. This was accompanied by gradual decrease of intracellular thyroglobulin content. Follicles rearrangement was accompanied by the changes in cytoplasm/nucleus distribution of S6K. At the 3rd day of cultivation the S6K was found only in the cytoplasm, while after 6 and 10 days of cultivation, both S6K1 and S6K2 were detected in both cytoplasm and nuclei. Moreover, the cells with S6K1/2-positive nuclei appeared at the leading edge of outspreading follicle, whereas in the centre of sheet the nuclei were still non-stained. These results strongly suggest that the loss of thyrocyte follicular organization in monolayer culture is accompanied by subcellular redistribution of S6K1 and S6K2.

B2.27
Subcellular redistribution of rpS6 kinase is related to functional activity of rat thyrocytes in 2D and 3D culture

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The kinase of ribosomal protein S6 is an important member of PI3K signal transduction pathway involved in control of protein synthesis and G1/S transition of the cell cycle. There are two forms of this kinase S6K1 and S6K2. Previously we have shown that in normal thyroid tissue they are detected predominantly in the cytoplasm of thyrocytes whereas in monolayer culture in course of follicle outspreading S6K1 and S6K2 appeared in nuclei as well. The goal of presented work was to detect which processes involved in follicle transformation from 3D structure to monolayer colony (migration, proliferation, loss of follicle organization) is related to S6K subcellular redistribution. The cultivation of thyrocytes resulted in activation of cell proliferation. But there was not the correlation between S6K1/2 subcellular relocalization and Ki-67 appearance in proliferating cells. To study possible effect of migration on subcellular localization of S6K1 and S6K2, cultured thyrocytes were stimulated to penetrate a porous membrane of Transwell. But immunocytochemical analysis revealed cytoplasmic localization S6K1/2. Detection of the content of these kinases in thyrocytes in 3 D culture with retention of follicle structure shown that in thyroglobulin-positive cells from follicle like structures S6K1/2 localized in cytoplasm, but in thyroglobulin-negative cells from solid areas of cultured aggregates of follicles S6K1/2 were observed in nuclei as well (like in monolayer cultures). Thus, the change of subcellular localization of S6K1/2 in cultured thyrocytes is directly related to change of level of functional activity, unlike the processes of proliferation and migration.

B2.28
Loss of RAF kinase inhibitor protein is a somatic event in the pathogenesis of therapy-related acute myeloid leukemias with C-RAF germline mutations

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While B-RAF is frequently involved in human tumor development, the first oncogenic C-RAF mutations have been described only recently by us in patients with therapy-related acute myeloid leukemia (t-AML). Despite germline presence of mutant C-RAF normal development in carriers may have been possible because of the weak nature of this mutation and the restriction of RAF signaling to the transformed cells. In the work presented here we searched for cooperating events in leukemogenesis and identified by immunoblotting the loss of the tumor and metastasis suppressor RAF kinase inhibitor protein (RKIP) in blast cells carrying mutant RAF. Since primary tumors had normal RKIP expression levels the loss of RKIP is most likely a somatic, t-AML-specific event. In focus formation assays the number of colonies formed by C-RAF S427G was significantly increased by RKIP silencing and the opposite was observed after RKIP overexpression. We therefore speculate that the loss of RKIP is a functional somatic event contributing to the development of t-AML, which manifests itself only in carriers of C-RAF germline mutations.

B2.29
Life/death decisions in growth factor signaling: Role of mitochondrial events

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Prolonged growth factor deprivation is a common physiological stimulus for the induction of apoptosis. Cell death under these conditions can be delayed through survival kinases or antiapoptotic members of Bcl-2 protein family. However, it is still largely unclear how the lack of a growth factor is translated to the death stimulus and how precisely survival proteins counteract these processes. We have been able to show that increased mitochondrial ROS production and mitochondrial Ca^{2+} overload following IL-3 withdrawal in 32D cells are essential intermediates in cell death induction, whose actions can be prevented by activated RAF and AKT or the overexpression of Bcl-2. To further define possible steps along the mitochondrial pathway to apoptotic cell death, which are susceptible to regulation by survival proteins, we analyzed changes in the mitochondrial membrane potential following growth factor removal. The drop in mitochondrial membrane potential ($\Delta\Psi$) after IL-3 removal was suppressed by activated RAF and overexpressed Bcl-2. Additionally, the treatment with the antioxidant *N-acetylcysteine* (NAC), which rescued cells from apoptosis, also prevented a drop in $\Delta\Psi$ following IL-3 withdrawal in 32D cells. These findings further support the critical role of mitochondrial events as targets for survival pathways.

B2.30
Optic Atrophy 1 is an A-kinase anchoring protein that mediates adrenergic control of lipolysis

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Adrenergic stimulation of adipocytes yields a cAMP signal that activates protein kinase A (PKA). PKA phosphorylates perilipin, a protein localised on the surface of lipid droplets that serves as a gate-keeper to regulate access of lipases converting stored triglycerides to free fatty acids and glycerol in a phosphorylation-dependent manner. Here, we report a new function for Optic Atrophy 1 (OPA1), a protein known to regulate mitochondrial dynamics, as a dual-specificity A-kinase anchoring protein associated with lipid droplets. By a variety of protein interaction assays, immunoprecipitation and immunolocalization experiments, we show that OPA1 organizes a supramolecular complex containing both PKA and perilipin. Furthermore, by a combination of siRNA-mediated knockdown, reconstitution experiments using OPA1 with or without the ability to bind PKA and cellular delivery of PKA anchoring disruptor peptides we demonstrate that OPA1 targeting of PKA to lipid droplets is necessary for hormonal control of perilipin phosphorylation and lipolysis.

B2.31
Regulation of calcium signaling in non-excitable polarized exocrine cells from Homer2-Deficient mice

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The Homers are scaffold proteins and consist of an N-terminal Ena/VASP homology 1 (EVH) protein-binding domain and

C-terminal leucin zipper/coiled-coil domain. The EVH domain recognizes the proline-rich motifs and binds many Ca^{2+} signaling proteins including G protein-coupled receptors, inositol 1,4,5-triphosphate (IP_3) receptors (IP_3Rs), ryanodine receptors, and TRP channels. However, their role in Ca^{2+} signaling of non-excitable cells is not well known. In the present work, we investigated the role of Homer2 in Ca^{2+} signaling in parotid gland and pancreas acinar cells using $Homer2^{-/-}$ mice. Homer2 showed polarized luminal localization in parotid acinar cells, but the deletion of Homer2 did not affect a localization of IP_3Rs or IP_3R channel activity. The protein expression level of plasma membrane Ca^{2+} ATPase (PMCA) was increased in parotid glands, whereas sarco/endoplasmic reticulum Ca^{2+} ATPase was increased in pancreas of $Homer2^{-/-}$ mice. We found that deletion of Homer2 increased PMCA activity and Homer2 interacted with PMCAs in parotid cells. Moreover, the mRNA expression levels of TRPC3, Orai1 and STIM1 were increased and inhibitory effects of blockers on Ca^{2+} entry were diminished in pancreas cells of $Homer2^{-/-}$ mice. These findings suggest that Homer2 may play an important role in regulation of Ca^{2+} signaling in non-excitable cells. This work was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Korea (A084007 and A090807).

B2.32
An integrated experimental workflow to increase throughput and data robustness for analysis of mammalian protein interaction networks

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To understand the organization of protein complexes and how biological information is propagated via protein-protein interaction is becoming increasingly important to get new functional insights on cellular processes. Affinity purification coupled to mass spectrometry (AP-MS) has been successfully used in the past to characterize protein complexes. However, mainly due to significant experimental limitations the success and power of the AP-MS strategy has been underrated to date. Here, we describe an integrated workflow for characterization of mammalian protein complexes. The experimental pipeline comprises rapid generation of isogenic mammalian cell lines, protein complex analysis using an efficient double affinity purification strategy followed by state-of-the-art nano-LC-Orbitrap-MS analysis. We have used the system to study interactions associated with the human protein phosphatase 2A and NF-kB systems. Analysis of these systems revealed new components of protein network structures that are linked to a number of important biological processes such as transcription, the cell proliferation, apoptosis and inflammation. The high performance of our strategy is well suited to identifying protein interactions partners that can subsequently be used as (i) entry points for focused research projects and (ii) to more globally map out whole signaling systems linked to important biological processes.

B2.33
Identification and functional study of CDK14/CCNY interacting proteins

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The CDKs are a family of Ser/Thr protein kinases that share a highly conserved PSTAIRE motif which is involved in cyclin binding and has been used to classify CDK-related kinases. We have reported human PFTK1 (CDK14), which is highly expressed in brain, pancreas, kidney, heart, testis and ovary. To search its substrate and regulatory proteins, we screened a two-hybrid library and identified seven PFTK1 interacting proteins including four 14-3-3 isoforms, a septin family member KIAA0202 (SEPT8), PLZF protein and a novel cyclin, cyclin Y (CCNY). Identification of the CCNY interacting proteins by two-hybrid screening revealed PFTK1 (CDK14) and four 14-3-3 isoforms. The CCNY is a regulatory partner of PFTK1 (CDK14), which recruited PFTK1 (CDK14) to the plasma membrane and activated its associated kinase activity. Membrane asssoaciated but not cytoplasm or nucleus localized CCNY is a substrate of CDK14-CCNY complex. 14-3-3s are a family of highly conserved acid proteins, which are expressed ubiquitously in all eukaryotic cells. The interaction between the 14-3-3 and CCNY or 14-3-3 and CDK14 was confirmed by co-immunoprecipitation and yeast two hybrid assay. The CCNY contains two putative 14-3-3 binding consensus motifs, RAS66T67IFLS and RKRS324AS326, the later is required for their interactions. The CDK14 contains a putative 14-3-3 binding consensus motif (RHS119S120PSS), which overlapped with its second NLS. The Ser119 is crucial for the interaction between CDK14 and the 14-3-3 proteins. The effects of 14-3-3/CCNY, 14-3-3/CDK14 and CCNY/CDK14 interactions on their subcellular localization and phosphorylation were investigated.

B2.34
Roles of human Cyclin Y (CCNY) in cell cycle

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Cyclins are regulatory subunits that associate with specific CDKs through the cyclin box to form functional protein kinase complexes. PFTK1 belongs to the subgroup of Cdc2- related kinase family and was characterized as a CDK that regulates cell cycle progression and cell proliferation. We identified a novel protein CCNY (Cyclin Y) as a PFTK1 (CDK14) interacting protein in a yeast two-hybrid screen. The cyclin box in CCNY and the PFTAIRE motif in PFTK1 (CDK14) are both required for the interaction which was confirmed by *in vivo* and *in vitro* assays. Two CCNY transcripts were detected in all tissues and cell lines. A 4.0 kb transcript is ubiquitously expressed at low level in human tissues, but 2.0 kb transcript is particularly abundant in testis, also expressed at high level in heart and skeleton muscle, and at low level in the other tissues. In cell lines, both the 4.0 kb and 2.0 kb transcripts are expressed at low level including 293T and NIH3T3, but higher level in HepG2. The CCNY was enriched at the plasma membrane due to an N-terminal myristoylation signal. The CCNY was also shown to affect the cell cycle progression. Ectopic expressed CCNY in 293T and HepG2 accumulated cells in S-phase and G2/M phase. Our data indicate that the CCNY is a regulatory partner of PFTK1 (CDK14), binding of CCNY to PFTK1 (CDK14) changes its intracellular location and regulates the PFTK1 (CDK14) activity.

B2.35
Vip induces NF-κB1 transactivation by different signaling pathways in human tumor and non-tumor prostate cells

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Introduction: Vasoactive intestinal peptide (VIP) behaves as a pro-metastatic factor in human prostate cancer cells. The involvement of nuclear factor-κB (NF-B) was demonstrated since the agent curcumin blocked VIP effects on the biomarkers related with the different steps of the metastatic cascade (Fernandez-Martinez et al., 2009). The main aim of this study was to test the signaling pathways involved in VIP-NF-κ B1 (p50) transactivation in human prostate cells in order to characterize the VIP mechanism of action in the prostate gland.
Methods: We evaluated the effect of VIP on transactivation of NF-kB1 (p50) in three human prostate cell lines: RWPE1 (non-tumor cells) and LNCaP and PC3 (tumor cells). In our study, ELISA and confocal immunofluorescence microscopy were performed.
Results: VIP increases the binding of NF-κB1 to the promoter of its target genes in three human prostate cells and provokes faster responses according to the most aggressive status (RWPE1 < LNCaP < PC3 cells). We show, using specific kinase inhibitors and the EPAC agonist (O-CPT-2Met cAMP), that in control cells (RWPE1), the effect is mediated by activation of cAMP-dependent protein kinase (PKA) and implicates neither the ERK1/2 nor the PI3K pathways as occurs in tumor cells, LNCaP y PC3. The PKA-independent GEF/EPAC pathway is involved in the transformed cells but not in the control cells. Curcumine inhibits NF-kB1 transactivation in all three cell lines.
Conclusions: Our results indicate that VIP uses different pathways for NF-κB1 (p50) transactivation, transformed cells resort on pro-survival and pro-proliferative signaling pathways including ERK1/2, PI3-K and cAMP/EPAC.

B2.36
Thrombin induces inducible nitric oxide synthase expression via Ras, Raf-1, ERK, and NF-κB signaling pathways in NR8383 lung macrophages

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Thrombin, a serine protease, is a well-known coagulation factor generated in vascular injury and which also plays an important role in lung inflammatory diseases. However, little is known about the signaling pathway in thrombin-induced iNOS expression in lung macrophages. In this study, we investigated the Ras/Raf-1/ERK and IKKα/β/NF-κB in thrombin-induced iNOS expression in NR8383 lung macrophages. Thrombin caused time- and dose-dependent increases in iNOS expression, which was attenuated by a Ras inhibitor (manumycin A), dominant negative mutant of Ras (RasN17), a Raf-1 inhibitor (GW 5074), and a mitogen-activated protein kinase kinase (MEK) inhibitor (PD 98059). Treatment of NR8383 macrophages with thrombin caused time-dependent activations of Ras, Raf-1, and extracellular signal-regulated kinase (ERK). The thrombin-induced increase in Ras activity was inhibited by manumycin A. Raf-1 phosphorylation at Ser338 by thrombin was inhibited by manumycin A and GW 5074. The thrombin-induced increase in ERK activity was inhibited by manumycin A, GW 5074, and PD 98059. Treatment of macrophages with a NF-κB inhibitor (PDTC), an IκBα phosphorylation inhibitor (Bay 117082), and IκBM all inhibited thrombin-induced iNOS expression. Stimulation of cells with thrombin activated IKKα/β, IκBα phosphorylation, IκBα degradation, and κB-luciferase activity. The thrombin-mediated increase in the activities of IKKα/β and κB-luciferase were also inhibited by the manumycin A, GW 5074, and PD 98059.*f*nThese results indicated the Ras/Raf-1/ERK pathway, which in turn initiates IKKα/β and NF-κB activation, and ultimately induces iNOS expression in NR8383 macrophages.

B2.37
Ganoderma tsugae extracts induce cell cycle arrest and apoptosis in lung cancer cells

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Background: Lung cancer ranks the 1st cancer death in Taiwan. Improved therapeutic methods are thus especially needed. Bioactive botanical products are found to be useful in treatment of clinical diseases. Ganoderma, also known as Lingzhi, has long been used for human health in oriental countries. We have demonstrated that Tien-Shen-Lingzhi (TSL), an improved species of Ganoderma tsugae (Gt) in Taiwan, is effective against colon and epidermoid carcinomas. Here, we focus on the growth inhibition effects of TSL extracts (TSLE) on lung cancer cells.
Objective: To evaluate the anticancer potential of TSLE on lung cancer cells via *in vitro* and *in vivo* experiments.
Methods: The quality control (QC) of TSLE was monitored by chemical fingerprints (CF) using high performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry (ESI-MS). A non-small cell lung cancer cell line, H23/0.3, was used for testing the anticancer effects of TSLE. In addition, MTT assay, flow cytometry, confocal microscopy, western blotting, and xenograft mouse model were also applied for this study.
Results: The CF profiles revealed identical patterns for TSLE from three individual batches of Gt. TSLE inhibited H23/0.3 cell proliferation with an IC50 of 0.37 mg/ml. TSLE-induced G1/S accumulation followed by cell apoptosis were observed. Western blotting results demonstrated decrease of cyclin A2 and increase of caspase-3 after TSLE treatment in a dose-dependent manner. Retardation of tumor growth was found in TSLE-treated mice.
Conclusion: TSLE demonstrated anticancer effects on lung cancer H23/0.3 cells. The active anticancer ingredient in TSLE remains to be investigated.

B2.38
A whole-genome microarray approach to signal pathway of growth inhibition effects of Ganoderma tsugae extracts on lung cancer cells

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Background: Lung cancers are among the neoplastic diseases with the worst prognosis. More treatment modalities are needed to improve clinical outcome. It's being a new trend to search for bioactive traditional Chinese medicines (TCM). Ganoderma, also known as Lingzhi, is a TCM and has been widely used for improving human health for centuries. We have addressed the anticancer effects of Ganoderma on various cancers. Here, a comprehensive genomic profiling was used to further explore the signal pathway for the anticancer effects of Ganoderma tsugae (Gt).
Objectives: To unravel the cellular effects of Tien-Shen-Lingzhi (TSL), an improved species of Gt in Taiwan, on non-small cell lung cancer (NSCLC) H23/0.3 cells.
Methods: A comparative genomics study was performed to search for TSL-mediated differential expressions on H23/0.3 cells. Bioinformatics tool, e.g. GeneGo MetaCore, was used to analyze the experimental results based on Affymetrix Human Genome U133 Plus 2.0 microarray.
Results: The whole-genome expression profiles of H23/0.3 with or without TSL treatment were resolved by multidimentional scaling (MDS) analysis. It indicated a TSL-mediated cell cycle perturbation. Bioinformatics analysis showed cell cycle was the most affected pathway. Genego MetaCore-derived cellular pathway map revealed dramatic hits (17/26) on the DNA replication. Some selected genes were confirmed by Q-PCR showing in concordance with the microarray results.
Conclusion: The genomic microarray data demonstrates the TSL-induced cell cycle deregulation in H23/0.3 cells. TSL-mediated cell cycle perturbation on H23/0.3 cells is consistent with the cellular pathway map resulting from Genego analysis.

B2.39
Molecular pathway of growth inhibition effects on breast cancer cells by Tien-Shen-Lingzhi

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Traditional Chinese Medicine (TCM) has been used to preserve the human vitality, to promote longevity, and for the prevention or treatment of a variety of diseases including cancer. Ganoderma, also known as Lingzhi, is a TCM and has been used for medicinal purposes for centuries. Among the eight common species of Ganoderma in Taiwan, Ganoderma tsugae (Gt) has been used in our study for fighting cancers. We have demonstrated that Tien-Shen-Lingzhi (TSL), an improved species of Gt in Taiwan, is effective against human breast cancer cell lines MCF7. The purpose of this study is to further unravel its molecular mechanism. Our results demonstrate that the ethanol extract of TSL (TSLEE) inhibits the proliferation of human breast cancer cells through the induction of cell cycle arrest. TSLEE-induced cell cycle arrest is associated with a dramatic decrease at the protein expression level of cyclin D1 and cyclin E in a dose-dependent manner, i.e. G1 arrest. Upon exposure of human breast cancer cells to TSLEE results in growth inhibition as evidenced by inhibiting PI3K/Akt pathway. Moreover, our data show that TSLEE can also reduce the chemoresistance of the anti-cancer drug Taxol, thereby increasing the clinical effectiveness of the treatment of breast cancer. In conclusion, our findings establish a mechanistic link between the PI3K/Akt pathway and TSLEE-induced cell cycle arrest. Our results also provide insightful understanding of using the TSL as a therapeutic TCM for the clinical treatment of human breast cancer.

B2.40
Polymethinium salts as new type ligands for polysulfate VEGF coreceptors

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VEGF (Vascular endothelial growth factor) signaling pathway play one of the key roles in many physiological and pathological processes such as cancer. Important parts of this pathway are VEGF receptors and their polysulfate saccharide coreceptors. Their modulation and can be effective strategy of cancer treatment. Their sensing can be neccessery for control cancer treatment. Therefore we study recognition polysulfate saccharide motive by polymethinium salts. We observed strongly selectivity of polymethinium salts for this structure motive coupled with strongly spectral change. This work was funded by grant from the Grant Agency of the Czech Republic (Grant No. 203/09/131) and supported in part by project LC06077 awarded by the Ministry of Education of the Czech Republic, by project AV0Z50520514 awarded by the Academy of Sciences of the Czech Republic to J. Králová and by project MSM6046137307 to V. Kral.

B2.41
Sumoylation of ultraspiracle protein from *Drosophila melanogaster*

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The ultraspiracle (USP) heterodimerizes with the ecdysone receptor (EcR) to form a functional complex EcR/Usp that mediates the effects of the steroid molting hormone ecdysone by activating and repressing expression of ecdysone response genes in insects. Complex EcR/Usp from *Drosophila melanogaster* provides an ideal model system for construct gene switches in humans. However, there is no information about posttranslational modification of Usp such as sumoylation. To investigate sumoylation of Usp we used Ubc9 fusion-directed sumoylation (UFDS) system [Jakobs *et al.*, 2007] which is based on fusion of conjugating enzyme Ubc9 with substrate protein. We prepared constructs coding fusion proteins where Ubc9 was placed to the N- or C-terminus of Usp (Ubc9-Usp, Usp-Ubc9), expressed them in HEK293 cells and analyzed the protein extracts by western blotting with an Ubc9 or an Usp antibody. The results revealed that Ubc9-Usp is observed as two different migrating bands, of which the upper migrated slower when EGFP-SUMO1 was coexpressed, suggesting that the higher-molecular-weight protein consist of SUMO conjugated Ubc9 fusion protein. Usp-Ubc9 is also sumoylated although less efficiently. To determine region of the modification, we prepared fragments of Usp including A/B region, DNA binding domain

(DBD) and ligand binding domain (LBD) fused to Ubc9. For A/B region and LBD we showed that there are potential residues that can be sumoylated. We also used bio-informatics tools (SUMOplot, SUMOsp and PCI-SUMO) to predict and score potential sumoylation sites in Usp. Using this data, we prepared some point mutants. Identification of Usp sumoylation sites is currently underway.

B2.42
Effect of Ang II on the generation of superoxide anion via NAD(P)H oxidase enzyme complex in primary cultured vascular smooth muscle cells isolated from spontaneously hypertensive and normotensive rat aortas, and role of Src in this metabolic pathway

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Ang II contributes to altered vascular tone, endothelial dysfunction, structural remodeling and vascular inflammation, characteristic features of vascular damage in hypertension. Ang II induces its effects via G-protein-coupled transmembrane receptor, Ang II receptor 1 (AT1R). The best characterized system in vascular cells is Ang II-stimulated NAD(P)H oxidase-mediated generation of superoxide anion, which appears to be upregulated in hypertension. Vascular smooth muscle cells (VSMC) were isolated from thoracic aortas of spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto rats (WKY). VSMC, to determine the AT1R-mediated Src phosphorylation and superoxide anion production via NADPH oxidase, were stimulated with Ang II with or without Losartan, diphenyl iodonium, PP1 and GF109203X. Src phosphorylation was determined by western blot method. Superoxide anion production was measured by cytochrom c reduction method. Src phosphorylation increased both SHR and WKY groups after Ang II stimulation compared with controls. On the other hand, Src phosphorylations were decreased both SHR and WKY groups after Ang II stimulation with inhibitors except DPI. Superoxide anion production was enhanced in both two groups in Ang II-stimulated cells. However superoxide anion production in SHR was significantly greater than WKY. All inhibitors reduced superoxide anion production both SHR and WKY groups in spite of Ang II stimulation. We found that Src and PKC phosphorylation may occur before the NAD(P)H activation. We showed that Ang II induced NADPH oxidase via Src.

B2.43
p38 MAPK phosphorylation by angiotensin II stimulation via NADPH oxidase in primary cultured vascular smooth muscle cells

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Angiotensin II (Ang II) has an important role in atherosclerosis, hypertension, pathogenesis of cardiovascular diseases and regulating blood pressure. It was shown that, in vascular smooth muscle cells (VSMC), Ang II activates various signal transduction pathways of p38 mitogen activated protein kinases (MAPKs). Ras can activate p38 MAPK through signal transduction pathway of NAD(P)H oxidase. VSMC cultured, used in the experiments, showed 99% positive immunostaining of smooth muscle α-actin antibody. This study was aimed to investigate whether or not Ras or NAD(P)H oxidase activation have a role in p38 MAPK phosphorylation after stimulation with Ang II in VSMC cultured. Phosphorylation was shown using western-blott techniques with specific phospho-antibodies against p38 MAPK proteins. In cultured rat vascular smooth muscle cells, angiotensin II (Ang II) induced a rapid increase in p38 mitogen activated protein kinase (MAPK) activity through the Ang II type 1 receptor. Ang II induced MAPK activity was abolished by the Ras inhibitor, FTS and NAD(P)H oxidase inhibitor DPI.

B2.44
Intracellular signaling cascades triggered by FPRL1 in human prostate cells

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We investigated intracellular signaling pathways triggered by stimulation of formyl peptide receptor-like 1 (FPRL1) with WKYMVm in human prostate cell line PNT1A. In growth-arrested cells, exposure to WKYMVm induced MEK-dependent ERKs activation as well as pertussis toxin-dependent (PTX) and ERK-dependent p47phox phosphorylation. We also examined the ability of the FPRL1-induced signaling cascade to transactivate Met receptor, that is expressed on cancer cells and is implicated in cancer progression. We demonstrated that PNT1A cell line express a biologically functional FPRL1 and that incubation of these cells with WKYMVm induce transactivation of Met receptor. Western blot experiments, performed with specific anti-pY antibody, showed that following FPRL1 agonist stimulation Met receptor resulted phosphorylated on Y1313, Y1349 and Y1356 residues. These events are prevented by pretreatment with WRW4, an antagonist of FPRL1, and by PTX, a selective inhibitor of Gi proteins. Moreover, the transphosphorylation of Met receptor on Y1313, Y1349 and Y1356 residues are also prevented by pretreatment with DPI, an inhibitor of NADPH oxidase, suggesting a key role of ROS in this process. We also demonstrated that stimulation with WKYMVm of PNT1A cells triggered the activation of further signaling pathways, involved in cell survivor, proliferation and neoangiogenesis such as PI3K/Akt and STAT3 pathway. These intracellular signaling cascades are prevented by pretreatment with WRW4, PTX and SU11274, a selective inhibitor of TK-activity of Met receptor. These studies suggest that stimulation of FPRL1 with a specific agonist contribute to the progression of tumorigenesis in prostate.

B2.45
Acute modulation of the proteome by physiological hypoxia

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Suitable provision of the oxygen (O_2) required for the aerobic synthesis of adenosine triphosphate (ATP) is a major physiologic challenge since O_2 deficit, even transient, can produce

irreversible cellular damage. Hypoxia has a critical role in the pathogenesis of major causes of mortality, such as myocardial infarction, stroke, and chronic lung disease, as well as in reperfusion injury of transplanted organs and carcinogenesis. Acute hypoxia triggers respiratory and cardiovascular systemic reflexes to ensure fast O_2 delivery to the brain or the heart, however upon exposure to protracted hypoxia cells generate homeostatic responses to minimize the deleterious effect of O_2 deficiency. Cell adaptation to hypoxia depends on transcriptional and non-transcriptional mechanisms whose nature is as yet only partially known. In order to characterize the initial homeostatic responses elicited by the lack of O_2 we have evaluated the proteomic changes associated with an acute and physiological drop in oxygen tension. Hypoxia leads to adaptive changes in the proteome that results in a tight control of the ATP expenditure. To evaluate the role of the well-know O_2 sensors prolyl-4-hydroxylases (PHDs) in this fast regulation we have estimated the changes in the proteome produced by acute PHDs inhibition. A subset of the modifications observed under hypoxia was mimicked by PHDs activity inhibition. Here we present the results obtained with these two proteomics approaches and the initial characterization of the mechanisms underlying the acute responses to low O_2 circumstances.

B2.46
Deriving Ca(2+) signals starting from puffs: The mechanism buffers genetic and environmental variability by functional robustness

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Puffs are elemental events of Ca(2+) release from the ER through clusters of Ca(2+) channels. Much evidence now supports the idea that intracellular Ca(2+) dynamics are stochastic. However, stochastic mathematical models have so far been limited by the large number of states which emerge from the spatial heterogeneity of Ca(2+) channels. Furthermore, most existing models fail to match statistical properties of measured Ca(2+) signals. As a new concept, we formulate a theory in terms of interpuff interval and puff duration distributions, and verify it with a series of long-time measurements of single cell Ca(2+) spikes in HEK-293 cells. Our theory reproduces the typical spectrum of measured Ca(2+) signals like puffs, spiking and bursting, in analytically treatable test cases as well as in more realistic setups. We find criteria for spiking in terms of the strength of spatial coupling and the channel closing rate. As a main result, we formulate functional robustness in terms of the relation between average and standard deviation of interspike intervals. This relation describes the function of Ca(2+) signaling to spike faster and more regular upon stimulation. In the model, it does not depend on cluster properties and cluster arrangement, and is determined only by global characteristics of the Ca(2+) signaling pathway. This unifies robustness of function with sensitivity for feedback and control. Our findings were confirmed by *in vivo* measurements of Ca(2+) spikes. In particular, we show that pharmacological reagents like PTH, U73122 and CPA fail to modulate spike statistics, as predicted by the model.

B2.47
Cancer cell-derived microvesicles constitute a novel mediator of hypoxia dependent induction of pro-angiogenic signaling

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Hypoxia is a hallmark of solid tumors and a major driving force in tumor angiogenesis and metastasis. Although, the role of several hypoxia-induced growth factors, cytokines, and proteases has been clearly established, the exact mechanisms of how hypoxia promotes tumor development remain to be defined. Membrane-derived microvesicles (MVs) released from malignant cells may promote tumor progression by re-modelling the tumor microenvironment through effects on stromal cells. Here, we show that hypoxic alterations in the molecular composition of MVs derived from human glioblastoma cells (U87MG) are associated with a pro-angiogenic signalling response. Microarray analysis of mRNAs (approx. 27.000 sequences) in normoxic and hypoxic MVs demonstrated hypoxic regulation of a subset of transcripts, several of which have been implicated in angiogenesis. Moreover, comparative analysis of angiogenesis-related proteins showed up-regulation of specific pro-angiogenic proteins in hypoxic MVs in comparison to normoxic ones. MV stimulation of primary, human endothelial cells (HUVECs) resulted in the activation of key signalling pathways, most notably mTOR, p70 S6 Kinase and STATs. Interestingly, hypoxic alterations of the molecular composition of MVs correlated with significantly greater effects on proliferation and survival of HUVECs by hypoxic MVs as compared with normoxic MVs. These results provide important insight into how the oxygenation status of the tumor microenvironment may affect the composition of cancer cell-derived MVs and implicate MVs as an important communication pathway in the adaptive response of malignant cells to hypoxic stress.

B2.48
The PKCα-Plectin interaction controls the cytoskeleton assembly in PC12 cells differentiated with NGF and ATP

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PKCα is a key mediator of neuronal differentiation mediated by NGF and ATP, but its downstream pathways largely remains to be elucidated. To identify signaling partners of PKCα, we analyzed proteins co-immunoprecipitated with this enzyme in PC12 cells differentiated with NGF and ATP and compared them with those obtained with NGF and growing media. Mass spectrometry analysis (LC-MS/MS) allowed the identification of several potential interacting proteins, being plectin and actin some of them. These putative PKCα-binding partners were validated by double-immunofluorescence and co-localization analysis of the endogenous proteins in the same cells. An increase in the co-localization of PKCα and plectin was detected when PC12 cells were differentiated with NGF and ATP. Furthermore, the intermediate filament organization of these cells was altered by small interfering RNA directed against PKCα, suggesting that this enzyme is participating in the intermediate filaments assembly during neuronal differentiation through plectin. In addition, cortical remodeling of actin associated with cell migration was reversed by downregulating PKCα synthesis, indicating the involvement of the enzyme in the dynamic reorganization of actin cytoskeleton during this process. Together, these data identify

plectin and actin as new functional targets of the ATP-dependent PKCα signaling and suggest that this enzyme might control neuronal development at different levels by interacting with several cytoskeletal components. [This work was supported by grants from the Fundación Médica Mutua Madrileña, Fundación Séneca 08700/PI/08 and MICINN-Dirección General de Investigación (BFU2008-01010)].

B2.49
Study of proteasome implication in TGF-β1 and IGF-I effects on the production of IL-6, TIMP-1 and type-I collagen by nasal polyps fibroblasts

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Nasal polyposis is a chronic inflammatory disease of the nasal mucosa, that characterized by inflammatory cells infiltration, ECM accumulation and oedema. High expression of TGF-β1 and IGF-I, factors which are implicated in fibrosis, in nasal polyps, has been reported. Proteasome is a large intracellular multisubunit protease complex that selectively degrades intracellular proteins. The aim of this work was to study the effect of TGF-β1 and IGF-I on the production of factors, which may be implicated in nasal polyposis pathogenesis, such as IL-6, TIMP-1, and type-I collagen, as well as the contribution of proteasome in this effect. High expression of IL-6, TIMP-1 and type-I collagen in nasal polyps was ascertained by RT-PCR. Both TGF-β1 and IGF-I caused significant dose- and time-dependent stimulation of IL-6, TIMP-1 and type-I collagen expression in nasal polyps fibroblasts. When fibroblasts were cultured in the presence of proteasome inhibitors, a significant suppression of constitutive as well as of TGF-β1- and IGF-I-enhanced production of TIMP-1 and type-I collagen was observed, whereas the IL-6 expression was enhanced. Both TGF-β1 and IGF-I were able to enhance the proteasome activity and its subunits expression in a dose-depended manner. When polyps explants were subjected to tissue culture in the presence of proteasome inhibitors, a significant suppression of IL-6, TIMP-1 and type-I collagen production was observed. In conclusion, it appears that TGF-β1 and IGF-I may be responsible for the high expression of IL-6, TIMP-1 and type-I collagen in nasal polyps, factors which are associated with ECM accumulation, through a process where the proteasome may be implicated.

B2.50
The cAMP and TGF-β1 pathways, suppress the IL-1β and TNF-α-induced production of Matrix Metalloproteinase-1 from nasal polyps fibroblasts Aacting on the NO and PKC pathways

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IL-1β and TNF-α, the main pro-inflammatory cytokines, stimulate the production of many MMPs by a variety of cells, whereas TGF-β1, an anti-inflammatory factor, induces the expression of TIMP-1 and in some cases it antagonizes the stimulatory effect of IL-1β on the expression of MMP-1 and MMP-3. The same effect has been also exerted by cAMP pathway. Nasal polyposis is a chronic inflammatory disease of the upper airways, in which the IL-1β, TNF-α, TGF-β1, MMPs and their inhibitors, are implicated. When polyps fibroblasts were cultured with IL-1β and TNF-α, a significant stimulation of MMP-1 expression was observed, which significantly inhibited by calphostin C, genistein, U0126, SP600125 and SB203580. The IL-1β- and TNF-α-induced production of MMP-1 was strongly suppressed in the presence of TGF-β1, forskolin and IBMX. The PKA inhibitor H-89 partially reversed the suppressive effect of forskolin but not that of TGF-β1. When fibroblasts were cultured for 2 or 48 hour with PMA, in the presence of TGF-β1 or forskolin, a significant suppression of MMP-1 expression was observed. The nitroprusside, a donor of NO, was also able to induce the MMP-1 expression in polyps fibroblasts, which was significantly inhibited by TGF-β1 or forskolin, while they had no effect on PMA-induced production of NO. In conclusion, TGF-β1 via of an unknown mechanism, and cAMP, possibly via activation of PKA, suppress the IL-1β- and TNF-α-induced production of MMP-1 by polyps fibroblasts. This effect may be mediated by influence on PKC and (or) on other factors which are involved in pathways activated by PKC and NO, but not directly on NO synthase.

B2.51
The role of GCN2 in cell-cycle regulation in mammalian cells

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We have shown that fission yeast cells delay entry into S phase after ultraviolet (UVC) irradiation in G1 phase [1]. More recently, we found that the G1/S transition is totally dependent on the Gcn2 kinase, which phosphorylates the translation initiation factor eIF2 and mediates a general depression of translation [2]. We are currently investigating how UVC irradiation affects the events of G1 phase in mammalian cells, and in particular the activation of GCN2 and its effects on cell cycle progression. It has previously been shown that also in mammalian cells translational repression in response to UV irradiation is dependent on GCN2 and phosphorylated eIF2α [3]. We have UVC-irradiated human U2OS and BJ cells, and confirmed previous findings of eIF2α phosphorylation after irradiation. In addition, we have begun investigating the role of GCN2 in the G1/S transition. GCN2 siRNA knockdown experiments indicate that eIF2α phosphorylation after UVC irradiation is indeed dependent on GCN2. These findings will be discussed and related to the possible checkpoint function of GCN2 in mammalian cells.

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B2.52
Characterization of insulin receptor substrate-4 expression in human colon tumors and tumoral colon lines

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Background and aims: Insulin receptor substrate proteins (IRSs), cytoplasmic adaptors that organize signalling complexes downstream of activated cell surface receptors, are implicated in mediating signals of tumour cells. Recently, it has been shown that IRS-1, IRS-2, and IRS-4 were each overexpressed in 80% of the HCC samples and we have demonstrated that IRS-4 is an essential protein for the proliferation/differentiation of HepG2 human hepatoblastome cells. In the other hand, colon-rectum cancers are one of the major ones in developed countries and their treatments remain poor and systemic therapies have not been efficient. Taking account the important role that IRS-4 shows in HCC and in other types of cancer, we decided to characterize for the first time IRS-4 expression in human colon tumours and tumour colon lines.
Methods: We have employed several tumour colon lines (HCT116, HT29, LOVO, MAWI, COLO205, RKO16) and samples of human colon tumours and adjacent tissue. IRS-4 was analysed by Western blotting using specific antibodies. Immunoprecipitation assays were carried out with anti-IRS-4, anti-pTyr and anti-p85. Immunohistochemistry experiments were performed using anti-IRS-4.
Results and conclusions: Immunohistochemistry results show that in healthy adjacent tissue IRS-4 is located in colon crypts, mainly in crypt colonocytes nucleus. Interesting, IRS-4 is expressed in every colon segments (ascending, transverse and descending colon) and dramatically expressed in tumour colon lines. Moreover, in human colon samples, IRS-4 is constitutively phosphorylated in tyrosine residues. Our results point to a potential role of IRS-4 in human colon tumours development.

B2.53
Comparative analysis of Sp1 gene regulation in an alternating oxygenating microenvironment of human brain tumors

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Sp1 is a human transcription factor involved in gene expression in the early development of an organism. It belongs to the Sp/KLF family of transcription factors. The protein is 785 amino acids long, with a molecular weight of 81 KDa. HIF-1α is the main the regulator most hypoxia induced genes. Studies related to the Sp1 regulation under hypoxic conditions where not conducted untill now in human brain cancer.
Materials and Methods: Sp1 regulation level was examined in human glioblastoma cells lines like U373, U251, GaMG and U87-MG under extreme hxpoxic oxygenation conditions (0.1% oxygen), reoxygenation after hypoxia (24 and 48 hour) and oxygenated conditions (21% oxygen and 5% carbon dioxide) *in vitro*. Protein and mRNA level were detected via western blots and RT-PCR. Cells incubated for 24 hour with 100 μM DFO served positive control for hypoxia and β-tubulin and β-actin served as loading control, respectively. *In vitro* hypoxic conditions were induced by incubation into the Ruskin Hypoxia Chamber.
Results: Sp1 was over expressed via hypoxic oxygenation development in different glioblastoma cells micromilieu *in vitro* under extreme hypoxic conditions (0.1% oxygen) or reoxygenation after hypoxia, both on protein and mRNA level. an A corelation between Sp1 expression and the hypoxia induced HIF-1α and the regulated genes in human glioblastoma tumor tissue specimes examined, *in vitro*.
Conclusions: Sp1 upregulation as an answer to hypoxic development in glioblastoma micromileu, both on protein and mRNA are considered as a coregulator of HIF-1α and as a consequence the genes that are regulated by the hypoxic HIF-1α pathway in cancer human cells.

B2.54
Luteolin induces apoptosis by ROS-mediated endoplasmic reticulum stress and mitochondrial dysfunction in mouse neuroblastoma cells

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Neuroblastoma is the most common solid cancer that forms in nerve tissue and it is currently being treated by chemotheraphy. In the present study, we investigated the anti-cancer effect of luteolin, a common flavonoid in Neuro2a mouse neuroblastoma cells. Luteolin induced activation of caspase-12, -9, and -3 and cleavage of poly (ADP-ribose) polymerase. Also, luteolin induced expression of ER stress-associated proteins, including glucose-regulated protein (GRP) 78 and 94, C/EBP homologous protein (CHOP/GADD153), the phosphorylation of eIF2α and the cleavage of ATF6α, and the activation of MAP kinases, such as p38, JNK, and ERK. We also found that luteolin induced ROS generation at early point and luteolin induced mitochondrial apoptotic pathway and induced mitochondrial dysfunction. In addition, antioxidants such as N-acetylcysteine and glutathione blocked luteolin-induced ROS generation, cell death, expression of CHOP and GRP78, and mitochondrial dysfunction. These results showed that luteolin-induced reactive oxygen species (ROS) accumulation might play an important role in ER stress-induced apoptosis and mitochondrial dysfunction in Neuro2a mouse neuroblastoma cells.

B2.55
JAK2/STAT pathway regulates HIF-1 activation and hypoxic gene expression via ROS and PI 3-kinase/Akt dependent pathways in hypoxia-stimulated microglial cells

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Hypoxia induces an inflammatory activation of microglia during cerebral ischemia. The transcription factor of hypoxia-inducible genes hypoxia-inducible factor-1 (HIF-1) is known to be involved in inflammation and immune response. JAK/STAT pathway is

shown to be activated in microglial cells from cytokines (IFN-γ) but its activation and effect on inflammatory responses are not understood in hypoxic microglia. Thus, we examined the roles of JAK2/STAT signaling in HIF-1 activation and inflammatory responses of hypoxia-stimulated microglial cells. Hypoxia increased phosphorylation of JAK2 and STAT pathways in microglia and treatment with a specific inhibitor of JAK2 (AG490) significantly inhibited hypoxia-induced expression of HIF-1α protein levels and transcription activity. Transfection with dominant-negative mutant of JAK2 (DN-JAK2) or STAT3 siRNA also blocked HIF-1 transactivation and expression of HIF-1 responsive genes such as iNOS, COX-2, and VEGF, suggesting that JAK2/STAT3 regulates hypoxia-induced HIF-1 activation. Treatment with antioxidant reduced hypoxia-induced JAK2/STAT3 phosphorylation. Moreover, we found that JAK2 pathway cross-talks with PI 3-kinase/Akt pathway in hypoxic BV2 microglia. Taken together, these results suggest that JAK2/STAT pathway is involved in hypoxia-induced HIF-1 activation and hypoxic gene expression and that ROS appears to act upstream of JAK2/STAT in hypoxic BV2 microglia.

B2.56

Abstract withdrawn

B2.57
Apicidin induces endoplasmic reticulum stress via reactive oxygen species generation in mouse Neuro 2A neuroblastoma cells

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Neuroblastoma is the most common solid cancer of childhood that forms in nerve tissue. In the present study, we investigated the anti-cancer effect of apicidin, one of HDAC inhibitors, in Neuro 2a mouse neuroblastoma cells. Apicidin induced activation of caspase-12, a specific caspase in ER, as well as caspase-3. Apicidin induced unfolded protein responses (UPR) of ER, including expression of CHOP and splicing of XBP-1 and phosphorylation of eIF2α, JNK, p38MAPK, and ERK and the cleavage of ATF-6. Apicidin increased ROS accumulation and mitochondrial dysfunction in Neuro 2a cells. We found that treatment with salubrinal or CHOP siRNA blocked luteolin-induced cell death. We also found that an anti-oxidant N-acetyl cysteine (NAC) and a chelator of intrcellular calcium ion BAPTA-AM inhibited apicidin-induced apoptosis and changes in UPR-associated proteins. Taken together, the results suggest that apicidin induces apoptotic cell death via ER stress through ROS accumulation and calcium ion.

B2.58
BDNF/TrkB axis regulates proliferation and invasion of cervical cancer cells

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Neurotrophin BDNF and Tropomyosin-related kinase B (TrkB) is a key signaling molecule in the development of the nervous system. BDNF and its receptor TrkB are often overexpressed in a variety of human cancers such as neuroblastoma and hepatocarcinama, in which these are involved in tumor expansion and resistance to anti-tumor agents. In the present study, we examined the roles of BDNF/TrkB signaling pathways in proliferation and invasion of human cervical cancer cells. We found that BDNF and TrkB were overexpressed in human cervical cancer cell lines including HeLa, SiHa, Caski, and ME180 cells. Treatment with specific inhibitors of PKA (H89), TrkB (K252a), and NF-kappaB (SN50) significantly inhibited the expression of BDNF and TrkB, suggesting the involvement of these molecules in BDNF/TrkB axis in cervical carcinoma. We found that a CaMKII inhibitor KN93 increased BDNF secretion and increased the invasion of cervical cancer cells. We also found that inhibition of TrkB receptor by K252a inhibited the invasion of ME180 cervical cancer cells. Taken together, these results suggest that BDNF/TrkB axis may play roles in tumor growth and expansion in cervical cancer cells.

B2.59
Inhibiton of TRAIL-mediated apoptosis in non-adherently growing colon epithelial cells is connected with activation of pro-survival pathways

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In healthy colon, the colonic epithelial cells are shed into intestinal lumen at the top of the crypt and die by detachment-induced apoptosis (anoikis). Resistance of transformed epithelial cells to the anoikis promotes cancer cell invasion and metastasis. We studied the effects of TNF-related apoptosis inducing ligand (TRAIL) on cytokinetic parameters and adhesive properties of the cell lines derived from human foetal (FHC cells) and human adenocarcinoma (HT-29 cells) colon tissues in association with anoikis induction. We detected the significant decrease of TRAIL-induced apoptosis in the non-adherently growing HT-29 cells in comparison with the adherent cultivation. Based on this finding we focused our attention to detail mechanisms of the cell survival under TRAIL treatment. We confirmed our hypothesis of activation of pro-survival pathways, actually PI3K/Akt and MAPK/ERK, which are connected with focal adhesion kinase (FAK) phosphorylation. Increased phosphorylation of Akt and ERK kinases and also enhanced expression of FLIP and Mcl-1 proteins as downstream molecules of PI3K/Akt pathway were observed during non-adherent cultivation. Moreover, we detected significantly enhanced apoptotic parameters after treatment with TRAIL in combination with specific PI3K/Akt and MAPK/ERK inhibitors during non-adherent cultivation. Taken together, our data suggested that the decrease of the TRAIL-mediated apoptosis of colon epithelial cells induced by non-adherent type of cultivation is connected with the anchorage loss, FAK phosphorylation and with activation of pro-survival pathways. This work was supported by grants 305/09/1526 GACR, 303/09/H048 GACR, and 524/07/1178 GACR.

B2.60
Hypoxic induction of protease activated receptor-2 promotes angiogenic signaling in endothelial cells

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Coagulation-dependent signaling through G-protein coupled protease activated receptors (PARs) has been implicated in tumor

angiogenesis. The pro-coagulant response in the tumor microenvironment has been linked to hypoxic induction of tissue factor (TF) in malignant cells with implications for angiogenesis-dependent tumor progression through platelet activation, fibrin formation, and triggering of PARs; however, the direct role of TF and PARs in the hypoxic response of endothelial cells (ECs) remains ill-defined. Here, we show that hypoxia specifically up-regulates PAR-2 in primary, human ECs. Interestingly, whereas TF was substantially induced by hypoxia in several cancer cell lines, ECs displayed insignificant TF expression both at normoxic and hypoxic conditions. We further provide evidence for an important role of PAR-2 in the hypoxic-signaling response in ECs; PAR-2 activation by the well established PAR-2 agonist peptide, SLIGRL, substantially promoted the migration and survival of hypoxic ECs, and PAR-2 blocking antibody or RNAi-mediated PAR-2 inhibition attenuated hypoxia-induced apoptosis and EC tube disintegration. PAR-1 expression was unaltered by hypoxia and its stimulation did not significantly affect EC survival and migration, altogether suggesting a specific role of PAR-2. It is further shown that hypoxia-induced pro-angiogenic signaling through PAR-2 may involve transactivation of epidermal growth factor receptor (EGFR) through heparin-binding EGF-like growth factor (HB-EGF). This novel pathway provides insights into the hypoxic signaling response of ECs, and identifies PAR-2 as a potential target in hypoxia-driven tumor angiogenesis.

B2.61
In vitro growth inhibitory effects of arsenic trioxide in non-small cell lung cancer with different k-ras mutations

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K-Ras mutation is frequently related to resistance of tyrosine kinase inhibitor (TKI) therapy for non-small cell lung cancer (NSCLC). Arsenic trioxide (ATO), as a novel anticancer drug, is primarily used to cure acute promyolocytic leukemia. However, for other types of cancers the effects of ATO are still unveiled. Here we test *in vitro* growth inhibitory effects of ATO in two NSCLC cell lines: H23 (K-Ras mutant) and HCC827 (K-Ras wild type) in hope of shedding light on overcoming the de novo resistance resulted by TKI treatments. ATO induces more cell death compared to Erlotinib (one small-molecule TKI in clinical use) as well as cell apoptosis. ATO leads to down-regulations of both phosphor-Akt and Akt while Erlotinib just diminishes phosphor-Akt. Of the Ras/Raf/Erk pathway, ATO enhances the phosphorylation of Erk1/2 significantly in H23 but not in HCC827. In order to prove that downstream signalings following activation of Erk1/2 are driven to apoptosis instead of cell proliferation in H23, we examined the change of Bax which is believed to play an important role at intrinsic apoptosis. Surprisingly, Bax is down-regulated in a dose- and time-dependent manner. Before the further explorations of this phenomenon, current acceptable explanation would be either that ATO is working through extrinsic apoptotic pathways or that ATO effects diversified signalings which combined together lead to final apoptosis at K-Ras mutant cell lines of NSCLC.

B2.62
Intracellular calcium content, phospholipase C and protein kinase C activity in rat colonocytes under colitis-associated carcinogenesis

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The signaling pathway that increases cytoplasmic calcium concentration is the phospholipase C (PLC) pathway. PLC hydrolyses the membrane phospholipid PIP2 to form IP3 and diacylglycerol (DAG). DAG and Ca^{2+} activate the protein kinase C (PKC). A new study shows that calcium may lower the risk of colon cancer, but mechanisms of this effect remain vague. The aim of this study is to determine cytoplasmic Ca^{2+} concentration, PLC and PKC activity in colonocytes during experimental colitis-associated carcinogenesis (CAC). CAC was modeled by water containing 1, 5% dextransulfatsodium salt (DSS) and weekly injections of 1,2-dimethylhydrazine (DMG) during 10 weeks. The Ca^{2+} content was determined by Indo-1. The PKC and PLC activity was assessed by the standard methods. Changes of intracellular Ca^{2+} content were not determined on the 1-st day of DSS-colitis induction but PLC and PKC activities slightly increased. Maximal accumulation of Ca^{2+} was observed on the 3-rd and 7th day of experiment, when the parameter was two times more than control value. PLC and PKC activities increased and were half as large (for PLC) and three times as much (for PKC) against the control on the 7-th day of DSS treatment. The DMG were accompanied by Ca^{2+} content gradually decreasing comparing with inflammation state but it remained significantly above the control. The PLC and PKC activity during DMG influence increased and reached maximum on the 4-th and 2-nd week respectively of carcinogenesis development. The changes in Ca^{2+} pathway may contribute to alterations in colonocyte functions and cell cycle associated with colonic inflammation-associated carcinogenesis.

B2.63
Nitric oxide synthase activity and expression its isoforms in gastric mucosa under stress-induced ulceration

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Nitric oxide (NO) as a secondary messenger has been shown to protect the gastrointestinal mucosa from a variety factors. However, production of excessive amount of NO has been implicated as a cytotoxic factor in a variety of pathophysiological processes. The exact role of NO in processes of gastric ulcer development induced by stress has not been established. The aim of the study was to determine the nitric oxide synthase (NOS) activity and expression its isoforms in gastric mucosa during development of ulcer induced by stress. Male rats weighing 250–270 g were starved for 24 hour prior to experiments, but were allowed free access to water. Gastric damages were induced by water immersion restraint stress. The animals were killed after application of 0.5; 1; 2; 3 hour WIR. NOS activity was measured in homogenate of gastric mucosa. Expression of NOS isoforms was analyzed by RT-PCR. The significant damages of gastric mucosa were not found after 0.5 hour of stress. However, the quantity and areas of ulcer increased on 1, 2, 3 hour of stress. The elevation of NOS activity in 3, 6, 7 times was observed during this experiment. Basal levels of endothelial and neuronal NOS mRNA expression were detected in normal tissue, but inducible mRNA expression wasn't

observed in this case. The levels of eNOS and nNOS mRNA expression hadn't changed during ulcer development. Expression of iNOS was detected only after application 2 hours WIR. Further level of iNOS expression was increased.

B2.64
Functional analysis of Epithelial cell adhesion molecule (EpCAM) in breast cancer cell lines

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The Epithelial cell adhesion molecule (EpCAM) is a transmembrane molecule that inhibits cell-cell contact and adhesion by antagonizing E-cadherins. In primary and metastatic breast cancer EpCAM gene expression has been shown to be increased up to 1000 fold. In particular, overexpression in primary breast carcinomas correlates with poor overall survival in node-positive breast cancer patients. These observations suggest that EpCAM overexpression correlates with tumor metastasis and aggressive progression. In this study we investigated, whether EpCAM overexpression or downregulation by shRNA affects *in vitro* proliferation, invasion and can promote tumor growth. Moreover, we screened for molecular targets that are modulated by overexpression or downregulation of EpCAM. Therefore, different breast cancer cell lines (n = 6) were analyzed for EpCAM, E-Cadherin and c-myc genes expression. Based on these data cell lines with low EpCAM expression were selected for overexpression by the use of a transposon based or adenoviral system. On the other hand, cell lines with a strong EpCAM expression were transfected by a lentiviral system to generate a downregulation of this antigen. Subsequently, all generated cell lines will be used to evaluate differences in cell morphology, *in vitro* proliferation and cell adhesion. Furthermore, we are planning to test these cell lines also *in vivo* to study invasion, three-dimensional tumor growth, angiogenesis and tumor metastasis. All these investigations will help us to gain to better understanding of the role of EpCAM in tumor biology and bring more light into its function, signal transduction and molecular interaction partners.

B2.65
Cytoplasm and organelle transfer between mesenchymal multipotent stromal cells and renal tubular cells in co-culture

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One of the modern directions of nephroprotection is regenerative cell technology, i.e. transplantation of some stem and progenitor cells for improving kidney function after acute kidney injury. Co-culture of human mesenchymal multipotent stromal cells (MMSC) and rat renal tubular cells (RTC) were explored. We observed formation of different types of intercellular contacts between MMSC and RTC, including gap junction and so-called tunneling nanotubes. In a non-confluent monolayer when cells were at a distance one from another, TNT were formed very extensively, whereas after culture had nearly formed a monolayer, numerous short TNT between cells resembling threads connecting cloth patches were observedWe documented intercellular exchange with fluorescent probes specific to cytosol, plasmalemma and mitochondria. Initial transport of cellular components was revealed after 3 hour of co-culturing, and occurred in two directions – both direct and retrograde as referred to RTC. However, transport of probes toward MMSC was more efficient than toward RTC. Blocking of gap junction transport or nanotubes formation prevented transef of cytoplasm content. One significant result of coculturing was appearance of renal-specific Tamm-Horsfall protein and cytokeratin in MMSC, indicating induction of their differentiation into kidney tubular cells. We conclude that transfer of cellular compartments between renal and stem cells could provide differentiation of MMSC when transplanted into kidney and result in therapeutic benefits in renal failure. Supported by Russian Foundation of Basic Research (# 08-04-01667 and 09-04-13663-ofi_c).

B2.66
Overexpressed Cyclophlin B protects cells from H_2O_2-mediated programed death

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Cyclophilins (Cyps) were identified as cellular binding proteins for the immunosuppressive drug cyclosporine A (CsA) and are constitutively expressed in most tissues. Cyclophilins are multifunctional proteins that are involved in protein folding, mitochondrial functions, interaction with CD147, the immune system, and cancers. One of these family, Cyclophilin B is found mainly in the ER lumen, and it has peptidyl-prolyl cis-trans isomerase (PPIase) activity that catalyzes protein folding reactions in cells.. Reactive oxygen species (ROS) are generated as by-products of the mitochondrial respiratory process. They have emerged as an important signaling molecule based on their unique biochemical properties. It consists of various radicals, which might exert different effects on cellular signaling. Low levels of ROS regulate cellular signaling and play and important role in normal cell proliferation. In this study, we determined the potential role of overexpressed Cyclophilin B as a protector cancer cells from oxidative stress. With Huh7 (Human Hepatoma), we performed several experiments including Cell viability assay and western blot. According to our results, overexpressed CypB increased cell viability though activation of ERK pathways. And PPIase activity was required for this function.

B2.67
5′-nitro-indirubinoxime induces G1 cell cycle arrest and apoptosis in salivary gland adenocarcinoma cells through the inhibition of Notch-1 signaling

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5'-nitro-indirubinoxime (5'-NIO) is a novel indirubin derivative which exhibits anti-cancer activity in a variety of human cancer cells. Here, we report that 5'-NIO has a significant effect on growth inhibition and the induction of apoptotic processes in human salivary gland adenocarcinoma (SGT) cells by inhibiting the Notch-1 pathway. 5'-NIO significantly inhibited the mRNA levels of Notch-1 and Notch-3 and their ligands (Delta1, 2, 3 and Jagged-2) in SGT cells. Immunocytochemistry analysis showed that 5'-NIO specifically decreased the level of Notch-1 in the

nucleus. In addition, 5'-NIO induced G1 cell cycle arrest by reducing the levels of CDK4 and CDK6 in SGT cells. Using flow cytometry and immunoblotting analysis, we found that 5'-NIO induces apoptosis following the secretion of cytochrome C and the activation of caspase-3 and caspase-7. Intracellular Notch-1 overexpression led to a decrease in G1 phase arrest and the inhibition of 5'-NIO-induced apoptosis. These results demonstrated that the anti-tumor effect of 5'-NIO is mediated by blocking the Notch-1 signaling pathway, which can be useful for developing novel therapeutic strategies for salivary gland adenocarcinoma.

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B2.68
The interplay between benzo[a]pyrene and proinflammatory cytokine affects both metabolic activation of the procarcinogen and the inflammatory response in lung epithelial cells

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Lung cancer is among the leading worldwide causes of the cancer-related death. Deregulated chronic lung inflammation plays an important role in cancer development and it can be induced both during pulmonary diseases and other external factors, such as tobacco smoking. Extensive production of proinflammatory cytokines, such as TNF, plays a key role in the pathophysiological events related to deregulate inflammatory reaction. In the present study, we analyzed mutual deregulation of enzymes involved both in xenobiotic metabolism and inflammatory responses, caused by environmental pollutant and important tobacco smoke constituent benzo[a]pyrene (BaP) and TNF in rat lung RLE-6TN alveolar type II cell line. Our results show that TNF may significantly modulate the expression of cytochrome P450 superfamily members, such as CYP1A1/1B1, and thereby affect xenobiotic clearance and toxicity. In similar manner, BaP modulates the expression/activity of enzymes induced by TNF that are involved in inflammatory responses such as prostaglandin-endoperoxide synthase 2 (COX-2) or inducible nitric oxide synthase (iNOS), leading to enhanced release of their products such as prostaglandin E2. Moreover, BaP increases expression of inflammatory cytokines induced by TNF in target cells. These results demonstrate that proinflammatory cytokines may significantly modulate metabolism of environmental pollutants, possibly contributing to tumor initiation. On the other hand, environmental pollutants affect signaling pathways related to inflammatory response and may thus contribute to deregulated inflammation in lung. [Supported by grant No. 524/09/1337 from the Czech Science Foundation.]

B2.69
The vitamin E analogue γ-Tocotrienol blocks osteocalstogenesis and promotes osteoblast maturation through the NF-KB and p38/MAPK pathway: evidence for combined anti-osteolytic and bone reforming activity

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We investigated the effects of the vitamin E analogue, γ-Tocotrienol (γ-T) on bone metastatic breast cancer cells survival and osteoclast differentiation and bone resorptive activity using three independent *in vitro*-model systems of osteoclastogenesis. When human peripheral blood mononuclear cells (PBMCs) and the RAW264.7 murine monocytic cell line were cultured with the receptor activator of nuclear factor kappa B-ligand (RANKL), formation of tartrate-resistant acid phosphatase (TRAP) positive multinucleated cells and bone resorption were increased compared with untreated cells. γ-T dose-dependently inhibited RANKL-induced osteoclastic differentiation and bone resorption in both cell models. Similarly, it inhibited the bone resorptive activity of mature osteoclasts that were isolated from human Giant Cell Tumours of bone when cultured on dentine slices. RANKL activated osteoclastogenesis essential pathways NFKB, ERK1/2 and p38/MAPK, whereas γ-T profoundly inhibited RAKNL-induced activation of NFKB and P38/MAPK. Effect of γ-T on osteoblast function was investigated in mineralized bone nodule-forming primary human osteoblast cultures. γ-T progressively increased matrix-containing mineralized nodules in osteoblast cultures with a concomitant increase in alkaline phosphatase activity. Comparable concentrations of γ-T induced profound apoptosis in highly metastatic breast cancer cell lines. Taken together these results demonstrate for the first time that γ-T has a combined inhibitory effect on osteoclast activity and on survival of bone metastatic cancer cells whereas it significantly promotes osteoblast activity, serving as promising treatment for osteolytic bone disease.

B2.70
Fluorescence polarization used in HTS in order to identify new antagonists of cAMP to inhibit PKA activity

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The second messenger cAMP (cyclic adenosine 3',5'-monophosphate) is generated by ATP hydrolysis due to the G-protein-mediated activation of adenylate cyclase. An elevated level of cAMP leads to the inhibition of TCR signaling. The binding of cAMP to PKA regulatory subunits dissociates the holo-PKA, releasing active catalytic subunits. The active PKA C-subunits are then able to phosphorylate Csk, which phosphorylates Lck, leading to inhibition of T cell proliferation. Thus, antagonists able to keep the PKA under inactive form could play important role in treatment based on immunomodulation. We previously showed that the impaired proliferation of T cells from HIV infected patients can be improved up to 300% by the use of selective antagonist of PKA type I. To determine new antagonists of PKA type I, we optimized a Fluorescence Polarization High-Throughput Screening (FP-HTS) assay (Saldanha et al. 2006) to test two types of small molecules: rationally designed analogues of cAMP and structural-based selected non-nucleotidic compounds. Using this method, several hits were identified after the primary screen; cell based assays have been pursued to test the toxicity of the hits and their effect on the T cell proliferation.

B2.71
Design, synthesis and characterization of a highly effective Hog1 inhibitor: a powerful tool for analyzing MAP kinase signaling in yeast

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Cells utilize signal transduction pathways to sense and respond to internal and external cues. The Saccharomyces cerevisiae High-Osmolarity Glycerol (HOG) pathway is a conserved mitogen-activated protein kinase (MAPK) signal transduction system that is required for yeast to adapt to various stress conditions. Hog1, the MAPK of the HOG-pathway, is the yeast orthologue of mammalian p38, and it controls transcription, translation and cell cycle adaptations in response to environmental stress. Herein we describe design, synthesis, and biological application of a small molecule inhibitor that is cell-permeable, fast-acting, and highly selective against Hog1. We show that this molecule is a potent inhibitor of Hog1 kinase activity both *in vitro* and i, and use it to demonstrate a crucial role of Hog1 in controlling the exit from stress-induced cell cycle arrest. Hence, this chemical genetic approach of signaling characterization provided novel insight into the role of Hog1.

B2.72
Chitinase 3-like 2 gene as a potential marker of human glial tumors

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Identification and characterization of differentially expressed genes in astrocytic gliomas can be expected to provide important insights into the molecular determinants of carcinogenesis. Serial Analysis of Gene Expression found CHI3L1 (YKL-40) and CHI3L2 (YKL-39) genes among the most abundant transcripts in glioblastoma. Overexpression of CHI3L1 at the mRNA and protein levels in glioblastoma was shown in our previous study. Recklies et al., (2002) reported that CHI3L1 stimulated DNA synthesis and proliferation by activation of extracellular signal-regulated kinases (ERK1/ERK2) and protein kinase B (AKT)-mediated signaling cascades. Taking into account the lack of similar data for CHI3L2, closely related in amino acid and nucleotide sequences (52 and 56% homology) to CHI3L1, this study was initiated to analyze the expression and functional role of CHI3L2 in glial tumors. Northern hybridization and Western blot analysis showed the expression of CHI3L2 in the majority of glioblastomas. CHI3L2 activates ERK1/ERK2 in human embryonic kidney (HEK293) and human glioblastoma (U87 MG) cells. Unexpectedly, dose dependent decreases in total DNA content and [3H]thymidine incorporation were observed in HEK293 cells treated with CHI3L2. CHI3L2 induced sustained phosphorylation of ERK1/ERK2 in HEK293 cells in a very similar way to that was shown for PC12 cells treated with nerve growth factor (NGF). These data suggest that CHI3L2 may cause differentiation phenotype, but not proliferation. Thus, increased expression of CHI3L2 at the mRNA and protein levels in glioblastoma and its involvement in activation of MAPK signaling cascade may suggest its potentional role in carcinogenesis.

B2.73
Abscisic acid released by quartz-stimulated macrophages plays a key role in the silica-induced inflammatory process

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Exposure to airborne crystalline silica particles induces silicosis, an inflammatory disease of the lungs where alveolar macrophages release inflammatory mediators, like prostaglandin-E2 (PGE2) and tumor necrosis factor-α (TNF-α) eventually leading to tissue damage. The phytohormone Abscisic acid (ABA) has been recently discovered as a new human hormone involved in inflammation. Here we report the pivotal role of ABA in the silica-induced inflammatory process in murine RAW264.7 cells and in primary culture rat alveolar macrophages. Stimulation of RAW264.7 cells with quartz powder generates plasma membrane peroxidation responsible for ABA release. ABA then activates an autocrine loop sequentially involving binding of the hormone to its plasma membrane receptor LANCL2, activation of NADPH oxidase, production of reactive oxygen species, intracellular calcium rise, nuclear translocation of NF-κB and consequent increase of cyclooxygenase-2 expression, PGE2 production and TNF-αrelease. Silencing LANCL2 expression in RAW264.7 prior to stimulation with quartz results in an almost complete inhibition of PGE2 and TNF-α release, underlining a fundamental role of ABA in the early stage of quartz-induced inflammation. Studies on rat alveolar macrophages revealed early and late steps of ABA-induced responses strictly comparable to patterns of ABA signaling in murine RAW264.7 cells. These results identify ABA as a key mediator in quartz-induced pulmonary inflammation and provide a new possible target for anti-silicotic therapies.

B2.74
The Ser/Thr protein kinase activities in rat parietal cells under chronic atrophic gastritis development

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Chronic atrophic gastritis (CAG) has been associated with an increased risk of gastric cancer. Thus, investigation of mechanisms of gastric atrophic changes development remain problems of high importance. Disorders of apoptosis and cell proliferation in mucous are underlined in atrophy changes. Since the serine/threonine protein kinases play a commanding role in many signal pathways. The phosphorylation of chloric channel proteins by cAMP-dependent protein kinase A (PKA) results in increasing of its conductivity. Besides PKA Ca^{2+}-dependent protein kinase C (PKC) phosphorylates cytoskeleton and membrane proteins thereby implicating in the processes for the regulation of parietal cell HC1 secretion. Rat model of CAG was performed by intragastric administration of 2% sodium salicylate, 20 mM/L deoxycholate sodium daily for 6 weeks. The parietal cells were isolated on 1st, 2nd, 3rd, 4th 5th and 6th weeks of model. Cell PKC and PKA activities have been evaluated by incorporation of 32P from labeled ATP to specific protein substrate. Histological findings showed a significant decrease of mucus gland thickness in CAG rats than that of control. The increasing of investigated PK activities was shown during initial four weeks of pathology development. These results suggested important role of Ser/Thr

phosphorylation in initiation of gastritis. Such disorders in intracellular signal transduction might result in functional changes, including HC1 secretion and proliferation intensification. Besides, these effects served as additional pathogenesis factors of chronic atrophic gastritis development. Protein kinase A and C activities were decreased in a five week of pathology development.

B2.75
Sumoylation of the 20-hydroxyecdysone receptor from *Drosophila melanogaster*

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The 20-hydroxyecdysone receptor (EcR) is an insect transcription factor belonging to the nuclear receptor superfamily. EcR and its ligands, absent in vertebrates, are used in commercially available ecdysone-inducible expression systems and may have potential usage as gene switches with therapeutic application. It is little known about EcR activity regulation especially by posttranslational modifications in mammalian cells and till now there has been no research about EcR sumoylation. In order to investigate SUMO attachment to EcR, we used the Ubc9 fusion-directed SUMOylation (UFDS) system. Series of constructs coding all three isoforms of *Drosophila melanogaster* EcR (EcR-A, EcR-B1 and EcR-B2) fused with sumoylation conjugation enzyme (Ubc9) on N - or C - termini were prepared, expressed in HEK293 cell line and examined by Western blotting. We observe electrophoretic mobility changes for all proteins suggesting SUMO conjugation, both with the endogenous SUMO and with the coexpressed EGFP-SUMO1. Dependent on the Ubc9 fusion this sumoylation requires neither SUMO ligase nor any extracellular stimulation. We also observe diverse sumoylation pattern depending on location of Ubc9 on N - or C - termini. Moreover we present here results of bioinformatic prediction of sumoylation sites of *Drosophila* EcR obtained with four predictors: PCI-SUMO, SUMOplot™, SUMOpre, SUMOsp 2.0.

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B2.76
Role of intracellular calcium in the control of boar sperm functions

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To successfully achieve the fertilization of the oocyte, sperm cells undergo several processes that include activated motility, capacitation and the acrosome reaction. Our aim was to study the involvement of intracellular calcium in the regulation of these cellular processes in porcine spermatozoa. Semen from 8 Duroc boars was used and motility parameters were evaluated by computer assisted ISAS® program and the plasma membrane fluidity (MF), acrosome reaction (AR), cell viability and mitochondrial membrane potential (MMP) were analyzed by flow cytometry using as dyes Merocyanine-540/YO-PRO-1, FITC-PNA/PI, SYBR-14/PI and JC-1, respectively. Spermatozoa were incubated in an extracellular calcium (EGTA)-free medium (TBM) for different times at 39°C and 5% CO_2 in presence/absence of the cell-permeable calcium chelator BAPTA-AM and cell permeable cAMP analogue 8Br-cAMP. BAPTA treatment led to a full inhibition of spermatozoa motility in TBM or stimulated by 8Br-cAMP. The reduction in spermatozoa viability and in the percentage of spermatozoa with high MMP observed in TBM or in presence of 8Br-cAMP is prevented by BAPTA incubation. Moreover, spermatozoa AR induced by 8Br-cAMP in TBM is inhibited by BAPTA treatment. However, intracellular calcium depletion did not modify the degree of MF in spermatozoa induced by 8Br-cAMP. In summary, our results suggest that intracellular calcium is necessary and sufficient to maintain the spermatozoa motility in a calcium free medium. Moreover, the intracellular calcium diminishes the spermatozoa viability and the MMP. However, the degree of MF in the porcine spermatozoa is independent of intracellular calcium.

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B2.77
Unique peptide sequence inducing inflammatory response of macrophagemacrophage

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The main allergen for food allergy against crustacean and shellfish is a myofibrillar protein, tropomyosin. We have recently found an inflammatory peptide sequence in tropomyosin for macrophage. On the other hands, we have recently demonstrated that cycloartenyl ferulate (CAF), one of gamma-oryzanol rich in grain bran, inhibits nuclear factor kappa B (NF-kappa B) activation in RAW264.7 cells. In this research, effects of CAF on inflammatory responses of RAW264.7 cells were evaluated. An inflammatory peptide sequence of tropomyosin (T2) was loaded on RAW264.7 cells and the levels of inflammatory cytokine mRNAs, tumor necrosis factor alpha (TNF-alpha), cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS) and interleukin 1 beta (IL-1 beta) mRNAs expressed in RAW264.7 cells, were then estimated through a real-time PCR. No apparent mRNA changes in TNF-alpha, COX-2 and iNOS was observed in the T2-treated cell, but only IL-1 beta mRNA expression was enhanced by T2 treatment. On the other hands, CAF failed to inhibit T2-induced IL-1 beta expression. These results suggest that the T2-peptide sequence should induce inflammatory response of RAW264.7 macrophages not via the NF- kappa B cascade.

B2.78

Abstract withdrawn

B2.79
Effects of glutathione on taste sensation in mammals

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We usually eat many kinds of food with each peculiar taste. Some chemical compounds stimulate our taste sensation and present specific and complex taste figures for individual foods. The resulting taste figures affect our acceptability of the foods. Glutathione is a tripeptide (Glu-Cys-Gly) that exists ubiquitously in food. GSH shows no basic taste, but is believed to affect some flavor characteristics, such as thickness, mouthfulness, and continuity. The paper deals with the effects of GSH on mouse taste

sensation. Licking response of GSH was measured using the conditioned taste aversion mouse. The lingual epithelium of c57BL mouse was applied to in situ calcium imaging using Calcium Green 1. Fluorescein-5-maleimide-labelled GSH was alternatively loaded on the mouse lingual epithelium to monitor GSH distribution in taste buds. Ligand docking was simulated in a simulation software Sybyl 8.1 with a Surflex Dock program. GSH slightly elicited intracellular calcium responses in MSG responsive taste receptor cells. One type of the GSH responsive cells failed to respond MSG during GSH-inactivation state. Some taste bud cells were well stained with the fluorescein-labelled GSH. The conditioned taste aversion tests revealed that GSH synergistically enhanced mouse response to IMP. These results suggest that GSH would probably share the receptor on taste cell membrane with MSG.

B2.80
The growth and differentiation regulation of ornithine decarboxylase in *Dictyostelium discoideum*

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The alphatic polyamine such as putrescine, spermidine and spermine are normal cell constituents that play an important role in cell proliferation and differentiation. Although the precise action modes of polyamines are not understood until now, an observation has emerged which suggests the specific interaction of polyamine with nucleic acids to be very crucial in bring about the actions in the cells at the molecular level. Ornithine decarboxylase (ODC, EC 4.1.1.17) catalyses the rate-limiting step of polyamine synthesis, in which L-ornithine is converted into putrescine. *Dictyostelium discoideum* offers an excellent model system to study the role of polyamines on the aspects of growth, differentiation and morphogenesis. This offered an interesting system for the present study roles of ODC in growth and differentiation and its relationship with methylglyoxal To find out the role of ODC, *odc* was knockout in wide type cell by disruption. The *odc* knockout cells showed lower growth rate than the wild type. Moreover, when developed, differentiation of *odc* knockout cells changed. To understand role of methylglyoxal by polyamine, endogenous level of methylglyoxal was measured by HPLC in both *odc* knockout and wild type cells.

B2.81
Imatinib mesylate plus lithium chloride failed to treat glioblastoma *in vitro*: the effect and mechanism of midkine

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Glioblastoma (GBM) is the most malignant primary brain tumor, characterized by its resistance to treatments and median survival times not much longer than one year. Failure of treatments relies on protective mechanisms against apoptosis. A heparin-binding growth factor Midkine (MK) has significant roles in anti-apoptotic processes and is highly expressed in GBM. In the present study, we aimed to investigate whether the combination of an effective and commonly used drugs in chemotherapy imatinib mesylate (IM) (10 μM) with an antipsychotic drug lithium chloride (LiCl) (100 μM) with new identified chemotherapeutic effects can be an effective treatment model for GBM by using monolayer cultures of human T98G human glioblastoma cell line and the role of MK levels in this treatment. The effects of drugs were investigated by evaluating cell proliferation index, MK levels by ELISA, and phospho glycogen synthase kinase 3-beta (p-GSK-3β), multi-dug resistance protein-1 (MRP-1) levels by western blotting at the 24th and 72nd hours. All drug treatments reduced cell numbers, and the levels of MK, MRP-1 and p-GSK-3β for 72 hours. LiCl was the most effective group to reduce cell numbers, MRP-1 and MK levels, and the combination group was the second one. The effective inhibition of p-GSK-3β levels were done by IM, the combination group and LiCl, respecively. Our results showed that IM plus LiCl failed to treat glioblastoma *in vitro* and IM decreased MK levels for the first time. Pleiotrophic effects of IM and LiCl on the crosstalk between MK/PI3K/AKT/GSK-3β/β-catenin signaling pathway and Wnt/Fz/GSK-3β/β-catenin pathway could lead to this failure.

B2.82
Crosstalk between the Ah receptor and cAMP signaling in an *in vitro* model of oval cells

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Aryl hydrocarbon receptor (AhR) has been originally described as a transcription factor mediating the toxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds. Apart from its role in xenobiotic metabolism, AhR has been found to be important for liver development and it plays a crucial role in carcinogenic effects of AhR ligands. The AhR signaling can be triggered also by endogenous molecules like the second messenger cyclic adenosine monophosphate (cAMP). Using an *in vitro* model of liver progenitor cells, participating both in liver regeneration and in hepatocarcinogenesis, we tested the potency of cAMP and another protein kinase A activator, forskolin, to activate AhR signaling pathway, as analyzed at the level of: (i) AhR nuclear uptake; (ii) AhR protein degradation; and iii) induction of model target gene *Cyp1a1*. As the expression of genes involved in intercellular communication can be deregulated by TCDD exposure, we also evaluated the potency of cAMP and forskolin, alone or in combination with TCDD, to modulate expression of *Jup* gene encoding adherens junctions/desmosomal protein plakoglobin. Our results indicate that cAMP and forskolin transiently activate AhR signaling in WB-F344 cells; however, no *Cyp1a1* induction has been detected and changes in *Jup* expression were contrary to those elicited by TCDD. Consequently, both compounds were able to modulate TCDD-induced changes in *Cyp1a1* and *Jup* expression. These results suggest a possible crosstalk between cAMP/forskolin-activated signaling and AhR in regulation of functions of liver progenitor cells. [This study is funded by the Czech Science Foundation, grant No. 524/09/1337].

B2.83
Sphingosine kinase mediates resistance to the synthetic retinoid n-(4-hydroxyphenyl) retinamide in human ovarian cancer cells

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A2780/HPR is a HPR-resistant clonal cell line derived from A2780 human ovarian carcinoma cell line treated with the synthetic retinoid N-(4-hydroxyphenyl)retinamide (HPR). HPR induces in A2780 cells the production of (dihydro)ceramide with a concomitant reduction of cell proliferation and onset of apoptosis. Sphingosine kinase (SK) activity and SK-1 mRNA and protein levels resulted higher in A2780/HPR cells versus A2780. Beside this, the production of sphingosine-1-phosphate (S1P) was significantly higher in A2780/HPR versus A2780 cells, while the treatment with a potent and highly selective pharmacological sphingosine kinase inhibitor effectively reduced S1P production and resulted in a marked reduction of cell proliferation of A2780 and A2780/HPR cells. Moreover, the SK inhibitor sensitized A2780/HPR cells to the cytotoxic effect of HPR, due to the alteration of intracellular S1P and ceramide levels. On the other hand, the overexpression of SK-1 in A2780 cells was sufficient to induce HPR resistance in these cells. The role of SK in HPR resistance is not mediated by the S1P receptors, as suggested by the absence of effects after treatment of A2780 and A2780/HPR cells with agonists and antagonists of S1P receptors on the sensitivity to the drug. These data clearly demonstrate a role for SK in determining resistance to HPR in ovarian carcinoma cells, due to its effect in the regulation of intracellular ceramide/S1P ratio which is critical in the control of cell death and proliferation.

B2.84
Quantitative analysis of the SNF1-pathway in *Saccharomyces cerevisiae*

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In eukaryotic cells the SNF1/AMPK family of protein kinases plays an essential role in the control of the cellular response to changes in the energy balance. The activity of the baker's yeast homologue Snf1 is dependent on the phosphorylation state of a single threonine (T210), which is regulated by a trio of activating kinase (Sak1, Tos3 and Elm1) and a single Type I phosphatase (Reg1-Glc7). In an effort to extend our knowledge of this process, we have integrated a large body of novel data into a mathematical model. First, we have analysed the effect on Snf1 activation of *SAK1* and *REG1* over expression. We observed that over production of Sak1 provokes a constitutive Snf1 phosphorylation and activation. However, in presence of glucose, the artificial Snf1 activation elicited by the increased *SAK1* gene dosage does not have any effect on Mig1 phosphorylation and localisation. Regulation of Snf1 phosphorylation was partially restored if *REG1* was concomitantly over expressed. Second, we undertook time course studies on the activation/deactivation state of Snf1. Third, we quantified the main protein components of the pathway by means of immuno-qPCR revealing a 1:1:1 ratio amongst the SNF1 kinases components. Our mathematical model reliably simulated our observations and has been employed to study the mechanisms that control Snf1 activation/deactivation.

B2.85
Mapping cellular signal transduction

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The survival, fitness and health of a living organism require the ability to perceive and adapt to changing external and internal conditions. On a cellular level, this ability is provided by the signal transduction network. The network encompasses all the interconnected signalling processes in the cell, and consists of a large number of proteins that convey signals as chains of conformational changes propagating through this network. Due to the combinatorial nature of these state changes, the number of states that can potentially be assumed within the signal transduction network is staggering. This problem is referred to as the combinatorial explosion, and is perhaps the principal challenge in both visual representation and mathematical modelling of the signal transduction network. Here, we present a framework for comprehensive and unambiguous documentation of signal transduction systems, which is consistent with the format in which most biological data is generated. Furthermore, our framework can be used to derive an equation system for mathematical modelling of the network or selected parts. The framework breaks down the network into fundamental, binary reactions and states, and both known and unknown contingencies can be explicitly defined in a way that avoids any implicit assumptions. An important strength of our approach is that it allows for the automated conversion between different visualisation strategies. We illustrate the power of our framework by mapping the core characterised signalling system in the yeast *Saccharomyces cerevisiae*.

B2.86
Genetic screening for suppressor mutations of the hyper- or non-filamentous growth of the yeast osmotic signalling mutants

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In certain yeast *Saccharomyces cerevisiae* strains, diploid cells develop pseudohyphae under nitrogen starvation, while haploid cells produce invasive filaments that penetrate the agar in rich medium. We have recently reported that these morphological developments are strongly inhibited under hyper-osmotic condition through the high-osmolarity glycerol (HOG) response MAPK pathway (Furukawa *et al.*, 2009, *Mol Microbiol*). Deletion of the *HOG1* MAPK gene enhances morphological developments, while expression of active Hog1 inhibits those even in the absence of hyper-osmotic stress. Moreover, it has been reported that *HOG1* affects morphology and pathogenicity also in other fungal species. *Candida albicans hog1* mutant cells form (pseudo)-hyphae and show related transcriptome changes even in the absence of morphogenetic signals. *Cryptococcus neoformans hog1* mutant cells enhance production of capsule and melanin, which are crucial virulence factors. Hence, fungal Hog1 orthologues are

thought to be central negative regulators of many aspects of morphological developments and virulence, and studies on the underlying mechanisms are relevant for the identification of novel targets for antifungal therapy. In order to more deeply understand the inhibitory role of Hog1 in morphological developments, the proteins or signalling pathways affected by Hog1 need to be identified. In this study, we attempted to screen suppressor mutations of the hyper- or non-filamentous growth which are caused by *HOG1* deletion or active Hog1, respectively. On the basis of the screening results, the mechanism by which Hog1 negatively regulates morphological developments will be discussed.

B2.87
Long-term inhibition of androgen receptor expression induces growth arrest in androgen sensitive and castration resistant prostate cancer cells through activating the intrinsic and extrinsic apoptotic pathways

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The androgen receptor (AR) signaling pathway is one of the main mediators of prostate tumor growth and progression. Hence, androgen ablation is the standard treatment for patients in advanced stages, although most tumors eventually relapse by developing castration resistance. We have shown previously that blocking AR expression by small antisense molecules inhibits prostate tumor growth. The aim of this study was to elucidate cell death mechanisms after long-term inhibition of AR expression in androgen sensitive LNCaP and castration resistant LNCaPabl prostate cancer cells. AR expression was inhibited with a siRNA specifically targeting the AR (siAR). After short term treatment over 72 hours 10 nM of the siAR resulted in a significant knockdown of AR, associated with about 55% growth arrest in both cell lines and an increase in cleaved PARP and caspase 3 activity (2-fold induction). To investigate whether resistance mechanisms are induced upon long-term AR knockdown we treated the cells up to 7 weeks with very low siRNA doses of 0.5 nM. In both cell lines, growth inhibition of 44% was evident as from the second week of treatment and lasted over the whole treatment period reaching a peak of 62% growth inhibition after 7 weeks. This inhibition was accompanied with induction of caspases 8 and 9 (1.6-fold) in the third week of treatment, followed by a 3-fold increase of caspases 3/7 activity. Our data show that AR knockdown induces growth arrest through activation of intrinsic and extrinsic apoptotic pathways and is likewise effective in androgen sensitive and castration resistant prostate cancer cells without apparent signs of resistance during long term treatment over 7 weeks.

B2.88
Cyclin A/Cdk1 and CK2 cooperate to trigger degradation of the Stem-loop binding protein (SLBP) at the end of S phase inhibiting histone mRNA biosynthesis

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In metazoans, bulk of histone proteins is encoded by replication-dependent histone mRNAs. Expression of these histone mRNAs is cell cycle regulated and a major regulatory mechanism is S phase restriction of Stem-loop binding protein (SLBP) which is a necessary factor for histone pre-mRNA processing. SLBP is degraded at the end of S phase and this inhibits the histone mRNA biosynthesis until SLBP accumulates just before the beginning of the next S phase. SLBP degradation is signaled by phosphorylation of T60 and T61 in TTP (60–62) motif and a KRKL (95–98) sequence, which is a putative cyclin binding site. A fusion protein with 58-aa fragment of SLBP (51–108) fused to glutathione S-transferase, is sufficient to mimic SLBP degradation at the end of S phase. Using GST-SLBP fusion protein as substrate, we identified cyclinA/Cdk1 as the major kinase for T61 in late S phase lysates. We also show that cyclinA/Cdk1 but not cyclinA/Cdk2 efficiently phosphorylates T61. Furthermore, knock down of Cdk1 stabilizes SLBP at the end of S phase. As a possible kinase for T60, CK2 inhibitors also prevent the degradation of SLBP at the end of S phase. *In vitro* phosphorylation of T60 by CK2 requires prior phosphorylation of T61 suggesting that T61 phosphorylation by cyclinA/cdk1 primes the phosphorylation of T60 by CK2 and signals SLBP degradation. Based on these findings, we conclude that towards to end of S phase, increase in cyclinA/Cdk1 activity in cooperation with CK2 triggers degradation of SLBP as a mechanism to shutdown histone mRNA biosynthesis.

B2.89
C-cell hyperplasia in thyroid from Sprouty1 knockout mice

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Sprouty family members (Spry1-4) are negative regulators of receptor tyrosine kinase signaling, especially of the ERK pathway. *In vivo* experiments have demonstrated that two members of the family, namely Spry1 and Spry2, regulate Ret activity in the developing kidneys and enteric nervous system, respectively. Ret functions as a receptor for the Glial-Derived Neurotrophic Factor (GDNF) family ligands (GFLs), which regulate several aspects of development of the autonomic nervous system and the urogenital system. Oncogenic mutations of Ret lead to development of medullary thyroid carcinoma (MTC), a malignancy of the calcitonin-producing cells of the thryroid (parafollicular cell or C-cells). In the present work we sought to elucidate whether Spry1 restricts signaling by oncogenic Ret in MTC. Lentiviral-mediated expression of moderate amounts of Spry1 in a MTC-derived human cell line (TT) reduced cell growth both *in vitro* and in subcutaneous xenografts in SCID mice. Expression of Spry1 in TT cells resulted in up-regulation of p16 INK4a and down-regulation of several angiogenesis-related genes. Finally, genetic ablation of Spry1 in mice led to development of C-cell hyperplasia, a precancerous lesion that precedes development of MTC. Taken together, these data suggest that Spry1 might function as a tumor suppressor in MTC by acting as a cellular brake to oncogenic Ret signaling.

B2.90

Abstract withdrawn

B2.91
Network-based reconstruction of the glucose repression signaling pathway of yeast

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Modeling of signaling pathways has proven to be a challenging task due to the large extent of the crosstalk between individual pathways. In most of the cases where the layout of the associated pathway is not believed to be explicitly shown, network-based approaches are excellent tools for the analysis of these signaling networks. A key element of the glucose repression signaling pathway which is subject to several ambiguities in its mechanism; Snf1 kinase is composed of a catalytic subunit Snf1p, a regulatory subunit Snf4p and a scaffolding β-subunit (Sip1p, Sip2p or Gal83p). In this study a network-based algorithm is developed for the integrated analysis of the genome wide expression data from SNF1 Δ, SNF4 Δ and SNF1 ΔSNF4 Δ mutants and the global PPI network of yeast. The linear paths between several key elements of the glucose repression signaling pathway were identified in the PPI network of yeast, following the elimination of the biases in the network. In the next step, the linear paths were scored based on the genome wide expression data. And, the significance of each binary interaction was calculated based on its occurrence frequency in the top scoring linear paths. The robustness of the algorithm was tested by using different PPI networks with varying coverage. On the other hand, the results were evaluated in a comparative manner through the identification of the functional modules in the network. As the final objective, the overview of the glucose repression mechanism in the absence of the Snf1 complex was achieved without being lost in the complexity of commonly used graph-theoretical approaches.

B2.92
A correlation between TGF-β1-mediated JAM-A downregulation and cell invasiveness

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Transforming growth factor-beta (TGF-β) and junctional adhesion molecule-A (JAM-A) have been indicated as key regulators of cell migration, and an inverse relationship of JAM-A expression and the invasiveness has been reported in breast cancer cells. Whether TGF-β1 signaling induces JAM-A downregulation leading to cancer cell invasion has not been investigated. In this study, we report that TGF-β1 significantly downregulates JAM-A expression at both mRNA and protein levels in MCF-7 cells that is slightly migratory and normally expresses high level of JAM-A. We showed that TGF-β1 downregulates JAM-A mRNA level via transcriptional regulation and the promoter region responsible to TGF-β1 stimulation is located between nt -367 and -1. In addition, TGF-β1 exerted its negative effect on JAM-A expression via post-translational control. There was a significant reduction (~60% reduction) in JAM-A protein levels in cyclohexamide-pretreated TGF-β1-treated cells, indicating that TGF-β1 promotes JAM-A protein turnover. We showed that JNK inhibitor, but not Smad3 and Smad4 siRNA, could effectively abolish TGF-β1-mediated JAM-A protein degradation, although Smad activation alone could abolish JAM-A degradation in the absence of TGF-β1 stimulation. Furthermore, clathrin siRNA abolished TGF-β1-mediated JAM-A degradation, indicating that the degradation is mediated via clathrin-dependent endocytosis. Reduction of JAM-A in MCF-7 cells upon TGF-β1 treatment led to enhanced invasion in the invasion assays. Taken together, these results indicate a strong correlation between TGF-β1-mediated JAM-A downregulation and cell invasiveness. [Supported by Hong Kong Research Grants Council (HKU771507)

B2.93
Inhibition of TGF-β signaling by nuclear receptor TLX in neuroblastoma

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Transforming growth factor beta (TGF-β) is a multifunctional cytokine that functions both as tumor suppressor and as inducer of tumor growth, depending on cancer type and stage of tumor development. TGF-β activates intracellular signaling through phosphorylation of Smad2 and Smad3 that form a complex with Smad4 and translocate to the nucleus, where they regulate transcription of various genes. TLX is an orphan nuclear receptor that is important for vertebrate brain functions. TLX has been shown to maintain adult neural stem cells in an undifferentiated and self-renewable state. We found that TLX also is highly expressed in several neuroblastoma cell lines. Although the role of TLX in adult neural stem cells is well established, its function in neuroblastoma is not well known. TGF-β does not have a significant effect on growth of wild-type IMR-32 neuroblastoma cells, but in IMR-32 cells where TLX expression was knocked down TGF-β treatment led to growth inhibition. Further, in TLX knockdown cells there is a significantly higher increase in mRNA levels of TGF-β target genes like Smad7 and p21 upon TGF-β treatment compared to control cells. TLX also binds several components of the TGF-β signal pathway, including Smad2, -3 and -4. We are now trying to unravel the mechanisms by which TLX regulates TGF-β signaling. Our results suggest that TGF-β and TLX have opposing effects on proliferation in neuroblastoma and insensitivity to TGF-β dependent growth inhibition due to high levels of TLX might be an important reason behind the high rate of proliferation of these cells.

B2.94

Abstract withdrawn

B2.95
Linearity range of the hyperosmotic stress response in *Saccharomyces cerevisiae*

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The HOG (High Osmolarity Glycerol) pathway in *Saccharomyces cerevisiae* is a MAP kinase cascade activated upon hyperosmotic stress. The core element of this cascade is the Hog1 MAP kinase, which resides in the cytoplasm in the absence of hyperosmotic stress and is rapidly accumulated inside the nucleus, when the external osmolarity increases. Using fluorescent protein fusions we followed the sub-cellular localisation of Hog1 over time. We employed a single cell analysis platform, based on a fluorescence microscope coupled with micro-fluidics and optical tweezers, to acquire images of osmotically stressed cells. Using different treatments, which included a wide range of osmoticum concentrations

(100 mM to 1000 mM NaCl), applied to approximately 25 cells, we investigated threshold levels of the HOG response. We found that the widely claimed linear ratio between stress intensity and Hog1 nuclear enrichment is valid only up to a certain level of stress. Beyond this level the maximum in Hog1 nuclear retention is delayed. This delay is also reflected in cell population data (immuno-blotting) on Hog1 phosphorylation, as well as the indirect transcriptional read-out (Gpd1 protein levels). At present we can only speculate that this delay could be caused by the properties of the Hog1 translocation machinery.

B2.96
Protein-protein interactions: identification, modulation and validation

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Yeast two-hybrid (Y2H) protein interaction screening is a key technology to identify new protein partners. To ensure reproducible and exhaustive Y2H results, our highly complex fragment libraries are screened to saturation using an optimized mating procedure that allows for testing on average 100 million interactions per screen, corresponding to a 10-fold coverage of the library. As a consequence, multiple, independent fragments are isolated for each interactant, enabling the immediate delineation of a minimal interacting domain and the computation of a confidence score. We have developed several methods to validate and further characterize interactions identified using Y2H screens or other protein interaction techniques. ''One-by-One'' assays allow for the pair-wise testing of the interaction between two selected proteins, either *in vivo* (in yeast) or *in vitro* using the powerful HTRF® (homogeneous time resolved fluorescence) technology of Cisbio International, which is based on time-resolved FRET. The smallest interacting domain on both the bait and the prey proteins, or individual amino acids critical for the interaction can be also rapidly delineated using yeast-based tools. This characterization is highly relevant for functional studies and drug discovery projects where a protein-protein interaction can be used as a target for the development of small therapeutic molecules. Here, we will present results that illustrate the strength of our Y2H screening and validation techniques.

B2.97
Effect of quercetin on phosphorylation of mitogen-activated protein kinases in malignant mesothelioma cells

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Malignant Mesothelioma (MM) is an aggressive tumour of serosal surfaces including pleura, peritoneum and pericardium. Although several chemotherapeutic agents and tyrosine kinase inhibitors are currently used to treat patients of MM, treatments are not always successful. Quercetin (QU), flavonoid has an effect on anti-proliferative and/or apoptotic mechanisms and the relative cell signal proteins, which are involved in both processes at several cancers. We have previously reported that QU reduced the proliferation of SPC212 and SPC111 cell lines and resulted in S phase arrest. In present study, in order to investigate QU treatments affect the mitogen-activated protein kinase (MAPK) cascade, the phosphorylation of extracellular signal-regulated kinases (ERKs) was analysed in MM cell lines. Our results indicate that QU, and MAPKK (MEK) inhibitor, PD98059 inhibited ERK 1/2 phosphorylation in dose and time dependent manner. Effect of QU on phosphorylation of c-jun NH2-terminal kinases (JNK) or stress-activated protein kinases (SAPK), and p38 kinases are still under investigation.

B2.98
Hydrogen peroxide generated during the RTKs signaling produces and acts locally confined

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Cells produce hydrogen peroxide (H_2O_2) as a part of physiological response to many growth factors and cytokines. For instance, binding of epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) to their receptors, EGFR and PDGFR, respectively, activates superoxide anion radical and H_2O_2 production by NADPH oxidases. H_2O_2 acts as second messenger and inactivates tyrosine phosphatases. However, it remains unknown which cellular compartment is responsible for H_2O_2 production and what the diffusion properties of H_2O_2 within the cell contribute. In our work we aimed to study the intracellular localization of H_2O_2, which synthesized during the RTK stimulation of 3T3 fibroblast and HeLa cells. We produced the collection of HyPer biosensor variants with different intracellular localization. HyPer is genetically encoded ratiometric biosensor with a high H_2O_2 sensitivity and specificity. We found, that different cell types demonstrate different subcellular patterns of ROS production. In epithelial cells during the EGF stimulation H_2O_2 synthesize near endosomes with internalized EGFR. H_2O_2 level near the plasmic membrane is not changed. Fibroblasts after their PDGF stimulation generate H_2O_2 in the region of the plasmic membrane. Therefore cells generate H_2O_2 locally and it isn't spread to other cell compartments. Both types of cells generate H_2O_2 also on endoplasmic reticulum membrane. Thus, our results show that during the stimulation of receptor tyrosine kinases H_2O_2 produces and acts locally in the particular cell compartments. H_2O_2 diffusion between compartments is limited. Our results provide the first visible evidence for a localized redox signaling model.

B2.99
RGS2 and RGS4 proteins differentially modulate delta and kappa opioid receptor signaling

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Regulators of G protein Signaling (RGS) comprise a large multifunctional protein family whose members possess intrinsic GAP activity that accelerates GTP hydrolysis from Ga subunits, thus modulating G protein coupled receptor (GPCR) signaling and also act as effector antagonists. We have previously shown that the RGS4 directly interacts with mu and delta opioid receptors to regulate their signaling (1-2). To deduce whether there is selectivity in coupling between members of RGS proteins and opioid receptors we tested the ability of other members of B/R4 RGS subfamily to interact with kappa (KOR) and delta (DOR) opioid receptors. Pull down experiments and co-immunoprecipitation studies indicated that RGS2 and RGS4 interact within C-terminus and third intracellular loop of both

DOR and KOR. Expression of RGS2 or RGS4 in cells expressing the DOR or KOR showed that the two RGS proteins differentially modulated opioid receptor-mediated adenylyl cyclase inhibition and MAPK phosphorylation. RGS4, but not RGS2, was also found to accelerate DOR internalization. Collectively, our data show that although RGS2 and RGS4 are negative modulators of DOR and KOR, they differentially regulate their signaling.

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B2.100
Analysis of carbonic anhydrase9 (CA9) expression in human brain cancer under different oxygenation conditions including hypoxia

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The hypoxia-inducible enzyme carbonic anhydrase 9 has recently been applied as a tumor hypoxia marker and an indicator of prognosis and a potential therapeutic target in malignant glioma.

Material and methods: To characterize patterns of expression of CA9 in human malignant glioma cells, we studied CA9 protein, CA9 mRNA and hypoxia-inducible factor-1a (HIF-1a) protein levels in U87-MG, U251, U373 and GaMG cells exposed to *in vitro* hypoxia (1, 6 or 24 hour at 5%, 1% or 0.1% Oxygen). Also CA9 mRNA and protein expression were examined in two groups of tumor patient specimens, namely low-grade astrocytoma (LGA; n = 15) and glioblastoma (GBM; n = 15) and compared to a negative control group of normal brain samples (n = 3). CA9 and HIF-1a protein expression were detected via Western blot while CA9 and HIF-1α mRNA expression detection was via Northern blot.

Results: In the series of *in vitro* experiments, all cell lines displayed a strong hypoxic induction of CA9 mRNA in response to prolonged severe hypoxia with cell-line specific patterns at moderate to mild hypoxia and shorter treatment times. CA9 changes under severe hypoxia and the inhibitory effect of the glycolysis inhibitor iodoacetate (IAA, 50 μM) on hypoxic CA9 overexpression were paralleled by the results for HIF-1a protein. In the patient tumor specimens, CA9 protein was only overexpressed in (GBM). CA9 mRNA was predominantly overexpressed in GBM (12/15) patients compared to LGA patients (3/15).

Conclusions: CA9 expression in (GBM) tumors occurs at a higher frequency, both on protein or mRNA levels, rendering CA9 as a suitable hypoxia-related diagnostic marker or target for therapeutic approaches.

B2.101

Abstract withdrawn

B2.102
The activity of interefron dependent 2′,5′-oligoadenylate-synthetase in rat spleen lymphoid cells under the chronic alcohol intoxication

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The investigation of alcohol influence on immune system functioning is very urgent recently. The role of interferon induced signaling pathway in formation of immune response to ethanol action is not clarified yet. The key enzyme of interferon system is 2',5'-oligoadenylate-synthetase (2',5'-OAS). Chronic alcohol consumption causes the zinc deficiency, that's why the search of new zinc preparations is necessary for the purpose of correction of ethanol-caused metabolic lesions. We have investigated the 2',5'-OAS activity in rat spleen lymphocytes against a background of chronic alcohol intoxication at introduction of zinc acetate. 40% ethanol was administrated orally in a dose of 2 ml per 100 g. The 2',5'-OAS activity decreased after 4-and 7-daily alcohol consumption. The values of enzyme activity were minimal under the long-term action of ethanol (21-daily) and were lower than control level on 80%. The combined administration to rats of zinc acetate in a dose of 2 ml per 100 g and ethanol causes the increase of 2',5'-OAS activity. The effect was most expressed on the late stages of alcohol intoxication development: the enzyme activity rose on 40% and 450% relative to control on the 16-th and 21-th day accordingly. The decrease of 2',5'-OAS activity during chronic alcohol intoxication development might be a result of interferon synthesis depression due to damaging action of ethanol. It also might be connected with interferon signaling injury in immunocompetent cells. The effect of zinc on 2',5'-OAS activity under the alcohol action may be a result of metabolic processes recovery, which correlates with our previous data concerning zinc influence on cell membranes.

B2.103
Effect of L-carnitine on NF-kB activity in lipopolysaccharide stimulated RAW 264.7 macrophage cell line

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L-carnitine is an important molecule in fat metabolism, it participates in the transport of long–chain fatty acids into mitochondrial matrix. It is an antioxidant molecule that exhibits free radical scavenging activity and enhances antioxidant status in rats. NF-kB is an inducible transcription factor and has a central role for regulation of proinflammatory gene expression. Stimulation of macrophages by LPS leads to activation of various transcripiton factors, including NF-kB. NF-kB participates in the regulation of inflammatory genes such as iNOS, COX-2, IL-1β. Excess production of nitric oxide by iNOS has been implicated in some inflammatory diseases. In this study we investigated the effect of L-carnitine on NF-kB activity in LPS stimulated RAW 264.7 macrophages. For this purpose, RAW 264.7 cells were first transfected with pNF-kB-luc plasmid and then stimulated with 1 μg/ml LPS either in the presence or

absence of 8.3–13.09 mM L-carnitine. Unstimulated cells acted as negative control. When stimulated with LPS, NF-kB activity increased by 2.19 ± 0.41-fold, compared to negative control ($p < 0.001$). Different L-carnitine concentrations (8.3, 9.8 and 13.09 mM) reduced the NF-kB transcriptional activity (27%, 28% and 31% respectively)($p < 0.005$). Since excess nitric oxide production through NF-kB activation plays an important role in inflammatory diseases, these results suggest that L-carnitine exerts an antiinflammatory effect by blocking NF-kB activation in LPS activated macrophages.

B2.104
Effects of pistacia atlantica extract on cell cycle regulatory proteins in breast cancer T47D cells

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Breast cancer is one of the most common cancers among Iranian women. Uncontrolled cell cycle plays a pivotal role in cancer incidence. Cell cycle is regulated by cyclins and cyclin-dependent kinases (cdks). Plants are important sources of active natural products which differ widely in terms of structure and biological properties. As a part of our studies on Iranian medicinal plants for new anticancer agents, in this project we evaluated the effects of Pistacia atlantica sub kurdica extract (with the local name of Baneh) on breast cancer T47D cells. The T47D cells were treated with 1 mg/ml of Baneh extract (IC50) and Doxorubicin 250 nM in the MTT cytotoxicity assay. Flow cytometry analysis of DAPI-stained cells showed significant arrest of cell cycle in G0/G1 phase in Baneh treated cells and G2/M accumulation of Dox-treated cells in comparison to the control cells. In another investigation, western blot analysis was done with the IC50 concentrations of Baneh extract and Dox for 12–72 hour. The G0/G1 arrest of cell cycle was confirmed by the sharp down regulation of cyclin D1 and cdk4 in comparison to the control cells by increasing time. Moreover, protein level of cyclin E, cyclin A, cyclin B, cdk1 and cdk2 was reduced in Baneh treated cells. The effect of Dox on the level of these proteins was not as strong as the effect of Baneh. On the basis of these findings, Baneh extract showed antiproliferative activity led by arresting of the cells in G1 phase of the cell cycle through down regulation of the regulatory proteins of G1/S transition.

B2.105
NF-kappaB activation and complex control of NOS_2 gene promoter in K562 imatinib mesylate resistant CML cell line

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Abnormal Wnt/β-catenin signaling pathway and increased expression of nitric oxide synthase2(NOS_2) are both implicated in the development of various human cancers. Imatinib mesylate which is an Abl kinase-selective inhibitor is a treatment option for most of the Chronic myeloid leukemia (CML) patients. Recently it has been identified that Bcr-Abl-mediated Y phosphorylation of β-catenin is a cause of its increased protein stability and transcriptional signalling activity.In this study we aimed to analyse if NOS_2 and cyclin D1 are target genes of β-catenin in K562 imatinib mesylate resistant CML cell line.For this purpose we first performed ChIP assay with anti β-catenin antibody and then PCR reactions were performed to examine β-catenin/Tcf-4 binding to NOS_2 and cyclinD1 promoters.Using transfection assays we also investigated activation of NF-kappaB in K562 imatinib mesylate resistant CML cell line.Our results show that NOS_2 and cyclinD1 are target genes of β-catenin. We also determined significantly increased NF-kB activation in K562 imatinib mesylate resistant CML cell line compared with non-resistant cells.In the present study,we have described the role of Wnt/β-catenin signaling in the regulation of NOS_2 in K562 imatinib mesylate resistant CML cell line. And we have provided strong evidence that NOS_2 is a novel downstream target of β-catenin suggesting the involvement of NO in Wnt/β-catenin signaling pathway that could contribute to imatinib resistant of CML.Increased NF-kB activation and binding of β-catenin to NOS_2 gene indicates that human NOS_2 promoter is under complex control and these observations present new targets to prevent imatinib resistance in CML.

B2.106
Effect of wnt3a or lithium chloride preconditioning on nitric oxide production in RAW264.7 macrophages and K562 leukemic cell line

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Wnt pathway regulates cell migration, proliferation and differentiation.In the absence of Wnt signal,β-catenin is degraded by a multiprotein complex containing GSK-3β.Binding of Wnt protein to its receptor leads to GSK-3β inhibition and nuclear accumulation of β-catenin,which binds to TCF/LEF to increase expression of Wnt target genes.LiCl is a mimicker of Wnt pathway via potent inhibition of GSK-3β.Wnt3A is also an activator of Wnt pathway.LPS/IFN-gamma synergically induce macrophages to produce various inflammatory mediators including nitric oxide.It was suggested that presence of LiCl potentiates nitrite production cell type selectively and LPS-dependent production of nitrite was modestly increased in RAW264.7 macrophages.In this study we aimed to analyse the effect of Wnt pathway activation on LPS/IFN-gamma induced or basal nitrite production in RAW 264.7 macrophages and K562 cell line.Our results show that LiCl induces nitrite production in both cytokine stimulated and unstimulated macrophages.28 mM LiCl increased nitrite production by nearly two fold in both stimulated and unstimulated macrophages($p < 0.05$).Wnt3A preconditioning did not significantly effect nitrite production when compared with LPS/IFN-gamma treated macrophages.K562 leukemic cells were also treated with LPS,LPS plus LiCl,nitrite production was not significantly different between unstimulated,LPS and LPS plus LiCl stimulated cells which means that LiCl cell specifically effects nitrite production.It can be concluded that there is a strong link between Wnt β-catenin pathway and iNOS activation which must be kept in mind

considering treatment options for diseases such as chronic inflammation and cancer.

B2.107
Hyperosmotic stress triggers the degradation of microtubule- associated protein Tau

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Tau is a microtubule-associated protein which plays an important role in the structure and function of the neuronal cytoskeleton. Tau is also a major component of paired helical filaments (PHF), one of the pathological hallmarks of Alzheimer's disease. Several alterations in the microtubule-associated protein Tau metabolism have been described in neurodegenerative disorders such as Alzheimer disease. Among these, hyperphosphorylation and cleavage are two of the most studied features. Although PHF formation mechanisms remain largely unknown it has been suggested that impaired proteolysis of Tau may be associated to its deposition. In the present work, we have studied the mechanisms underlying Tau degradation in response to hyperosmotic stress in the neuroblastoma cell line SH-SY5Y. Sorbitol treatment induces a time-dependent degradation of Tau and this effect is partially prevented by MG-132, suggesting a role for proteasome in this process. In addition, sorbitol also increases both calpain and caspase-3 activity and p38 MAPKs and ERK1/2 phosphorylation in a time dependent manner. Pre- treatment of cells with different concentrations of p38 MAPKs inhibitors (SB203580 and BIRB0796) does not prevent sorbitol-induced Tau degradation. However, this effect is partially prevented when cells are pretreated with ERKs inhibitors (PD98059, PD184352 and U0126), which indicate the involvement of the ERK pathway in sorbitol-induced Tau degradation. Work supported by grants: PRI06A202, GRU09056 and BFU2007-67577-C02-02/ BMC. MCB and MOSC are recipients of Junta de Extremadura fellowships. S. S. P belongs to Erasmus Program.

B2.108
Estradiol as stimulator of syndecan-4 gene expression in human breast and colon cancer cells

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Syndecans are transmembrane cell surface heparan sulfate proteoglycans (HSPG) implicated in cancer development. Syndecan-4 is believed to have regulatory roles in cell adhesion and in the regulation of cell migration. Estrogens and particularly 17β-estradiol (E2) regulate several physiological processes and are implicated in the growth of solid tumors, such as breast and colon. E2 also influences estrogen receptor (ER) expression. ER is synthesized in many cell types as two protein forms, ERα and ERβ, which are products of separate genes. E2 signaling is supposed to be mediated via linear pathway involving insulin-like growth factor receptor (IGF-R) and epidermal growth factor receptor (EGFR). The aim of this study was therefore to examine whether estradiol affects the expression of ERs and proteoglycans (PGs) implicated in breast and colon cancers as well as whether this effect is associated with ER/IGF-R/EGFR system. *In vitro* studies were performed on a panel of breast and colon cell lines in the presence or absence of E2 and specific IGF-R, EGFR tyrosine kinase inhibitors. The results showed that E2 affected the gene expression of syndecan-4 via genomic and nongenomic pathway and is closely related with the expression balance between ERα/ERβ and the crosstalk between crucial ER/IGF-R/EGFR pathways.

B3 – Global Networks

B3.01
Androgen receptor signaling induced by supraphysiological doses of dihydrotestosterone in human peripheral blood lymphocytes

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Anabolic androgenic steroids (AASs), a class of steroid hormones related to testosterone, are natural ligands of androgen receptor (AR), a member of the nuclear receptor superfamily of ligand-activated transcription factors. AR binds specific DNA elements, known as androgen response elements (AREs). Testosterone, the main male sexual hormone, binds AR directly and indirectly, through conversion into dihydrotestosterone (DHT), its more active metabolite. AASs are frequently detected in the urine of doped athletes; their consumption is growing also among sport amateurs and adolescents. The effects of androgens can differ depending on the target cells and/or tissues. To gain insight into transcription activation mechanisms of AR, we investigated AR protein signaling in human peripheral blood lymphocytes treated with supraphysiological doses of DHT. We performed a comparative proteomic analysis and we identified about 30 differentially expressed proteins. At least five species contained a consensus ARE sequence in the promoter region of related coding genes. The analysis also revealed that high doses of DHT activate the drug detoxification process, stimulate cell motility and exert a prosurvival effect rather than an apoptotic one.

B3.02
Systems-level analysis of mitotic feedback controls

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Entry into mitosis depends on the activation of the Mitosis Promoting Factor (MPF), a complex made up of cyclin-dependent kinase-1 (CDK1) and cyclin-B. In many organisms, MPF activity is regulated by inhibitory phosphorylation of a CDK1-tyrosine residue by the Wee1 kinase. This phosphorylation is removed by the Cdc25 phosphatase once the cyclin component of MPF reaches a critical threshold. MPF in turn activates Cdc25 and inhibits Wee1, thus creating a positive and a double-negative feedback loop. These feedbacks are known to be important for proper mitotic control, since elimination of the Wee1 phosphorylation site on CDK1 can compromise the cell cycle control system. However, the individual contributions of the two feedback loops have not been separately assessed. We have constructed a mathematical model for the MPF tyrosine-phosphorylation switch that takes into account the multiple phosphorylations of Wee1 and Cdc25 by MPF and studied the effects of disrupting the feedback loops independently. We show that the Wee1-MPF double-negative feedback loop and the Cdc25-MPF positive feedback loop are not functionally equivalent. We also propose that MPF regulation of the Cdc25 phosphatase might have a more significant role than Wee1 regulation in setting the cyclin threshold for MPF activation and thus mitotic entry.

B3.03
Protein-protein interactions (PPIs) and disease

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While the complete sequence of the human genome is now available, the same state of completeness cannot be said to exist for the interactome or the proteome. Protein complexes are responsible for most processes within the cell, and modifications to their interactions perturbs the normal sequence of events, and contributes to many diseases. A more complete coverage of PPIs would help enable us to discover connections between disordered PPI networks and disease processes, identify new targets for drug therapy, and generate novel approaches to treatment or disease modification. IntAct is an freely available (open source) database for molecular interactions, containing over 210,000 curated binary protein-protein interactions (PPIs) from at least 63,000 proteins from many organisms. The captured data can displayed in a variety of formats and now includes drug-protein, and DNA-protein interactions. IntAct is a member of the IMEx consortium; a network of participating major public domain databases sharing information, and aiming to jointly capture all published molecular interaction data in a standardised format. IntAct source code and data are freely available at http://www.ebi.ac.uk/intact

B3.04
Comparison of three detection methods of Y chromosomal short tandem repeats (Y-STRs) in population genetics and forensic investigations

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Introduction: One of the most frequently used approaches in forensic investigations and population genetics is detection of Y chromosome short tandem repeats (Y-STRs). A wide spectrum of analyzed polymprphic loci exhibits a high level of heterogeneity at the inter- and intra-population level. Molecular techniques typing for a length variation of Y-STRs are mostly based on multiplex PCR of 12 to 17 loci. The aim of the present study was to compare several multiplex Y-STRs detection methods.
Subjects and methods: Ninety unrelated males were analyzed using AmpFlSTR® Y-filer™ (Applied Biosystems) for forensic investigations as a routine procedure and 158 unrelated males for population genetics studies were typed using PowerPlex Y System (Promega). DYS392 was genotyped separately in several cases.
Results: Out of analyzed 248 samples, in 8.4% cases it was not possible to detect DYS392 using multiplex PCR reactions. Therefore, these samples were checked individually by one-step PCR reaction and genotyped. The most frequent alleles of DYS392 were 11 (46.2%) and 13 (35.4%), but genetic heterogeneity was 0.6425 ± 0.0245. Genetic heterogeneity was ranged from 0.42 for DYS385a to 0.70 for DYS390 for the rest 11 Y-STRs in the Latvian population. The haplotype diversity for 12 Y-STRs was

observed 0.9923 demonstrating the usefulness and informative power of this Y-STR set.
Conlusions: Based on series multiplex PCR, Y-STRs detection not always provide sufficient results for all loci. Additional molecular approaches in several cases should be applied, which include smaller number of analyzed loci.
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B3.05
A global protein Kinase and phosphatase interaction network in yeast

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Kinases and phosphatases regulate virtually all cellular processes through reversible phosphorylation of myriad substrates. The systematic identification of the proteins that associate with kinases is crucial for understanding the global regulatory network of the cell. However, due to the difficulty in detecting the often weak associations between kinases and their substrates or regulators, the kinase interactome (kinome) is only poorly charted. We systematically interrogated the complete set of kinases and most phosphatases in budding yeast using multiple epitope tags in small scale cultures. Protein complexes were captured on magnetic beads, followed by on-bead trypsin digestion and nano-scale LC-MS. We identified a kinase and phosphatase interaction (KPI) network of 1844 statistically significant interactions. The KPI network contained many dense local regions of interactions that suggested new functions. Notably, the cell cycle phosphatase Cdc14 associated with multiple kinases that revealed roles for Cdc14 in mitogen-activated protein kinase signaling, the DNA damage response and metabolism, while interactions of the target of rapamycin complex 1 (TORC1) uncovered new effector kinases in nitrogen and carbon metabolism. Our analysis reveals that kinase-kinase interactions were significantly enriched compared to all other kinase interaction partners and collectively formed a highly interconnected kinase network. This network was extremely robust and was far less modular than previous less complete networks. Our results suggest that yeast phosphorylation-based signaling has integrated, non-modular signal processing architecture.

B3.06
Do pneumatic tube systems affect hemolysis index?

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Introduction: Pneumatic tube systems provide rapid specimen transportation to the laboratories as point-to-point services. With these systems, specimen damage due to rapid acceleration or decelaration is still in debate. Majority of unsuitable laboratory samples are attributable to *in vitro* hemolysis which often results from incorrect sampling procedures or transportation. Hemolysis index is the quantification of free hemoglobin in specimens, which can produce chemical interference in a variety of analytical reactions. In this retrospective study, the affect of pneumatic tube system on specimens hemolysis indeces were evaluated in a university hospital settings.
Methods: In our laboratory, measurement of H-index is an automated test for every single sample and the method is preinstalled in the chemistry analyzers. In the laboratory, the pneumatic system was installed on the 21th of January 2009. Previously, for specimens transport to the laboratory, courier services were used. Sample hemolysis indices were retrieved from laboratory information system for the time period 3 months before and after the installation. Pre- and post- installation values were compared using SPSS, and the results were reported as mean ± standard deviation.
Results: Before the pneumatic tube system, hemolysis index was 8.21 ± 16.17 mg/dl (n = 43,278) and after the installation it was 7.81 ± 20.83 mg/dl (n = 48,102). The difference was statistically significant ($p = 0.001$).
Conclusion: The level of hemolysis index is a crucial step towards reducing errors in laboratory testing. Pneumatic tube system technology significantly improves hemolysis indices.

B3.07
The PSICQUIC interface – a portal into to the world of the interactome

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Molecular interactions are key to our understanding of biological systems and there are many data resources, such as the IntAct molecular interaction database (www.ebi.ac.uk/intact) dedicated to capturing such experimental data. This increasing number of resources, however, presents the research worker interested in a specific biological domain with a significant data access challenge. To instantaneously access multiple interaction data resources, a common interface for computational access allowing software clients to interact with multiple sources using the same interface is required. This has lead to the development of PSICQUIC, the PSI Common Query InterfaCe, a community standard for computational access to molecular interaction resources. PSICQUIC has been jointly developed by major interaction data and tool providers in the context of the HUPO Proteomics Standards Initiative using the standard formats developed by the PSI Molecular interaction workgroup. This initiative is supported by an increasing number of major interaction databases and a number of practical applications are demonstrated.

B3.08
Sulfate assimilation mediates tellurite accumulation and toxicity in yeast

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Despite a century of research and increasing environmental and human health concerns, the mechanistic basis for the toxicity and intracellular accumulation of derivates of the metalloid tellurium, in particular the oxyanion tellurite, remains an unsolved question in microbiology. We provide an unbiased view of the mechanisms of tellurium metabolism in the yeast Saccharomyces cerevisiae by

measuring deviations in tellurium tolerance and accumulation of a complete collection of gene knockouts. Reduction of tellurite and concomitant intracellular accumulation as metallic tellurium strongly correlated with loss of cellular fitness, demonstrating that tellurite reduction and toxicity are causally linked. The sulfate assimilation pathway upstream of Met17 and in particular the sulfite reductase and its cofactor siroheme were shown to be central to tellurite toxicity and its reduction to elemental tellurium. Gene knockouts with a deviation in tellurite tolerance also showed a similar deviation in the tolerance to both selenite and, interestingly, selenomethionine. This suggests that the bioassimilation of selenium and tellurium into seleno- and telluromethionine via the sulfate assimilation pathway, with potential further incorporation into proteins, is an important cause of tellurite and selenite toxicity in yeast. The here reported results represent a robust base from which to attack the mechanistic details of this molecular enigma.

B3.09
Comparison of histone H2A.X (Ser139) and ATM kinase (Ser1981) phosphorylation after one hour treatment of HL-60 cells with vanadocene dichloride and cisplatin; relation to the cell-cycle phases

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Aim: To assess difference in extent of DNA damage that involves formation of dsDNA breaks after 1 hour treatment of HL-60 cells with metallocene agent vanadocene dichloride (VDC) and cisplatin (CP).
Methods: Phosphorylation of H2AX on Ser139 and ATM on Ser1981 in HL-60 cells were detected immunocytochemically, DNA was counterstained with propidium iodide; the cell immunofluorescence (IF) was measured by flow cytometry. The intensity of H2AX-Ser139P (termed gammaH2AX) and ATM-Ser1981P IF, or the percentage of gammaH2AX and ATM-Ser1981P – labeled cells were evaluated by flow cytometry software Summit v4.3.
Results: VDC intensely induce formation of gammaH2AX foci. In cells treated with CP, compared with VDC, the gammaH2AX IF was less intense. Mean of gammaH2AX IF increase linearly with the dose of VDC in all cell-cycle phases. CP caused weak increase of gammaH2AX IF, in S phase was decrease observed. The cells treated with e.g. 1 and 0.5 mM/L VDC showed 99 resp. 96% gammaH2AX positivity. In case of same doses of cisplatin were 34 resp 29% cells gammaH2AX positive. Control cells 2% gammaH2AX positive, 98% negative. Slight increase of ATM-Ser1981P IF was observed after VDC treatment, whereas CP did not promote IF increase. Cells exposed to 1 and 0.5 mM/L VDC were 9 resp. 4% ATM-Ser1981P positive, control 2%.
Conclusions: The strong correlation between phosphorylation of ATM and H2AX after VDC treatment was observed. The data indicate that VDC cytotoxic effect can be particularly due to formation of dsDNA breaks in cells.
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B3.10
Mechanisms underlying anticancer effect of vanadocene complexes in comparison to cisplatin in p53 negative cells HL-60

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Aim: The aim of the present study was to determine differences in molecular mechanism in action of known cytostatic drug cisplatin (CP) and a compound vanadocene dichloride (VD) in HL-60 leukemia cell line.
Methods: We used WST test to evaluate toxicity of cytostatics (IC50 value) and trypan blue exclusion technique to determine cell viability. Changes in protein expression were analyzed by Western blotting. Caspase activity was determined by Caspase-Glo Assay set. Nuclei of influenced cells were stained by DAPI.
Results: IC50 value was determined after 72 hours treatment as $3*10^{-6}$mol/l for CP and as $35*10^{-6}$mol/l for VD. Changes in protein expression of p21, Bid, Mcl-1 and gammaH2AX were evaluated. Expression of the protein p21 was detected mainly after VD treatment in the time interval 24, 48 and 72 hours. The induction of gammaH2AX was detected in samples influenced by CP after 48 and 72 hours of incubation, expression of Mcl-1 appeared after treatment by both drugs and the induction of the protein Bid was the most visible after influence by CP in the time interval 72 hours. The activity of caspases 3 and 9 in cells influenced by CP increased after 48 hours incubation, the activity of caspases 3, 8 and 9 stayed approximately the same in the same time interval after treatment by VD.
Conclusion: According to results of our study, there are differences in mechanism of action of both cytostatics.
Acknowledgement: This work was supported by The Ministry of Education of Czech Republic, project MSM 0021620820.

B3.11
The deletion of Raf Kinase-1 Inhibitor Protein (RKIP) produces an increase in the pancreas volume during development

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RKIP is a docking protein with the capacity to inhibit MAPK, GPCR and NFκB pathways, participating in cell proliferation and apoptosis. Previous studies, have demonstrated a downregulation of RKIP in a model of β cell regeneration. Also, we have seen the RKIP KO presents a bigger pancreas at 8-weeks old and a higher sensitivity to the glucose at 15 and 30 minutes in a glucose tolerance test. To understand the role of RKIP in the plasticity of the β cell, and a possible role in the inhibition in the replication, we have studied the pancreas in the whole KO mouse of RKIP during the development. The pancreas begins bigger in the KO mouse since the 28-days of development (1.11% ± 0.03 of BW in KO and 0.98% ± 0.05 in WT). Through morphometrics studies we can observed that there are differences in the total beta cell mass (KO: 0.7 mg ± 0.01, WT: 0.41 mg ± 0.08) and in the distribution of different β cell areas. KO animals present a

higher percentage of insulin-positive cluster cells smaller than 1.000 μm^2 (KO: 70,4% ± 42.5, WT: 61.5% ± 2.6) than de WT. The proliferation in KO mouse is 6-fold higher in β cell and 10-fold higher in all pancreatic cells (KO: 2.6% ± 0.25, WT: 0.42% ± 0.3 for β cell; KO: 3% ± 1.0, WT: 0.28% ± 0.14 for pancreatic cell). We suggest that RKIP is implicated in β cell plasticity and its absence increases the formation of new β cell clusters since the development, maybe, allows an highest signaling in proliferation pathways. Therefore, we propose that RKIP should be considered as a gene which controls the formation of cluster processes in the pancreas and its modulation with some pharmacologic agents could be considered as a new target for diabetes.

B3.12
A comprehensive genetic-interaction map of a eukaryotic kinome

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We have used functional genomic screens to interrogate the yeast kinome using combinatorial genetic perturbations. We systematically assessed the effects of either gene overexpression or gene deletion on the fitness of non-essential kinase deletion mutants to generate a meta-network of Synthetic Dosage Lethal (SDL) and Synthetic Lethal (SL) interactions of yeast kinases. The datasets are enriched for pathways known to be regulated by cognate kinases and analysis of the network schema reveals considerable new information about kinase function. The kinases that yielded informative SDL interactions were largely those with significant cell cycle or cell polarity roles and the SDL hits were enriched for phosphoproteins, cell cycle regulated genes and inhibitory pathways regulated by the kinases. Condition-specific screens and analysis of kinase double mutants suggests that the apparent resistance of many kinases to genetic perturbation cannot be solely attributed to kinase redundancy but most likely reflects the requirement for many kinases in certain activating conditions. We also present the most complete list of literature-curated kinase targets to date. Using the curated kinase-substrate gold standard and the integrated SDL-SL meta-network we have identified triplet regulatory motifs, unraveling novel pathways regulated by kinases. Our functional map of the yeast kinome will provide a valuable resource that can be mined to identify roles of this important class of regulatory proteins.

B3.13
Systems biology approaches towards dynamic complexes of monogenic, aggregation-prone disorders: case study NCLs

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Protein-protein interaction networks are relevant to systems biology because they elucidate both spatial and temporal organization principles of the cellular networks. The Neuronal ceroid lipofuscinoses (NCLs) are the most common cause of inherited progressive encephalopathy of childhood, characterized by accrual of autofluorescent, ceroid-like lipopigment and degeneration of cortical neurons. NCLs are caused by mutations in 10 postulated genes (CLN1-10), eight of which have been cloned. Bioinformatic analyses and literature information demonstrates that NCL proteins are connected at the molecular level. Juvenile NCL (JNCL, Juvenile Batten disease) is caused by mutations in the CLN3 gene. To systematically characterize the NCL protein complexes we have created a ''Gateway®-compatible NCL entries library'', including all available disease genes and their direct interacting partners. At present our ''NCL destination library'' contains approximately 600 clones, with tags either at the N- or C-terminus of the human ORFs. As a proof of principle study, we chose to characterize CLN3 dynamic interactome by validating available binary yeast-two hybrid (Y2H) interaction data with affinity purifications combined with mass spectrometry. For validation of Y2H data we are utilizing various biochemical approaches, i.e. LUMIER-based coimmunoprecipitation assay (i). Human SH-SY5Y cells, stably expressing CLN3 protein with dual affinity tags (Protein G-Strep) have been generated as described (ii). The identity of isolated proteins will be determined by LC-MS/MS, and brought forward for comparative bioinformatic analyses and systematic RNAi studies to assess the dynamics of CLN3 complexes.

B4 – Gene Regulation

B4.01
Investigation of benzodiazepines and zolpidem effects on the expression of cychromes P450 CYP1A1, CYP1A2 and CYP3A4 in primary cultures of human hepatocytes

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We have examined the potency of Alprazolam, Bromazepam, Chlordiazepoxide, Clonazepam, Diazepam, Lorazepam, Medazepam, Midazolam, Nitrazepam, Oxazepam, Tetrazepam, Triazolam and Zolpidem to induce CYP1A1, CYP1A2 and CYP3A4 in primary cultures of human hepatocytes. Compounds were tested in therapeutic concentrations and in concentrations corresponding to their plasma levels in intoxicated patients. We found weak but significant induction of CYP3A4 mRNA by Midazolam and Medazepam, while other drugs did not induce CYP3A4 expression. None of the tested compounds induced CYP1A1 and CYP1A2 mRNAs in three different human hepatocytes cultures. In addition, employing gene reporter assays with transiently transfected hepatocarcinoma cells, we found that tested compounds did not activate aryl hydrocarbon receptor (AhR), whereas Midazolam and Medazepam slightly activated pregnane X receptor (PXR). In conclusion, Medazepam and Midazolam are weak activators of pregnane X receptor and displayed weak potency to induce CYP3A4 mRNA in human hepatocytes. Our laboratory is supported by the grants from the Grant Agency of the Czech Republic GACR304/10/0149, GACR303/07/0128 and GACR305/08/P089.

B4.02
Valproic acid potentiates vitamin D receptor-mediated induction of CYP24 gene – A consequence for drug-induced osteomalacia

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Valproic acid (VPA) found its use as a mood-stabilizing agent for treatment of epilepsy or bipolar disorders (brand names like Depakote, Depacon or Stavzor). On the molecular level, it was found to be an inhibitor of GABA transaminase or histone deacetylase 1 (HDAC1). Recently, our group described that VPA activated pregnane X receptor (PXR), which led to induction of cytochrome P450 3A4 (CYP3A4) in human hepatocytes. Since there are many drugs causing so-called drug-induced osteomalacia and these drugs act mainly via the PXR, we decided to investigate, whether the molecular mechanism might involve a vitamin D receptor (VDR)-mediated induction of CYP24, one of the major enzymes involved in the catabolism of active form of vitamin D (VD3). We found that VPA did not induce CYP24 mRNA in human hepatocytes next to the VD3, a typical inducer of CYP24. However, VPA potentiated CYP24 mRNA induction mediated by VD3. In human carcinoma cells, HepG2, it was able to transactivate a CYP24 promoter and this effect was independent on the presence of co-transfected expression vectors for VDR or PXR. The same potentiation was observed for endogenously present VDR in human embryonal kidney cells, HEK293. We conclude that VPA significantly potentiates ligand-triggered induction of CYP24s and this may disturb the vitamin D homeostasis via increased catabolism. This work was supported by the grants from the Grant Agency of the Czech Republic GACR303/07/0128, GACR305/08/P089 and GACR304/10/0149.

B4.03
Evolutionary expansion of SPOP and associated TD/POZ gene family via chromosomal segmental duplication, retrotransposition and exonization of transposed elements

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Evolutionary expansion of a gene family may occur at both the DNA and RNA levels and may involve the highly abundant transposed elements (TEs). The rat testis-specific Rtdpoz-T2 and -T1 (rT2 and rT1) retrogenes are members of the TD/POZ gene family which also includes the well-characterized SPOP gene. We have previously shown frequent TE exonization in rT2/rT1 transcripts in developing embryos resulting in a complex set of transcripts as opposed to the relatively simple and TE-free transcripts in the testis. In this study, rT2/rT1 transcriptional activation in cancer cells is demonstrated; the cancer rT2/rT1 transcripts are structurally similar to the developmental transcripts in frequent TE exonization. An uncharacterized rT2/rT1-like SPOP paralog, designated as SPOP-like (SPOPL), and related retro-sequences have also been identified by sequence interrogation of the GenBank databases. Sequence alignment, synteny and phylogenetic analyses implicate that evolution of the TD/POZ family of genes and retrogenes from a common ancestor involves (i) at the DNA level: chromosomal segmental duplication and recruitment of TEs as tissue-specific promoters, and retrotransposition, and (ii) at the RNA level: TE exonization through alternative splicing to further expand the transcript population. TE involvement is particularly profound in embryonic and cancer cells and may have biological significance.

B4.04
Chromosomes open into two opposing semi-cones with projection of active translatable genes into the interchromatin domain: novel geometrical model explaining speed of recognition of binding sites by transcription factors TF

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Objective: Explaining the fast recognition of appropriate sequences by TFs Designing a 3d model highlighting microscopic organization of interphase chromosomes.
Materials and Methods: Incorporating nucleosome/beads on string model, we designed a topological model with six preconditions:
(1) Easy opening and condensation without risk of aberration.
(2) Retention of two longitudinal axes for long and short arms.
(3) Easy access of the TFs to the transcribed genes.
(4) Neighborhood of genes (chromosomes) that are translocated in cancer to each other.
(5) Availability of an interconnected channel system (interchromatin domain) in continuity with the nuclear membrane pores for TFs.
(6) Dynamic property of DNA molecule sensing the entrance of TFs and extending the transcribed genes to reduce the time of search.
Results: A topological model of two angled semi-cones (one for each chromosome arm) is the best fit. The potential space harbors genes for transcription. The central axes comprise of non transcribed genes seen as heterochromatin during interphase. The DNA molecule has alternating double and single bounds (nucleotide distance = 3.3 Angstrom) which is the major prerequisite for being electricity conductive. This property is used to extend specific genes outwards for easier recognition by transcription factors. Based on four color problem in topology, 63 frequent translocation seen in cancer were incorporated into a three dimensional neighborhood of chromosomes.
Conclusions: (1) Try and error of TFs is not an economic strategy.
(2) The interphase chromosome has a geometrical hierarchy.
(3) Two opposing angled semi-cone model best explains the potential space of transcribed genes.

B4.05
Overexpression of arabidopsis transcription factor *AtICE2* enhance the expression of genes involved in the ABA-dependent cold response

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An overexpression of *AtICE2* (as we named the At1g12860 locus) in transgenic arabidopsis plants results not only in increased tolerance to deep freezing stress after cold acclimation (Fursova et.al., 2009), but in development of stomatal cell lineages also (Novokreshchenova et.al., 2009). In the current study, we characterized an expression pattern of arabidopsis genes *CBF1-3, RAB18, NCED3, RD29A, RD29B* in arabidopsis plants constitutively over-expressed full-sized *AtICE2* gene sequence (F-ICE2 line) and the deleted variant without 1st exon (D-ICE2). The expression level was examined by Real Time PCR analysis of cDNA using standard protocols. The mRNA level of *CBF2* and *CBF3* genes in both transgenic lines was the same as in wild type (WT) plants. The *CBF1* mRNA was 32 times higher in F-ICE2 line and 8 times higher in D-ICE2 line than in WT. The expression level of *RAB18,* which regulated by ABA, was 18 times higher in F-ICE2 line (in D-ICE2 line was the same as in WT). The mRNA level of *NCED3* that involved in ABA synthesis was increased (108 times) in F-ICE2; in D-ICE2 it is increased 14 times over WT. The same pattern was observed in the changes of expression level of ABA-regulated *RD29a* and *RD29b* genes. This fact suggests that *AtICE2* gene regulates ABA-dependent cold response pathway. The first F-box coding exone of *AtICE2* plays an important role in this regulation. The results of splice variants for *ICE2* gene was studied also. The study was supported by grants from RFBR program (09-04-12216-ofi-m).
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B4.06
Micro-RNA regulation of SPOP in cancer cells

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SPOP, speckle-type POZ protein, is a highly conserved protein found ubiquitously in human tissues, and has been shown to down-regulate the pancreatic transcription factor, PDX-1, in pancreas development. SPOP also participates in protein ubiquitination and degradation. This study aims to investigate regulation of SPOP expression. When real-time RT-PCR was performed to determine relative SPOP mRNA levels in a number of human cancer cell lines, a 14-fold difference was observed between different cell lines with the breast cancer MCF7 and cervical cancer SiHa cell lines expressing the highest and the lowest SPOP mRNA levels, respectively. Moreover, western blot analysis indicated relatively constant SPOP protein levels in all the cell lines analyzed except for the colon cancer cell line SK-CO1 which showed very low SPOP protein levels suggesting post-transcriptional regulation. miRNA database searches of the ~1.5-kb 3'-UTR predict involvement of miRNAs 106 and 145 in SPOP regulation which was verified by luciferase reporter assays and direct miRNA knockdown and over-expression in selected cancer cell lines.

B4.07
Methylation status of miR-124a gene promoter and its tumor suppressor role in cervical oncogenesis

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Identification of genes that undergo cancer-specific CpG island hypermethylation and correlation of these data with pre-neoplastic lesions, tumor stage, progression, and long-term prognosis are becoming increasingly common. Subsequent analysis proved that miR-124a is also frequently methylated in colon, breast and lung carcinoma cell lines. The hypermethylation of miR-124a promoter in cancer cells results in an increased expression of its target CDK6. This study was conducted to investigate the promoter methylation status of the miR-124a gene promoter in pre-neoplastic lesions and cervical cancer and his possible implication in regulation of p21 mRNA levels, and the CDK6 partner-cyclinD1. Promoter methylation was evaluated using a MS-PCR for bisulphite treated DNA (EpiTect Bisulfite Kit - Qiagen) samples. The DNA samples were isolated from 50 cervical swabs with abnormal cytology and 10 from normal patients. (High Pure PCR Template - Roche). RNA was isolated using Trizol and reverse-transcribed with Access Quick (Promega). The level of p21 and

cyclinD1 gene expression was quantified using Applied Biosystems primers and probes. We found significantly higher methylation frequencies for the miR-124a gene promoter in cervical cancer lesions (78%). The methylated promoter was presented also in pre-neoplastic lesions. Regarding the p21 gene we observed a significant decrease of gene expression in cervical cancer (p = 0,045, CI = 95%), but the level of cyclin D1 expression was higher in pre-neoplastic lesions and in cancer (p = 0,02, CI = 95%). The 124 miRNA seems to have an important role in cell cycle regulation acting like a tumor suppressor.

B4.08
Human genome enhancers show tissue-specific and promoter-specific activity

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The mammalian genome contains a great number of functional cis-regulatory non-transcribed elements, such as promoters, enhancers, silencers, and insulators which play a major part in regulation of genome activity. Enhancers are the DNA elements capable to increase the transcription initiated by the cognate promoter, and their activity is in most cases insignificantly depends on the promoter-enhancer distance and orientation. At the same time, it is believed that the activity of enhancer critically depends on tissue or cell type where this element is functioning. Earlier we identified and cloned 15 enhancers within one megabase region of human chromosome 19. Here we tested the activity of four of them in 5 cell lines of different origin and with respect to two different promoters (CMV and SV40). We demonstrated that the enhancers possess both pronounced tissue- and promoter-specificity. In addition, one of the enhancers was subjected to deletion analysis which allowed us to isolate its short core sequence responsible for the enhancer activity.

B4.09
The role of nuclear receptors in statin-induced molecular mechanisms

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Statins, cholesterol lowering drugs, act as competitive inhibitors of HMGCR which catalyses the rate limiting step of cholesterol synthesis. They activate different pathways not only via PXR (atorvastatin) but also via CAR-mediated mechanisms (rosuvastatin). The aim of this study is to get a better insight into the cross-talk between cholesterol homeostasis and metabolism of selected drugs in human primary hepatocytes and to define novel PXR and CAR-modulated genes. The effect of CAR on expression of cholesterol biosynthesis pathway will also be investigated. Human hepatocytes have been treated with rosuvastatin or atorvastatin for 12, 24 or 48 hour. Rifampicin has been applied as a well known PXR activator. Expression profiling was performed with Steroltalk v2 and Affymetrix HG-U133 Plus 2.0 arrays and data analysed with Orange and R Bioconductor software. To discriminate between targets of the two statins partitioning of differentially expressed genes and enriched genesets into Venn diagrams has been applied. Selected genes were confirmed by qRT-PCR. Microarray analyses showed that changes in human primary hepatocytes induced by both statins at 48 hours are extensive. The overlap between rifampicin and atorvastatin exposed genes activated by PXR. The overlap between both statins underlined genes common for the activity of both and may be activated by CAR. Overlap of the atorvastatin, rosuvastatin and rifampicin exposed genes involved in drug metabolism, cancer pathway and regulation of autophagy. Using expression profiling, bioinformatics and data mining we started to reveal novel pathways that are targeted by statins. More detailed information will be presented on the meeting.

B4.10
The role of orphan and ligand-dependent nuclear receptors in the regulation of the human apoM gene in the liver

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Apolipoprotein M (apoM) plays an important role in High Density Lipoprotein (HDL) remodeling in plasma. Mice lacking HNF-1, the major regulator of apoM in the liver, are characterized by absence of apoM and preb HDL particles in plasma. We have previously reported that the proximal promoter of the human apoM gene is additionally regulated by orphan and ligand-dependent nuclear receptors such as HNF-4, RXR homodimers and RXR heterodimers with RAR, T3R, PPAR or LXR. In the present study, we further examined their binding characteristics to apoM promoter using site-directed mutagenesis, DNA affinity precipitation and chromatin immunoprecipitation assays. We show here that all receptors bind to a DR1 hormone response element (HRE) at position -33 to -21 albeit with different affinities while binding was abolished upon mutagenesis of this HRE. Interestingly, the same mutation did not affect the binding of RXR/T3R heterodimers or of LRH-1 shown previously by others to occupy the same region, suggesting the presence of two adjacent HREs in the proximal apoM promoter. In agreement with these, the HRE mutation abolished transactivation by all receptors except LRH-1. Adenovirus-mediated overexpression of RXR or treatment of hepatic cells with retinoids, fibrates, oxysterols and T3 induced apoM gene expression. Retinoids, however, exhibited a biphasic mode of regulation with an early activation phase at 1–3 hour followed by a repression phase at 8–24 hour. In summary, our findings indicate that the apoM gene, similar to other genes of the HDL pathway, is a direct target of hormone nuclear receptors in hepatic cells.

B4.11
Macrophage specific regulation of apoCII gene expression via distal regulatory elements

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Purpose: Apolipoprotein CII is involved in the conversion of VLDL into LDL by activating lipoprotein lipase, an enzyme found on the surface of endothelial cells. In atherosclerotic inflammation, monocyte derived macrophages become foam cells by ingesting ox-LDL and eventually die, further propagating the inflammatory process. This study aims to understand the mechanisms of apoCII gene regulation in macrophages; the data obtained may be useful for future therapeutic strategy.

Methods and Results: Transient transfection experiments on RAW 264.7 cells showed that the multienhancer 2 region (ME2) interacts with the proximal promoter of apoCII (-545/+18) and with its deletion mutant (−388/+18). Chromosome conformation capture experiments revealed that ME2 and apoCII promoter interact in antisense direction. Transient co-transfection experiments indicated that STAT1 transcription factor has a binding site in the region −545/−388. The interaction between ME2 and apoCII promoter allowed STAT1 to induce the transactivation of the proximal promoter of apoCII. DNA precipitation experiments demonstrated the presence of a STAT1 binding site in the region 145-160 of ME2 that was also predicted by in silico analysis.
Conclusions: In macrophages, ApoCII gene expression is controlled by the cooperation between the promoter with ME2, which can bring different transcription factors in the proximity of apoCII promoter, but their effect depends on the interactions with factors acting on the proximal promoter.
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B4.12
Inhibition of cornea angiogenesis by small interference RNA targeting vascular endothelial growth factor receptor2

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Introduction: Cornea neovascularization (NV) occurs in various ocular disorders including proliferate diabetic retinopathy, retinopathy of prematurety and secondary neovascular glaucoma and chemical injuries which often result in blindness. Frequently vascular endothelial cell growth factors (VEGFs) are mainly responsible for the pathological neovascularization. Therefore, VEGF & VEGF Receptors (VEGFRs) could provide targets for therapeutic intervention on Cornea NV.
Material and method: In this study, we inspected the inhibitory effects of the specific small interfering RNAs (siRNA) targeting VEGFR (VEGFR2) on the expression of VEGFR in human umbilical vein endothelial cells (HUVEC) *in vitro*. Three different SiRNAs against the VEGFR2 were designed and chemically synthesized. Using GFP expression vector the specificity of SiRNAs were investigated in Hela cell and the suitable SiRNA which significantly silenced the target mRNA were transfected HUVEC cell using lipfecthaminth 2000. 24 hour after transfection the cells were harvested ant total RNA purified. RT-PCR and PCR Using VEGFR2 specific primers were done and PCR products were electrophoreses.
Results: The result has shown significantly decreased in VEGFR2 and mRNA. The clinical examination shown significantly reduces in corneal neovascularization.
Conclusion: These results indicate that VEGFR2-specific siRNAs can be use as a suitable therapy against corneal neovascularization in eye diseases.

B4.13
Characterization of a new signalling pathway controlling virulence in the bacterium Dickeya dadantii

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Production of plant cell wall degrading enzymes (CWDE), the major virulence factors of Dickeya dadantii, is a highly regulated process involving multiple regulators that enable the information from different inputs to be assessed before virulence genes are expressed. We have characterized a 25-kb vfm cluster which is required for D. dadantii pathogenesis. The vfm cluster encodes a biosynthetic pathway for a metabolite composed of both amino acid and fatty acid moities. Indeed, it encodes proteins displaying similarities with enzymes involved in amino acid activating process such as Ala-Ala ligases and amino acid decarboxylase (VfmO, VfmP, VfmK) and with enzymes involved in fatty acid biosynthesis such as acyl-CoA synthase, 3-oxoacyl-ACP synthases, acyl-CoA dehydrogenases, acyl-carrier proteins, 4-phosphopantetheinyl transferase (VfmM, VfmA, VfmT, VfmW, VfmR, VfmB, VfmU, VfmN, VfmZ, VfmQ, VfmJ). We suggested that the metabolite produced via the vfm cluster might act as a signal controlling virulence factor production in D. dadantii. This assertion is supported by the fact that, fatty acid derivatives serve as environmental cues to regulate virulence in several bacteria including Proteus mirabilis, Vibrio cholerae and Xanthomonas campestris. The vfm cluster contains two transcriptional regulators, VfmE of the AraC family and VfmH a two component response regulator which is associated to the VfmI sensor histidine kinase. Preliminary results suggested that VfmE controls signal production while VfmH-VfmI may sense the signal and induce transcriptional activation of pathogenicity genes.

B4.14
Expression analysis of genes involved in epigenetic regulation and apoptosis in human myelodysplastic syndrome cell line treated by 5-azacitidine

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PC-MDS is the first therapy-related myelodysplastic syndrome derived cell line, and based on its immunological, cytogenetic and molecular characterization, PC-MDS may be used as a new tool in evaluation of complex biology of MDS and a model for methylation studies. Therefore, our aim was to study the modulation of the expression status of 10 different genes involved in epigenetic regulation and apoptosis by the DNMT inhibitor 5-azacitidine (5-AZA), as markers of response to inhibition.
In our analysis we have used the SybrGreen technology and gene-specific primers for the qRT-PCR analysis of 10 genes, in cDNA of PC-MDS and K562 cells, treated by 1 micromole of 5-AZA for 24 hour. DNMT1 and DNMT3A showed statistically significant decrease of expression in 5-AZA treated PC-MDS cells, whereas DNMT3B showed significantly decreased expression in 5-AZA treated K562 cells. The members of the bcl-2 fam-

ily of apoptosis-regulating genes bcl-2 and Bax, showed the statistically significant differences in expression, in comparison with non-treated PC-MDS cells. Our most interesting result was the significant re-expression of p15 in 5-AZA treated PC-MDS cells. p15, the inhibitor of cyclin-dependent kinases, is frequently hypermethylated in MDS. 5-AZA, reverts hypermethylation of this gene *in vitro*. Evaluation of our qRT-PCR results showed that the re-expression of p15 in PC-MDS cell line, implicate this novel cell line as a suitable model for the studies of pharmacologic demethylation as a possible mechanism resulting in hematologic response in MDS.

B4.15
Expression of the main virulence genes in Dickeya datantii is sensitive to the DNA supercoiling state

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Dickeya dadantii (Erwinia chrysanthemi) is a bacterium that attacks a wide range of plant species. Soft rot, the visible symptom, is mainly due to the production of pectate lyases (Pels) that can destroy the cell walls. Transcription of the pel genes is modulated by various regulators in response to different stimuli such as KdgR (pectic compounds), Fis (growth phase), CRP (catabolic repression), H-NS (temperature and nutritional starvation). Most of these stimuli (growth phase, temperature, nutritional starvation,...) also modulate the structural organisation of DNA and remarkably, two regulators of the pel genes, H-NS and Fis, are chromatin structuring proteins. We report that the expression of the pel genes is modulated *in vivo* by changes in the DNA topology. However the impact of the structural organization of DNA on the expression of these genes is less pronounced in the hns and fis mutants than in the parental strain. These findings therefore suggest that H-NS and Fis might adjust the global structural modifications of the chromatin at the pel gene promoters in order to optimize their expression. These data obtained *in vivo* were further confirmed *in vitro* by comparing the transcriptional capacity of DNA templates with various supercoiling states. Together, these data indicate that the transcription of the pel gene is regulated by a dynamic cooperation between the transcription machinery and the topological state of DNA.

B4.16
WT1 and its molecular partners modulate bag3 gene expression

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Wilms' tumour 1 protein, WT1, is a zinc finger transcriptional factor that controls the expression of a wide number of genes involved in tissue differentiation, in apoptosis, or in cell cycle control. Moreover, the transcriptional regulatory properties of WT1 are known to be influenced by interaction with an increasing number of molecular partners. Recently, we identified the Krüppel-like zinc finger protein, ZNF224, as a novel human WT1-associating protein and demonstrated that ZNF224 may act as a transcriptional co-regulator of WT1 in order to modulate target gene activity. In the present study, we identify bag 3 as a novel target gene of WT1 protein, involved in apoptosis regulation. Bag3 gene expression is constitutive in some tumour types, including leukemias; in these cells, BAG3 protein has been demonstrated to sustain cell survival and down modulate cell apoptotic response to drugs. We report that WT1 positively regulates bag3 gene expression through a transcriptional mechanism. WT1 knockdown affects bag3 expression and enhances apoptosis by 30% in the human erythroleukemia cell line K562. Instead, in HEK293 clones stably over-expressing ZNF224 we observe that WT1 is unable to activate bag3 gene expression, thus indicating that ZNF224 behaves as a transcriptional co-repressor of WT1 on bag 3 promoter. The study of the role of the proteins WT1 and ZNF224 in regulation of bag3 gene expression will provide novel insight into the understanding of apoptosis regulation.

B4.17
Gene expression profile in human white blood cells after ramp-type progressive exercise

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It is well known that high intensity (˜ 80% peak O_2 uptake) endurance exercises alter gene expression in human white blood cells (WBCs). The purpose of present study was to investigate the effects of short ramp-type progressive exercise on immediate early gene (IEG) expression. Ten well trained skiers (21–24 year old) participated in ramp-type progressive exercise on treadmill until exhaustion. Average time in test and after anaerobic threshold was 14:30 and 5:15 minute respectively. Blood samples were obtained preexercise (T0) and immediately after exercise (T1). Exercise induced significant increase in extracellular heat-shock protein 70kDa (eHSP70), growth hormone (GH), cortisol concentration and creatine kinase (CK) activity in serum, so subjects endure high intensity physiological stress. Gene expression in WBCs was measured using GeneChip 1.0 ST Array (Affymetrix). With false discovery rate (FDR) <0.05 with 95% confidence, a total of 153 genes were differentially expressed (117 up regulated, 36 down regulated). Exercise influenced a variety of established gene pathways related to inflammation, stress response, signal transduction and apoptosis. But in present study we find a large group of differentially expressed ribosomal proteins (RP) and imprinted small nucleolar RNAs (snoRNA). In summary, even very short physiological stress (up to 7-15 min) lead to activation of stress response genes and previously unknown significant alteration of RP and snoRNA gene expression.

B4.18
E(y)2/Sus1 is required for PRE-blocking activity of the Wari insulator in *Drosophila melanogaster*

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Insulators are classically described by two experimentally defined properties, enhancer-blocking activity and barrier activity. Insulators operate in a position-dependent manner (enhancer-blocking activity), interfering with the activity of enhancers when inserted between these regulatory elements and a promoter but not when located upstream or downstream from them. The barrier activity of insulators blocks heterochromatinization and consequent silencing of a gene. Both activities of insulators are required for the anti-silencing function, i.e. for blocking the repressive effect

of a Polycomb response element (PRE). Recently we have found an insulator, named Wari, located between the *white* gene, which is responsible for eye pigmentation, and the CG32795 gene. The Wari insulator is also present downstream of the *mini-white* gene, which is widely used as a marker in transposons. Here, we show that the previously identified 368-bp core of this insulator is sufficient for blocking PRE-mediated silencing. Although Wari does not contain binding sites for known insulator proteins, the E(y)2 and CP190 proteins bind to Wari as well as to the Su(Hw)-containing insulators *in vivo*. It may well be that these proteins are recruited to the insulator by as yet unidentified DNA-binding protein. Partial inactivation of E(y)2 in a weak $e(y)2^{u1}$ mutation impairs only the anti-silencing but not the enhancer-blocking activity of the Wari insulator. Thus, the E(y)2 protein in different *Drosophila* insulators serves to protect gene expression from silencing. This study is supported by the Russian Foundation for Basic Research (project no. 09-04-00903-a).

B4.19
Interaction of insulators with promoters results in gene looping in *Drosophila melanogaster*

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Chromatin insulators are special regulatory elements involved in modulation of enhancer promoter interaction. Although much of the research was made to understand the main role of insulators in the regulation of gene expression *in vivo*, it's still poorly known. Previously it was shown that *Drosophila yellow* and *white* genes contain insulators placed immediately downstream them, 1A2 and Wari, respectively. Here we report that both insulators are able to interact with their target promoters forming gene loops. Moreover, we show the interchangeability of interacting insulator-promoter pairs supporting that this could be a general property of insulators. We show that deletion of the Wari insulator downstream the *white* gene in transgenic constructs leads to significant decrease of the *white* gene expression what argues the important role of at least some insulators in gene transcription. Our results provide new insights in mechanism of insulator function and suggest their basic role in gene transcription. This study is supported by the Russian Foundation for Basic Research (project no. 09-04-00903-á).

B4.20
Genes expression in biofilm of Thermus spp

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At least 50% Thermophilic isolates from hot spring of Taiwan with biofilm forming ability. Biofilm is community of microorganism embedded in extracellular matrix which was secreted by the microorganism and it formed a physical and chemical barrier to keep clinical isolates from antibiotics and of macrophage attack. Following two-dimensional gel electrophoresis was applied to study the molecular mechanism of biofilm formation of Thermus spp. Results revealed that protein involved in general function prediction, transcription, and hypothetical proteins shared 37% of differential expressed proteins between the 8th hour planktonic and sessile cells. Meanwhile, hypothetical protein or protein with unknown function also played a role of 15% while comparing total protein from the 18th hour cells. We also found that 8% and 10% of protein involved in carbohydrate transport and metabolism were observed in the 8th and 18th hour samples, respectively. GalE protein is an UDP-galactose-4'-epimerase which involved in conversion UDP-galactose and UDP-glucose was found expressed differentially in biofilm cells. Chaperonin (GroESL) also expressed differentially between biofilm and planktonic cells. Overexpression of galE gene and groESL showed increasing biofilm formation ability indicating these genes wree involved in biofilm forming. Meanwhile, we also found a two component regulator like protein expressed in sessile cells only while biofilm was formed. In order to study the complex regulatory networks of biofilm formation, we will clarify the function of those differentiate proteins and their contribution to biofilm formation.

B4.21
Rhythmic interaction between *Period1* mRNA and HnRNP Q leads to circadian time-dependent translation

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The mouse PERIOD1 (mPER1) plays a crucial role in the circadian rhythm, and also provides an important link between the circadian and the cell cycle system. To date, the important issue of how mPER1 expression is rhythmic is explained by only limited information, transcriptional and posttranslational regulation. Here we show that the expression of mPER1 is regulated by rhythmic translation of m*Per1* mRNA regardless of its mRNA profiles. This time-dependent translation is controlled by an internal ribosomal entry site (IRES) element in the 5'-untranslated region (5'UTR) of m*Per1* mRNA along with the trans-acting factor, mouse heterogeneous nuclear ribonucleoprotein Q (mHnRNP Q). Knockdown of mHnRNP Q caused a decrease and a delay in mPER1 expression. The rate of IRES-mediated translation exhibits phase-dependent characteristics through rhythmic interaction between m*Per1* mRNA and mHnRNP Q. A mathematical model for describing the mPER1 expression also suggests that mHnRNP Q-mediated rhythmic translation is a key process in its circadian oscillation. Here, we demonstrate 5'UTR-mediated rhythmic m*Per1* translation, and provide evidence for posttranscriptional regulation of the circadian rhythmicity of core clock genes.

B4.22
Nicotinamide N-Methyltransferase: a promising biomarker for early diagnosis and prognostic monitoring of oral squamous cell carcinoma

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Oral squamous cell carcinoma (OSCC) is the most common malignancy of oral cavity. Up to now, reliable biomarkers for OSCC are still lacking. Therefore, it is necessary to identify target molecules for effective therapy and early diagnosis of OSCC. We analysed the expression of Nicotinamide N-Methyltransferase (NNMT), which catalyses the N-methylation of nicotinamide, in paired tumour and non-tumour tissues by RT-PCR, Real-Time PCR, and western blot. Compared with normal mucosa, OSCC exhibited significantly increased expression of NNMT in 50% of examined patients. NNMT was upregulated in most of the favourable OSCCs (N0). pT, pathological staging and lymph node metastasis showed an inverse correlation with NNMT mRNA levels. Immunohistochemical analyses seem to indicate a favourable prognosis in patients with tumours expressing high NNMT levels, whereas the absence of marked NNMT expression seems to constitute a hallmark of aggressive biological behaviour. We also evaluated the effect of shRNA-mediated inhibition of NNMT on the proliferation and apoptosis of oral cancer cell line PE/CA-PJ15. ShRNA vectors efficiently suppressed NNMT expression. The shRNA-mediated gene silencing of NNMT resulted in a significant rise in apoptosis rate. Preliminary studies on salivary NNMT by western blot showed strong immunoreactive bands in samples of OSCC patients and negative or weakly positive bands in normal saliva samples. The present data support the hypothesis that NNMT plays a role in tumour expansion and represents a highly promising marker for early detection of oral cancer. Moreover, knowing the status of NNMT expression would be important in predicting the patient prognosis.

B4.23
Up-regulation of p21WAF1/CIP1 expression stimulates G1-phase arrest of the cell cycle in cladosporol A-treated HT-29 cells

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Chemoprevention is a clinical practice that is gaining favour in the treatment of degenerative diseases including cancer. The list of compounds able to interfere with the various steps of the tumorigenic process is growing and includes new molecules isolated from plants, fungi and microorganisms. Cladosporols, a series of compounds purified as secondary metabolites from Cladosporium tenuissimum, display antifungal activity. In this study we sought to investigate the antiproliferative properties of cladosporol A, the major isoform of this family of compounds. By assessing cell viability we show that cladosporol A inhibits the growth of several human colorectal cancer-derived cell lines in a time- and dose-dependent manner. Administered to HT-29 cells, cladosporol A causes a G1-phase arrest of the cell cycle, accompained by an early and robust induction of p21WAF1/CIP1. By RT-qPCR assays and transient transfections of a luciferase reporter gene, under the control of the p21WAF1/CIP1 promoter region, we demonstrate that the induction takes place at transcriptional level. Molecular dissection of this region, by deletion analysis, revealed the shortest fragment able to drive the full transcription. A series of Sp1 binding sites located at nucleotides were identified as crucial for the induction. Their site directed mutagenesis abolishes the induction. This is the first report of an antiproliferative effect induced by cladosporol A, mediated by transcription induction of p21WAF1/CIP1 in a p53-independent manner.

B4.24
Dynamics of histone modifications in actively transcribed locus

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Considering the importance of histone modifications in transcription control, the aim of our study is to determine the area and impact of transcription coupled histone modifications in the proximity of an actively transcribed locus. For that purpose we have constructed a yeast (S. cerevisiae) strain carrying a strong transcription termination sequence in the middle of an inducible model gene (VPS13) to detect different nucleosome modifications found on chromatin before and after RNA polymerase II transcription through the region. As shown previously, in actively transcribed loci, nucleosomes are constantly being exchanged giving rise to the question of the origin and maintenance of histone marks in that area. We show the spread of different acetylation and methylation marks on histones H3 and H4 on transcribed and untranscribed gene regions hence also indicating the origin of histone modifications and their relationship to RNA polymerase II dependent transcription. We also discuss the activity of histone deacetylase Rpd3 during transcription and its role in chromatin mark dynamics.

B4.25
Protein kinase Cδ downregulates vaccinia related kinase 1 activity in DNA damage induced apoptosis

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Vaccinia-related kinase 1 (VRK1) is a novel serine/threonine kinase, which plays an important role in cell proliferation. However, little is known about the upstream regulator of VRK1 activity. Here we provide evidence for a role of protein kinase C (PKC) in the regulation of murine VRK1. We show that PKC interacts with VRK1, phosphorylates its Ser355 residue in the putative regulatory region and negatively regulates its kinase activity *in vitro*. Intriguingly, PKCδ-induced cell death is facilitated by phosphorylation of VRK1 when cells were exposed to DNA damage agent. In addition, p53 plays a critical role in the regulation of DNA damage-induced cell death accompanied with PKCδ-mediated modulation of VRK1. In p53-deficient cells, the effect of PKCδ-mediated phosphorylation of VRK1 on cell viability did not appear. However, the cells overexpressing p53 exhibited significant reduction of cell viability when the cells were cotransfected with both VRK1 and PKCδ. Taken together, these results indicate that PKC regulates phosphorylation and downregulation of VRK1, thereby contributing to the cell cycle arrest and apoptotic cell death in p53-dependent manner.

B4.26
Decitabine in combination with SAHA causes apoptosis in cancer cells as well as in normal PBMC

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Decitabine (DAC) causing DNA hypomethylation and SAHA (vorinostat) inhibiting histone deacetylases represent epigenetic forces regulating gene and protein expression in cells, affecting cell cycle and cell death. Epigenetic agents are often used for developing novel methods of cancer therapy because of the reversibility and relatively slight mode of their action. Both DAC and SAHA has been approved by FDA for cancer therapy and combination of these two drugs has a great potential to make a considerable epigenetic changes. In our experiments, DAC alone causes substantial increase of p21 expression, higher levels of proteins p53 and Puma vere also detected, but the viability of the cells, as measured by MTT test, decreased only minimally, and we did not detect any remarkable apoptotic effect. While in CML-T1 cells we observed slight decrease in Mcl-1 expression and PARP fragmentation, none of these effects have been found in lymphocytes. Addition of SAHA simultaneously with DAC supressed DAC-induced p21 upregulation, p53 induction partially persisted only in lymphocytes. Substantial downregulation of anti-apoptotic Mcl-1 mRNA and protein expression and apoptosis-related features like mitochondrial membrane (MM) depolarization and PARP fragmentation were also detected. The extent of MM depolarization and viability decrease observed in CML-T1 cells after DAC + SAHA treatment implies that this combination has synergistic effect. Higher concentration of SAHA added with DAC was required for lymphocytes to lead them to apoptosis, designating this combination to be a promising therapeutic tool.

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B4.27
Tumor hypoxia induced HIF-α regulated genes expression and its potential functionality as tumor marker genes in human brain cancer

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Tumor hypoxia is a crucial factor in its aggressiveness and resistance to treatment, particularly modalities in brain cancer. HIF-1α regulates a set of genes under hypoxic oxygenation condition in the tumor microenvironment that are responsible for cancer disease development in human.

Methods: There was an *in vitro* as well an *in vivo* experimental approach, where in experiments series the hypoxia induced HIF-1α regulated genes were analyzed for their protein and mRNA levels under different oxygenation and reoxygenation conditions in GaMG, U373, U87-MG, and U251 brain tumor cell lines exposed to different oxygenation conditions including induced hypoxia as well as in tissue specimens groups from human brain cancer patients. Analyzed genes were; Ca9, NDRG1, EPO, OPN, VEGF, HIF-1α and Sp1.

Results: *In vitro*, Ca9, NDRG1 and HIF-1α showed a hypoxia regulated protein overexpression pattern in all cell lines. VEGF was overexpressed at 0.1% Oxygen. Epo level expressed was less than Ca9 in all cell lines. On mRNA level, OPN, Ca9 and NDRG1 were strongly expressed at 0.1% oxygen, but there was no elevated HIF-1α expression *In vivo*, Ca9, NDRG1, OPN, Epo and VEGF were overexpressed at protein and mRNA levels, where the first three had the highest expression rates.

Conclusion: Ca9, NDRG1 and OPN overexpression is associated with brain tumors development in a tumor stage specific manner which occurs at a higher frequency in glioblastoma both, on protein or mRNA level making them as ultimate brain cancer diagnostic markers when used as single or combinatory screening marker. Sp1 co regulates together with HIF-1α the expression of these genes under hypoxic conditions.

B4.28
CEBP-1α is induced in human glioblastoma as a consequence of the hypoxic oxygenation development in the brain tumor microenviroment

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CCAAT-enhancer-binding proteins (C/EBPs) are a family of transcription factors that promote the expression of certain genes through interaction with their promoter and comprise a family of basic region leucine zipper (bZIP) transcription factors that bind DNA through their basic regions, dimerize by their adjacent C-terminal leucine zippers to form homodimers or heterodimers with other C/EBP family members. Hypoxia induced activation of this regulatory protein in brain cancer had not been examined yet extensively.

Materials and Methods: C/EBP-1α regulation level was examined in human glioblastoma cells lines like U373, U251, GaMG and U87-MG under extreme hxpoxic oxygenation conditions (0.1% oxygen), reoxygenation after hypoxia for 24 and 48 hour and oxygenated conditions (21% oxygen and 5% carbon dioxide *in vitro*. Protein and mRNA level were detected via western blots and RT-PCR. Cells incubated for 24 hour with 100 μM DFO served as positive control for hypoxia and β-tubulin and β-actin served as loading control.

Results: C/EBP-1α was up-regulated via hypoxic development in different glioblastoma cells *in vitro* under extreme hypoxic conditions (0.1% oxygen) or reoxygenation after hypoxia, both on protein and mRNA level. Further, there was an association between expression and the expression of hypoxia induced, HIF-1α regulated genes in human glioblastoma tumour specimens examined, *in vitro*.

Conclusions: C/EBP-1α regulation as an answer to hypoxic development in glioblastoma, both on protein and mRNA level is a tumor cell specific hypoxic conditions influenced regulation in glioblastoma. HIF-1α is the main regulator under this conditions in human tumors.

B4.29
Genetic polymorphism detection of acute lymphoblastic leukemia in a group of eastern mediterranean patients

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Acute lymphoblastic Leukemia (ALL) is a severe blood disorder in which abnormal leukocytes are identified as immature forms of lymphocytes and is considered responsible for 25–30% of pediatric malignancies. Molecular approaches are used to detect various cancers types' genomic alterations; one of these approaches is random amplified polymorphic DNA analysis (RAPD).
Methods: Whole blood genomic DNA extraction from ALL patients was performed with the later use of twenty three different arbitrary primers for RAPD-PCR analysis. Loss, gain, shift, and detectable band's intensity changes were scored and subjected to statistical analysis. Cluster analysis methods were used for grouping bands into respective categories.
Results: 10¨M 23 RAPD primers used in the study were able to amplify genomic sequences of ALL leukemia patients. The ten amplifying primers varied in their ability to detect genomic DNA instability of ALL patients. The obtained results revealed that one of amplifying primers showed novel DNA bands pattern in ALL patients, band number one (3162 bp) was absent in 78% of ALL patients, while this band was present in normal individuals. On the other hand, band number four (1 Kb) was absent in normal individuals but it was present in 55% of ALL patients. Moreover, dissimilarity matrix analysis of obtained RAPD-PCR bands of genomic DNA from whole blood of ALL patients provided essential information on genetic polymorphism among genomic DNA of ALL patients.
Conclusions: Genetic instabilities are frequent in ALL genomic DNA and could be detected quickly and accurate by using suitable RAPD primer.

B4.30
Potassium mediates Escherichia coli enzyme IIANtriVdependent regulation of sigma factor selectivity

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An Escherichia coli mutant devoid of enzyme IIANtr (EIIANtr) of the nitrogen PTS is extremely sensitive to leucine-containing peptides due to decreased expression of acetohydroxy acid synthase. This decreased expression is due to defective potassium homeostasis. We report here the mechanism for regulation of gene expression by the intracellular K+ level. The leucine hypersensitivity of a ptsN (encoding EIIANtr) mutant was suppressed by deleting the rpoS gene, encoding the stationary phase σ factor. Despite intracellular levels of sigma factors comparable to the wild-type strain, most of the genes down-regulated in a ptsN mutant are controlled by σ70, while all the up-regulated genes are controlled by σS, implying that the balance of sigma activities is modified by ptsN deletion. This change of sigma factor activity was found to be due to increased levels of K+. *In vitro* transcription assays showed that a σ70 controlled gene and a σS controlled gene were differentially affected by potassium concentration. Biochemical studies revealed that K+ is responsible for sigma factor competition by differentially influencing the binding of σ70 and σS to core RNA polymerase. Taken together, the data indicate that EIIANtr controls sigma factor selectivity by regulating the intracellular K+ level.

B4.31
The effects of chemotherapeutic agents on differentiated chordoma cells

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Background: Chordoma is a rare type of malignant bone tumor and is known to arise from the remnants of the notochord. Resistance to chemotherapy makes the treatment even more inconvenient therefore new approaches need to be developed in curing chordoma. Differentiation therapy with various differentiating agents is a common method that is used in the treatment of several types of tumors. Based on the enforcement of cells to commit and maturate into other lineages, differentiation therapy might be a promising technology in order to treat chordomas.
Methods: In this study a chordoma cell line, U-CHI, was treated with all trans retinoic acid and the efficacy of the chemotherapeutic agents including doxorubucine, cisplatin, etoposid, fludarabin, cyclyphosphamid, meplhalan, paclitaxel, and methotraxate after the differentiation was assessed.
Results: The number of chordoma cells, after the differentiation therapy, was decreased when treated with chemotherapeutic agents. The percentages of viable chordoma cells over untreated counterparts were significantly reduced when treated with cisplatin (from 87% to 45%), vincristine (from 65% to 15%), etoposide (from 95% to 35%), doxorubicin (from 61% to 4%), and fludarabine (from 100% to 34%). Other chemotherapeutic agents including, cyclophosphamide, melphalan, paclitaxel, and methotrexate had showed no effect on the number of chordoma cells.
Conclusion: The results suggest that chemotherapeutics might be more effective against chordoma cells when combined with retinoic acid. This new approach is promising to develop an alternative method to conventional therapies.

B4.32
Dengue viral core protein induces Interleukin-8 (IL-8) via interacting with positive transcription elongation factor b (P-TEFb)

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It is well known that Dengue viral (DENV) infection induces the elevation of IL-8 expression, however, little is known about this molecular mechanism. The positive transcription elongation factor b (P-TEFb) not only functions as a basic transcriptional elongation factor, but also activates certain genes bearing specific DNA elements recognized by transcriptional activators that may interact with P-TEFb. In this study, we explored the possibility

that P-TEFb may contribute to the stimulation of IL-8 gene expression by interacting with dengue viral core protein. The results of Immunostaining and co-immunoprecipitation assays demonstrated the association between P-TEFb and DENV core protein. The chromatin immunoprecipitation data provided evidence that the associated complex of P-TEFb and DENV core protein is recruited directly to the IL-8 gene promoter region, resulting in the activation of IL-8 gene expression. To our knowledge, these data provided the first evidence that P-TEFb and DENV core protein participate in the induction of IL-8 gene expression by DENV infection.

B4.33
Using siRNA and miRNA mimetics to obtain RNA-interference in cancer cells

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It is a challenge to try to understand how gene expression is regulated in various eukaryotic organisms, including the human body. The cells in eukaryotic organisms contain small double-stranded hairpin-like RNA structures coded for by microRNA (miRNA) genes in the cell nucleus (Carthew and Sontheimer, 2009). These miRNA structures specifically base pair with coding mRNA after being processed, and hence hinder further expression of the targeted genes. This kind of gene silencing can also arise if a small double-stranded exogenous RNA, so called small interfering RNA (siRNA), is introduced into a eukaryotic cell. It would therefore be of interest to design and construct siRNA and miRNA mimetics and to specifically target their silencing effect on genes associated with the development of cancer. In the present project, we initially intend to explore the role of different miRNA candidates for the survival of various types of cancer cells. miR-146a is for example an interesting candidate for investigation, since this miRNA seems to influence the expression of the genes BRCA1 and BRCA2. These genes code for proteins that are associated with the development of not only female breast cancers, but for example also male breast cancers (Shen et al., 2008).

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B4.34
Role of cytokines in repair of DNA damages induced by alkylating agents

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Repair of primary DNA damage induced by alkylating agents is realized by repair enzyme O6-alkylguanine-DNA alkyltransferase (or MGMT). MGMT repairs the most dangerous damages – formation of O6-alkylguanine in DNA. Mechanisms regulating expression of this enzyme are complicated and not studied enough nowadays. But the expression of the MGMT has been already known to be influenced by DNA hypermethylation and DNA breaks and also can be regulated through the mediated way, for example by protein p53, phosphorylation, ubiquitination. Moreover there are literature data that MGMT expression can be regulated by cytokines and growth factors IF-β, IL-3, GM-CSF. The purpose of our work was to research the possible influence of cytokines LIF, SCF and IL-3 on the MGMT expression in the human cell cultures. There were used the cells of different origin: original human cell lines BKF, 4BL2, standard tumor cell line Hep-2. Identification of MGMT protein in the cellular extract was carried out by the Western blot analysis. In our early works it has been shown that the MGMT protein may exist in cell cultures in two forms: the unmodified one with the molecular weight 22–24 kDa, and the modified of 48–50 kDa. All the cytokines studied have been shown to inhibit MGMT expression in different way. The cytokin LIF almost did not affect on the amount of the modified protein, but caused the disappearance of the unmodified form. After the treatment of cells with IL-3 and SCF the protein in the unmodified form disappeared completely, and the amount of the modified protein considerably decreased.

B4.35
Next generation sequencing to reveal new sight into cancer research

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The human genome lies at the core of human disease research. Using next generation sequencing technology such as Illumina/ Solexa, we could get an all-round view of complex diseases including cancer at DNA, RNA and epigenetic level. With genome resequencing, somatic mutations from cancer genome can be obtained, including all point mutations, rearrangements and copy number changes. Exome capture sequencing is a relatively economical strategy. Cancer-specific genetic mutations will be identified by directly comparing exon sequences from a patient's tumor to its non-disease exons. In addition, SNP genotypes will be calculated for each patient (both non disease and tumor tissue) as well as structural variations. Using transcriptome sequencing, digital gene expression profiling and small RNA sequencing technologies, cancer-specific alterations in expression levels, aberrant splicing and fusion transcripts could easily be defined. Whole-genome bisulfite sequencing, MeDIP-sequencing and ChIP-sequencing are used to discover aberrant DNA methylation and histone modifications. Epigenetic changes are now established in development and progression of cancer and other complex diseases. We are applying the strategies above to several solid tumors research. We are intended to identify the full range of somatically acquired genetic alteration, including point mutations on a genome-wide basis, insertions and deletions, copy number changes and genomic rearrangements, as well as characterizing the cancer cell transcriptome and epigenome. All these discoveries will promises a new era for cancer genomics.

B4.36
Peptidyl-prolyl isomerase cyclophilin B suppresses gastric carcinoma against hypoxia-induced apoptosis through attenuation of endoplasmic reticulum stress

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Cyclophilins are protein chaperones that accelerate the rate of protein folding through their peptidyl-prolyl cis-trans isomerase (PPIase) activity. Cyclophilin B (CypB) is a 21-kDa protein with peptidyl-prolyl cis-trans isomerase activity. CypB is detected mainly in the ER lumen. Although there has been some speculation that CypB plays a role in protein folding in the endoplasmic

reticulum (ER), there is currently no direct information supporting the notion that it provides a significant role in response to ER stress. Low oxygen tension (hypoxia) is a common feature of solid tumors and stimulates the expressions of a variety of genes including those related to angiogenesis, apoptosis and ER stress response. To determine the role of CypB in tumorigenesis, we investigated the induction of CypB as well as the role it plays in cancer cells. We found that hypoxia induced ER stress, as assessed by the expression of GRP78 and CHOP. Recent studies have shown diverse functions of cyclophilin family and we suggest that CypB might play like an antioxidant. Overexpression of wild-type CypB attenuated ER stress-induced cell death, whereas overexpression of an isomerase activitydefective mutant, CypB/R95A, not only increased apoptosis-associated with proteins from the ER and ROS generation. Here, we have shown that induction of CypB is associated with hypoxia in AGS cells.

B4.37
Overexpression of modified cyclophilin a regulated apoptosis through endoplasmic reticulum stress

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The protein is guided to the ER by a signal sequence. signal sequence of preprolactin, after translocation into microsomes and cleavage by signal peptidase, is converted to an intermediate form. The KDEL sequence(lys-asp-glu-leu) is a signal for permanent retention of proteins in the endoplasmicreticulum(ER). Cyclophilin A(CypA) is a relatively abundant small immunophilin exist in the cytoplasm of all mammalian cells. These immunophilins link to cytoplasmic dynein indirectly through the association of the immunophilin peptidylprolyl isomerase (PPIase) domain with dynamitin, a component of the dynein-associated dynactin complex. our study showed that the localization of cyclophilin A(cypA) is regulated by prolactin signal sequence and the KDEL sequence. The ER stress can be induced not only by alterations in physiological conditions such asproteins undergo post-translational modifications in the ER, which requires high levels of luminal Ca^{2+} and oxidative components. Exposure of cells to glucose starvation, inhibition of protein glycosylation, disturbance of Ca^{2+} homeostasis, or oxygen deprivation devastates ER environments and causes unfolded proteins to accumulate in the ER (ER stress). We identified ER existence of a modified cyclophilin A expressed in the Endoplasmic reticulum directed by the prolactin signal sequence and the KDEL sequence that resisted ER-stress-indeced cell death in chang cells.

B4.38
T-cadherin overexpression in culture of murine melanoma cells suppresses proliferation

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T-cadherin has a lower expression profile in many types of cancer and tumour cells. Tumour cells transfected with T-cadherin cDNA exhibit decreased proliferative and invasive potential. We analyzed the effect of T-cadherin overexpression on proliferation of murine melanoma B16F10 cells. Melanoma cells were stably transfected with the plasmid encoding human T-cadherin or control plasmid. For proliferation assay, cell numbers were determined daily for up to 6 days by counting in a cell counter. Distribution of melanoma cells at different stages of the cell cycle was determined with propidium iodide staining by flow cytometry. Expression of TGF-beta was measured by quantitative real-time RT-PCR. T-cadherin overexpression reduced proliferation rate of melanoma cells and decreased expression of TGF-beta as compared to control. Flow cytometry confirmed the delay in the cell cycle progression in T-cadherin overexpressing melanoma cells since the percentage of those cells in G2/M phase was significantly lower than of control cells. In normal cells TGF-beta is known to inhibit proliferation, but in cancer various components of the TGF-beta signaling pathway could be mutated, making the cell resistant to the effects of TGF-beta. Although the mechanisms of T-cadherin-dependent suppression of melanoma cell growth have yet to be elucidated, our data indicate that there is a correlation between elevated T-cadherin and reduced TGF-beta expression. The downregulation of TGF-beta mRNA upon overexpression of T-cadherin suggests the possible implication of TGF-beta signaling in T-cadherin-mediated suppression of melanoma cell proliferation.

B4.39
Opposite effect of HIF-1 and HIF-2 on regulation of IL-8 expression in endothelial cells

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Hypoxic transcriptional response is primarily mediated by hypoxia-inducible factor (HIF)-1 and HIF-2, consisting of α and β subunits. The HIF-1α and HIF-2α are similar in their DNA binding and dimerization domains, but differ in transactivation domains, implying they may regulate distinct genes and require different transcriptional cofactors. It is well known that hypoxia is a potent inducer of angiogenesis by enhancing the expression of VEGF. Interestingly, recently we showed that interleukin-8 (IL-8), the other mediator of angiogenesis, tumor growth and metastasis, is down-regulated by HIF-1α. As HIFs may differ in their functions, we aimed to examine the effect of HIF-2α on IL-8 expression. Human microvascular endothelial cells (HMEC-1) were transduced with adenoviral vectors to express HIF-1α or HIF-2α. Our results indicate that in contrast to HIF-1α, overexpression of HIF-2α resulted in significantly increased expression of IL-8, as revealed by analysis of the IL-8 promoter activity as well as mRNA and protein level. Accordingly, HIF-2α was found to positively affect the activity of SP-1, transcription factor involved in IL-8 regulation, as shown by reporter gene analysis and EMSA assay. Moreover, exemplary gene, thymidine phosphorylase (TP), containing binding sites for SP-1, was shown to be upregulated by HIF-2α as well. Importantly, effect of HIF-2α on IL-8 as well as TP expression was reversed by SP-1 inhibitor, mithramycin A. Current study revealed for the first time opposite role of HIF-1α and HIF-2α in regulation of IL-8 expression in endothelial cells and the involvement of SP-1 in the positive regulation of IL-8 by HIF-2α.

B4.40
Heme oxygenase-1 overexpression leads to melanoma de-differentiation

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Heme oxygenase-1 (HO-1) is an inducible enzyme that degrades heme to biliverdin, carbon monoxide and iron. In our previous experiments we have demonstrated that HO-1 exerted pleiotropic protumoral activities in B16(F10) melanoma cells both *in vitro* and *in vivo*. Therefore, the aim of the present study was to investigate mechanisms responsible for observed effects. Melanoma cell line B16(F10) were transduced with retroviral vectors encoding for luciferase, GFP and HO-1 [B16(F10)-luc-GFP-HO-1]. B16(F10)-luc-GFP cells were used as controls. B16(F10)-luc-GFP-HO-1 displayed a slight upregulation of antioxidative genes: biliverdin reductase, catalase and glutathione S-transferase. On the other hand, they expressed lower numbers of transcripts for p21 and Mdm2. Of importance, B16(F10)-luc-GFP-HO-1 cells exhibited less dendritic morphology and weaker pigmentation than their control counterparts. In parallel, they were characterized by significantly lower expression of melanoma markers: tyrosinase, TRP-1, TRP-2, gp100 and MART-1. Interestingly, de-differentiative activities of HO-1 were notably enhanced by hypoxic conditions. Finally, basal production of reactive oxygen species (ROS) was lower in B16(F10)-luc-GFP-HO-1 cells than in B16(F10)-luc-GFP cells and prolonged N-acetylcysteine (NAC) treatment reversed highly pigmented phenotype of B16(F10)-luc-GFP cells, suggesting role of HO-1 as a ROS scavenger in differentiation of melanoma cells. Altogether, these data suggest that HO-1 exerts strong de-differentiative activity probably through antioxidative mechanisms and in this way may induce more aggressive melanoma phenotype.

B4.41
The interaction of Snf1 with Tor1 in *Saccharomyces cerevisiae*

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The yeast *Saccharomyces cerevisiae* attains energy homeostasis through complex regulatory events that are predominantly controlled by the Snf1 protein kinase. Snf1 senses the stress and energy starvation and activates the metabolic processes to produce ATP and inhibits anabolism, e.g., the biosynthesis of protein and lipid. Since its mammalian ortholog AMPK, a drug target for obesity and type II diabetes, also exerts analogous control of metabolism, there has been extensive interest to understand Snf1 activation and regulation in yeast to expedite human disease studies as well as fundamental understanding. Snf1 regulates the cellular growth and development in coordination with other signaling pathways such as the Tor1 pathway. When abundant and high quality nitrogen sources are present, Tor1 is active and initiates transcription of genes that are involved in ribosome biogenesis and protein translation. Although both Snf1 and Tor1 are key components in nutrition sensing and regulation of the cellular growth, little is known about how these two kinases coordinate in the regulation of cell growth.
Here we apply a systems biology approach to investigate the interaction between Snf1 and Tor1. The yeast reference strain CEN.PK 113-7D and its derivative strains snf1Δ, tor1Δ and snf1Δtor1Δ were grown in chemostat in both carbon (glucose) and nitrogen (ammonia) -limited media. Samples were taken at the steady state for the analysis of transcriptome, phosphor-proteome and metabolites such as free amino acids and lipids as well as glycogen/trehalose. Integrative analysis was performed to combine the datasets at different levels and the preliminary results will be presented.

B4.42
MGST1 downregulation affects genes expression in PC12 cells

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The microsomal glutathione transferase 1 (MGST1) belongs to the super family of Membrane Associated Proteins in Eicosanoid and Glutathione Metabolism (MAPEG). The enzyme is present in high amounts in the endoplasmic reticulum, mitochondrial and plasma membranes and possesses transferase and peroxidase activity. It may also play an important anti-apoptotic role. To test the MGST1 role in cell development we examined the effect of MGST1 suppression in PC12 cells. After transfection with eukaryotic vector containing specific antisense sequence, the expression of MGST1 mRNA decreased by 60% as was quantified by real-time PCR. The flow cytometry analyses revealed a higher percentage of necrotic cells. The total activity of glutathione transferases determined with non-specific substrate CDNB decreased by 15–20%, whereas in the presence of DCNB, a substrate more specific for cytosolic GSTs, the activity was similar to control cells. It may indicate that the decline in MGST1 cannot be compensated by a respective increase in cytosolic GSTs activity. Using microarray that profiles the expression of 263 genes we detected the altered expression of several groups of genes involved in the processes such as cell cycle and cell proliferation (S100A6, Rasgfr2, Cdk5), differentiation (Efna1, Ncoa6, Pax2), cell adhesion (Dscam, L1cam, Ninj1, Ret, Spg7) and apoptosis (Mtch1, Ngfr, Rtn4, Ywhah). MGST1 appears to be essential for a proper PC12 cell development and its reduction enhanced necrosis; however, some adaptive processes caused by changes in genes expression could promote cell survival. Supported by the grants 502-16-809, 502-16-810 and 503-6086-2 from the Medical University of Lodz.

B4.43
Experimental rat model of heart failure induced by chronic hypoxia: gene expression profiles of HIF-1α and BNP-45

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Despite significant therapeutic advances, incidence and mortality in heart failure (HF) remain unacceptably high. Therefore, novel insights into molecular mechanism of HF are required to develop novel therapeutic approaches. The progress made in our understanding of the pathophysiology of heart failure would not have been possible without a number of animal models, each one having unique advantages as well as disadvantages. The aim of study is to develop a rat model of HF induced by chronic hypoxia and

explored the gene expression profiles of HIF-1α and BNP-45 as master regulatory gene of oxygen homeostasis and biomarker of HF, respectively. Rats, male, were randomized into seven groups (n = 4 per group), control normoxia exposed to room air, hypoxia group were housed in hypoxic chamber (028%) for 1, 3, 7, 14, 21 and 28 days respectively.

Result: Histopathologic examination shows: Massive hypertrophy of cardiomyocytes accompanied by alterations of intercalated disk, necrosis, fibrosis and apoptosis as a hallmark ventricular remodeling. A drastic increase in plasma BNP-45 levels at 21-day exposure. The BNP-45 mRNA and HIF-1α mRNA expression increased and the activities of HIF-1α were significantly increased compared with control group.

Conclusions: Chronic hypoxia causes: Ventricular hypertrophy accompanied by myocardial structural damage and then progress to heart failure. There were changes in the gene expression either in transcription and post-transcription level.

B4.44
An early zygotic genome activator in tissue patterning formation, a multipotent Drosophila nuclear protein

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The zinc finger protein Zelda, a key activator of the early zygotic genome in Drosophila, is shown to be necessary for patterning the wing structure during late developmental stages of the fly. This protein has been shown to play crucial roles during embryogenesis. Our data suggest that Zelda affects major components of signalling pathways such as Hedgehog and Notch. Spatiotemporal overexpression and knockout of zelda's gene product in the larval stages of the fly, impair wing development resulting in abnormal adult tissue formation. In these experiments several changes in the production patterns of proteins such as Patched and Wingless are observed, indicating a direct involvement of Zelda in the signalling cascade involving these proteins. A prominent role of Zelda in cell cycle progression has been suggested by previous experiments. However, cell cycle promotion could be linked to or divorced from patterning decisions. Zelda is known to be involved in both processes and it remains to be elucidated the exact mechanism of this involvement.

B4.45
Nuclear myc promoter-binding protein-1 (MBP-1) expression is a prognostic factor in invasive ductal breast carcinoma

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Myc Promoter-Binding Protein-1 (MBP-1) is a transcriptional repressor, generated by alternative translation of the α-enolase mRNA, that negatively regulates c-Myc gene expression (1) and plays a suppressive role on tumorogenesis (2, 3). We analyzed α-enolase and MBP-1 expression and localization in normal breast epithelium and primary infiltrating ductal breast carcinoma (IDC) from 177 patients by Western blotting and immunohistochemical analyses, using specific anti-α-enolase mAbs. A significantly increase in α-enolase expression was observed in 98% of the analysed tumors, compared to normal tissues. Nuclear MBP-1 has been found in almost all normal tissues while its expression is retained in only 35% of the matched tumours. Statistically significant inverse correlation was observed between expression and nuclear localization of MBP-1 and ErbB2 and Ki-67 expression, node positivity and tumor grade. Furthermore, nuclear MBP-1 is associated with good disease-free survival of patients with primary IDC. Transfection experiments in human breast SKBr3 cells (ErbB2+) demonstrated that MBP-1 ectopic expression results in down regulation of ErbB2 expression, and led us to identify the ErbB2 promoter region involved in the binding, indicating that, like c-Myc gene, ErbB2 is a direct target of MBP-1 transcriptional repression fuctions in breast cancer. Owing to the prognostic influence of nuclear-MBP-1 expression in a subgroup of small tumors and among patients with node-negative and ErbB2- cancers, where the need for prognostication is the greatest, MBP-1 nuclear expression may prove to be a clinically valuable prognostic variable for breast cancer patients.

B4.46
GAPDH regulation in human tumor cells under hypoxic oxygenation conditions from different origin and their suitability as experimental housekeeping genes

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Gene expression studies related to cancer diagnosis and treatment are important. In order to conduct such experiment accurately, absolutely reliable housekeeping genes are essential to normalize cancer related gene expression. However, no single gene of this group of genes manifests always stable expression levels under all experimental conditions. Incorrect choice of housekeeping genes leads to interpretation errors of experimental results including evaluation and quantification of pathological gene expression. Here, we examined (a) the degree of GAPDH expression regulation in Hep-1-6 mouse hepatoma and Hep-3-B and HepG2 human hepatocellular carcinoma cell lines as well as in human lung adenocarcinoma epithelial cell line (A-549) in addition to both HT-29, and HCT-116 colon cancer cell lines, and 4 glioblastoma cell lines under hypoxic conditions *in vitro* in comparison to other housekeeping genes like β-actin, serving as experimental loading controls.

Results: No hypoxia-induced regulatory effect on GAPDH expression was observed in cell lines studied *in vitro* that were; Hep-1-6 mouse hepatoma and Hep-3-B and HepG2 human hepatocellular carcinoma cell lines, Human lung adenocarcinoma epithelial cell line (A-549), both colon cancer cell lines HT-29, and HCT-116, glioblastoma U373, U251,U87-MG and GaMG.

Conclusions: As it is the case for human hepatocellular carcinoma, mouse hepatoma, human colon cancer, and human lung adenocarcinoma, brain cancer GAPDH represents an optimal choice of a housekeeping gene and / (or) loading control to determine the expression of hypoxia induced genes in tumors of different origin.

B4.47
Glia/ischemia dual specific gene expression system for glioblastoma gene therapy

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Glioblastoma multiforme (GBM) is the most aggressive malignant primary brain tumor in human. However, glioblastoma has a very poor prognosis despite advances of multimodality treatment such as surgical resection and radio-chemotherapy. In this study, we developed the glia and ischemia dual specific gene expression system for glioblastoma gene therapy. pNI2-SV-Luc was constructed with the nestin intron 2 (NI2)-SV40 promoter for glia cell specific gene expression. To evaluate the efficiency of the plasmid, pNI2-SV-Luc was transfected into rat C6 and human U87 glioblastoma cells. Branched polyethylenimine (PEI, 25KDa) was used as a gene carrier. Transfection assay showed that luciferase activity in the pNI2-SV-Luc transfected cells was much higher than that in the pSV-Luc transfected cells. pEpo-NI2-SV-Luc was constructed with an erythropoietin (Epo) enhancer for hypoxia specific gene expression. To confirm the glia and ischemia dual specific gene expression, pEpo-NI2-SV-Luc was transfected into C6 cells and the cells were incubated under normoxia or hypoxia condition. The results showed that pEpo-NI2-SV-Luc had higher gene expression than pNI2-SV-Luc under hypoxia. Herpes simplex virus thymidine kinase/ganciclovir (HSV-TK/GCV) is the most common strategy for cancer suicide gene therapy. pEpo-NI2-SV-TK was constructed and transfected into C6 cells and the cells were maintained with GCV. As a result, the growth of the pEpo-NI2-SV-TK transfected cells was significantly suppressed. Therefore, the glia and ischemia dual specific gene expression system with the NI2-SV40 promoter and the Epo enhancer will be useful for glioblastoma specific gene therapy.

B4.48
Hypoxia-specific gene expression system using oxygen dependent degradation domain of activating transcription factor 4 for ischemia specific gene therapy

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Angiogenic growth factor genes have been used for ischemic disease gene therapy. They are effective to protect the cells in ischemic tissues. However, they may induce tumor growth when they are expressed in normal tissues. Therefore, it is important to develop a gene regulatory system for hypoxia specific gene expression. In this study, the oxygen dependent degradation (ODD) domain from activating transcription factor 4 (ATF4) was evaluated for hypoxia specific gene expression. ATF4 is a member of the ATF/CREB family of the basic-leucine zipper transcription factors. ATF4 is induced post-translationally under hypoxia, which was mediated by the ODD domain. To evaluate the ATF4 ODD domain for gene therapy application, pSV-Luc-ATF4 ODD was constructed. For the construction, the luciferase cDNA without the stop codon was inserted into pSV vector. Then, the ODD domain was chemically synthesized and inserted at the downstream of the luciferase cDNA. *In vitro* transfection assay was performed and the cells were incubated under hypoxia or normoxia. The results showed that luciferase expression was induced under hypoxia in the pSV-Luc-ATF4 ODD transfected cells. The ATF4 ODD domain stabilized luciferase under hypoxia and promoted degradation of luciferase under normoxia. However, the pSV-Luc transfected cells did not show this effect. RT-PCR showed that the luciferase mRNA levels were not different between normoxia and hypoxia, suggesting that the induction of luciferase expression was post-translational event. In conclusion, a post-translaitonal regulation system using the ATF4 ODD domain may be useful for the development of hypoxia-specific gene therapy systems.

B4.49
Gene regulation by histone-like modification of transcription factors

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Gene regulation drives the development and homeostasis of every living organism; deregulation of this process has been implicated in the development of a range of severe diseases, including numerous cancers. It has become clear in recent years that an essential level of control of gene expression resides in reversible post-translational modifications of histone proteins that package DNA (''the histone code''). Recent data provide hints that analogous modifications in DNA-binding transcription factors exist and might provide a whole new level of complexity in gene regulation. However, our understanding of the molecular mechanisms through which these processes are regulated is far from complete. To address this problem, we focused on the essential transcription factor GATA-1 that regulates the expression of all erythroid and megakaryocyte-specific genes. It has recently been shown that GATA-1 is post-translationally acetylated at lysine residues located in the C-terminal portion of the protein and that mutations at these sites abrogate its biological activity. We have identified a bromodomain-containing protein that interacts with an acetylated GATA-1 peptide using two distinct acetylated lysines. We have characterized the binding mechanism structurally and biophysically using a combination of nuclear magnetic resonance spectroscopy (NMR) and surface plasmon resonance (SPR). Furthermore, we have used chromatin immunoprecipitation experiments (ChIP) to show that increases in GATA-1 occupancy are accompanied by increases in the bromodomain at important target sites confirming the biological significance of this interaction.

B4.50
Co-delivery of drug and gene using R3V6 and R3L6 peptides

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R3V6 or R3L6 peptide, which is composed of 3 arginines and 6 valines or 6 leucines, formed self-assembled micelles in aqueous solution with a cationic arginine surface and a hydrophobic valine or leucine core. In this study, R3V6, R3A6, R3L6, and R3F6 peptides were synthesized and transfection efficiencies of the peptides were evaluated. *In vitro* transfection assays into 293 cells showed that R3V6 and R3L6 peptides had higher transfection efficiency than R3A6, R3F6 and poly-L-lysine (PLL). Since the peptide micelles had hydrophobic core, hydrophobic drug may be loaded into the core of the micelles. The incorporation of hydrophobic drug into the core of the peptide micelles may have two effects. First, the micelles will be more stable with tighter core in the presence of hydrophobic drug, which will increase the transfection efficiency. Second, the drug will be delivered into the cells more efficiently by endocytosis of the micelles rather than simple diffusion. *In vitro* transfection assay with the mixture of hydrophobic

drug, BCNU, and the peptide micelles showed that the transfection efficiency of the micelles, suggesting that incorporation of hydrophobic drug increased the stability of the peptide micelles was improved, compared with the micelles without BCNU. The weight ratio between BCNU and R3V6 or R3L6 peptides for transfection was optimized. BCNU and the peptide mixture had the highest transfection at a 0.8:1 weight ratio (BCNU:R3V6) and a 1.2:1 weight ratio (BCNU:R3L6). BCNU is a strong anti-tumor drug. Therefore, the R3V6 and R3L6 peptides with BCNU may be useful for anti-cancer drug and gene combinational therapy.

B4.51
TISU, a common transcription and translation element specific to mRNAs with extremely short 5'UTR

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Regulation of gene expression at the transcriptional and translational levels occurs primarily at their initiation stages. Some proximal transcription regulatory elements are localized downstream to the transcription start site (TSS), and are also present in the mRNA where they could also influence translation. However there is little evidence for such composite transcription and translation elements. We have identified an element downstream to the TSS, which, remarkably, controls the initiation stages of both transcription and translation. This composite element is present in genes encoding for proteins involved in basic cellular functions such as respiration, protein metabolism and RNA synthesis. We showed this element to be essential for transcription and promoter strength. The core of the motif has an invariable ATG sequence, which serves also as the translation initiation codon. In these genes the initiating AUG is preceded by an unusually short 5'UTR. We demonstrated that translation *in vitro* and *in vivo* is initiated exclusively from the AUG of this motif, and that the AUG flanking sequences create a strong translation initiation context that is distinguished from the well-known Kozak in its unique ability to direct accurate translation initiation from mRNAs with a very short 5'UTR. We therefore named it TISU for Translation Initiator of Short 5'UTR. Our data suggest that TISU directs a new mode of translation initiation, which is cap-dependent but scanning independent. Moreover TISU is differentially regulated by translation initiation factor and under cellular stress. Thus coordinated transcription and translation can be mediated by a common regulatory element.

B4.52
Regulation of expression of human intersectin 1 gene by alternative splicing and alternative transcription

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Alternative splicing has recently become one of major mechanisms, increasing the diversity of transcriptom and has important application in physiology, development and genesis of diseases. Intersectin 1 (ITSN1) is an evolutionary conserved, multidomain protein involved in clathrin-associated endocytosis, signal transduction, and cytoskeleton rearragement. ITSN1 mRNAs encode two major isoforms, a short and a long one. The short form is ubiquitously expressed and consists of two EH domains, a coiled-coil region, and five SH3(A-E) domains. The long form is predominantly expressed in neurons and includes three additional C-terminal domains, namely, DH, PH, and C2. Recently, it has been identified different variants of alternative splicing events affecting ITSN1 transcripts. Five of human transcripts have deletions in resulting protein and may affect binding properties and functions of ITSN1. Here we present results of cloning and characterization of fifteen full-length human ITSN1 transcripts comprising different combinations of alternatively spliced exons. Many eukaryotic genes contain multiple promoters. We have found alternative promoter in intron 5 of human ITSN1 gene that generates transcripts which encode proteins without first EH-domain. Their expression was found mostly in kidney, lung and liver tissues. We have cloned two full-length transcripts and found critical promoter region in LINE1-repeat using deletion mutants. Thus, tissue and development specific splicing may influence the interaction of ITSN1 with their partners and contribute to the regulation of ITSN1 protein function in endocytosis and signal transduction.

B4.53
Elucidation of regulatory behaviour of Spo0A protein on *bac* operon

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In *Bacillus subtilis*, a variety of responses are stimulated to allow the bacteria survive in the increasingly hostile environment. A very crucial response that makes *B. subtilis* form specialized cells, is sporulation. Sporulation initiation signals in *B. subtilis* result in the activation of the master regulatory protein Spo0A, by phosporylation. Under such circumstances, phosphorylated and hence active Spo0A works directly or indirectly on the activation or inhibiton of the transcription of many genes as sporulation accelerates. Besides sporulation, production of antibiotics is among the responses that lead bacteria to adapt the changing environment. In spo0A disrupted mutants of *B. subtilis*, the synthesis of antimicrobials and also resistance against other antibiotics were found to be deficient. These effects of spo0A mutations were associated with AbrB global regulator protein which is repressed by Spo0A. Bacilysin is a dipeptide antibiotic and *bacABCDE* gene cluster of *B. subtilis* carries biosynthetic core functions on bacilysin production. In this study, *spoOA* gene, amplified from *B. subtilis*, was ligated to pQE60 expression vector and this recombinant plasmid was used to transform competent *E. coli* cells and SpoOA protein was isolated from these cells. Following purification, using electrophoretic mobility shift assay, direct or indirect regulatory behaviour of Spo0A protein was elucidated through the analysis of protein-DNA interaction between Spo0A protein and *bac* operon promoter region. This work is supported by The Scientific and Technological Research Council of Turkey (TUBITAK)(TBAG, 109T569).

B4.54
HMG-box protein Ixr1 is required for DNA-damage-induced expression of ribonucleotide reductase Rnr1

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In *Saccharomyces cerevisiae*, Mec1 and Rad53 are essential and required, via the nonessential kinase Dun1, to activate ribonucleotide reductase (RNR), a key enzyme in the production of all four dNTPs. The activation of RNR by the Mec1-Rad53-Dun1

pathway leads to increased dNTP levels. Dun1 phosphorylates RNR inhibitors Sml1 and Dif1 promoting their degradation. In addition, Dun1 phosphorylates transcriptional repressor Crt1, leading to its dissociation from the promoters of *RNR2*, *RNR3*, and *RNR4* genes. *RNR1*, which encodes the large subunit of yeast RNR, is also DNA-damage-inducible, but its promoter does not contain the Crt1 binding sites, and the mechanism of its activation by DNA damage has not been identified. We show that the induction of the Rnr1 levels by DNA damage requires transcription factor Ixr1. The Rnr1 levels decrease in *ixr1* mutants after DNA damage, instead of increasing as in the wild-type. In contrast, the DNA-damage-dependent elevation of the Rnr2, Rnr3 and Rnr4 levels is not affected by *ixr1* mutation. In the absence of DNA damage, inactivation of *IXR1* results in a moderate decrease of Rnr1 and dNTP levels, which is compensated by the Mec1-Rad53-Dun1-dependent upregulation of Rnr3 and Rnr4. The activation of RNR by Dun1 in *ixr1* mutants is essential for cell viability. Deletion of *SML1* or overexpression of *RNR1* or *RNR3* increases dNTP levels and rescues the *ixr1 dun1* synthetic lethality. This work identifies Ixr1 as a novel player in regulation of DNA- damage-inducible genes in yeast.

B4.55
Determination of regulatory mechanism of AbrB protein on *bac* operon

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Bacilysin is a simple dipeptide antibiotic which is produced extracellularly by certain species of *Bacillus subtilis* that consists of L-alanine and L-anticapsin. Its synthesis seems to be under transcription regulation via the stringent response as well as under feedback regulation, and moreover it is a part of the global quorum-sensing control system. The loss of bacilysin production in *spo0H* and *spo0A*-blocked mutants as well as an increase in the production of bacilysin in *abrB*-disrupted mutants and the suppression of bacilysin-negative phenotype by an *abrB* mutation in *spo0A*-blocked mutants revealed that the transcription of some gene(s) involved in bacilysin formation is under the negative control of *abrB* gene product which is relieved by Spo0A protein. Recently, it was found that *ywfBCDEFG* operon and a monocistronic gene *ywfH* are required for the biosynthesis of bacilysin and renamed as *bacABCDE* operon. Under the light of these findings in this study, it was aimed to identify the binding properties of quorum sensing regulatory protein AbrB with the promoter region of *bac* operon whether it directly binds to the promoter region or not. For this purpose, *abrB* gene from *B. subtilis* genome was cloned into expression vector pQE60 and AbrB was expressed in *E. coli*. Obtained AbrB protein was used in Electrophoretic Mobility Shift Assay in order to identify the binding properties between AbrB and *bac* operon promoter. This work is supported by The Scientific and Technological Research Council of Turkey (TUBITAK)(TBAG, 109T569).

B4.56
Identificaton of SASP and spore coat protein profiles in *bacA* spores

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When exposed to nutrient deprivation, *Bacillus subtilis* cells cease growing exponentially and enter sporulation. During sporulation, cell division is asymmetric, generating two different cell types, forespore and mother cell. In subsequent stages, a variety of complex biosynthetic and morphogenic changes take place in the mother cell, in the forespore, and in the space between them. The proteinaceous coat which is produced by the mother cell, provides a thick, protective barrier that encases the mature spore. Bacterial spores are surrounded by a mechanically flexible protein coat that consists of many coat proteins, which protects the spore from environmental factors. Notwithstanding, forespore produces small-acid soluble proteins, SASPs. The chromosome of forespore is coated by some of these proteins that makes the spore gain resistance against UV. SASP's are also functional in dormancy and germination processes. In this study, a previously constructed *B. subtilis* strain (*bacA::lacZ::erm*), namely OGU1 containing transcriptional *bacA-lacZ* fusion at *bacA* locus was used for the ultimate purpose of the characterization of spore coat proteins and SASP profiles in OGU1 (*bacA::lacZ::erm*) and wild type PY79. DSM sporulation medium was used to drive cells into sporulation and then sporulated cells were used for SASP and coat protein isolations. In order to isolate SASP proteins, cells were ruptured in acidic environment and concentrated using low cut-off ultracentrifugation tubes and profiling of SASPs was done by using low pH polyacrilamide gel. Moreover, dialyzed and lyophilized spore coat proteins were also visualised by SDS-PAGE.

B4.57
SASP content and spore coat protein profile in *yvfI* spores

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Under unfavorable nutritional conditions, *Bacillus subtilis* cells exhibit many differential behaviours, including sporulation. Sporulation in *B. subtilis* is intimately tied to the cell cycle. Sporulation process involves sequential and complex interactions between a large number of genes. Sporulation begins with an asymmetric cell division and then, the specialized spore septum is formed. Following that, the smaller prespore is then engulfed by mother cell. In forespore, small acid-soluble proteins, known as SASPs, start to accumulate. These proteins, then act as amino acid storage proteins that to be used in case of germination for the resynthesis of biomolecules and they are stored to saturate spore DNA, therefore preventing DNA damage. Moreover, the proteinaceaus spore coat begins to be deposited on the outside surface of the spore that consist of at least a dozen different proteins. Under the light of above-mentioned findings, the present project aims for the comparative clarification of the deposited SASP profiles and spore coat proteins between *yvfI::Tn10::spc* mutant and wild type PY79 strains. In this study, SASP proteins and spore coat proteins were isolated from the spores grown in DSM sporulation medium. Extracted spore coat proteins were concentrated using dialysis and visualized using SDS gel electrophoresis. Additionally, SASP proteins were extracted from cells through rupturing and proteins were ultrafiltrated using low cut-off ultrafiltration tubes in order to obtain a concentrated SASP protein mixture. Comparative analysis of SASP protein profiles were done after running those proteins on polyacrylamide gel electrophoresis at low pH.

B4.58
Regulatory profile of CodY protein on bacilysin biosynthetic operon

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Bacilysin is one of the simplest peptide antibiotics which nonribosomally synthesized by certain strains of the *Bacillus subtilis*. Earlier studies revealed that quorum sensing mechanism and signal transduction phosphorelay control its biosynthesis. Bacilysin production is negatively regulated by GTP via the transcriptional regulator CodY and AbrB. In wild-type cells, a forced reduction of intracellular GTP enhances the expression of these genes and the disruption of CodY which regulates stationary phase genes by detecting intracellular level of GTP results in an increase in their transcription. Moreover, it was demonstrated that bacilysin production in rel^+ increased when the CodY protein was distrupted. Recently, *ywfBCDEFG* operon and a monocistronic gene (*ywfH*) was shown to be essential biosynthetic genes taking role in the production of bacilysin. In this present study, the regulatory behaviour of CodY protein on bacilysin biosynthetic operon was identified by using Electrophoretic Mobility Shift Assay(EMSA) with the purpose of determination if CodY effects bacilysin biosynthesis through a direct or indirect interaction with the bac operon promoter. For this aim, *codY* gene was amplified from *B. subtilis* and cloned into pQE60 expression vector. CodY protein was expressed in *E. coli* cells and was purified. Interaction of this purified CodY protein with *bac* operon promoter was identified using EMSA, in order to reveal out regulatory pattern of CodY on this operon. This work is supported by The Scientific and Technological Research Council of Turkey (TUBITAK) (TBAG, 109T569).

B4.59
The effect of ComK transcriptional regulator on the expression of *yvfI* gene in *Bacillus subtilis*

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Quorum sensing mechanism is used by many bacterial species for establishing different bacterial behaviors. *Bacillus subtilis* is a good example in order to understand the signaling and regulation mechanism managed through a cell-density dependent manner. In *B. subtilis*, in the QS control of competence system, ComX binds to and activate a protein kinase, ComP. Then, ComP donates its phosphate group to ComA, ComA˜P activates the production of ComS. ComS inhibits the proteolytic degradation of a transcriptional activator, ComK. After ComS transcription, ComK concentration increases quickly and competence genes become expressed which results in DNA-uptake into the cell. Recent studies showed that synthesis of bacilysin was controlled as a part of the global quorum-sensing control system. Recently, it has been showed that *yvfI* gene of *B. subtilis* was essential for the bacilysin biosynthesis. In this present study, we aimed to determine the effect of ComK, on the expression of *yvfI* gene. For this purpose, a *comK* insertional inactivation vector was constructed through the ligation of *comK* gene into a spectinomycin cassette harbouring pGEMT vector. In order to analyze the effects of comK inactivation on *yvfI* expression, a *Bacillus* strain carrying *lacZ* fusion at *yvfI* locus, TEK7 (*yvfI::lacZ::erm*) was transformed by the constructed vector and this vector was driven into the chromosomal DNA of TEK7 by a single crossover event. In the following step, TEK7 and its *comK* inactivated derivative were grown in PA medium and *yvfI*-directed β-galactosidase activities were determined.

B4.60
The expressions of ADAMTS-1 and VEGF in DU-145, PC-3, MCF-7 and HT-29 cell lines

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The formation of new blood vessels from preexisting capillaries by the invasion and sprouting of the endothelial cells is called as angiogenesis. Angiogenesis is essential for tumor growth, progression and metastasis. Several studies proved that the microvascular intensity of the primer tumor is a very valuable prognostic determinant for breast, prostate and colorectal tumors. Angiogenesis is controlled by several endogenous stimulators and inhibitors of endothelial cell growth. Among these stimulators, the vascular endothelial growth factor (VEGF) owns a private value because of its importance and specificity in angiogenesis. As a result, suppression of the signal produced by VEGF inhibits angiogenesis and reduces tumor severity. The first described member of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin) family, ADAMTS-1, is an endogenous inhibitor of the angiogenesis. It is demonstrated that ADAMTS-1 binds and sequestrates VEGF and this results with the suppression of endothelial cell proliferation. There are several studies about the expression of ADAMTS-1 in different tissues and cell lines. The aim of this study was to determine the expression of ADAMTS-1 in breast, prostate and colorectal cancer cell lines namely MCF-7, DU-145, PC-3 and HT-29. The expression of the VEGF was also determined for the same cells. Total cellular RNAs were prepared from MCF-7, DU-145, PC-3 and HT-29 cells and subjected to RT-PCR using ADAMTS-1 and VEGF primers. GAPDH amplification was carried out as a positive control. VEGF expression was detected in all cells. ADAMTS-1 expression was also detected in PC-3 cell line.

B4.61

Abstract withdrawn

B4.62
Regulation of the genes for chlorate reductase (*Clr*) and chlorite dismutase (*Cld*) in the chlorate-respiring bacterium *Ideonella dechloratans*

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Enzyme activities and mRNA levels of chlorate reductase and chlorite dismutase was investigated in whole cell extracts of *Ideonella dechloratans* grown under different growth conditions. This bacterium grows well both at aerobic and anaerobic conditions, using oxygen and chlorate, respectively, as a terminal electron acceptor. It was found that preparations from cells grown in the absence of chlorate under aerobic conditions showed activity of both chlorate reductase, measured as chlorate dependent reduction of methyl viologen, and chlorite dismutase, measured as chlorite dependent oxygen production. At aerobic growth conditions, the addition of chlorate resulted in an increased activity of chlorate reductase. The highest activity of chlorate reductase was found in preparations from cells grown anaerobically in the pres-

ence of chlorate. No increase in enzyme activity could be detected for chlorite dismutase during anaerobic or aerobic growth in the presence of chlorate, compared to aerobic growth in the absence of chlorate. The mRNA levels for *Clr* and *Cld*, measured by real-time quantitative PCR using 16SrRNA as an intern standard, was found to be equal in preparations from cells grown anaerobically in the presence of chlorate compared to cells grown under aerobic conditions in the absence of chlorate. The results suggest that, in *I. dechloratans*, the activity of chlorate reductase is up-regulated by at least two factors, anaerobiosis and the presence of chlorate. Interestingly, the results also indicate that the studied regulation occurs at post-transcriptional level, while most examples of oxygen regulation in bacteria are reported to occur at transcriptional level.

B4.63
Effect of *Bacillus thuringiensis* Cry3Aa intoxication on the expression of cysteine and serine peptidases in the midgut of the yellow mealworm, *Tenebrio molitor*

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The yellow mealworm, *Tenebrio molitor*, is a stored-product pest found in stored grain products and is sensitive to the coleopteran-specific Cry3Aa toxin from *Bacillus thuringiensis* (Bt). Because insect digestive peptidases catalyze the processing of Cry protoxins to toxins, we evaluated the effect of Cry3Aa toxin on the expression of cysteine and serine midgut peptidases genes in *T. molitor* larvae. High-throughput sequencing was used to obtain EST databases from the midguts of one month old *T. molitor* larvae fed for 24 hour with either a control diet or diet containing 0.1% Cry3Aa. Sequences encoding midgut cysteine peptidases from the C1 family were similar to four cathepsin B, four cathepsin L, and one cathepsin K. The expression of cysteine cathepsins was decreased overall in Bt-treated larvae, although one cathepsin L was found only in the treated animals and the expression of cathepsin K was 1.7-fold enhanced. Serine peptidases from the S1 family were represented by 18 predicted active peptidases and 19 serine peptidase homologs lacking functional amino acids. Three active peptidases were predicted as trypsins and five as chymotrypsins. Among the active serine peptidase sequences, five were found in both control and Bt-treated larvae, but eight were unique to control and five were unique to Bt-treated larvae. Serine peptidases common to both groups overall were expressed 1.5–10-fold lower in Bt-treated larvae. The data is the first application of high-throughput sequencing to the study of Bt intoxication and demonstrates that Cry3Aa intoxication in *T. molitor* induces widespread changes in peptidase gene expression. The work was supported by the RFBR.

B4.64

Abstract withdrawn

B4.65
Tissue transglutaminase contributes to the all-trans retinoic acid induced differentiation syndrome phenotype in the NB4 model of acute promyelocytic leukemia

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Therapy of acute promyelocytic leukemia (APL) primarily consists of an all-trans-retinoic acid/ATRA-based treatment. Administration of ATRA leads to gene expression remodelling and results in the terminal differentiation of leukemic cells toward neutrophil granulocytes. One of the most induced genes by ATRA in APL NB4 cells is transglutaminase 2 (TG2). RNA interference-mediated stable silencing of TG2 in NB4 cells (TG2-KD NB4) coupled with whole genome microarray analysis revealed that TG2 contributes to the expression of large number of ATRA-regulated genes. In the TG2-KD NB4 cells several genes, related to neutrophil granulocyte function, stayed suppressed during ATRA-induced differentiation. Low induction of these genes leaded to reduced adhesive, migratory and phagocytic capacity of neutrophils and less superoxide production. ATRA-controlled down-regulation of those genes which are involved in cell cycle control and cell proliferation were held at higher expression level and found to be manifested in a higher proliferative rate of TG2-KD NB4 cells. Furthermore, in TG2-silenced NB4 cells we observed significantly lesser induction of CC-chemokines (CCL2, -3, -22, -24), which are responsible for the development of differentiation syndrome (DC) in ATRA treated APL patients. Based on our findings we suppose a complex regulatory effect of TG2 upon the ATRA-mediated differentiation of myeloid cells and propose that a reduced expression of TG2 in differentiating APL cells may suppress effector functions of neutrophil granulocytes and therefore moderate ATRA-induced hyper-inflammatory response in DS.

B5 – Regulation of Protein Function

B5.01
Abstract withdrawn

B5.02
β7-Strand of α-crystallin domain is important for monomer-monomer interaction in homo- and heterooligomers of human small heat shock protein

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Members of the small heat shock proteins (sHsp) family play multiple roles in different cellular processes and αB-crystallin, Hsp27, Hsp20, Hsp22 are expressed in practically all human tissues. All sHsp contain conservative α-crystallin domain and form small (dimers, tetramers) or large (16 or more subunits) oligomers. Heterogeneity and high mobility prevent successful crystallization of full-size human sHsp and hamper structural investigations. This makes desirable a search of new approaches for investigation of sHsp structure. Trying to solve this problem we used the methods of site-directed mutagenesis and replaced endogenous Cys residues of αB-crystallin, Hsp20 and Hsp22 by Ser and simultaneously introduced a single Cys residue in position homologous to that of Cys137 of Hsp27. This residue is located in putative site involved in intersubunit interaction within sHsp oligomers. Point mutations do not affect the secondary, tertiary or quaternary structure and chaperone-like activity of investigated proteins and therefore mutated sHsp could be used for the study of monomer interaction. The wild type Hsp27 and the Cys-mutants of all other sHsp effectively form disulfide crosslinked homodimers after mild oxidation. αB-crystallin, Hsp27, Hsp20 also effectively form disulfide crosslinked heterodimers, whereas formation of heterodimers containing Hsp22 and any other sHsp was markedly reduced. Thus, the β7-strand is essential for monomer interaction in homo- and heterooligomers of sHsp and Hsp22 is less effective than any other sHsp in formation of heterooligomeric complexes.

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B5.03
Alleles of *APETALA1* gene with truncated C and K domains differently affect on the reproductive floral organ development in *Arabidopsis thaliana abruptus* mutant

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The *AP1* gene is involved in determining floral organ development. It encodes a transcription factor with a MADS, L, K and C domains and expresses in flower primordia. The functional role of the AP1 C-terminal domain is not well understood. The mutations which destroy different domains can help to elucidating their functions. The *ap1-1* and *ap1-3* mutations are the result of splice site acceptor changes at the third and fifth introns, respectively (Mandel et al., 1992); *ap1-20* has deletion (21 bp) at the K domain region (Ondar et al., 2008). In the *ap1-6* allele we have found the mutation in acceptor site in third intron (similar to *ap1-1*). PCR-amplified cDNA from *ap1-6* mutant shows two splice variants: 671 bp without 4 exon; 626 bp without 4, 5 and GAG motif from 6th exon. Both cDNA variants contains 1-3 exons, which coding functional MADS, I and a part of K domain. The lack of 4 exon leads to the frameshift and translation of incorrect amino acids of the C domain before a premature stop codon. A truncation of the C domain in *ap1-1*, *ap1-6* and *ap1-3* mutants correlates with the phenotype displayed by these mutants on the *abruptus* background (*abr*, allele of *PID*). The *abr* mutation leads to ectopic *AP1* expression in the inner floral whorls. All double mutants lack floral organs in the 1 and 2d whorls and demonstrate strong reduction in stamen and carpel number. In contrast, double mutant *ap1-20 abr* does not demonstrate reproductive organ reduction. Since *ap1-20* has functional C domain, it is suggested that this domain prevents the alteration of the process of determination of stamens and carpels when ectopically expressed in the inner whorls of *abr* mutant.

B5.04
Study of degradation processes of the AtNUDT7 Nudix regulatory protein from *Arabidopsis thaliana*

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Arabidopsis thaliana pyrophosphohydrolase AtNUDT7 belongs to a widely distributed Nudix family of proteins. It is a homodimeric protein that *in-vitro* preferentially hydrolyzes ADP-ribose and NADH. It has recently been shown that AtNUDT7 is a very important factor modulating cellular response to biotic and abiotic stresses in *Arabidopsis*. It is well established that the cellular content of essential proteins is firmly regulated at different levels including controlled degradation of the proteins. Despite indications that some Nudix hydrolases may play compelling functions in the cell, no data were presented on mechanisms that regulate the cellular level of these proteins. During previous *in-vitro* studies we have established that the 26S proteasome non-ATPase regulatory subunit interacts with AtNUDT7 suggesting that the cellular level of this hydrolase may be regulated by proteasome. Therefore, the aim of this work was to show whether the AtNUDT7 protein is processed by this degradating complex. To address this question a cell-free degradation assay was used. We show here that in the presence of plant cell extract the AtNUDT7 protein diminish within two hours. The influence of ATP, protease inhibitors (PMSG, Complete, leupeptin) and specific proteasome inhibitor (MG115) on this process was studied. The obtained data led us to the conclusion that the AtNUDT7 Nudix hydrolase is degradated by the proteasomal complex.

B5.05
Hydrogen producing hydrogenases in *Escherichia coli* under glycerol fermentation at different pH

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H2 is produced by *E. coli* Hyd-3 (*hyc*) and Hyd-4 (*hyf*) hydrogenases whereas Hyd-1 (*hya*) and Hyd-2 (*hyb*) are uptake hydrogen-

ases. At pH 7.5, under fermentation of glucose, Hyd-4 is responsible for H2 production but under fermentation of glycerol, Hyd-2 rather than Hyd-1 is responsible for H2 production. Hyd-1 and Hyd-2 have been suggested to work in a reverse mode which is likely with Hyd-3. In this study, H2 production was determined under glycerol fermentation at pH 7.5 and 5.5 (compared with that under glucose fermentation) and that was inhibited by N,N'-dicyclohexylcarbodiimide (DCCD). H2 production rate (VH2) by *hyfG fhlA* and *hycG fhlA* double mutants provided at pH 7.5 and by *hybC*, *hyfG* and *hycG fhlA* at pH 5.5 was shown to be of the same value as wild type. VH2 by *hyaB* and *hyfG fhlA* double mutants at pH 5.5 was ˜2 fold higher and in *fhllA* mutant ˜2.5 fold lower than in wild type. DCCD inhibited VH2 in all strains but strongly (˜28-fold) in *hyaB*. These results might indicate that Hyd-3 is responsible for H2 production and *fhlA* gene or its product has relationship with Hyd-3 under glycerol fermentation at pH. Hyd-1 and Hyd-4 are likely to be uptake hydrogenases at the above conditions.

B5.06
Inhibitory potential of short collagen-related peptides against extracellular proteolytic enzymes involved in regulation of blood tension and coagulation

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Introduction: Short collagen-related (SCR) peptides similar to collagen repeating sequences are known to have multiple biological effects including hypotensive, anticoagulant and antiulcerogenic. However molecular basis of such effects has not been elucidated so far. We supposed proteolytic enzymes to be potential targets for SCR peptides. Substrate-inhibitor specificity of human matrix metalloproteinase-2 (MMP-2), human thrombin and bovine angiotensin-converting enzyme (ACE) for thirteen SCR synthetic peptides predominantly consisting of glycine and proline residues was investigated.
Methods: Protease activities were assayed using chromogenic and fluorogenic substrates. To determine protease hydrolysis products HPLC-analysis of dansylated SCR peptides and their derivates has been performed.
Results: As collagen breakdown can be a crucial event in SCR peptides formation we examined their inhibitory activity for MMP-2. All of SCR peptides (1 and 100 μM) tested appeared not to inhibit MMP-2. Meanwhile, several SCR peptides (10 and 1000 μM) reduced thrombin activity up to 80%. This effect occurred only in the presence of heparin, otherwise inhibition was decreased or lost. We have also shown that ACE breaks down peptides containing the C-terminal sequence Gly-Pro with release of H-Gly-Pro-OH as the major product.
Conclusions: SCR peptides are likely involved in regulating of blood tension and coagulation by extracellular proteases. Our data provide a new insight into molecular mechanisms of action of SCR peptides.
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B5.07
Whether changes a plant extract of inducing protein modifications in free radical processes

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In our presented work we investigated the influence of plant extract on free-radical oxidizing processes by changing of some enzymes activity. One of many aspects of oxidative damage to proteins includes mechanisms and consequences of oxidative stress. SOD is one of enzymes from antioxidant enzyme system for defense of cell from harmful. With this aim was investigated the value both the level of spontan chemiluminescence (ChL) and SOD activity by influence on biological target.Many attempts have been made to better characterize therapeutic properties of drug plant extracts for using in future by diseases. Herbs are harmless sources for obtaining natural antioxidants. Natural antioxidants are extensively studies for their capacity to protect organisms and cell from damage induced by oxidative stress. As experimental plant we were taken only drug store samples of Artemisia Absinthium L. Extraction of plant was carried out with using extractor of Soxlet in during long-term extraction. As experimental approaches had used a spontan and induced ChL analyze of lipid contained biological target Activity SOD was determinate by reaction of nitro blue tetrazolenium reaction. We obtained that oxidation in plant extracts accompany by level of its ChL and SOD activity. Those results have high level of correlation. So, those investigations showed that these plant extracts were inhibitors of free radical oxidation with different power, and that must be provided by enzyme activity. These results were statistical processing with special computer program MatLab, and can be useful in clinical evaluation of actualities of drug plants by antioxidant therapy.

B5.08

Abstract withdrawn

B5.09
LRP-1-mediated endocytosis of CD44: a new molecular mechanism to control cancer cell adhesion and invasion?

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LRP-1 (Low-density lipoprotein Receptor-related Protein-1) is a scavenger receptor belonging to the LDL receptor family. It can regulate extracellular proteolysis and tumour invasion though its capacity to internalize many molecules including matrix macromolecules and proteases. Beyond endocytosis, LRP-1 could also trigger intracellular signalling pathways through its intracellular NPxY domains to regulate cell proliferation, differentiation or migration. We recently demonstrated that LRP-1 supports tumour cell invasion by controlling cytoskeleton organization and adhesive complexes turnover in malignant cells (Dedieu et al., MCB 2008). To investigate the molecular mechanisms by which LRP-1 controls the malignant cell adhesion and invasion, we attempted to identify a membrane co-receptor for LRP-1. We found that inhibition of LRP-1-mediated endocytosis by selective antagonist (RAP) induces the accumulation of the hyaluronan receptor CD44 at the plasma membrane. Multiple coimmunoprecipitation experiments demonstrated for the first time that LRP-1

and CD44 exist as a tight biomolecular complex. Interestingly, this complex occurs in a RAP-independent manner. Moreover, we established by immunofluorescence experiments that LRP-1 and CD44 are colocalised at the plasma membrane and are partially located into cholesterol-rich membrane microdomains (caveolae). Inhibition of LRP-1 by RAP or by validated siRNA approach accelerates cell attachment. We demonstrated that the LRP-1-mediated control of cell adhesion occurs in a CD44 dependant manner. Finally, our results revealed for the first time that LRP-1 may act as an endocytic receptor for CD44 to support cell invasion.

B5.10
Polymorphisms of apolipoprotien(a) isoforms and lipoprotein(a) in a Macedonian population

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High plasma levels of Lipoprotein(a) [Lp(a)] increase the risk of premature atherosclerosis. Lp(a) contains a unique protein, apolipoprotein(a) [apo(a)]. Apo(a) shows a high degree of genetic polymorphisms, resulting from variable number of tandem repeats of K-IV type 2 in the LPA gene. 180 healthy blood donors, aged between 18 and 60 years (94 males, 86 females), and 100 healthy children aged between 9 and 18 years (51 boys and 49 girls) were included in the study. Denaturing 3–15% gradient SDS-PAGE was used for the apo(a) isoforms separation. The results showed that the frequency distribution of apo(a) isoforms (B, S1, S3, S4, >S4) fit the expectation of the Hardy-Weinberg equilibrium. The frequencies of the six alleles were: B = 0.022, S1 = 0.028, S3 = 0.201, S4 = 0.397, >S4 = 0.110, and 0 = 0.242. The distribution of alleles was skewed towards alleles encoding large apo(a) isoforms associated with low Lp(a) levels. Large apo(a) isoforms (S4 and S3 from single banded and S4S3 from double-banded) were most prevalent in children and in adults. 164 of the adults (91.1%) and 98% of the children had a Lp(a) level beneath the 30 mg/dL, with 77% showing levels of 10 mg/dL or less. We found a significant inverse correlation ($r = -0.3477$, $p < 0.001$) between the Mr of apo(a) isoforms and plasma levels of Lp(a) in adults and in children ($r = -0.4257$, $p < 0.001$). Our results indicate that apo(a) isoforms are primarily genetically-determined, and are not affected by the plasma lipid and apolipoprotein concentration.

B5.11

Abstract withdrawn

B5.12
Distinct regulation of p53-mediated apoptosis by protein kinase C isoforms revealed by a yeast cell system

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Apoptosis induced by p53 protein is firmly established as a central mechanism of tumour suppression. Therefore, the activation of p53 in tumour cells has been recognised as a promising strategy for cancer treatment. Several studies revealed that protein kinase C (PKC) isoforms are major regulators of p53. However, the complexity of the PKC family, namely the coexistence of several isoforms in a same mammalian cell, has hampered the elucidation of the role of individual PKC isoforms in the regulation of p53-mediated apoptosis. Hence, we used H_2O_2-treated yeast cells co-expressing the human wild-type p53 and a PKC isoform of the classical (PKCα), novel (PKCδ and -ε) and atypical (PKCε) subfamilies. Effects of PKC isoforms on p53-mediated apoptosis were evaluated by analysis of cell viability, plasma membrane integrity, DNA fragmentation, metacaspase activation, reactive oxygen species production, mitochondrial transmembrane potential modification, mitochondrial network fragmentation, p53 phosphorylation and subcellular localization. A distinct regulation of p53-mediated apoptosis by PKC isoforms was detected. Whereas PKCα and -ε had no effect, PKCδ and -ε stimulated a p53-mediated metacaspase and mitochondria-dependent apoptosis through p53 phosphorylation and partial mitochondrial p53 translocation. Together, this work reveals the conservation in yeast of a functional p53 transcription-independent mitochondria-mediated apoptosis. Also, it identifies PKCδ and -ε as activators of p53, and therefore as major targets in cancer therapy. We thank REQUIMTE/CEQUP, FCT (I&D/No 8/94) for financial support. I. Coutinho is recipient of a FCT PhD fellowship (SFRH/BD/36066/2007).

B5.13
Searching for small-molecule modulators of p53 activity and inhibitors of p53-MDM2 interaction, using yeast expression systems

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The p53 tumour suppressor is a major regulator of cell proliferation and death. Activation of wild-type (wt) p53 and inhibition of p53 interaction to its endogenous negative regulator MDM2 represent attractive strategies in anticancer therapy [1]. Due to the high complexity of the mammalian p53 pathway, yeasts expressing the human wt p53 and co-expressing the human wt p53 and MDM2

proteins were used as simpler eukaryotic cell systems for a more efficient first-line screening of small-molecule modulators of p53 activity and inhibitors of p53-MDM2 interaction. We previously showed that expression of human wt p53 caused a marked growth inhibition [2]. Using this yeast p53 expression system, the effects of several flavonoids and xanthones on p53 activity were evaluated by growth assays and by analysis of several cell death markers. Some of the small-molecules tested behaved as potential p53 activators. Moreover, we detected that co-expression of human MDM2 with wt p53 significantly reduced the p53-induced growth arrest. The use of this yeast assay to search for small-molecule inhibitors of p53-MDM2 interaction was further validated by testing known inhibitors of p53-MDM2 interaction, RITA and Nutlin-3a [1]. Together, the developed yeast expression assays revealed to be promising drug screening approaches to identify modulators of p53 activity as well as inhibitors of p53-MDM2 interaction.

References:
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B5.14
Sic1 plays a role in timing and oscillatory behaviour of B-type cyclins in yeast

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A number of dynamic models of cellular processes focus on cell cycle regulation. These models explain whether the dynamics of a network can be understood combining *in vitro* kinetics of its individual reactions. Moreover, biochemical feedback and feed-forward loops coordinate cell cycle oscillations occurring over a wide range of time scales.
The budding yeast cell cycle oscillates between states of low and high cyclin-dependent kinase activity, driven by the association of Cdk1 kinase with B-type (Clb) cyclins. Various Cdk1-Clb complexes are activated and inactivated in a fixed, temporally regulated sequence during cell cycle progression, inducing the characteristic behaviour known as ''waves of cyclins''. The mechanism that regulates the appearance on schedule of these complexes is currently unknown. Here, we analyse the molecular basis of the oscillations of the Clbs, with major focus on the role of their inhibitor Sic1. We compare mathematical networks differing in the regulatory interactions that Sic1 may establish with Cdk1-Clb complexes. Our analysis suggests that the wave-like cyclins pattern derives from the binding of Sic1 to all three Clb pairs rather than from the degradation of Clbs. We show that a specific sequence of molecular interactions reproduces the oscillation-like behaviour of the Clb cyclins waves and verify experimentally the model predictions by protein-protein interaction techniques and time course experiments. Our results highlight a feed-forward regulation of Sic1 to synchronize the Cdk1-Clb complexes, acting as a timer in their appearance.

B5.15
Synthesis and biological activity of a new synthetic tea-derived compound that inhibits Dihydrofolate reductase in melanoma and breast cancer cells

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Tea catechins have emerged as promising chemopreventive agents because of their observed efficacy in various animal models. Tea polyphenols have shown antiproliferative and proapoptotic effects on human cancer cells. Recently, we have shown that the ester-bonded gallate catechins isolated from green tea, epigallocatechin-3-gallate (EGCG) and epicatechin-3-gallate (ECG), are potent inhibitors of Dihydrofolate reductase (DHFR) activity. However, despite these excellent anticancer properties, tea catechins have at least one limitation: their low bioavailability, which is related to their low stability in neutral or slightly alkaline solutions and their inability to cross cellular membranes. In our search to improve the stability and bioavailability of green tea polyphenols for cancer therapy, we successfully synthesized a new catechin-derived compound following three-step reaction sequence starting from the commercially available catechin. 3-O-(3,4,5-trimethoxybenzoyl)-(-)-catechin (TMCG) has shown significant antiproliferative activity against several cancer cell lines from breast, lung, colon and melanoma in a dose-dependent manner. As regards its action mechanism, we demonstrated that TMCG binds efficiently to DHFR and downregulates its expression in MDA-MB-231 breast cells and SK-MEL-28 melanoma cells. Disruption of the folate cycle by TMCG is a plausible explanation for its observed biological effects and suggests that, like other antifolate compounds, TMCG could be of clinical value in cancer therapy.

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B5.16
Characterization of the phosphoinositide-binding site of C2 domains

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C2 domains are considered peripheral proteins that are water-soluble and associate reversibly with lipid bilayers. Several evidences have demonstrated that some of these domains are able to interact with the inositol phospholipid PtdIns(4,5)P_2, which is able to directly participate in a myriad of functions, including cell signaling at the plasma membrane, regulation of membrane traffic and transport, cytoskeleton dynamics, and nuclear events. Recently, the crystal structure of PKCα-C2 domain in complex with Ca^{2+}, PtdSer and PtdIns(4,5)P_2 has been solved showing that the C2 domain of PKCα interacts specifically with PtdSer and PtdIns(4,5)P_2 through two independent motifs. Structure reveals that aromatic and cationic residues are able to interact directly with the PtdIns(4,5)P_2 moieties. Another important issue revealed in this work appeared when we analyzed the similarity of the C2 domains of classical PKCs with other C2 domains by structure-based sequence alignment enabling us to predict that there is a potential PtdIns(4,5)P_2 interacting-site. Moreover, we have characterized the binding of different C2

domains to PtdIns(4,5)P_2 and PtdSer using isothermal titration calorimetry and protein-to-membrane FRET, confirming the existence of a consensus sequence. This work was supported by grants form the Fundación Médica Mutua Madrileña and Fundación Séneca 08700/PI/08 (to S.C.-G.), and Ministerio de Ciencia e Innovacion BFU2008-01010 (to J.C.G.-F.). J.G.-C. belongs to the Programa Juan de la Cierva from the Ministerio de Ciencia e Innovacion.

B5.17
Cyt1Aa and Cyt2Ba toxins from *Bacillus thuringiensis* subsp. *israelensis*: activation by endogeneous and exogeneous proteases

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The Gram-positive soil entomopathogenic bacterium *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) produces three parasporal Cyt δ-endotoxin species: Cyt1Aa, Cyt2Ba and Cyt1Ca in the form of protoxins. Cyt1Aa is the major and the most active component of the Bti parasporal crystal, comprising almost 50% of the toxin proteins. The other crystal component, Cyt2Ba, is highly homologous to Cyt1Aa. The Cyt1Aa protoxin exhibits some hemolytic and cytolytic activity, while the Cyt2Ba protoxin is almost completely inactive. Native activation of the protoxins occurs upon alkaline solubilization and further proteolysis from both the N- and the C-termini, which converts the protoxins into active toxins. We report on the activation of Cyt1Aa and Cyt2Ba by the endogeneous zinc-containing metalloprotease camelysin, isolated from the *Bti*. Camelysin (23 kDa with a pI of 6.2) was extracted from intact bacterial cells by monolamellar liposomes and separated on a sucrose gradient. When treated with camelysin, Cyt protoxins were truncated to 22–23 kDa polypeptides and their hemolytic activity increased from 40% to 70% for Cyt1Aa and from 6% to 50% for Cyt2Ba. With the aim to produce shorter active peptide fragments Cyt1Aa was processed by a number of exogeneous proteases in partially denaturating conditions. After pre-incubation with SDS and proteolysis with proteinase K a 4.9 kDa peptide was purified, identified and shown to be active in the hemolytic test. The isolated peptide can be applied as a natural cytotoxic non-immunogenic agent.

B5.18
A pH-dependent structural rearrangement of the human aromatase active site: implications in catalysis

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Aromatase is a cytochrome P450 catalyzing the conversion of androgens to estrogens. This enzyme, known to be an important target for cancer therapy, plays also a crucial role in the neuroendocrine brain system where is involved in the brain sexual differentiation, in the control of sexual behavior, neuroplasticity, neuroprotection, modulation of mood and affective status, aggressive behaviour, memory and cognitive function. The central role of aromatase in neuroprotection has been demonstrated in the cases of stroke, Alzheimer's, Parkinson's diseases and epilepsy. Like other neuroactive steroids, local estrogens levels may be controlled in the brain by a combination of both aromatase expression and activity. Nevertheless, the mechanisms responsible for aromatase activity modulation and therefore rapid changes in estrogens production are still unknown.
In this work, the effect of subtle changes in pH, known to be associated with neuronal activity, on human aromatase was investigated *in vitro* by using a recombinant form of human aromatase. A change of pH from 6.5 to 7.4 resulted in increase of the substrate dissociation and Michaelis-Menten constants, and conformational changes in the active site, as detected by circular dichroism and dynamic fluorescence spectroscopy. A pH dependence of substrate dissociation was observed as a high to low spin transition with an apparent pKa of 8.2 ± 0.3. The Asp309Asn mutant did not exhibit such pH dependence. These findings are consistent with protonation/deprotonation of Asp309 and structural rearrangements of the active site induced by subtle pH changes and altering substrate binding ability and catalysis of the enzyme.

B5.19
The protein N-terminal acetyltransferase NatA: A novel target for cancer chemotherapy?

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NAA15 (NATH) was originally identified by our group as upregulated in thyroid papillary carcinomas and has lately also been found to correlate with aggressiveness of neuroblastic tumours. hNaa15p protein interacts with the catalytic subunit hNaa10p (hARD1) forming the human NatA complex responsible for protein N-terminal acetylation in human cells. By using siRNA-knockdown of NatA in cancer cell lines combined with phenotype analysis and drug sensitization experiments we have characterized the biological impact of NatA. Knockdown of NatA in cancer cell lines induces apoptosis and G1-cell cycle arrest. We screened 11 different thyroid cell lines for hNAA10 RNAi phenotypes and observed mostly growth inhibition, which was independent of TP53 functional status and developed by several different mechanisms involving i) downregulation of cyclin D1, ii) increase in p27/Kip1 and iii) inactivation of Rb/E2F pathway. hNatA depletion in aggressive thyroid cancer cell lines (8305C, CAL-62, FTC-133) with mutated TP53 increased sensitivity to drug-induced cytotoxicity, but in a cell-type specific manner: 8305C (TRAIL), CAL-62 (Daunorubicin), and FTC-133 (Troglitazone). Cells harbouring wild-type TP53 were also prone to apoptosis via the p53 pathway after hNatA downregulation. Importantly, in hNatA-depleted cells DNA-damage signalling was activated in the absence of exogenous DNA damage independent of TP53 status. Our findings indicate that several mechanisms of growth inhibition and apoptosis may be induced by hNatA knockdown and that hNatA knockdown could be exploited for use in combinatorial cancer chemotherapy.

B5.20
The AtNUDT7 Nudix hydrolase from Arabidopsis thaliana interacts with two regulatory proteins RACK1A and 14.3.3

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The AtNUDT7 protein belongs to a widely distributed Nudix hydrolases that catalyze hydrolysis of a variety of nucleoside diphosphate derivatives. Previously, it has been shown that the protein exists as a dimer and that ADP-ribose and NADH are preferred substrates of this enzyme. Genetic analyses have indicated that the AtNUDT7 protein exerts negative control on the EDS1 complex that controls defense activation and programmed cell death. However the molecular mechanism of the AtNUDT7 role in this process remains elusive. Finding proteins that interact with AtNUDT7 can help to understand this protein biological function. To address the question whether the AtNUDT7 protein interacts with other cellular polypeptides we have applied several *in vitro* and *in vivo* techniques. Using pull-down analysis we have identified two regulatory proteins, 14.3.3 and RACK1A, as the potential partners of AtNUDT7. We confirmed these interactions in yeast two- hybrid system. With a help of the bimolecular fluorescence complementation (BiFC) analysis, the interaction between AtNUDT7 and was also observed in Arabidopsis protoplasts. These data suggest that AtNUDT7 may function as part of a complex of regulatory proteins.

B5.21
Targeting the BAG-1/Hsp70 axis to inhibit tumor growth

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BAG-1 is a multifunctional protein, which regulates cell growth, survival, intracellular signaling, and protein folding. Important for its function is the ability to associate with different proteins. These interactions are mainly mediated by the C-terminal BAG-domain comprising approx. 100 amino acids. Known interaction partners include the survival protein Bcl-2, heat shock protein 70 (Hsp70) and RAF kinases. BAG-1 expression is frequently altered in human cancer and depletion of BAG-1 negatively affected growth and survival of several cancer cell lines and prevented transformation by activated RAF *in vivo* and *in vitro*. Hsp70 is present at constitutively elevated levels in many cancers and promotes tumor growth. Recently first evidence has been obtained that the disruption of BAG-1/Hsp70 interaction compromised tumor cell growth. Here we created a structure-based 3D-pharmacophore model combined with a GRID-based model using the crystal structure of BAG-1/Hsp70 as a basis to screen commercially available libraries of small molecular weight compounds. We were able to identify a putative ligand binding site in the interface of the BAG-1/Hsp70 complex and using several pharmacophore models with different degrees of restriction 40 virtual hits were selected following visual inspection and re-docking into the proposed binding site. These substances are currently screened in biochemical and cell-based assays for their ability to block BAG-1/Hsp70 interaction.

B5.22
Acetylcholinesterase expression in human colorectal adenocarcinoma cells (Caco-2)

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Although the best known function of cholinesterases is the hydrolysis of the neurotransmitter acetylcholine, many studies indicate that acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) play non-catalytic actions, being implicated in morphogenesis, hematopoiesis, osteogenesis, apoptosis, and probably in tumorigenesis. We have previously reported that the expression of AChE and BuChE changes in brain tumours (meningiomas, gliomas, and neurinomas), as well as in those developed in breast, lymph node, gut, lung and kidney. These results prompted us to study AChE and BuChE expression on cell lines derived from human colon tumours and the effects that the under- and over-expression of AChE and BuChE genes have on cell proliferation, differentiation and apoptosis. For this purpose, cancerous intestinal cell lines (Caco-2 and HT-29) were transfected with AChE-siRNA and the effects of the silencing on the cell survival and apoptosis were studied. Our results show that Caco-2 cells contain little AChE activity and still less BuChE activity, whereas HT-29 cells have no detectable AChE or BuChE activity. Sedimentation analysis in sucrose gradients showed AChE activity of Caco-2 cells distributed between principal 4.6S forms and less 3.1S forms. According to our previous results, the above AChE forms were assigned to dimers and monomers. Caco-2 cells exhibited abundant AChE-H mRNA, four times lower levels of AChE-R and AChE-T mRNAs, and very few BuChE mRNA. Transfection with AChE-siRNA decreased AChE activity by about 90% and the content of AChE mRNAs by 70%. Current observations do not support severe effects of AChE silencing on Caco-2 survival.

B5.23
Phosducin family proteins in thyroid

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Phosducin-like orphan protein 1 (PhLOP1, 22,317 kDa), phosducin (Phd, 28,246 kDa) and phosducin-like protein 1 (PhLP1, 32,118 kDa) belong to phosducin-related regulatory phosphoprotein family. Their middle and C-terminal sequences are identical while differences occur in the length of their N termini. Despite of structure similarities, in the cell, each of the proteins plays different role. Phd binds and sequestrates heterotrimeric G protein's beta subunit, PhLP1 is involved in chaperoning of protein folding and PhLOP1, detected in nucleus, plays a role in transcription regulation. Although in various tissues the presence of the above phosducin family proteins was reported, their occurrence and function in the thyroid was never investigated. The aim of the present study was to determine the presence of phosducin-related proteins in postoperative thyroid and cultured thyroid cells. Two methods were used: the PCR which demon-

strated the presence of PhLP1 transcripts in thyroid, and Western blotting. Rabbit ap33 antibodies, against Phd and PhLP1 SQSLEEDFEGQATHTGPK sequence and ap34 as well as ap36 antibodies recognizing CKIKASNTGAGDRFSLDVLPT and CMQDMHQKLSFGPRYGFVYE peptides respectively, present in Phd, PhLP1 and also PhLOP1, were obtained. In partially purified thyroid homogenate fractions Western blotting have shown the presence of 33 kDa immunoreactive band corresponding probably to PhLP1. Additionally a few other immunoreactive bands, stained also with anti 14-3-3 antibodies were found. They may reflect 14-3-3 - phosducin-like protein complexes present in thyroid *in vivo* or forms arisen during samples purification.

B5.24
Looking for a new anti-ipertension therapy based on direct renin inhibition

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Renin is a proteolytic enzyme of the hydrolases-class (aspartic proteases) appointed to the activation of the process in which ansiotensinogen is converted in angiotensin I and, successively, into the biologically active angiotensin II. Angiotensin II is the molecule stimulating the aldosterone secretion. Renin is a 37kD molecule constituted by 340 aminoacidic residues. Circulating are both pro-renin, which lacks enzymatic activity, and renin (Pro-Renin/Renin, 9:1 molar ratio). The renin-angiotensin system is often the target for clinical interventions relative to the hypertension treatment. Renin plays a primary role in the regulation of arterial pressure and it is involved in physiological processes inducing arterial hypertension. This explains the continuous research aimed to find new molecules to use as efficient renin inhibitors. Direct inhibition of renin represents a relatively new tool devoted to block the renin-angiotensin system and the body of cascade reactions leading to secretion of aldosterone. To this issue, we are undertaken a study on renin focused to achieve new renin inhibitors for therapeutic application in the hypertension field. At present, the structural and functional properties of the protein are under investigation under different experimental conditions; further, an expression system for renin will be realized soon, with the aim to achieve new mutants to be used as targets for specific inhibitors. From the results obtained, it will be evaluated the possibility to extend the study to the renin-angiotensin-aldosterone system for a therapeutic approach.

B5.25

Abstract withdrawn

B5.26
Distinct effects of caspase inhibitors on caspase-3 activity, DNA fragmentation and cell adhesion to fibronectin in apoptotic cells

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We studied in detail the effects of three caspase inhibitors (a relatively specific caspase-3 inhibitor zDEVDfmk, a general caspase inhibitor zVADfmk and a novel inhibitor Q-VD-OPh) on different apoptosis-related processes. The apoptosis was induced in the leukemic cell lines JURL-MK1 and HL60 using two unrelated drugs (SAHA and Glivec). In all these experimental systems, the inhibitor effects were markedly dose-dependent in the concentration range from 0.1 to 50 μM. All three inhibitors irreversibly bound to caspase-3 (as shown by anti-caspase-3 immunoblots) and prevented caspase-3, -9 and -2 activity in an *in vitro* assay at concentrations lower than 0.5 μM. On the other hand, the individual inhibitors greatly differed in their ability to inhibit the apoptotic DNA fragmentation (2 to 10 μM Q-VD-OPh had larger effect than 50 μM zVADfmk while zDEVDfmk had no effect). We also analyzed the cellular adhesivity using a sensitive assay and found that only Q-VD-OPh (at least 10 μM) was able to prevent the apoptosis-related loss of the cell adhesivity to fibronectin. This detailed analysis shows that ''caspase'' inhibitors have multiple targets in apoptotic cells and that all results obtained using these inhibitors at high concentrations have to be interpreted with caution. The work was supported by grant No 301/09/1026 from the Grant Agency of the Czech Republic.

B5.27
The analog of diadenosine tetraphosphate (JB 419) induces apoptosis in FHIT-positive cells

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The fragile histidine triad (FHIT) protein is a diadenosine polyphosphate hydrolase belonging to the histidine triad family of nucleotide-binding proteins. FHIT is a tumor suppressor protein which plays an important role in pro-apoptotic signaling, cell cycle control and sensitivity to DNA damaging agents. Loss of FHIT is observed in a variety of tumors and premalignant states. Viral-mediated FHIT gene therapy induces apoptosis and suppresses tumors in preclinical models. Some reports suggest that formation of a FHIT-substrate complex, without subsequent hydrolysis of the substrate, induces cell death by apoptosis [1]. We have synthesized an analog of Ap4A called JB419 of the structure: ApsxpsA, where ''ps'' is a phosphorothioate linkage and ''x'' is the glycerol moiety. Also a fluorescent derivative of JB419 of the structure Fl-psxpsA (where Fl is a fluorescein residue) was prepared. ApsxpsA occurred to be an efficient inhibitor of FHIT hydrolase activity [2]. We observed decrease of viability (MTT test) and induction of apoptosis (caspase-3 assay) after incubation of HEK293T cells with ApsxpsA. Fluorescence microscopy visualization showed that the fluorescent analog of JB419 accumulates in cytoplasmic compartments of the transfected cells. Moreover, co-localization studies indicated that Fl-psxpsA is partially present in mitochondrion. Our finding is relevant to recent findings that FHIT may participate in mitochondrion-mediated apoptosis.

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B5.28
Crystal structure of the N-terminal region of brain spectrin reveals a helical junction region, and a stable structural domain

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The crystal structure of a recombinant protein consisting of the first 147 residues of brain α-spectrin was solved to 2.3 A. The N-terminal region consists of the partial domain (Helix C') and the anti-parallel, triple helical coiled-coil first structural domain (helices A_1, B_1, and C_1). The data revealed that each asymmetric unit contained two crystallographically independent structures (1 and 2). The crystal structure of the first structural domain resembled that of the first structural domain of erythroid α-spectrin, determined before by solution NMR studies, with some specific differences, especially at the N-terminal region, including Helix C' and the region connecting Helix C' with the first structural domain (the junction region). The first ten residues are in a disordered conformation, followed by Helix C' with an apparent, flexible bend. The junction region exhibits a helical conformation in contrast with an unstructured junction region in erythroid α-spectrin. A special feature that has not been reported in other spectrin domains is the long and flexible A1B1 loop of 13 residues. This loop is likely the recognition site for interaction with other proteins. Hydrogen bonds and hydrogen bond networks were identified in the first structural domain and compared with those in erythroid α-spectrin. We suggest that these hydrogen bonds might contribute toward the stability of brain and erythroid spectrin.

B5.29
Characterization of protein complexes subunits - using interaction and sequence identity

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Protein complexes play important roles in many cellular processes. There are several approaches have been developed for protein complexes prediction. Most of the approaches make use of the assumptions that (i) protein complexes have dense protein-protein interactions among their subunits, and (ii) high functional similarity for the subunits. In this paper, we suggest to investigate those assumptions by studying the subunits' interaction topology and sequence similarity. Two topological parameters, density of protein-protein interactions (PPI) and connectedness, are defined to test whether protein complex are found in PPI dense region or not. The present data indicated that interaction dense regions represent protein complexes up to 20% of all the cases. A rather large proportion of protein complexes have a lower density of PPI, and connectedness. It is conjectured that prediction approaches based on the assumption that complexes are composed of highly PPI dense regions, connectedness can predict a rather limited numbers of the complexes. Computational approaches making use of the subunit sequence identity can identify complexes of a size less than 13 with reasonable evidence. In the future, we suggest computing variety aspects of protein complexes properties, i.e. static as well as the physiochemical properties, to describe protein complexes. These attributes include mRNA expression data, amino acid composition, length of protein subunits, half-life or instability index, and the dissociation constant, pKa, of a protein complex subunit. Such consideration can possibly provide better insights for our understanding of protein complexes architecture. NSC98-2221-E-150-062

B5.30
Dephosphorylated NPr of the nitrogen-metabolic PTS regulates lipid A biosynthesis by direct interaction with LpxD

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Bacterial phosphoenolpyruvate-dependent phosphotransferase systems (PTS) play multiple roles in addition to sugar transport. Recent studies revealed that enzyme IIANtr of the nitrogen-metabolic PTS regulates the intracellular concentration of $K+$ by direct interaction with TrkA and KdpD. In this study, we show that NPr of the nitrogen-metabolic PTS directly interacts with and regulates Escherichia coli LpxD which catalyzes biosynthesis of lipid A of the lipopolysaccharide (LPS) layer and therefore is essential for growth. LpxD showed a preferential interaction with unphosphorylated NPr. Mutations in lipid A biosynthetic genes such as lpxD are known to confer hypersensitivity to hydrophobic antibiotics such as rifampin; in contrast, a ptsO (encoding NPr) deletion mutant showed increased resistance to rifampin and increased LPS biosynthesis. Taken together, our data show that unphosphorylated NPr decreases LPS biosynthesis by inhibiting LpxD activity.

B5.31
Biochemical and physiochemical characterization of thermostable ditrect hemolysin from grimontia hollisae

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Thermostable direct hemolysin (TDH), a major virulence factor, is widely present in the clinical strain of Vibrio parahaemolyticus and a few other strains of Vibrio species. In this study, we describe biochemical and biophysical characterization of the recombinant TDH from Grimontia hollisae, and physiochemical determination of its cytotoxicity and virulence. The native polyacrylamide gel electrophoresis, gel filtration chromatography, analytical ultracentrifugation, and transmission electron microscopy (TEM) analysis revealed a possible tetrameric structure of Gh-rTDH in the absence of the host cell membrane environment, similar to that of the TDH purified from V. parahaemolyticus. The Gh-TDH exhibited the Arrhenius effect and changed its conformation into fibril form at around 57°C, as determined by the thermostability assay, circular dichroism, differential scanning calorimetry, and TEM. Further, apparent *in vitro* cytotoxicity and morphological changes including membrane blebbing, cell

detachment, and loss of cell cytoplasm with cell shrinkage were observed with AGS and HeLa cells. Finally, injection of Gh-rTDH protein into BALB/c mice confirmed the virulence function of this protein. The putative functional residues involved in affecting protein's conformational stability, Arrhenius effect, and hemolytic activity will be discussed.

B5.32
Effect of pseudophosphorylations on the structure and properties of 14-3-3ε

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Adapter proteins 14-3-3 are ubiquitously expressed in eukaryotes. The members of 14-3-3 family form dimers and predominantly interact with phosphorylated proteins thus regulating multiple intracellular processes. 14-3-3 itself also undergoes phosphorylation catalyzed by a number of different protein kinases. For instance, 14-3-3ε is phosphorylated at Ser58, Ser184 and Thr232 both *in vitro* and *in vivo*. Phosphorylation affects interaction of 14-3-3 with certain client proteins, however effect of phosphorylation on physico-chemical properties of 14-3-3 remained uninvestigated. By replacing phosphorylated Ser/Thr residues by Glu we introduced mutations mimicking phosphorylation and analyzed effect of these mutations on the properties of purified recombinant 14-3-3. Mutation S58E increased intrinsic Trp fluorescence and binding of bis-ANS to 14-3-3. At low protein concentration mutation S58E induced dissociation of 14-3-3 dimer, increased its susceptibility to proteolysis and decreased its thermal stability, thus indicating potential role of Ser58 in regulation of 14-3-3 degradation in the cell. Mutation S184E slightly increased Stokes radius and thermal stability of 14-3-3. Mutation T232E induced only small changes in hydrodynamic properties probably reflecting the changes in the size or shape of 14-3-3 dimer. The properties of triple mutant (S58E/S184E/T232E) were intermediate between those of the wild type protein and its S58E mutant. Thus, pseudophosphorylation of 14-3-3ε at different sites has different effects on its structure and this might be important for regulation of interaction of 14-3-3 with different protein targets. This study was supported by RFBS grant.

B5.33
The modulation of the antioxidative system in MRC-5 cell line exposed to Fe_3O_4 nanoparticles

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Ambient particulate matter (including nanoparticles) is an environmental factor that has been associated with respiratory morbidity and mortality. It is well known that the oxidative stress they generate induces pro-inflammatory effects in the pulmonary system. The aim of our study was to investigate the way of operation of the antioxidative system of MRC-5 lung fibroblast cell line after exposure to Fe_3O_4 nanoparticles for up to 72 hour. The MTT test revealed that a concentration of 12.5 micrograms of magnetite nanoparticles/ml has led to a decrease of MRC-5 cells viability of about 25% after 72 hours of exposure. The malondialdehyde concentration increased 3, 5.5 and 9 times referred to control after 24, 48, respectively 72 hour. The reduced glutathione level was almost unchanged after 24 hour but decreased by 46% and 63% after 48 hour respectively 72 hours. The total SOD activity slightly increased after 72 hour due to MnSOD contribution whereas CAT activity was not significantly up-regulated during 72 hour of exposure. The GPx and GST specific activities were unchanged, whereas the GR activity was downregulated after 72 hour and almost unchanged at shorter exposure periods. The expression of Hsp 27decreased by about 30% and of Hsp 60 increased with 15% compared to control after 72 hour of treatment. Taking into account the increase of malondialdehyde level and the decrease of the reduced glutathione one and expression of the two Hsps, it can be concluded that the MRC-5 cells cannot efficiently counteract the Fe_3O_4 nanoparticles induced oxidative stress and pathological events could occur.

B5.34
Biochemical aspects of the cytotoxic effects of hematite nanoparticles in MRC-5 cells

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Particles generated from numerous anthropogenic and/or natural sources, such as crystalline alfa-Fe_2O_3 (hematite) nanoparticles, have the potential to damage the lung cells. In our study, the effects of these nanoparticles at a concentration of 12.5 μg/ml for 24, 48 and 72 hour on lipid peroxidation as well as, on the antioxidative system belonging to MRC-5 lung fibroblast cells were investigated. Exposure to alfa-Fe_2O_3 nanoparticles caused an increase in lipid peroxidation by 81%, 189% and 110% after 24, 48 respectively 72 hour of treatment. The reduced glutathione concentration decreased by 23.2% and 51.4% after 48 hour respectively 72 hour of exposure. An augmentation of superoxide dismutase, catalase, glutathione peroxidase, glutathione transferase and glutathione reductase within the interval between 48–72 hour was noticed. The proteic expression of Hsp 27decreased by 50% and of Hsp 60 increased with 30% compared to control after 72 hour of treatment. Taking into account that the GSH concentration decreased and MDA level remained highly increased up to 72 hour of exposure, it appeared that MRC-5 antioxidant defense mechanisms did not efficiently counteract the oxidative stress induced by exposure for 72 hour to hematite nanoparticles. The down-regulation of Hsp 27 corroborated with the up-regulation of Hsp 60 could suggest an anti-apoptotic effect of these nanoparticles on MRC-5 cells, associated with possible pathological effects.

B5.35
Functional analysis of the matrixmetalloproteinase-11 in tumor growth, angiogenesis and metastasis

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Angiogenesis and metastasis of tumors is strongly dependent on matrix metalloproteases that cleave basement membranes and

extracellular matrix proteins. Immunoreactive MMP-11 was detected in blood vessels and fibroblasts of the reactive tumor stroma. MMP-11 gene and protein expression was not upregulated after proangiogenic stimulation. Effects of MMP-11 overexpression on primary endothelial cells were studied by the use of recombinant adenoviruses. MMP-11 overexpression did neither affect *in vitro* proliferation of human endothelial cells nor angiogenic sprouting capacity in collagen. Although MMP-11 protein was clearly upregulated intracellularly, we did not observe an increase of the secreted active protease. To study the role of MMP-11 in tumor growth *in vivo* we generated MMP-11 overexpressing B16F10 melanoma cells by the sleeping beauty transposase system. B16F10 cells stably overexpressing MMP-11 secreted the active isoform of the protease into the supernatant. In comparison to mock-transfected cells, MMP-11 overexpressing B16F10 cells showed neither an increase in proliferation nor changes in expression levels of proangiogenic genes *in vitro*. Moreover, tumor cells were embedded in collagen and grafted into the chorioallantoic membrane of chicken embryos. In comparison to controls, MMP-11 overexpressing cells formed significantly larger tumors with strong invasion into host tissue as determined by IHC stainings for the melanoma marker S-100, alpha smooth muscle cell actin and desmin. Our data support the hypothesis, that MMP-11 secreted by stromal fibroblasts and endothelial cells supports invasion of tumor cells, tumor growth and metastasis *in vivo*.

B5.36
Understanding calcium induced conformational changes as revealed by an 8 KDa protein from Fasciola hepatica

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Ca^{2+} ions and calcium binding proteins (CaBP) are fundamental players in all aspects of cell function. The large majority of the CaBPs belong to the EF-hand family of proteins, which bind Ca^{2+} using a common helix-loop-helix structural motif. FH8 is a 8 KDa protein from Fasciola hepatica which contains two EF-hand motifs with canonical binding loops, indicating that it may represent a new type of CaBP. After cloning and expressing FH8 in E. coli we confirmed the ability of the protein to bind Ca^{2+} by equilibrium dialysis and native gel mobility shift. Moreover, ANS fluorescence measurements demonstrated that Ca^{2+} binding results in a large increase in the hydrophobic character of FH8 indicating that, as occurs for sensor proteins, the protein alters its conformation. The existence of a conformational modification was also supported by intrinsic fluorescence and DLS measurements, where changes in phenylalanine fluorescence and average protein radius were observed, respectively. As occurs for other family members, the far UV-CD spectrum of FH8 contains the shapes and amplitudes characteristic of proteins with a high percentage of helical structure and demonstrates a high thermo stability. A melting point of 74°C was observed for the apo state and no lost in secondary structure up to 98°C (the working range limit) for the Ca^{2+}-loaded state, revealing the stable and robust structure of the EF-hand domain. FH8 is the smallest EF-hand protein described until now, which along with its high solubility and stability, makes it an ideal model for a clear understanding of the main aspects that drive conformational changes in CaBP's.

B5.37
Neurotrophin 3 (NT-3) loses the immunomodulatory role in chronic diseases

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Introduction: Chronic obstructive pulmonary disease (COPD) is characterized by the airflow limitation associated with increases of inflammatory proteins in systemic circulation The inflammatory cells present in COPD have been shown to play important role in production of pro-inflammatory cytokines and neurotrophins who have been recognized as mediators of both inflammatory and/or airway remodeling processes. In the present work we investigated the possible correlation of NT-3 with pro-inflammatory cytokines IL-1β, TNF-α and IL-6 in serum of patients with COPD to find out if the expression of inflammatory cytokines, may be differently regulated by neurotrophins.
Patients and Methods: Twenty patients (four female and 16 male) with COPD (mean age 61.0 ± 14.19 years; range 17 to 77 years; mean smoking history, 28.4 ± 25.9 pack-years) were recruited to the study. Nine patients were classified as having stage II GOLD, seven patients as having stage III GOLD, and four patients as having stage IV GOLD. Serum samples were assessed by ELISA kits. The Pearson correlation coefficient (r) was calculated.
Results: NT-3 was positive correlated with IL-1β ($r = 0.83$ $p = 0.05$), TNF-α ($r = 0.93$ $p = 0.05$) and IL-6 ($r = 0.94$ $p = 0.05$) in patients with GOLD II. There are no correlation between these parameters in patients with GOLD III, IV.
Conclusions: Our results showed that NT-3 lost the immumomodulatory role on pro-inflammatory cytokines in patients with COPD. It suggests the potential pathophysiological role of NT-3 as mediators involved in airway inflammation and tissue remodeling in the pathogenesis COPD.

B5.38
Biological functions of Pir51

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Pir51(protein interacting with Rad51), is found highly expressed in aggressive mantle cell lymphoma (MCL) but not in indolent small lymphocytic lymphoma (SLL) patient samples. The expression level of Pir51 is regulated during the cell cycle in a pattern nearly identical to Rad51. RAD51 plays a major role in homologous recombination of DNA during double strand breaks and interacts with a lot of proteins. We find that pir51 interacts with RAD51. Therefore, we hypothesize that Pir51 exists in the protein complex. To explore the roles of the Pir51 protein complex, we used the BacterioMatch Two-Hybrid system in order to find the proteins that can interact with Pir51. After screening, we identified CDC20, one of the interesting clones. CDC20 is highly expressed in M phase of the cell cycle, and appears to act as a regulatory protein. To verify the interaction between Pir51 and CDC20, we performed IP-Western and immunostaining. Indeed, the data showed that CDC20 associates and colocalizes with Pir51. In addition, cdc20 functions as an activator of the anaphase promoting complex (APC). We are examining if Pir51 is ubiquitinated by APC and determning what roles of the ubiquitinated Pir51 protein are in the cells. In the long term goals, we will understand the biological functions of pir51, and may apply for the treatment of diseases.

B5.39
Enzyme IIA^{Glc} regulates a mammalian insulin-degrading enzyme homolog in *Vibrio vulnificus*

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Vibrio vulnificus is an opportunistic human pathogen that causes severe and often fatal infections in susceptible individuals including those suffering from liver disease or diabetes. The mortality rate exceeds 50% in septic patients, and most patients die within 48 h after infection. While the components of the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) in *Escherichia coli* have been shown to regulate numerous targets, little such information is available for the *V. vulnificus* PTS. Here we show that enzyme IIA^{Glc} of the *V. vulnificus* phosphoenolpyruvate:glucose phosphotransferase system interacts with and regulates the peptidase activity of a protein that has high sequence similarity to mammalian insulin-degrading enzyme (IDE). While the specific interaction with the IDE homolog is independent of the phosphorylation state of enzyme IIA^{Glc}, only unphosphorylated enzyme IIA^{Glc} activates the peptidase activity of the IDE homolog. Although an IDE homolog-deficient mutant of *V. vulnificus* shows no growth defect *in vitro*, it shows significantly lower degrees of survival and virulence than wild type in mice. Taken together, our results demonstrate that the insulinase-enzyme IIA^{Glc} complex of *V. vulnificus* can be activated by sensing glucose in host serum and this complex is an important virulence factor.

B5.40
Study of the interactions between 14-3-3 protein and phosducin

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Phosducin (Pd) is a regulator of G-protein-mediated signaling that is especially abundant in photoreceptors and pineal gland. In photoreceptors, Pd is phosphorylated in dark-adapted retina and undergoes dephosphorylation in response to light. Dephosphorylated Pd binds Gβγ, sequestering and translocating it away from disk membrane, which blocks the interaction between Gβγ and effectors or reasociation with Gα. When Pd is phosphorylated at Ser54 and Ser73 it binds 14-3-3 protein. 14-3-3 proteins are family of acidic regulatory proteins. Biological meaning of the interaction between 14-3-3 and Pd could be to sequester Pd from Gβ γ subunit or to protect phosporylated Pd from degradation. To elucidate the mechanism of 14-3-3 protein-dependent regulation of phosducin function, we performed biophysical characterization of Pd/14-3-3 complex. We prepared Pd/14-3-3 complex *in vitro*. Nondenaturating electrophoresis was used to verify that the formation of Pd/14-3-3 complex is phosporyalation dependent and phosphorylation at both serins (Ser54 and Ser73) is necessary for complex formation. Sedimentation equilibrium measurements revealed that the stoichiometry of 14-3-3/Pd complex is 2:1. Time resolved fluorescence spectroscopy was employed to characterize the conformational changes of Pd induced by 14-3-3 binding. Acknowledgements: This work was funded by Grant IAA501110801 of the Grant Agency of the Academy of Sciences of the Czech Republic, by Research Project MSM0021620857 and Centre of Neurosciences LC554 of the Ministry of Education, Youth, and Sports of the Czech Republic, and by Research Project AV0Z50110509 of the Academy of Sciences of the Czech Republic.

B5.41

Abstract withdrawn

B5.42
The study of endoderm markers expression in spontaneous differentiation of mouse embryonic stem cells in Compare to activin A

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To study the endodermal expression in spontaneous differentiation of mouse ES cells in compare to direct differentiation using activin A, we assessed the expression of endoderm markers by RT-PCR and flow cytometry in mouse ES spontaneous in compare to direct differentiation with activin A. The mouse ES cells were cultured in suspension state for 5 days to induce embryoid bodies (EBs). It is treating, Activin A (100 ng/ml) in DMEM containing BSA 5 mg/ml in activin group and no factors in DMEM containing FBS, 10% in spontaneous group. The EBs cells were transfer to gelatine coated dishes and cultured for a week in DMEM plus FBS 10% and Nicotin amide (10 mM). expression of GATA-4, PDX-1, SHH and TAT (endoderm) brachyary and nodal (mesoendoderm) and nestin (neuroectodermal) were compared in both by RT-PCR. The number of Pdx-1+, brachyury+ and nestin+ cells was compared in both by flow cytometry. There was A significant difference in mean of pdx-1+ cells (35.28 ± 5.23) in activin in compare to (12.6 ± 5.48) in spontaneous group. Also there was a significant difference in brachyury positive cells between both groups. The brachyury+ cells was 15.83 ± 2.99 and 7.82 ± 2.17 in activin and spontaneous groups respectively. There was a positive correlation between pdx-1+ and brachyury+ ($R = +0.722$, $p = .007$). The activin is capable of to enrich the endoderm during EBs induction. It also showed that endoderm marker increase along with an mesoendodermal makers increasing as a positive correlation observe between pdx-1+ and brachyury+ cells. So the results confirmed the positive role of Brachyury expression on promoting of ES cells into endoderm while there was no correlation with nestin.

B5.43
Characterization of genes with biotechnological relevance from *Thermus thermophilus* HB8

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Thermus thermophilus HB8 is a well-studied thermophilic bacterium with biotechnologically relevant enzymes. The increasing

interest in enzymes from extremophiles, as well as in their expression in mesophilic microorganisms is based not only on their thermostability but also on their resistance to chemical agents and extreme pH values. Esterases and lipases are widely used enzymes in a large number of industrial processes, while kinases play an important role in cell physiology. The aim of this work was to characterize TTHA0214, TTHA1187, TTHA1268 and TTHA1705 proteins of this bacterium and define their function. Gene products were heterologously expressed as full length His-tagged protein in *E. coli* host, purified with affinity chromatography and characterized. TTHA0214 possessed a dihydroxyacetone (DHA) kinase activity, with higher substrate specificity for DHA and to a smaller specificity for glyceraldehyde and not to glycerol. The activity of this recombinant enzyme was localized in the cytosol. The DHA kinase reaction showed an inhibition at high concentrations of glycerol. This enzyme function as a dimer/oligomer, as it was indicated by the cross-linking experiments, requires Mg^{2+} or and to a lesser extent Ca^{2+} and is inhibited by fluoride or ADP. Gene TTHA1187, TTHA1268 and TTHA1705 possessed a lipase/esterase activity. The functional characterization of these novel enzymes will be a valuable tool towards elucidating their mode of action and their involvement in important metabolic pathways in thermophilic bacteria. Acknowledgments: This work was carried out with the financial support of EC under the IP-project "BIOPRODUCTION/NMP-2-CT-2007-026515".

B5.44

Abstract withdrawn

B5.45

Human Naa50p (Nat5/San) displays both protein N^{α}- and N^{ε}-Acetyltransferase activity

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Protein acetylation is a widespread covalent modification for eukaryotic proteins mediated by two distinct types of acetyltransferases. KATs (lysine N^{ε}-acetyltransferases) modify the side chain of specific lysines on histones and other proteins, a central process in regulating gene expression, whereas NATs (N^{α}-terminal acetyltransferases) acetylate the amino group of nascent polypeptides as they extrude from the ribosome. In yeast and humans, three different NAT complexes have been identified, NatA, NatB, and NatC. These complexes exhibit distinct substrate specificities. NatA is composed of the catalytic subunit Naa10p, and the auxiliary subunit Naa15p. In addition, Naa50p is physically associated with NatA, but its role in the complex is unknown. In fruitfly and humans Naa50p was shown to have acetyltransferase activity and play an important role in chromosome segregation. We have used recombinant purified hNaa50p and multiple oligopeptide substrates in order to identify the substrate specificity and characterize the acetyltransferase activity of hNaa50p. The results suggest that peptides with N-termini Met-Leu-Xxx-Pro are acetylated by hNaa50p. In addition to this N^{α}-terminal acetyltransferase activity, we also found autoacetylation of hNaa50p in lysines 34, 37, and 140, which modulated hNaa50p substrate specificity. Autoacetylation is consistent with a lysine N^{ε}-acetyltransferase activity, and this was further supported by acetylation of histone H4 by hNaa50p *in vitro*. Our findings thus provide the first experimental evidence of an enzyme having both NAT and KAT activities.

B5.46

A novel cancer cell senescence-evasion mechanism involving GCIP inactivation by Rad

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Our work focuses on identifying factors critical to the carcinogenesis. Here, we investigated the possible role of Rad in this context. Rad is a Ras-related GTPase that promotes cell growth by accelerating cell cycle transitions. Rad knockdown induced cell cycle arrest and premature senescence without additional cellular stress in multiple cancer cells, indicating that Rad expression might be critical for the cell cycle. To investigate the precise function of Rad in this process, we used human Rad as bait in a yeast-two-hybrid screening system, and sought Rad-interacting proteins. We identified the Grap2 and Cyclin D Interacting Protein (GCIP)/DIP1/CCNDBP1/ HHM, a cell cycle inhibitory molecule, as a binding partner of Rad. Further analyses revealed that Rad binds directly to GCIP *in vitro* and coimmunoprecipitates with GCIP from cell lysates. Rad translocates GCIP from the nucleus to cytoplasm, thereby inhibiting the tumor suppressor activity of GCIP, which occurs in the nucleus. In the presence of Rad, GCIP loses its ability to reduce Rb phosphorylation and inhibit cyclin D1 activity. The function of Rad in transformation is also evidenced by increased telomerase activity and colony formation according to Rad expression level. *In vivo* tumorigenesis analyses revealed that tumors derived from Rad-knockdown cells were significantly smaller than those from control cells ($p = 0.0131$) and the pre-established tumors are reduced in size after the injection of siRad ($p = 0.0064$). Therefore we propose for the first time that Rad may promote carcinogenesis at least in part by inhibiting GCIP-mediated tumor suppression.

B5.47

Intramolecular control of the Ssy5 protease by its Pro-domain accounts for regulated Receptor-Activated Proteolysis of transcription factor Stp1

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Extracellular amino acids induce the yeast SPS-sensor to endoproteolytically activate transcription factors Stp1 and Stp2 in a process termed receptor-activated proteolysis (RAP). Ssy5, the activating endoprotease, is synthesized as a zymogen with a large N-terminal Pro-domain and a C-terminal chymotrypsin-like catalytic(Cat)-domain. Concomitant with translation, Ssy5 cleaves itself, however the Pro- and Cat-domains remain associated forming a primed protease that binds but does not process Stp1 or Stp2. Here we show that amino acid-induced signals trigger proteasome-dependent downregulation of the Pro-domain. Destabilizing mutations in the Pro-domain that result in reduced Pro-domain levels cause constitutive Stp1 processing. Conversely, mutations that stabilize the Pro-domain abolish Stp1 processing activity without affecting autolysis. We critically tested the functional significance of Pro-domain downregulation by synthetically targeting the Pro-domain for degradation in a temperature-controlled manner. Our data show that Pro-domain degradation suffices to liberate the Stp1 processing activity of the Cat-domain independent of SPS-sensor signaling. These findings indicate that the Pro-domain acts as a potent inhibitor of Cat-domain activity,

and its inactivation is a requisite for RAP. This regulatory mechanism is novel for proteases functioning within eukaryotic cells.

B5.48
Fish as a suitable model to unveil the function of the four and half LIM domains protein 2

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Four and a half LIM domains protein 2 (FHL2) is characterized by its structural LIM motifs, composed of two tandemly repeated zinc fingers, known to mediate protein-protein interactions. This particular structure is most probably responsible for FHL2 ability to function as a tissue-specific transcriptional co-regulator as well as a modulator of cytoskeleton architecture. Mammalian FHL2 gene expression is particularly abundant in the vascular system, namely in heart and blood vessels, but interestingly, knockout mice have been shown to develop an osteopenic (bone mass loss) phenotype. To better understand the physiologic relevance of FHL2, a vertebrate non-mammalian system was used. The gilthead seabream (Sparus aurata) is a marine teleost fish, economically relevant for aquaculture of Southern European countries, for which a large amount of molecular and cellular tools are available. Using a gain-of-function approach, by stably overexpressing FHL2 in seabream VSa16 pre-osteoblast cells, we have observed an increase in matrix Gla protein (MGP) and Gla-rich protein (GRP) gene expression. Both genes are typically expressed in vascular smooth muscle cells while poorly expressed or absent, respectively, in wild type VSa16 cells. Overexpressing clones also exhibit a clear change of phenotype and a significant decrease in mineralization rate. Through in situ hybridization, we have identified *in vivo* expression of FHL2 in myoblasts, during early development as well as in adult muscle tissue. We hypothesized that FHL2 could act as a key determinant for cell fate by directing mesenchymal progenitor cells or transdifferentiating pre-osteoblast cells towards the myocyte lineage.

B5.49
Expression of chromosomal passenger protein in thyroid cancer

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Survivin is a member of the inhibitor of apoptosis protein (IAP) family. Overexpression of survivin is commonly detected in various human cancers but not in normal adult tissue. The IAP function of survivin is believed to be critical for cancer progression. Survivin is also known to be a component of chromosome passenger protein (CPP) complex essential for chromosome segregation and cytokinesis as well as Aurora-B. Our previous study showed that the prognosis of colorectal cancer may depend on the localization of survivin. Here we show that by immnohistochemical analysis nuclear survivin and Aurora B overexpression in undifferentiated carcinoma of the thyroid were more frequently detected than those in papillary carcinoma and poorly differentiated carcinoma. Subcellular distribution of survivin in thyroid cancer was further demonstrated by Western blotting. Some of undifferentiated carcinomas with multiple metastasis expressed cytoplasmic and nuclear soluble survivin. These findings indicate that the expression of CPP may be deeply involved in the pathways of histogensis of thyroid cancer, suggesting that cytoplasmic survivin may have a certain function to survive cancer cells.

B5.50
Altered ribonucleotide reductase (RNR) activity in yeast superoxide dismutase 1 (SOD1) deletion mutant

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Deoxyribonucleoside triphosphates (dNTPs) as precursors for DNA synthesis play a fundamental role in DNA replication and repair. Their levels are precisely regulated during the cell cycle and after DNA damage, primarily by RNR, a key enzyme in dNTP biosynthesis. The transcription of the RNR subunits is highly upregulated after DNA damage and the activity of RNR is enhanced by degradation of its inhibitor, Sml1. The Sod1 protein is a cytosolic copper-zinc superoxide dismutase in yeast, which catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide and is important in antioxidant defence. Earlier, it has been demonstrated that loss of Sod1 leads to oxygen-dependent sensitivity to different DNA-damaging agents and that the induction of the RNR proteins after DNA damage was reduced in the *sod1*Δ mutant. We tested if lower dNTP levels in *sod1*Δ were responsible for the increased DNA damage sensitivity. Surprisingly, our preliminary data revealed that dNTP pools in the *sod1*Δ mutant were increased, even though the RNR protein levels were the same as in wild type. In this work we attempt to clarify what pathways are responsible for the elevated dNTP pools in *sod1*Δ and why the DNA damage checkpoint is not - or only partially - activated after damage.

B5.51
GABA-shunt enzymes mRNA expression in PMCA-depleted GH3 cells

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Plasma membrane calcium ATPase (PMCA) is an enzyme maintaining low cytosolic calcium concentration. Our previous study on PC12 cells showed that reduction of neuron-specific isoforms - PMCA2 and PMCA3 resulted in the increase of intracellular calcium level and changed GABA metabolism under oxidative stress. In the present work we focused on rat anterior pituitary tumor GH3 cells – a widely used model in studies concerning calcium homeostasis. Using two stably transfected GH3 cell lines with suppressed expression of PMCA2 and PMCA3 we examined the intracellular calcium concentration and mRNA expression of all PMCA isoforms. Also, we determined the expression of mRNAs of GABA-shunt enzymes: glutamate decarboxylase, GABA-transaminase and succinic semialdehyde dehydrogenase. This pathway is considered to be one of protective mechanisms mobilized in response to stress conditions. Real-time PCR analysis revealed that expression of PMCA2 and PMCA3 mRNAs decreased up to 50% in the transfected GH3 cells. However, a significant increase of PMCA4 expression in PMCA3- suppressed cells indicated on some compensatory mechanism. The mRNA of GABA-shunt enzymes was altered only in PMCA3-suppressed cells: about 50% increase of GAD65 and about 80% decrease of SSADH. The reduction of PMCA isoforms caused increase of

basal and thapsigargin-sensitive intracellular Ca^{2+} pool, as was determined using Fura-2/AM assay. We also observed differences in morphology of control and transfected cells. This work was supported by the Ministry of Science and Higher Education grant N N401 076337 and Medical University of Lodz grants 502-16-810 and 503-6086-2.

B5.52
Study of the interaction between furosemide and human carbonic anhydrase II by spectroscopic methods

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Human carbonic anhydrase (hCA, EC 4.2.1.1) is a Zn-dependent enzyme which catalyzes reversible interconversion of carbon dioxide to bicarbonate, with a proton release. In this study, the interaction of furosemide, an efficient diuretic and a sulfonamide drug, with hCA II was investigated by various spectroscopic methods such as intrinsic and extrinsic fluorescence, UV-Vis and circular dichroism (CD) spectroscopy, in 50 mM Tris buffer, pH 7.75. Fluorescence data indicated that furosemide acts as a potent quencher of hCA II emission. The Stern-Volmer analysis of quenching data at different temperatures elucidated that the quenching of intrinsic fluorescence of hCA II is occurred through a static quenching mechanism. Analysis of the thermodynamic parameters of binding indicated that hydrogen bonding and electrostatic forces play the major role in the interaction of furosemide with hCA II. Calculation of the protein surface hydrophobicity (PSH), using 1-anilinonaphthalene-8-sulfonic acid (ANS), indicated the decrement of PSH of hCA II in the presence of furosemide. The job's plot confirmed that the binding of furosemide to the enzyme occurred via a 1:1 stoichiometry. The Far-UV CD results showed that furosemide caused slight increment in the α-helix content of hCA II whereas near-UV CD experiments in the presence of the drug indicated some decrement in the flexibility of the enzyme tertiary structure. Binding of furosemide to hCA II is accompanied with quenching the intrinsic fluorescence of the enzyme, decreasing the PSH of the complex, inducing some α-helical structure in the secondary structure of the enzyme and reducing its tertiary structure flexibility.

B5.53
Identification of a molecular switch regulated by phosphorylation in E2 ubiquitin-conjugating enzymes

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Post-translational phosphorylation is an ubiquitous mechanism for cellular regulation and it is known it can modulate the proteins conformation by changing their energy landscape which is largely driven by the electrostatic perturbation induced by the phosphate group (1). Recently, it has been shown that several enzymes involved in the ubiquitination pathway can be affected by phosphorylations (2–4), as ubiquitin ligases and E2 ubiquitin conjugating enzymes. In the present contribution, we provide through molecular dynamics simulations, homology modelling, protein-protein docking, sequence comparisons and phylogenetic analysis of known E2 ubiquitin-conjugating enzymes, and biochemical assays, the first model of the structural effects and regulation mechanisms mediated by phosphorylation of the catalytic domain of E2 enzymes. Our investigation suggest that phosphorylations at catalytic domain of E2 ubiquitin conjugating enzymes can stabilize the whole protein structure and, in particular promote a conformation competent for ubiquitin binding of a loop in the surrounding of the catalytic cleft, which modulate the accessibility of the catalytic site.

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B5.54
Purification of the rat kidney glutathione reductase

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Glutathione reductase (GR, E.C. 1.8.1.7, NADP oxidoreductase) is a member of flavoenzyme family. It catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) in the expense of nicotinamide adenine dinucleotide phosphate (NADPH). Pentose phosphate pathway is the main source of NADPH. GSH is the most abundant antioxidant in cells, its concentration is 100 times more than GSSG. GSH and GSSG forms thiol buffer and GSH fights against reactive oxygen species (ROS). Glutathione reductase (GR) is the main enzyme to maintain the thiol buffer system against oxidative stress. Thus, GR is one of the most important enzymes against oxidative damage. GR was purified and characterized from various sources but, according to our knowledge, there is no report on the purification of GR from rat kidney. In this study, rat kidney GR was purified 2356 fold with a yield of 16% by conventional chromatographic methods; Sephadex G-25 gel filtration, 2′,5′-ADP agarose affinity, and Polybuffer-94 ion exchanger chromatographies. The purified enzyme gave single band on both native and sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE). The specific activity and the subunit molecular mass, Mr, of the purified GR were 250 units per miligram protein and 53 kDa, respectively.

B5.55
Comparative transcriptome and proteome analysis for the effect of berberine

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The emergence of widespread drug resistance of pathogenic bacteria is a major threat to public health. In this respect, products derived from natural sources serve as invaluable sources for the development of novel anti-microbial agents. Berberine is an important plant derived alkaloid found in many medicinal plants including Hydrastis canaddensis (goldenseal), Coptis chinensis, Berberis aquifolium, Berberis aristata, and Tinospora cordifolia, with medicinal uses focused on sub-acute and chronic inflammations including gastric disorders, respiratory affections and cancer during the 1800 second. In the late 1900 second, berberine was evaluated as a natural antibiotic to treat various bacteria-associated diarrheas. In the light of this information, the molecular response of Escherichia coli for the anti-microbial berberine has been investigated using comparative transcriptome and proteome analysis. Microarray analysis has been performed to study the transciptional profiles of the cells exposed to berberine for identification of genes that were induced in response to the anti-microbial agent. 2-dimensional gel electrophoresis followed by mass spectrometric analysis has been performed for the characterization of differentially expressed proteins of the cells treated with berberine. Proteins and genes repressed or induced in the presence of berberine included those that are involved in transport and binding, membrane repair and maintenance, proteins synthesis, energy metabolism, regulation and replication. In addition, the comparative analysis of the transcriptome and proteome showed that there is significant correlation between them.

B5.56
Characterization of glutathione reductase purified from rat kidney

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Glutathione reductase (GR, E.C. 1.8.1.7) is a flavoenzyme which belongs to the pyridine nucleotide: disulfide oxidoreductase family. It reduces oxidized glutathione (GSSG), using nicotinamide adenine dinucleotide phosphate (NADPH), formed mainly by glutathione peroxidases. The final product, reduced glutathione (GSH) is the major component of thiol buffer ([GSH]/[GSSG] = ˜100/1) and it is used fighting against oxidative stress created by the reactive oxygen species (ROS). Glutathione reductase is responsible from the maintenance of thiol buffer in cells. Recently, GR was purified from rat kidney, using conventional chromatographic procedures; Such as gel filtration, affinity chromatography and ion exchange chromatography. In this study, it was aimed to characterize some features of rat kidney GR. The optimum pH and temperature were found to be 6.5 and 65°C, respectively. These data were in accordance with the previous studies characterizing GR from other sources. The activation energy, Ea, and temperature coefficient, Q10, for GR purified from rat kidney, were found to be 7.02 Kcal/mole and 1.42, respectively. The Michaelis-Menten constants were determined in 0.1 M phosphate buffer at pH 7.4 and 37°C. The Km(NADPH), at fixed [GSSG], 1 mM, and varied [NADPH], 20–1200 μM, and the Km(GSSG), at fixed [NADPH], 0.1 mM, and varied [GSSG], 10–200 μM, were found to be 15.3 ± 1.4 and 53.1 ± 3.4 μM, respectively.

B5.57
Defining the mitotic functions of San/Naa50 during Drosophila development

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N^{α}-terminal acetylation is a highly conserved and widespread protein modification. Increasing amount of data has linked this modification to cell cycle progression and cancer. Yet, little is known about the biological role of N^{α}-terminal acetylation during development. Others and we showed that *san* (separation anxiety), which encoded an N-terminal acetyltransferase (NAT), was essential for mitosis in *Drosophila* (*in vivo* and *in vitro*) and in HeLa cells (*in vitro*). San function was originally associated to centromeric sister chromatid cohesion. However our work suggested that San was also important for chromosome condensation/ resolution, implying a more general mitotic function. Although the NAT activity of San is known to be required for its mitotic function, the relevant *in vivo* substrates are still unknown. We hypothesize that San regulates the activity (or stability) of unknown proteins whose function is crucial for mitosis. In order to identify these proteins we are currently using COFRADIC proteomics technology. Preliminary results comparing the N-terminal acetylation profile of *san* mutant and wild type embryos identified eighteen *in vivo* substrates of San. Two of these proteins are required for mitosis and we are currently investigating if the San-dependent N-terminal acetylation is important for their mitotic function. Our long-term goal is to define the mitotic functions of San and other related NATs, and better understand the role of N-terminal acetylation during development.

B5.58
Biophysical studies on the structure and function of molecules from the vertebrate myelin sheath

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Myelin is an insulating multilamellar membrane structure wrapped around selected axons in the vertebrate nervous system, allowing the rapid transmission of nerve impulses. Biochemically, myelin is a highly specialized plasma membrane produced by myelinating glial cells, harbouring several unique proteins and a specific lipid composition. De- or dysmyelination, caused e.g. by mutations in myelin-related genes or an autoimmune disease, can lead to severe neurological problems. Our goal is to use biophysical, biochemical, and structural biology methods to obtain information on the structure-function relationships in purified myelin proteins, and complexes thereof. Specifically, we mainly employ several synchrotron-based methods (X-ray crystallography, small-angle X-ray scattering, synchrotron radiation CD spectroscopy) together with complementary experiments, such as neutron scattering, fluorescence and oriented CD spectroscopy, chromatographic methods, calorimetry, surface plasmon resonance, enzyme kinetics, and light scattering to elucidate the details of molecular structure and interactions in myelin proteins. Eventually, we hope to better understand the details of the tightly packed myelin structure

and the etiology of myelin-related diseases. The presentation will provide background into the molecular structure of myelin, and recent results from our laboratory, dealing with the structure and function of selected myelin proteins, will be highlighted.

B5.59
Molecular properties of γ-glutamyl transpeptidases adapted to extreme environments

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γ-Glutamyl-transpeptidase (γ-GT) is an ubiquitous extracellular enzyme that plays a key role in glutathione metabolism. γ-GT catalyzes the hydrolysis of γ-glutamyl bonds in glutathione and glutamine and the transfer of the released γ-glutamyl group to amino acids and short peptides. γ-GT is synthesized as a precursor protein, which undergo a post-translational auto-cleavage to a mature enzyme composed of a large and a small subunit. γ-GTs from extremophiles, bacteria adapted to live in hostile environments, were selected as model systems to study the molecular underpinnings of their adaptation to extreme conditions and to find out special properties of biotechnological interest. Here we report the cloning, expression and purification to near homogeneity of three members of γ-GT family from different extremophilic sources, Thermus thermophilus, Deinococcus radiodurans and Geobacillus thermodenitrificans. We found that, as other γ-GTs, these enzymes are synthesized as precursor proteins of 60 kDa, undergoing an intramolecular auto-cleavage to yield two subunits of 40-kDa and 20-kDa, respectively. However, the three extremophilic enzymes display γ-glutamyl hydrolase activity, but no transpeptidase activity. The comparison of their sequences and structural models with those of other bacterial and eukaryotic γ-GTs suggests that several amino acids exchanges could be used by molecular evolution for enhancing the stability of proteins adapted to hostile environments. Moreover a phylogenetic analysis suggests that these γ-GTs specialized in γ-glutamyl hydrolase activity could represent the progenitors of the other bacterial and eukaryal counterparts.

B5.60
Phosphorylation is switch of L-PK cooperativity

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Pyruvate kinase (ATP-pyruvate 2-O-phosphotransferase, EC 2.7.1.40) is the key enzyme in glycolysis and modification of its catalytic properties is critical for regulation of this metabolic pathway. There are four pyruvate kinase isoenzymes M1, M2, R and L, whereas only M1 is considered as a non-allosteric enzyme. In our work we have shown that not only M1 is non-allosteric enzyme, but also non-phosphorylated L-type pyruvate kinase (L-PK) shows hyperbolic kinetics toward its substrate phosphoenolpyruvate (PEP) and can be characterized by hyperbolic Michaelis-Menten plot and KPEP = 0.11 mM. The L-type PK is the only one isoenzyme which activity can be regulated by phosphorylation. It takes place at Ser(12) residue, flanked by peptide sequence Arg(9)-Arg(10)-Ala(11)-Ser(12)-Val(13). Phosphorylation of L-PK by cAMP-dependent protein kinase changes the catalytic properties of pyruvate kinase. We have discovered that the non-phosphorylated enzyme is not allosterically regulated and through phosphorylation the enzyme obtains cooperativity. This means that phosphorylation is a molecular switch between non-allosteric and allosteric forms of the enzyme. The phosphorylated allosteric enzyme was characterized by KADP = 0.1 mM, KPEP = 2.2 mM, and the Hill coefficient n = 2.5. Also it was observed that phosphorylation of the first subunit of the tetrameric enzyme switches on the allosteric mechanism, while further phosphorylation only modulates this effect. The discovery that phosphorylation is a switch between allosteric and non-allosteric states of L-PK seems to be important for understanding the interrelationship between allostery and the regulatory phosphorylation in general.

B5.61
C-terminal segment of yeast BMH proteins exhibits different structure compared to other 14-3-3 protein isoforms

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14-3-3 proteins are abundant binding proteins involved in many biologically important processes. 14-3-3 proteins bind to other proteins in a phosphorylation-dependent manner and function as scaffold molecules modulating the activity of their binding partners. Yeast 14-3-3 protein isoforms BMH1 and BMH2 posses a distinctly variant C-terminal tail which differs them from the isoforms of higher eukaryotes. Their C-termini are longer and contains polyglutamine stretch of unknown function. It is now well established that the C-terminal segment of 14-3-3 proteins plays an important regulatory role by functioning as an autoinhibitor which can occupy the ligand binding groove and blocks the binding of inappropriate ligands. Whether the same holds true or not for the yeast isoforms is unclear. Therefore, we investigated the conformational behavior of the C-terminal segment of BMH proteins using various biophysical techniques. Dynamic light scattering, time-resolved fluorescence anisotropy decay and size exclusion chromatography measurements showed that the molecules of BMH proteins are significantly bigger compared to the human 14-3-3 zeta isoform. On the other hand, the sedimentation equilibrium analysis confirmed that BMH proteins form dimers. Time-resolved tryptophan fluorescence experiments revealed no dramatic structural changes of the C-terminal segment upon the ligand binding. Taken together, the C-terminal segment of BMH proteins adopts very open and extended conformation that increases their apparent molecular size. It seems, therefore, that the C-terminal segment of BMH proteins does not function as an autoinhibitor and does not block their ligand binding grooves.

B5.62
Relevance of hNatB mediated protein N-terminal acetylation for a proper actin cytoskeleton function

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N-α-terminal protein acetylation is one of the most common protein modifications in eukaryotic cells, occurring on approximately 80% of soluble human proteins. There are not many identified cases indicating the biological relevance of this protein modification but there is an increasing number of studies linking N-α-terminal acetylation to cell differentiation, cell cycle, cell survival, and cancer. The three major N-acetyltransferase complexes (NAT), NatA, NatB and NatC, are composed of a catalytic subunit and one or several accessory subunits, modifying a defined subset of cellular proteins in each one. NatB complex is characterized by acetylating proteins with Met-Asp, Met-Glu and Met-Gln termini. It consists of the catalytic subunit, Naa20p, and one accessory subunit, Naa25p, that are associated to the ribosome. It has been observed that this enzyme catalyzes N-terminal acetylation of actin and tropomyosin in yeast, being this modification necessary for a proper function of yeast actin cytoskeleton. It has also been demonstrated that mammalian actin and tropomyosin N-terminal acetylation is important for their proper biochemical activity. Therefore, we have analyzed hNatB downregulation effect on mammalian actin cytoskeleton function. We have observed that reduction of any of hNatB subunits, hNaa20 and hNaa25, expression induces actin stress fibers disorganization, thus, affecting cell mechanics. This is accompanied by a diminution of cellular focal adhesions and a decrease in cellular motility.

B5.63
A novel immunohistoenzymography approach for the in situ assessment of drug-target interactions

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A major challenge in mechanistic toxicology is to assess the functional relationships between drug-target interactions and target organ toxicity. Here we describe a novel quantitative functional approach for simultaneous in-situ assessment of pharmacological target activity and localization in tissues obtained from preclinical animal models. Using specific inhibitors of the prolyl dipeptidyl peptidase (DPP) enzyme family as a model, we have combined an enzyme activity assay with immunohistochemical localization of DPP proteins on the same tissue section. DPP enzymes catalyze the release of fluorescent AFC from the synthetic substrate Ala-Pro-AFC. When performed on histological slides, the resultant signal of this reaction can be localized and quantified using confocal microscopy. Concomitant immunohistochemical staining allows for the unequivocal assignment of enzyme activity to specific DPP proteins. DPP inhibitors with varying selectivity profiles can be used to discriminate between different enzyme forms. Our results show that rat kidney contains high levels of DPP activity that co-localizes with DPP4 protein in the proximal tubules and could be inhibited by pre-incubation with inhibitors. This immunohistoenzymography method can be applied to both frozen and formalin-fixed tissue samples, the latter providing superior spatial resolution. The ex vivo application of this approach to tissue samples from preclinical toxicity studies should provide novel insights into the mechanisms of action of DPP inhibitors in specific sub-cellular domains. Moreover, it can be applied to additional classes of enzymes for which suitable fluorescent synthetic substrates are available.

B5.64
dNTP pool imbalances: Mechanism of mutagenesis and effects on cell cycle

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Deoxyribonucleoside triphosphates–dNTPs (dATP, dTTP, dGTP, and dCTP)–are the building blocks of DNA and their balanced supply is essential for faithful genome duplication. Both the overall concentration and the balance among the individual dNTPs are tightly regulated, primarily by the enzyme ribonucleotide reductase (RNR). We introduced single amino acid substitutions in loop 2 of the allosteric specificity site of Saccharomyces cerevisiae RNR and obtained a collection of yeast strains with different but defined dNTP pool imbalances. We used these strains to study the mechanisms by which dNTP pool imbalances induce genome instability. Mutagenesis is enhanced even in the strains with mildly imbalanced dNTP pools, despite the availability of the two major replication error correction mechanisms: proofreading and mismatch repair. However, the mutagenic potential of different dNTP pool imbalances does not directly correlate with their severity and the locations of the mutations in a strain with elevated dTTP and dCTP are completely different from those in a strain with elevated dATP and dGTP. We further investigated whether dNTP pool imbalances interfere with cell cycle progression and are detected by the S-phase checkpoint, a genome surveillance mechanism activated in response to DNA damage or replication blocks. The S-phase checkpoint is activated by the depletion of one or several dNTPs. In contrast, when none of the dNTPs is limiting for DNA replication, even extreme and mutagenic dNTP pool imbalances do not activate the S-phase checkpoint and do not interfere with the cell cycle progression.

B5.65
Heat stress induced sumoylation by SUMO2/3

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SUMO (small ubiquitin related modifier) is a small protein modifier from a group of ubiquitin like (Ubl) proteins. Sumoylation, covalent attachment of SUMO to its target protein, is reversible and highly dynamic process. It plays role in many cellular processes such as subcellular localization of proteins, transcription regulation and DNA replication and repair. It was published recently that sumoylation also plays role in a protein degradation which was not formerly expected. There are three human SUMOs - SUMO1, SUMO2 and SUMO3, where SUMO2 and SUMO3, unlike SUMO1, contain internal sumoylation site and they are able to form covalent chains of SUMO2/3 units. Heat

stress causes changes in cellular sumoylation and high molecular sumoylated species cumulate in the cell. Sumoylation of proteins by SUMO2/3 is highly increased but sumoylation by SUMO1 is not that greatly altered. Furthermore, it was published that SUMO2/3 is important for cell survival of a heat stress. We will present changes in the sumoylation pattern during the heat stress and discuss the factors affecting the heat induced accumulation of high molecular sumoylated species. This work was supported by the specific university research (MSMT no. 21/2010, A2_FPBT_2010_027) and the Czech Ministry of Education Research Project grant MSM 6046137305.

B5.66
Determination of novel tumor markers in prostate carcinoma

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Prostate cancer is second leading cause of death among men. In the present time, prostate specific antigen (PSA) is the only tumor marker used for carcinoma detection. This tumor marker has high level of sensitivity, but lower level of specificity, so it can be increased either in inflammation or in prostate hyperplasia. It is desirable to find a new markers with higher level of specificity. Metallothionein (MT) and alpha-methylacyl-CoA (AMACR) racemase could be such markers. Expression of both of these proteins is highly unregulated in prostate cancer. MT levels are elevated most likely due to disbalance of zinc metabolism in this disease, and reason of elevation of AMACR is still unknown. In our work we analyzed levels of MT, PSA and AMACR in cell lines derived from prostate carcinoma – LNCaP, PC-3 and 22RVL, which were compared to prostate cell line PNT1A derived from normal prostate epithelium. For analysis we used immunoseparation techniques, western blotting and SDS-PAGE. In addition to this clasical methods of molecular biology we used novel electrochemical detection and capillary electrophoresis on the chip. We found significant elevation of MT, AMACR and PSA in prostate tumor cell lines similarly as in real blood samples from patients with prostate carcinoma. This study provides important new information about expression of MT, PSA and AMACR in prostate tumor cell lines and desribes application of electrochemical methods for their detection. Acknowledgement: Financial support from GACR 301/09/P436 and NS10200-3 is greatly acknowledged.

B5.67
Overexpressing 'difficult proteins' in escherichia coli

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E. coli is the most widely used vehicle for the overexpression of proteins. Protein overexpression is usually driven by the T7 RNA polymerase. Finding the right strain and optimal conditions for the overexpression of a protein can be very time consuming and cumbersome. Therefore, we have developed a generic solution for E. coli based protein overexpression, the so-called Lemo21(DE3) strain. In this strain T7 RNA polymerase activity can be modulated precisely by its natural inhibitor T7 lysozyme, which is expressed from the titratable rhamnose promoter. The Lemo21(DE3) strain conveniently allows optimizing the overexpression of any given protein using only a single strain rather than a multitude of strains and is compatible with any pET vector. The Lemo21(DE3) strain is the platform of choice for the successful overexpression of membrane proteins and other 'difficult-to-express' proteins. In addition, we have developed a tag that forces the overexpression of any given protein in inclusion bodies. This tag greatly facilitates the expression and isolation of e.g., very toxic proteins and antigens.

B5.68
A subgroup of Ulcerative colitis patients with active disease has an altered MUC2 *O*-glycosylation pattern in sigmoid colon

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The mucus barrier plays a key role in colonic barrier function. Glycosylated mucins form a matrix that prevents entry of bacteria. One possible mechanism behind impaired barrier function in ulcerative colitis is a defective glycosylation pattern. Thus, novel glycoproteomic methods were used to study sigmoid colon biopsies obtained from ulcerative colitis patients (active and inactive disease), and control patients (n = 50). Mucins representing the inner attached mucus layer of colon were extracted from single biopsies and further separated by SDS-polyacrylamide/agarose composite gel electrophoresis. The three major mucin bands were trypsin digested and identified as oligomeric forms of MUC2 by proteomics. MUC2 is a highly glycosylated mucin and the major component of both mucus layers in the colon. The MUC2 *O*-glycans of the sigmoid colon were analyzed by nanoLC/mass spectrometry and more than 100 complex *O*-linked oligosaccharides were identified, mainly based on core 3 structures. A high degree of sialylation was observed and the substructure Galβ1-3/4-GlcNAcβ1-3(NeuAc-6)GalNAcol was found in most glycans. The glycans present in more than 10 of the patients (26 glycans) were also semiquantified. In contrast to the *O*-glycans of other mucins, the sigmoid MUC2 *O*-glycan repertoire and relative amounts in normal individuals were relatively constant. However, a subpopulation of patients showed an accumulation of precursor glycans with a decrease of more complex glycans. This pattern was mainly found in active colitis but some patients in this group also had the normal pattern. The altered glycan pattern may contribute to deterioration of the barrier function in active colitis.

B5.69
Apoptosis-related cofilin phosphorylation at Ser3 is independent of ROCK activity

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The interactions of hematopoietic cells with the extracellular matrix (ECM) not only mechanically retain the unmature cells in the bone marrow microenvironment but also regulate the activity of different intracellular signal cascades. We found that several anti-leukemic drugs (e.g. SAHA, Imatinib) modulated the cellular adhesivity to fibronectin and we performed a proteomic analysis of JURL-MK1 cell line to uncover the proteins whose expression

level and/or posttranslational modifications are affected by these drugs. In this contribution, we focuse mainly on cofilin, the pivotal mediator of actin dynamics, whose activity is regulated by phosphorylation at Ser3. The phosphorylated (inactive) cofilin was concentrated in small distinct spots distributed throughout the cytoplasm. The fraction of cofilin phosphorylated at Ser3 was elevated by the cell treatment with adhesivity-stimulating drugs, in agreement with the need for preservation of strong actin structures. Surprisingly, cofilin phosphorylation level also increased when apoptosis was triggered, in spite of a marked F-actin disassembly. We speculate that some prominent actin structures have to be protected from cofilin-mediated destruction in order to assure the cell disintegration into apoptotic bodies. The apoptosis-related increase in cofilin phosphorylation was at least partly inhibited by the caspase inhibitor Q-VD-OPh but not by Y-27632, an inhibitor of the protein ROCK which is known to play a major role in cofilin regulation. This work was supported by the grant No 301/09/1026 from the Grant Agency of the Czech Republic and by the grant MZ0UHKT2005 from the Ministry of Health of the Czech Republic.

B5.70
Nuclear localization of hepatic FBPase is impaired in the liver from fasted rats and mice

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Here, we find that the subcellular distribution of hepatic fructose 1,6-bisphosphatase (FBPase) is modulated by the nutritional state and diabetes, whereas the liver zonation was not affected. In healthy starved rats, FBPase was mainly localized in the cytoplasm of hepatocytes, adopting a uniformly pattern. However, in healthy re-fed rats, its subcelullar localization dramatically changed and concentrates in the nucleus and the cell periphery. Interestingly, in hepatocytes of streptozotocin-induced diabetic rats FBPase was unable to translocate to the nucleus, indicating that insulin and not hyperglicemia plays a crucial role in the nuclear concentration of the enzyme. Moreover, hepatic FBPase was found phosphorylated only in the cytoplasm of both healthy and diabetic rats, suggesting that the phosphorylation state is involved in the nuclear translocation of FBPase. Despite the difference in primary structure between rat and mouse liver FBPase, the mouse enzyme showed a similar behaviour. Moreover, hepatic fructose 1,6 bisphosphate aldolase (aldolase B) presented virtually the same dynamic that FBPase in the liver of fasted and re-fed healthy rats and mice. The impaired nuclear translocation and the cytosolic FBPase concentration could account for the increased endogenous glucose production of liver of diabetic rats (Grants Funding: Fondecyt 1090740; 1090694; DID-UACh 2006-12; Fundación Marcelino Botin).

B5.71
Inadequate heat shock response of Mesothelial cells caused by Glucose degradation products contained in Peritoneal dialysis fluids

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Peritoneal dialysis (PD) is a convenient and cost-effective alternative to hemodialysis and the predominant treatment modality in pediatric patients. For decades glucose-based PD fluids (PDF) have been used in clinical practice. Because of their physicochemical properties these fluids harm the mesothelial cell (MC) layer. Glucose degradation products (GDP) are regarded as the major toxic compounds of conventional PDF, but their mode of cytotoxic action is yet unclear. Heat shock proteins (HSP) have been shown to essentially contribute to mesothelial cell protection and repair following PDF exposure in *in-vitro* and *in-vivo models*. Using a proteomics and bioinformatics approach we could identify involved biological processes and molecular pathways leading to MC injury. In this study MC were exposed to PDF with or without GDP at normal culture conditions or elevated temperature (42°C) for varying exposure times. Cellular damage or viability was assessed using standard assays and HSP expression was measured using Western blot analysis. For deeper biological insight proteomic and transcriptomic analyses were performed. Our results showed increased cell damage of MC following exposure to PDF containing GDP, whereas HSP expression was not further elevated. Under heat stress conditions MC were only able to induce HSP when GDP were not present. Proteomic analysis confirmed a dampening effect of GDP on HSP expression over a range of normally stress-inducible chaperones. In follow-up experiments we could assign this effect to defined chemical compounds occurring in commercial PDF. This finding represents a novel pathomechanism, likely relevant in several glucose-associated diseases.

B5.72
Serglycin interacts with type-1 collagen and affects the expression of matrix metalloprotinases from myeloma plasma cells

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Serglycin is a small proteoglycan which can carry four to eight chains of chondroitin sulphate, which is the major serglycin form expressed by cells of hematopoietic origin. We have previously showed that serglycin is constitutively secreted but also present on the cell surface of myeloma plasma cells. Moreover multiple myeloma, a B-cell malignancy, is characterized by clonal proliferation of plasma cells within the bone marrow, impaired hematopoiesis and osteolysis. The degradation of the extracellular matrix by proteolytic enzymes, such as matrix metalloproteinases, plays an important role in tumor progression, invasion, metastasis and bone remodeling. The aim of this study was to examine the role of serglycin, found on the membrane of multiple myeloma cells, as a surface receptor responsible for binding to type-1 collagen and to analyze the expression of matrix metalloproteinases mediated through this interaction. Our results derived from solid phase binding assays showed that purified serglycin from multiple myeloma cell lines, has the ability to interact with type-1 collagen. This interaction is mediated through the chondroitin sulphate chains of serglycin as after digestion with chondroitinase ABC the binding with collagen was decreased. In addition we have test the ability of three different multiple myeloma cell lines to adhere to type-1 collagen coated mictotiter plates in cell adhesion assays. Multiple myeloma cells can attach to type-1 collagen suggesting a role for serglycin found on the cell surface of these cells. Finally we have showed that the expression of matrix metaloproteinases was affected by this interaction.

B5.73

Abstract withdrawn

C1 – Molecular Machines

C1.01
Exploring the role of a conserved motif in the adenylation domain of a non-ribosomal peptide synthetase from *Bacillus brevis*

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Non-ribosomal peptide synthetases (NRPS) are molecular machines that synthesize peptide-based natural products, many of which are important pharmaceuticals with antibiotic, immunosuppressor and antitumor activities. A typical NRPS assembly line consists of a number of catalytic modules, each responsible for the incorporation of a single amino acid into the growing peptide product. Modules themselves comprise distinct catalytic domains, such as the adenylation domain (A) that, firstly, selects a specific amino acid monomer to be activated by ATP and, secondly, transfers it to the phosphopantetheinyl arm of the adjacent thiolation domain. Recent biochemical and structural evidence support the idea that the A domain uses a rotational movement of its two subdomains to adapt a single active site for the two partial reactions in the catalytic cycle. It has long been recognized that A domains share a set of 10 conserved motifs (A1-A10), most of which where assigned a particular role in substrate binding and/or catalysis. To our knowledge, this is the first study aimed at elucidating the role of the A9 motif. By analyzing homology models of the A domain from tyrocidine synthetase 1 from *Bacillus brevis*, it could be observed that during the rotational movement of the subdomains, the A9 motif drastically changes its position relative to the active site. The role of the conserved amino acid residues in this region and its importance for the two partial reactions of the catalytic cycle was examined by site-directed mutagenesis and biochemical characterization of mutant enzymes.

C1.02
Decavanadate effects in actin structure and function

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Decameric vanadate (V10) is a vanadium (V) oligomer known to interact with many proteins and to affect their structure/function (Aureliano, 2009). Particularly with actin, this interaction prevents G-actin polymerization into F-actin filaments whereas decavanadate molecule is stabilized during the process, avoiding their decomposition into monomeric vanadate, V1 (Ramos et al, 2006). More recently, it was observed that upon incubation with decavanadate, protein cysteine oxidation occurred in G- and F-actin, whereas no effects were detected with V1 (Ramos et al, 2009). Moreover, only with V10 a concomitant vanadate reduction to vanadyl (vanadium (IV)) was observed by EPR (Ramos et al, 2009). These studies were further explored and it was observed that both cysteine oxidation and vanadate reduction are blockaged by ATP. On contrary to V1, V10 up to 500 micraM (meaning 50 micraM decameric vanadate) induces an increase in G-actin intrinsic fluorescence, suggesting a change in protein conformation leading to tryptophan exposure to the solvent. An increase of actin hydrophobicity upon V10 incubation was confirmed by using the fluorescence probe ANSA. Upon incubation of F-actin with increasing V10 concentrations, a decrease of intrinsic fluorescence was detected, suggesting protein inactivation. Nucleotide exchange experiments point out that only decavanadate affects ATP interaction with actin. Putting it all together it is suggested that: (i) decavanadate interaction with actin induces cysteine oxidation and vanadate reduction being these effects prevented by ATP; (ii) decavanadate promotes actin conformational changes, iii) decavanadate have an effect on actin ATP binding site.

C1.03
Mycobacterial ubiquitin-like targeting – tagged for destruction

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In eukaryotes, proteasomes represent the main degradation route for cellular proteins, however, in bacteria, the occurrence of the 20S proteasome is limited to the group of actinobacteria, amongst them the highly pathogenic Mycobacterium tuberculosis (Mtb). Actinobacteria use a tagging system functionally analogous to eukaryotic ubiquitylation, termed pupylation, to recruit substrates for proteasomal degradation. The pupylation-degradation pathway contributes to the virulence of Mtb supporting its persistance and proliferation inside the host macrophages. We have recently reconstituted and characterized the pupylation pathway that leads to conjugation of the prokaryotic ubiquitin-like protein (Pup) to proteasomal substrate proteins. We showed that Pup is rendered coupling-competent by deamidation of its C-terminal glutamine through the action of a previously uncharacterized protein we now call Dop (deamidase of Pup), then PafA catalyzes formation of an isopeptide bond between the glutamate and a substrate lysine. The latter step turns over one ATP to ADP per coupled Pup, suggesting that Pup is activated for conjugation via phosphorylation of its C-terminal glutamte. We have also investigated the events downstream of the pupylation pathway, which lead to degradation of pupylated substrate proteins by the mycobacterial proteasome complex. Mechanistic and structure/function principles of the complete pupylation-degradation pathway will be presented and *in vivo* relevance will be discussed.

C1.04

Abstract withdrawn

C1.05
Ceramide down-regulates the Na+/K+ ATPase in HepG2 cells

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We showed previously that TNF-α down-regulates the Na+/K+ ATPase in HepG2 cells. TNF-α is known to cause the release of ceramide which enters the sphingomyelin pathway where it gets modified by several enzymes to other sphingolipids such as sphingosine, sphingosine 1-phospate (S1P) and ceramide 1-phospate (C1P) some of which have antagonistic effects to ceramide. The ratio of ceramide to the other sphingolipids determines the final outcome of the cell response. This work was

undertaken to study the role of ceramide and its metabolites on the Na+/ K+ ATPase in HepG2 cells. Cells were incubated for 15 minute with ceramide in presence and absence of CAY 10466 or SHKI, respective inhibitors of ceramidase and spingosine kinase (SPHK). The activity of the pump was assayed by measuring the amount of inorganic phosphate liberated, while changes in its expression were monitored by western blot analysis. Ceramide reduced the activity and protein expression of the Na+/ K+ ATPase. This effect disappeared when ceramidase and sphingosine kinase were inhibited, suggesting that the effect of ceramide is mediated via sphingosine or spingosine 1-phosphate. However, SPHKI added alone to cells, up-regulated the pump, revealing a stimulatory effect of the accumulated endogenous sphingosine. This hypothesis was confirmed by the observed up-regulation of the ATPase in HepG2 cells treated with sphingosine. The results suggest that S1P and ceramide (and/or ceramide -1P), exert an inhibitory effect on the pump while sphingosine has a stimulatory one.

C1.06
Nuclear import of RNA polymerase II requires the conserved GPN-loop GTPase XAB1/GPN1

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Over the past three decades, many efforts have been made to identify and characterize the factors that regulate the activity of RNA polymerase II (RNAPII), the enzyme that synthesizes all the mRNA and many small nuclear RNA in eukaryotes. Quite surprisingly, very little is known about the cell machinery that regulates the fate of RNAPII before or after transcription. In this study, we report that XAB1/GPN1, a member of a unique class of GTPases containing a conserved GPN-loop, is involved in the biogenesis of RNAPII by regulating its nuclear import. XAB1/GPN1 is physically associated with RNAPII in the soluble cell fraction and is essential for cell growth. Accumulation of RNAPII in the cell's cytoplasm was induced upon (i) depletion of XAB1/GPN1 by siRNA silencing, (ii) overexpression of a XAB1-GFP dominant negative fusion protein, (iii) mutation of the GTP-binding or GPN motifs of the XAB1/GPN1 homologue in yeast, NPA3, and (iv) treatment of cells with leptomycin B, an inhibitor of XPO1/CRM1-dependent nuclear export. Yeast strains with mutations in the NPA3 gene showed hypersensitivity to benomyl, a specific inhibitor of microtubule assembly, suggesting a role for microtubules in RNAPII biogenesis. Together, these results indicate that XAB1/GPN1 shuttles between the cytoplasm and the nucleus to mediate nuclear import of RNAPII.

C1.07
Nucleotide utilization requirements that render ClpB active as a chaperone

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ClpB (Bacterial Hsp100) is an AAA+ molecular machine that uses the energy of ATP binding and hydrolysis to extract unfolded proteins from aggregates, and to remodel and translocate them throught its axial channel for productive reactivation in colaboration with the DnaK system. As a member of class I of the Hsp100 chaperone family, ClpB contains two nucleotide binding sites (NBD) per protomer. Therefore, in the functional hexameric assembly there are 12 nucleotide binding sites arranged in two rings, each containing one of the two nucleotide binding sites of the protomers that most likely are allosterically communicated to thread the substrate protein through the central pore during aggregate reactivation. We extend here previous studies on ClpB nucleotide utilization requirements by using an experimental approach that maximizes random incorporation of different subunits into the protein hexamer. Incorporation of one subunit unable to bind or hydrolyze ATP knocks down the chaperone activity, while the wt hexamer can accommodate two mutant subunits that hydrolyze ATP in only one protein ring. Four subunits seem to build the functional cooperative unit, provided that one of the protein rings contains active nucleotide binding sites.

C1.08
Reconstitution into liposomes enhances nucleotide binding affinity of TrwB conjugative coupling protein

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Conjugative systems contain an essential membrane protein that couples the relaxosome to the DNA transport apparatus called coupling protein (T4CP). TrwB is the T4CP of the conjugative plasmid R388. This protein, purified in the presence of detergents, binds preferentially purine over pyrimidine nucleotides, NTPs over NDPs, and ribo- over deoxyribonucleotides. In contrast, a soluble mutant, TrwBΔN70, binds uniformly all tested nucleotides. Reconstitution of membrane proteins into liposomes is a widespread approach to analyze their biological function. Since, we are interested in the characterization of the activity of TrwB, we developed a reconstituted system that could be used to study the function of this protein. In this work, TrwB has been succesfully reconstituted into liposomes being the protein preferentially outside oriented. The functional analysis of TrwB proteoliposomes demonstrate that when the protein is inserted into the lipid bilayer, TrwB is selective for ATP and the affinity is enhanced compared with the protein purified in detergent and with the soluble deletion mutant, TrwBΔN70. From these results we conclude that the reconstitution of TrwB is the suitable way to study the biological activity of this coupling protein and that the transmembrane domain of TrwB could play a regulatory role in the biological activity of TrwB.

C1.09
Domain motions in skeletal G-actin

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Actin is involved in many cellular processes (force-generation, cell-division cycle etc.). The atomic structure of actin monomer (G-actin) consists of 4 subdomains. The interaction between the subdomains is dynamic; they might have different conformational and motional states depending on the bound cations and nucleo-

tides, and on the interactions with small molecules and larger entities. The different functions of actin require special internal organization and interactions with other molecules at well-defined regions. By DSC and EPR the global and local motional states of the G actin subdomains were studied in the presence of different cations. Two site-directed paramagnetic labels attached to two different regions of actin allowed to map the cation-induced motional changes, and how these states vary at different temperatures. By the Lumry-Eyring model the thermal transitions of G actin were analyzed, and by deconvolution of the DSC scans it was possible to study the interactions between larger subdomains affected by divalent cations. The molecules which are able to modify the dynamics and the local conformations of actin are usually bound to the barbed end of actin between subdomains 1 and 3. One of the reporter molecules was attached to Cys 374 site near to the barbed end, the second one to Lys 61 in subdomain 2. The EPR spectra of both bound labels reflected two different populations with different rotational correlation times. Removal of calcium or ATP resulted in significant change of rotational correlation times in both populations. Analysis of DSC transitions showed that the local internal motions detected by EPR are coupled with global motions measured by DSC.

C1.10
Comparative study of ZnO-MTCP and ZnO-CuMTCP nanoparticles in photodynamic therapy for breast cancer treatment in T47D cell line

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Photodynamic therapy (PDT) is a method of clinical treatment whereby diseased cells and tissues are destroyed by a combination of light and special drugs called photosensitizers. Both the light and the photosensitizing agent are relatively harmless by themselves but combined, can result in selective tumor destruction. In this method for cancer treatment upon UV light exposed, light will emit from nanoparticles and this light can activate photosensitizers to generate singlet oxygen and caused to killing cancerous cells. Zinc oxide nanoparticles were synthesized, coated by cysteine amino acid, as comparative study ZnO conjugated with meso-tetra(4-carboxyphenyl) porphine (MTCP) and CuMTCP (photosensitizer drugs for photoactivation) and folic acid (for cell recognition). Conjugated nanoparticles were characterized by FTIR, UV-Vis measurements and fluorescence spectrophotometry. Cytotoxic activity was evaluated by MTT assay and using the human breast cancer cell line (T47D). Our observations indicate that ZnO nanoparticle conjugates (30–60 iM, 48 hour) are efficient photodynamic agents that can be initiated by UV light (256 nm, 100 $\mu W/cm^2$) for 180 second. Free MTCP and CuMTCP and ZnO nanoparticles are shown little cytotoxic potential. Ultraviolet radiation alone exhibited little evidence of cytotoxicity either. Co-exposure to both UV radiation and ZnO nanoparticle conjugates elicited cytotoxicity. Comparative examination between ZnO-MTCP and ZnO-CuMTCP conjugated shown that ZnO-MTCP have more cytotoxic effect than ZnO-CuMTCP (63% and 40% respectively). the results indicate that either ZnO-MTCP and ZnO-CuMTCP conjugates may be useful in photodynamic therapy.

C1.11
The oxygen reactive species implications in the efficiency of antitumoral treatment with complex combinations of ruthenium

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Introduction: The aim of our paper is to identify if the free radicals of oxygen plays an important role in the signaling pathway for the apoptotic transformations when we use as an antitumoral treatment new complex combinations of ruthenium.
Materials and methods: Our *in vivo* studies were performed in Walker 256 carcinoma bearing Wistar rats treated with four new ruthenium complexes with fluorochinolones. The treatment started at 7 days from the tumor graft and we assayed in dynamics the apoptosis by flow-cytometry and the biochemical oxidative status parameters (such as lipid peroxides level, cooper-oxidize activity, total thiol-groups level, ferric reducing ability of sample), The biological samples are represented by cells removed from liver, kidney, brain, pulmonary and tumor tissues, compared with the untreated control group. Results. The results show an increase of apoptosis from 14, 79% to 59, 72% in tumor cells treated with the most active combination of ruthenium with norfloxacin. Also we registered an increase of the oxidative status and the free radicals of oxygen production during the treatment.
Conclusions: Even the new compounds biological activities are at the DNA levels and they act as the oncostatic molecules with platinum (cisplatin, carboplatin, oxaliplatin, which can develop also a high cross-resistance) we found than our new synthesized complexes are less toxic and their activity is based also on the induction of oxidative stress.

C1.12
The C-terminal domain of TrwK, the VirB4 protein of the conjugative plasmid R388, displays a critical regulatory function

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Type IV secretion systems (T4SS) translocate DNA and protein substrates across the cell envelope of bacteria to a widely distributed number of eukaryotic and prokaryotic target cells. Conjugative bacteria use T4SS to mediate the transfer of DNA and proteins to recipient cells. T4SS are macromolecular assemblies composed of 11 mating pair subunits (VirB1 to VirB11) and a coupling protein (VirD4) that span inner and outer bacterial membranes. Three of these subunits are ATPases that energize DNA and protein substrate transfer as well as pilus assembly. VirB4 proteins are the largest and most evolutionarily conserved proteins in T4SS and are essential for virulence and plasmid transfer.VirB4 proteins have been suggested to energize substrate translocation across the T4SS. Recently, we demonstrated that TrwK, the VirB4 homologue in the R388 conjugative system, is able to hydrolyze ATP in the absence of potential substrates. The atomic structure of VirB4 proteins is unknown. Based on computing predictions, a model of the C-terminal (residues 413 to 772) of Escherichia coli TrwK has been suggested using the atomic coordinates of the coupling protein TrwB of plasmid R388 as a template. Secondary structure predictions of TrwK

and TrwB revealed the presence of three α-helices in the C-terminus that are conserved in all VirB4 proteins but are absent in TrwB. Therefore, we decided to generate truncated variants of TrwK where these α-helical structures were sequentially removed and we have analyzed their *in vitro* and *in vivo* properties. The results suggest that the C-terminal end of VirB4 proteins could play a key functional regulatory role in the biological activity of T4SS

C1.13

Abstract withdrawn

C1.14
Actinoporin structure at the lipid-water interface

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Actinoporins are monomeric 20 kDa proteins produced by sea anemones that bind preferentially sphingomyelin-containing membranes and form oligomeric pores with a functional radius of ~ 10 Å. Pore formation leads to disruption of target cells and takes place in three steps: membrane binding, oligomerization and insertion of an amphipathic α-helix located at the porin N-terminus. Whereas monomer binding and insertion of the N-terminal helix have been extensively characterized, the structure of the oligomeric pore has remained elusive. The currently accepted model is the so-called toroidal pore in which the walls of tetrameric pores are lined by proteins and lipids. Fragaceatoxin C is a novel actinoporin isolated from the sea anemone Actinia fragacea. Here we report on the crystallographic 1.8 Å resolution structure of oligomeric FraC in complex with detergent. It consists of a crown-shaped oligomer with a diameter of about 110 Å and made up of 9 monomers. Cryo-electron microscopy studies of FraC pores in lipid bilayers show oligomers composed of 8 subunits. Our results challenge the toroidal model since a cluster of 8-9 α-helices can build the pore without lipids lining the walls. Actinoporins may thus be included in a new category of pore forming toxins: the α-helical barrel toxins.

C1.15
Anti-cancerous effects of LaF_3:Tb^{3+} nanoparticles on T47D cell line

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Cancer is a group of disease, in which cells become abnormal and divide without control. This accounts for 13% of human death. Recently nanotechnology is beginning to change the scientific landscape in terms of disease diagnosis, treatment and prevention. Nanoparticles are being used in nanomedicine as vehicles for drug delivery. One of the newly invented methods for cancer treatment is photodynamic therapy(PDT) which can be defined as the administration of a non-toxic drug known as a photosensitizer to a patient bearing a lesion, which is frequently, but not always cancer. After an incubation period this specific lesion is then usually illuminated with light which, in the presence of oxygen, leads to the generation of cytotoxic species and consequently to cell death and tissue devastation. In our experiment we used LaF_3:Tb^{3+} luminescent nanoparticles that get excited by UV lights so they would luminescence and activate a photosensitizer drug called "MTCP" [meso- tetra (4-carboxyphenyl) porphine] which has been conjugated to the nanoparticles. When MTCP absorbs visible light it produces singlet oxygen which is toxic for cells. We have produced these nanoparticles and purified them by gel-exclusion chromatography. Then we examined their spectrum via spectrofluorimeter and spectrophotometer. Also we observed their uptake in T47D human breast cancer cell line via fluorescence microscope after incubation with 1 mM nanoparticles for 24 hours. UV light(256 nm, 100 $\mu W/cm^2$) was exposed to cells for 180 second and cell viability was measured using MTT test. MTT test shows 40% death resulting from singlet oxygen produced by MTCP conjugated nanopaticles.

C1.16
Novel single stranded DNA binding proteins crucial for the DNA damage response

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Single stranded DNA binding (SSB) proteins are ubiquitous and essential for a variety of DNA metabolic processes including DNA replication, recombination, transcription and repair as well as for the recruitment of the repair machinery to sites of DNA damage. SSB proteins from the three domains of life are evolutionarily conserved and utilise oligonucleotide-binding (OB) domains for DNA binding. We describe two new human SSBs (hSSB1 and 2), with a domain organisation closer to the archaeal SSB than to the eukaryotic Replication Protein A (RPA). Building on our recent data (Richard et al. (2008) Nature 453(7195): 677–681), we show that hSSB1 is critical for the cellular response to double-stranded DNA breaks (DSBs) and directly interacts with the MRN (Mre11/Rad50/NBS1) complex to facilitate the repair of lethal DSBs. Preliminary data indicate that the second SSB, hSSB2 is involved in the cellular response to ultra violet radiation and we have embarked on the first biochemical characterisation of this newly discovered protein. We propose that these novel hSSBs are functional homologues of the bacterial and archaeal SSB family of proteins, placing them centrally in the DNA repair pathway. Like many early participants in the damage response pathway, hSSBs may therefore be involved in tumorigenesis and may significantly affect the response of patients to cancer therapies.

C1.17
A four-helix bundle model for *E. coli* PhoQ transmembrane domain

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In bacteria, two-component systems (TCS) detect the environmental changes via a sensor kinase at the periplasmic level, which leads to a phosphorelay cascade mediating specific gene transcription. PhoPQ is a TCS, which detects and responds to divalent cations and antimicrobial peptides. It is composed of a chemoreceptor (PhoQ) and a regulon (PhoP), which triggers gene regulation. PhoQ assembly is characterized by a homodimer transmembrane protein. The X-ray crystal structure is largely unresolved, and consequently the mechanism that transmits the signal across the membrane remains unclear. Here, we use molec-

ular simulations to assemble an atomistic model of PhoQ. First, the assembly of the transmembrane domain is investigated. We observed that few polar amino acids are crucial for the stability of this domain. Moreover, our results are consistent with new experimental cross-linking data, and support a four-helix bundle arrangement, which can be further exploited for the mechanistic understanding of PhoQ signaling response to external stimuli.

C1.18
Poly(A)-specific ribonuclease (PARN): Molecular mechanisms of mRNA cap and poly(A) tail recognition

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A critical step in eukaryotic mRNA degradation is removal of the poly(A) tail at the 3'- end of the mRNA. Poly(A)-specific ribonuclease (PARN) is an oligomeric, processive and cap-interacting 3'-5' exoribonuclease that efficiently degrades eukaryotic mRNA poly(A) tails. In addition to the exonuclease domain, PARN harbors two RNA-binding domains, an RNA recognition motif (RRM) and an R3H-domain. We investigated the RNA binding properties of PARN and we could show that PARN binds poly(A) with high affinity and specificity. Furthermore, we showed that the RRM and R3H domains of PARN separately could bind to poly(A). To investigate specificity for and recognition of poly(A) in the active site of PARN, we performed a kinetic analysis on a repertoire of trinucleotide substrates *in vitro*. We showed that PARN harbors affinity for adenosines in the active site and that both the penultimate and the 3' end located nucleotide play an important role for providing adenosine-specificity in the active site of PARN. Moreover, we solved a crystal structure of PARN in complex with m^7GpppG cap analogue and showed that the cap binding and active sites overlap both structurally and functionally. By mutational analysis we identified residues in the active site that specifically recognize adenosines. Furthermore, biochemical data showed that the adenosine specificity in the active site is lost when $Mn^{2+}$is used instead of Mg^{2+}as divalent metal ion. Taken together, these results demonstrate that both RNA-binding properties of the RRM and R3H-domains in addition to base recognition in the active site contributes to PARN poly(A)-specificity.

C1.19
A model for the assembly and activation process of the pore-forming toxin Aerolysin

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Pore-forming toxins are a complex virulence factor common to several bacterial families. These proteins are secreted as water-soluble but, upon binding to specific receptors on the target cell's membrane, they transform into a transmembrane complex via oligomerisation and important conformational changes. This results in the creation of a pore, which induces a reaction cascade usually leading to membrane lysis. By mean of all-atom molecular dynamics compared to *in vitro* experiments, we study the activation and assembly process of Aeromonas hydrophila aerolysin beta pore-forming toxin. We show here that the C-terminal peptide has a dual role. It is indeed both affecting the proper folding of the toxin and its activation. We ultimately identify specific point mutations affecting the correct functioning of this nanomachine.

C1.20
Transfection of cultured myoblast with mutant β-tropomyosin (*TPM2*$_{EGFP}$)

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Tropomyosins (TMs) belong to the family of actin-binding proteins. It provides stability and it is essential for myosin-actin interaction. The tropomyosin filament also plays an important role in maintaining the actin microfilament system. Mutations in the gene encoding β-tropomyosin (*TPM2*) result in a variety of inherited diseases, including nemaline myopathy, cap disease and distal arthrogryposis. We investigated the effects of E41K, K49del and G53ins mutations in *TPM2* in cultured myoblasts. The E41K mutation is associated with a congenital myopathy characterized by either cap structures or accumulation of nemaline rods. The pathogenesis in patients with cap disease associated with K49del and G53ins mutations in *TPM2* involves abnormal coarse-meshed intermyofibrillar network and the appearance of cap structures. A C-terminal tagged β-tropomyosin-enhanced green fluorescent protein (EGFP) fusion construct was generated. Human cultured myoblasts were transfected with wild-type and mutant β-tropomyosin-EGFP constructs (WT-$TPM2_{EGFP}$, E41K-$TPM2_{EGFP}$, K49del-$TPM2_{EGFP}$ and G53ins-$TPM2_{EGFP}$) and stained with TRITC-phalloidin and DAPI. We also examined the ability of WT and mutant TMs to contribute to the sarcomeric thin filament by differentiation of myoblasts transfected with EGFP-tagged constructs. Confocal microscopy was performed using a Zeiss LSM 510 META. The transfection studies in cultured myoblasts and myotubes may provide a better insight into the pathogenetic mechanisms involved in microfilament system of the myoblasts and myotubes, which may be a part of the pathogenesis involved in the assembly of myofilaments and sarcomere structure in patients with mutations in *TPM2*.

C1.21
Interactions between TrwB protein and DNA substrates with secondary structures

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TrwB is a DNA-dependent ATPase involved in DNA transport during bacterial conjugation. The protein presents structural similarity to hexameric molecular motors such as F1-ATPase, FtsK or ring helicases, suggesting that TrwB also operates as a motor, using energy released from ATP hydrolysis to pump ssDNA through its central channel. During this year, we have carried out an extensive analysis with various DNA substrates to determine the preferred substrate for TrwB. Oligonucleotides with G-rich sequences forming G4 DNA structures were the optimal substrates for TrwB ATPase activity. The protein bound with 100-fold higher affinity to G4 DNA than to ssDNA of the same sequence. Moreover, TrwB formed oligomeric protein complexes only with oligonucleotides presenting such a G-quadruplex DNA structure, consistent with stoichiometry of 6 TrwB monomers to G4 DNA, as demonstrated by gel filtration chromatography and analytical ultracentrifugation experiments. G-quadruplex structures are widespread in the genomes and are thought to play a biological function in transcriptional regulation. They form very stable structures that can obstruct DNA replication, requiring the action of specific helicases to resolve them. These observations are discussed in terms of a possible role for TrwB in resolving G4 secondary structures that arise during conjugative DNA processing.

C1.22
The main pathways for obtaining abiotic stress-tolerant transgenic poplars

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Populus is very important genus in biology as well as in industry. Firstly, they are model woody plants with genome sequenced, secondly poplars are trees actively used for energy, paper and wood production because of their fast-growth and wide native areal. Though many varieties were obtained through conventional breeding methods, genetic engineering has provided more effective tools for improving different traits. Creation the transgenic *Populus* lines is significant for fundamental physiological, biochemical and tree gene functions studies as well, as for applied fields, particularly for industry. One of the main fields for improving poplar phenotypes through gene transferring is tolerance to abiotic stresses – oxidative stress, drought, salinity, cold, environmental pollution etc. A number of processes are involved in the tolerance mechanism: osmolytes, polyamines, reactive oxygen species and antioxidant defense mechanisms (such as superoxide dismutase, catalase, peroxidase, ascorbate peroxidase, glutathione reductase), ion transport and compartmentalization of injurious ions. Thus, increasing the activity of antioxidant enzymes, levels of osmolites, metalothioneins at date are the main tasks for gene transformation of poplars to enhance stress-tolerance.

C1.23
Dissect and build: Reconstructing the cellulosome using X-ray crystallography and small angle X-ray scattering

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With increasing energy consumption and the depletion of fossil fuels, development of alternative fuel sources is paramount. Plant cell wall (PCW) polysaccharides are the most abundant renewable carbon source. Therefore, conversion of plant biomass into biofuels is an attractive option to reduce our dependence on fossil fuels. However, the PCW is an insoluble and recalcitrant substrate, which makes the efficient breakdown of PCW polysaccharides into monosaccharides a major obstacle in bioethanol production. Several anaerobic microbes possess large cell surface bound multienzyme complexes, dubbed cellulosomes, which are capable of the synergistic degradation of PCW polysaccharides. Enhanced cellulolytic activity is conferred to hydrolytic enzymes that bind the core non-catalytic scaffoldin subunit of the cellulosome. However, understanding the structural elements responsible for the enzymatic synergy of the cellulosome is hindered by both the large size and inherent flexibility of these multi-protein complexes. Herein, we describe ongoing work to elucidate the three dimensional structure of the cellulosome using a dissect and build approach, which combines X-ray crystallography and small angle X-ray scattering (SAXS). Specifically, the crystal structure of a multimodular fragment of the cellulosome from *Clostridium thermocellum* containing three polypeptides will be presented along with SAXS structures of cellulosomal complexes that reveal the modular arrangement and flexibility of these complexes in solution. Our findings provide valuable insight into cellulosome structure and function that will aid in engineering designer cellulosomes for large scale bioethanol production.

C2 – Biological Cycles

C2.01
The urea cycle enzymes and several aminotransferases in fruit bodies maturation process of fungus *Pleurotus ostreatus*

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In metabolism of some fungal species it was noted that ammonia (which is cellular poison) inhibits basidiospore formation. Ammonia removes from the organism by biosynthesis of urea in urea cycle. The urea cycle is integral part of ammonia- scavenging network. In higher fungi urea is source of reserve nitrogen but high levels of urea influence on taste and maturation process. The aspartate aminotransfarse (AST) and alanin aminotransfarse (ALT) play important role in nitrogen metabolism, particularly in nitrogen assimilation. In the present study activities of arginase, ornithine carbamoyltransferase (OCT), ALT, AST, content of urea and total protein in different stages of *Pleurotus ostreatus* fruit bodies formation have been investigated. The measurements were carried out every two days after primordia formation up to spore generation. We have noted significant changes of enzymes activities, content of urea and total protein during maturation. Arginase activity increased many times on the 2nd and 4th days of development. OCT and ALT activities increased up to basidispore generation phase, after which it sharply dropped. In the last stages of development considerable rising of AST activity has been noted. Generally, the arginase and AST activities in the cap and gills exceeded stem 2–3 times. In contrast to arginase and AST, high levels of ALT and OCT activities were found in fungal stem. Highest amount of urea was pointed out in basidispore generation phase. Total protein has increased throughout all stages of development.

C2.02
Prognostic significance of P53 abnormalities in Turkish colorectal cancer patients detected by PCR-SSCP

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Colorectal cancer is the second most frequent cause of cancer-related death. The p53 tumor suppressor gene, located on chromosome 17p13,is one of the most commonly mutated genes in all types of human cancer. The process of colorectal tumorigenesis is based on the concept of the 'adenoma-carcinoma sequence' and is believed to follow a multistep genetic model. Single-strand conformational polymorphism (SSCP) analysis can be used to detect abnormalities within the regions of p53. The study group comprised 40 patients admitted to Cerrahpasa Medical Faculty's Hospital, Istanbul for curative surgical resection of colorectal cancer. Archival formalin-fixed, paraffin wax-embedded tissue was used. A single block representative of the tumor was selected in each case and the same block was used for PCR-SSCP. Ten-micrometer-thick tissue sections were placed on a glass slide and stained with HE. Then the tissue sections were dehydrated in graded ethanol solutions and dried without a cover glass. DNA was extracted from the tissues with 100 μl of extraction buffer at 55°C overnight. The tubes were boiled for 7 minute to inactivate the proteinase K, and then 2 μl these extracts were used for polymerase chain reaction (PCR) amplification. All analyses were performed using the Statistical Package for the Social Sciences (SPSS). Non-parametric data were assessed using the Mann-Whitney *U*-test. Categorical variables were assessed by the x' test. In conclusion, we observed a statistically significant relationship between the evaluation of p53 alterations by SSCP/sequencing and stage of tumors.

C2.03
Computer controlled automated assay for comprehensive studies of S.cerevisiae growth kinetic in response to extracellular conditions

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A systematic and comprehensive assessment of the influence of extracellular parameters, such as culture conditions and metabolic by-product accumulation on growth kinetics, biomass production and glycolytic flux is a cumbersome task. In a setting where a large number of experimental parameters can be varied and the goal of the optimization can be expressed as a number, numerical methods allow to get closer to an optimal composition in a systematic manner. Such methods typically treat the relation between input parameters and experimental outcome as a black box with a few mild restrictive assumptions. Therefore, they can be applied even when no valid mathematical model of the process under investigation is known. Here we implemented a computer-controlled robot-system that combines automated variation of multiple culture/medium parameters with the seamless integration of mathematical tools for experimental planning and data processing. Our system is unique by the fact that it is based on abstract, object oriented descriptions of experiments written in the R programming language that provides a wide range of statistical tools. Providing a small set of defined stock solutions, yeast growth is automatically assayed in a multidimensional parameter space. The resulting datasets of growth kinetic parameters are visualized using multidimensional regression methods and response surface modelling that facilitate the determination of the optimum values for the factors under investigation. Using this automated procedure we demonstrate the dynamic model of biomass yield- and growth rate-dependence on extracellular glucose concentration, pH conditions and acetic acid concentration.

C3 – Extracellular Structures

C3.01

Abstract withdrawn

C3.02
Alasan from Acinetobacter radioresistens S13 is an N-linked glycoprotein with a peculiar consensus sequence

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Surfactants of biological origin are very requested in both the food industry and for cosmetic applications. In the present research the glycosylated nature of a surfactant protein, previously identified in the A. radioresistens S13 proteome, was demonstrated, and a multiplexed analysis was performed in order to establish the degree of homology with a known OmpA-like surfactant, produced by A. radioresistens KA53, named Alasan. The coverage study after N-terminal amino acid sequencing, peptide mass fingerprinting and MS-MS experiments revealed a 99,97% of identity, displaying only one residue change at the position 25 where a glycine of A. radioresistens KA53 alasan is replaced by a threonine in the protein isolated from A. radioresistens S13. Curiously, the mutation gives rise to a consensus N-glycosylation sequence absent in the previously characterized Alasan. Furthermore the residue 23 was detectable only after N-glycosidase treatment thus demonstrating that this position is consistent with the N-glycosylation site. The consensus sequence Asn-Asp-Thr proved to be peculiar since in general Asp, as well as Glu and Pro in the central position of the consensus site, gives rise to unstable glycosylations. All these results suggest that glycosylation processes in bacteria differ from those observed in eukaryotic cells and that the mechanisms and the significance of the glycosylations are far to be understood.

C3.03
NMR structural studies of the human interferon-α2 complex with human type I Interferon receptor

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Type I Interferons (IFNs) are a family of homologous helical cytokines initiating strong anti-viral and anti-proliferative activity. All type I IFNs bind to a common cell surface receptor consisting of two subunits, IFNAR1 and IFNAR2, associating upon binding of interferon. We studied inter-molecular interactions between IFNAR2-EC and IFNα2 using two novel approaches based on the use of deuteration and 2D homonuclear NOESY experiments for the detection of inter-molecular NOEs in large protein complexes which were developed and applied to the IFNAR2-EC/IFNα2 complex. Sequential and side-chain assignment of IFNAR2-EC and IFNα2 in their binary complex helped assign the inter-molecular NOEs to the corresponding protons. A docking model of the IFNAR2-EC/IFNα2 complex was calculated based on the inter-molecular interactions found in the present study as well as four double mutant cycle constraints, previously observed NOEs between a single pair of residues and the NMR mapping of the binding sites on IFNAR2-EC and IFNα2. Our docking model doubles the buried surface area of the previous model and significantly increases the number of inter-molecular hydrogen bonds, salt bridges and Van der-Waals interactions. Furthermore, the current model reveals participation of several new regions in the binding site such as the N-terminus and A-helix of IFNα2 and the C-domain of IFNAR2-EC. As a result of these additions, the orientation of IFNAR2-EC relative to IFNα2 has changed by 30° in comparison with the previous model. In addition, the new model strongly supports the recently proposed allosteric changes in IFNα2 upon IFNAR1-EC binding to the binary IFNα2/IFNAR2-EC complex.

C3.04
Cellulases catalysed cellulose polymerisation

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In bacteria, the cellulose biosynthesis requires at least four genes encoded by the Bacterial Cellulose Synthesis (Bcs) operon. Surprisingly, one of these genes, *bcsZ*, encode an enzyme referred to as endocellulase. The function of this enzyme is suspected to be the transglycosilation of newly produced cellooligosaccharides to the existing cellulose. Here two new cellulases, RBcel1 and Pst_2494, respectively derived from an Antarctic soil metagenomic library and from *Pseudomonas stutzeri*, are described and compared to the well characterized hydrolytic cellulase of *Thermoascus aurentiacus* (1GZJ). Biofinformatic analysis reveals changes in the substrate binding sites along the active site cleft. Indeed, both RBcel1 and Pst_2494 lack cellulose binding residues in subsites +2 and +3, downstream the active site. Such modifications are suspected to shift the enzyme activity from hydrolase to transglycosilase. These two new enzymes are so supposed to be required for cellulose synthesis by bacteria such as *Pseudomonas* species. In order to test our hypothesis, the tree enzymes (RBcel1, Pst_2494 and 1GZJ) were compared regarding both their hydrolytic and transglycolytic abilities.

C3.05
Hyaluronidases in colorectal cancer tissue samples

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Hyaluronan is a major glycosaminoglycan that plays crucial role the integrity of the extracellular matrix (ECM). Hyaluronidases (Hyal-1, -2, -3, -4, PH-20 and pHyal) are enzymes that degrade predominantly hyaluronan and are known to be involved in both physiological and pathological processes. Hyaluronidase-mediated degradation of hyaluronan increases the permeability of ECM and facilitates cancer progression. Our study focused on the activity and expression of hyaluronidases in colon cancer samples. Tissue samples (healthy, macroscopically normal and cancerous) were obtained from patients subjected to surgical operation due to colorectal carcinoma. Tissues were subjected to successive extraction using: PBS, 4M GdnHCl and 4M GdnHCl

- 1% Triton X-100. Hyaluronidases were detected using hyaluronan-zymography, Western Blotting and RT-PCR analysis. Hyaluronan-zymography revealed increased hyaluronidase activity mainly in cancerous samples. RT-PCR and Western Blotting confirmed high Hyal-1 and -2 expression in cancerous samples. PH-20 was found to be elevated mostly in normal samples. Hyal-1 and Hyal-2 seemed to be the major hyaluronidases expressed in cancerous samples, contributing to the formation of small angiogenic hyaluronan fragments. Hyal-3 was exclusively extracted in GdnHCl-Triton extracts of advanced cancer stage. These observations indicate that hyaluronidases are highly associated with tumor progression and may be used as targets in tumor therapy.

C3.06
Involvement of hepatic stellate cell-derived myofibroblasts and portal myofibroblasts in liver fibrosis: A comparative study using proteomics and transcriptomics

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In chronic liver diseases, the accumulation of extracellular matrix leading to fibrosis is caused by myofibroblasts, the origins of which are debatable. We compared rat hepatic stellate cell-derived myofibroblasts (MF-HSCs) and portal myofibroblasts (PMFs) using both proteomics (with two-dimensional electrophoresis and MALDI-TOF/TOF mass spectrometry) and transcriptomics (with DNA-microarrays). The aim of this study was to determine phenotypical and functional specificities of each kind of myofibroblasts and to define specific markers for each. Our results show that MF-HSCs would be involved in liver regeneration, angiogenesis, vascular tone, and express many proteins related to cellular response to stress, whereas MFPs showed proliferative and contractile features and could be involved in leucocytes chemoattraction. Using markers identified in this work, i.e. (i) cytoglobin and RGS4 for MF-HSCs; (ii) phosphocofilin-1, collagen-XV and calsequestrin-2 for PMFs, immunohistochemistry was performed in bile duct ligated rats and showed that PMFs are preferentially localized in the septa and the portal tract during cirrhosis. The microarray study also revealed that MF-HSCs expressed osteoprotegerin (OPG), which could be a serum marker of liver fibrosis. Score including OPG could be more reliable than existing scores for the diagnosis of significant fibrosis and cirrhosis in a population of HCV-chronically infected patients. This work highlights new features on MF-HSCs and PMFs specific functions in liver fibrosis and suggests that PMFs would greatly contribute to fibrogenesis.

C3.07
The content of glycosaminoglycans (GAG) as part of a diagnostic complex evaluation of treatment effect in ovarian cancer stages III-IV

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After the neoadjuvant polychemotherapy (NPChT) it becomes possible to make an operation to delete the original tumer in patients having ovarian cancer (OC) at the late (III-IV) stages. The complex of biochemical,clinical and ultrasound criteria for evaluating the effect of NPChT (1–6 courses) helps to find the optimal number of courses for each patient having OC stages III-IV and improve the efficiency of treatment. GAG fractions and total GAG in blood serum of 82 patients having OC at III-IV stages (serous adenocarcinoma) before treatment and after NPChT were determined by M. Stern et al. method. ELISA method were used to determine CA125 content. It was found that the decrease in level of total GAG in blood serum up to 75–70%, chondoitinsulphates up to 80–65%, II fraction GAG up to 75–65% of the original, I,III fractions, ratios of total GAGs and their fractions to normal, as well as the content of CA125 to 9,6–3,6% of its initial value suggests NPChT efficiency in patient having OC stages III-IV. These findings correlated with a reduction in size of a tumer conglomerate to 60–20%, of metastatic tumor in the recto-vaginal septum to 70–20%, the dissepearance of ascites and pleural fluids (according to clinical and ultrasound data). Using the level GAGs and their fractions, their ratios,CA125 in diagnostic complex evaluation of the NPChT effect as well as clinical and ultrasuond parameters improves the efficiency of this category patients treatment.

C3.08
Extracellutar synthesis of the polyol lipid - type bioemulsifiers by *Rhodotortula glutinis*

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Biosurfactants have attracted considerable interest in recent years, due to their unique properties e.g. mild production conditions and a variety of functions. Microbial emulsifiers have application in enhanced oil recovery operations (MEOR), in textiles, pharmaceuticals, cosmetics and the food industry. It was reported that production of surfactant can be stimulated in several microorganisms by deproving them essential nutrients different that carbon, such as phosphorus, iron, calcium and magnesium. This may lead to the synthesis and excretion of biosurfactants as a physiological response. Soil sample were collected at Polish sites historically contaminated by crude oil or its products. A strain *Rhodotortula glutinis*isolated in our laboratory was found to produce an extracellular emulsifier. The cultures grows rapidly after a lag time of about 12 hour but emulsifier production occurs during stationary phase. This observation suggest that compound production by isolated strain is a secondary microbial metabolic process. The highest bioemulsifier production was observed when C/N and C/Fe were combined. At high C/N of 30 and high C/Fe of 780, emulsification activity was 14U. High yeast extract concentration inhibited the production of emulsifier. It was found that emulsifier stabilizes the oil-in-water emulsions with aromatic hydrocarbons. With n-alkanes, the dispersed droplets separate into two phase and, therefore, the emulsion formed is unstable. The emulsifier was stable and active in wide pH range of 3.0–10.0. At 1000°C it was stable for 30 minutes. The present emulsifier was un affected by sodium chloride up to 7%.

C3.09
The central cysteine-rich CysD domain of the MUC2 mucin mediates protein-protein interactions by dimer formation

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MUC2 is the major gel-forming mucin of the colon mucus in human. The MUC2 mucin gel is a branched net-like polymer assembled from monomers via covalently linked dimers in its C-termini and trimers in its N-termini. N- and C-terminal oligomerization is mediated via cysteine-rich parts that form covalent interchain disulfide bridges. The large central region of MUC2 contains two highly O-glycosylated mucin domains that are interrupted by another cysteine-rich region (CysD domain) of unkown function. We aimed at studying its function in a recombinant fusion protein that contained the CysD domain and a removable immunoglobulin G (IgG2a) Fc domain. When expressed in Chinese hamster ovary cells the fusion protein was secreted into the culture medium and subsequently purified by affinity chromatography. Analysis of the purified fusion protein by native gel electrophoresis and gel filtration chromatography showed that it formed tetrameric, octameric, dodecameric and 16-meric complexes. Individual analysis of the isolated CysD part and IgG2a part revealed that both parts formed dimers only. This finding was confirmed by analyzing another recombinant CysD fusion protein that encoded twice the general tandem repeat of the MUC2 mucin domain. In conclusion, oligomeric complexes were multiples of tetramers linked to each other via protein-protein interactions mediated by the CysD domain. These findings show that CysD domains of gel-forming mucins are inserting additional cross-links into the polymeric gel thereby determining its pore size. Due to the varying number of CysD domains in other gel-forming mucins distinct gel types are formed with different properties.

C3.10
Extracellular reactive oxygen species in aphids

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Examples of reactive oxygen species, ROS are superoxide anion, $O_2^{\bullet-}$ and hydrogen peroxide, H_2O_2. ROS are intermediates in the reduction of O_2 into H_2O. In the red-ox homeostasis ROS are constantly/parallel released and removed. In general, the xenometabolism amplifies ROS production. ROS excess, inside/outside the cells is defined as oxidative stress. Although the dual nature of ROS is emphasized, in fact most research concern ROS toxicity. Recently, ROS appear as signaling molecules in biotic/abiotic stresses and plant-insect interactions. This paper presents extracellular ROS status in *Rhopalosiphum padi* and Sitobion avenae, in particular: (i) microscopic visualization of $O_2^{\bullet-}$ and H_2O_2 *in vitro* and *in vivo*; (ii) H_2O_2 production consequently exogenous phenolics; (iii) enzymatic sources of $O_2^{\bullet-}$ and H_2O_2 generation and decomposition. *In vitro*, H_2O_2 is produced in consequence of the salivary oxidative metabolism of dietary o-dihydroxyphenolics i.e. caffeic acid, catechin. *In vivo*, midgut appears major locus of $O_2^{\bullet-}$ and H_2O_2 production. The phenolic xenobiotics show different prooxidant impact, o-dihyroxyphenolics and polyphenolics i.e. DOPA, catechin, caffeic, gallic and chlorogenic acid, in contrast with the monophenolics, enhance ROS production. Polyphenol oxidase (PPO) biocatalysis, located in midgut and saliva is recognized as fundamental oxidation of phenolic xenobiotics and enzymatic supply of $O_2^{\bullet-}$, precursor of H_2O_2. Contrary to PPO, aphid peroxidase, POD eliminates H_2O_2. In addition, catalase, CAT decomposes H_2O_2 in midgut and impairs oxidative stress, *in vivo*. There is a direct link between ROS level/oxidative stress and PPO, POD and CAT of *Aphididae*.

C3.11
Isolation and characterization of α-Amylase obtained from thermophilic *Anoxybacillus sp*

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α-Amylase (α-1-4 D-glucan glucanohydrolase EC 3.2.1.1) catalyzes endohydrolysis of α-1-4 glucosidic linkages in starch and any related oligosaccharides to make oligosaccharides and glucose. Amylases have a significant commercial importance and they occupied about 25–33% of the world enzyme. These enzymes are used in the textile and paper industries, detergents in starch liquefaction, as a food adhesive, and in sugar production. In this study, the morphological, biochemical, and 16 S rRNA analyses of the bacterium that was isolated from hot water springs of Ömerli, Afyonkarahisar in Turkey have been studied. The gram positive, spore-forming, motile, moderately thermophilic bacterium was found to be a strain of *Anoxybacillus sp*.analyzed by 16S rRNA comparison. The optimum growth time of *Anoxybacillus sp*. was determined to be the 20th hour, pH as 6.0 and temperature as 55°C. The extracellular amylase production of the bacterium was studied through SmF method. The highest α-amylase production was obtained in the 24th hour (1509.3 U/ml). The optimum enzyme activity was determined as 55 oC and pH as 7.0. The thermostable α-amylase obtained from *Anoxybacillus sp*. can be used in industrial applications.

C3.12
Fluorophore-kodecytes – fluorescent function-spacer-lipid (FSL) modified cells for *in vitro* and *in vivo* analyses

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Most live cells are naturally poorly visible and so various secondary techniques, such as staining (including fluorescent tagging), are used to visualize them. However, a major limitation of most staining techniques is that they commonly compromise cell vitality and/or functionality because the stain either covalently attaches to functional molecules or has toxic interactions. In contrast, FSL (function-spacer-lipid) constructs are designed to be dispersible in biological media, they can insert into cell membranes, and a range of synthetic molecules can be attached, such as fluorophores, without affecting the cells functionality or vitality (1–3). Two constructs for fluorescent tagging of live cells were investigated. In the first, FSL incorporated FITC as its functional moiety (FSL-FITC), and the second approach used biotin (FSL-biotin), which was then secondarily reacted with fluorophore labeled avidin. Both approaches were used to successfully label a variety of living cells including murine embryos, spermatozoa, epithelial and endometrial cells. FSL-FITC was additionally used to stain the digestive tract and circulation of zebrafish embryos. Acknowledgement: Supported by KODE Biotech Ltd (kodebiotech.com).

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C3.13
Proteome of chondrocyte-alginate constructs and monolayer cultures in relation to extracellular matrix modulation

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Chondrocyte-alginate constructs have been shown to produce differences in extracellular matrix organization and composition when compared to the monolayer culture system. The construct has been shown to change the collagen fibres orientation, increase glycosaminoglycan synthesis and retain chondrocytes isogenous ovoid morphology. Identification of proteins expressed by chondrocytes in these two culture systems is important to understand the modulation of the extracellular matrix and also regulation of its gene expression, cellular physiology and characteristics. Therefore, in this study, proteomic analysis was performed to compare protein profiles between chondrocyte-alginate construct and monolayer chondrocyte cultures, aimed at identification of key and novel proteins involved in the modulation of extracellular matrix synthesis. Using this investigative modality, we were able to establish a standard chondrocyte protein profile of chondrcocyte monolayer cultures and chondrocyte-alginate constructs using 2-dimensional electrophoresis and mass spectrometry. Following the proteomic analysis, we have successfully determined expression changes on 8 proteins at lower molecular weight region (CAPL, S100-A6, CHA, HPUBQ, CA1, CA2, CA3 and CA4). CAPL, S100-A6, CHA and HPUBQ proteins could be involved directly or indirectly in modulating chondrocyte extracelluar matrix. Uncovering such specific proteins involved in chondrocyte matrix modulation will further lead to further identification of potential biomarkers for early detection of cartilage degeneration.

C3.14
Characterization of an extracellular β-galactosidase produced by thermophilic *Bacillus licheniformis* KG9 isolated from hot spring in Batman-Turkey

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In the present study, a thermophilic bacterial strain (KG9) was isolated from a hot spring in Batman (Turkey) and was found to be a member of *Bacillus licheniformis* following complete sequencing of 16S rRNA. This strain was found to produce high levels of extracellular β-galactosidase in NB at 50°C. The time course experiments of β-galactosidase synthesis in the cells show constitutive expression of enzyme, starting from 36 hour (with specific activity of 1.38 U/mg) up to 96 hour (2.45 U/mg) for lactose-free samples, whereas the enzyme production starts at 48 hour (1.39 U/mg) for lactose-containing samples, which increases sharply by increasing time up to 96 hour (3.25 U/mg) of cultivation at 50°C. The enzyme production levels were studied after addition of various carbon (galactose, glucose, lactose and soluble starch) and nitrogen sources (yeast extract glycine, ammonium sulphate, peptone, triptone and beef extract) into liquid media. Galactose, glucose and soluble starch at 1% concentrations were found to inhibit the extracellular enzyme production to a great extent, whereas the nitrogen sources such as glycin and ammonium sulphate caused a slight increase in enzyme activity. Moreover, the extracellular β-galactosidase was partially purified and some properties of the enzyme and electrophoretic analysis were studied. The optimum temperature, pH and thermal stability for enzyme were found as 55°C, 8.0 and 90% retained activity at 60°C in 120 minute, respectively. This study is the first to report for the extracellular enzyme production in *Bacillus licheniformis* species, which may well be used in biotechnological processes.

C3.15
Modeling and molecular dynamics of α-dystroglycan's C-terminus reveals the presence of an Ig-like domain encompassing its β-dystroglycan binding epitope

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Dystroglycan (DG) is a cell surface receptor which is composed of two subunits that interact noncovalently, namely α- and β-DG. Given its sarcolemmal localization at the interface between the cytoskeleton and the basal lamina, DG is thought to be involved in the pathogenesis of several congenital muscular disorders [1]. To date only the 3D-structure of the N-terminal domain of α-DG has been solved by X-ray crystallography [2], and the structures of the other DG domains need still to be determined. We generated a first theoretical 3D-model on a portion of the α-DG's C-terminal domain. An initial model was established using the I-TASSER algorithm which combines the methods of threading and ab initio modeling [3]. Subsequent refinement by molecular dynamics simulation led to an improved model structure which was analyzed by different protein analysis programs, including PROCHECK for the evaluation of Ramachandran plot quality, PROSA for testing interaction energies and VERIFY3D for identifying regions of improper folding. The resulting α-DG's C-terminal domain (between residues 505–596) model is remarkably similar to the N-terminal domain (residues 60–158) solved at high resolution (PDB 1u2c), revealing the presence of another Ig-like domain within α-DG. Such domain model includes the β-DG binding epitope of α-DG and, confirming our previous experimental results, suggests that the epitope assumes a β-strand conformation [4].

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C3.16
High Tenascin-C expression marks active breast cancer tissue in neoadjuvantly treated patients

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The extracellular matrix molecule Tenascin-C (TNC) in its many isoforms is highly expressed during tissue repair and pathological

conditions such as cancer. Utilising the selective pressure of neoadjuvant chemotherapy on carcinoma cells we screen for a subgroup of breast cancer patients with elevated TNC expression and a bad therapy response, evaluated by immunopathological markers, histopathological data (pTNM staging system), clinical data and F-18 FDG PET-CT. Sections of paraffin embedded samples from 68 preoperatively treated breast cancer patients are used for automated Immunohistochemical staining with appropriate antibodies targeting TNC, AE1/AE3, Ki67, CD3 and p53. In addition F-18 FDG PET-CT is performed to discriminate therapy response. We show that TNC expression in breast cancer is immensely reduced after neoadjuvant chemotherapy (43% of patients lost TNC expression) and that expression profiles after treatment correlate with histopathological data, especially the grade, indicating prognostic value. But we also show that TNC expression alone does not sufficiently correlate with therapy response. However, there is a strong correlation and colocalisation of the proliferation marker Ki67 in cancer cells and TNC in the surrounding tumour-stroma of patients having a less beneficial response to chemotherapy. The evaluation of expression levels revealed a direct linear correlation of TNC and Ki67. We expect TNC to be a dependable marker for active cancer tissue remaining after neoadjuvant treatment. These findings concur with the role TNC plays in tissue reorganisation, wound healing and its growth promoting properties in cancer.

C3.17
Glycoproteomic characterisation of O-glycosylated proteins from human cerebrospinal fluid

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The glycosylation of proteins represents the most complex but also the most common post-translational modification of proteins. The glycans are either O-glycosidically linked to Ser or Thr or N-glycosidically linked to Asn. We have developed a capture-and-release method to enrich both tryptic N- and O-glycosylated peptides from proteomic samples, and then characterise them with tandem mass spectrometry (1). We mapped the glycan structures and identified 36 N-linked and 44 O-linked glycosylation sites on proteins from human cerebrospinal fluid (CSF). In order to more specifically study O-glycans we treated CSF samples with glycosidase PNGase F prior to the capture protocol, which removed most N-glycans. With this strategy 22 additional O-glycosylation sites on CSF glycoproteins were characterized. For instance, we identified two previously unknown *minor* glycosylation sites on ApoE, (Thr-26 and Thr-36). In addition, we found that the *major* glycosylation site in ApoE (Thr-212) showed microheterogeneity with respect to the glycan core structures. By using electron capture dissociation for the fragmentation of glycopeptides with intact O-glycans we pinpointed the second *major* glycosylation site of ApoE (1) to Ser-308 and not to Thr-307. ApoE interacts with amyloid beta peptides and the E4 variant of the ApoE gene is an established risk factor for developing Alzheimer's disease. The biological implications of the presented ApoE glycosylations remain to be investigated.

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C3.18
Bacteriocin-like substances from the genus *Bacillus* bacteria: Production and antimicrobial activity

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Antimicrobial peptides – naturally occurring antibiotic molecules - both from eukaryotic and prokaryotic organisms are of great interest. Among them there are bacteriocins or bacteriocin-like inhibitory substances (BLIS) ribosomally synthesized by bacteria with activity towards species related to the producing strain. They are considered not only as potential therapeutics but as biological control agents in food industry. The aim of this work was to study the production of bacteriocin-like peptides by *Bacillus* spp. and to evaluate their antimicrobial activity. A group of *Bacilli* strains, isolated from milk or milk products, were screened for the synthesis of peptides in cross-antagonism tests, deferred antagonism method and disc-diffusion assay against gram-positive and gram-negative indicator bacteria. Two isolates, I_1 and I_2, during growth on solid and liquid media secreted proteinaceous, highly thermostable substances with relatively broad spectrum of activity against gram-positive microorganisms and also enteric rods *Escherichia coli* and *Serratia marcescens*. During batch cultivation antimicrobial activity was detected in the late exponential growth in the case of I_1 and at the beginning of this phase in the case of I_5, but maximum, stable activity was observed at t he stationary phase, proving that they are secondary metabolites. The effect of media composition, temperature or pH on the growth of the producing bacteria and synthesis of antagonistic metabolites were studied. Assay of mode of action on *Staphylococcus au.* of cell-free supernatants and substances partially purified by ammonium sulfate revealed that they are bactericidal but not bacteriolytic.

C3.19
Urinary glycoproteomics: Pinpointing *O*-linked glycans to specific attachment sites in human glycoproteins

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Urine is a complex mixture of proteins and waste products formed in the kidneys by ultrafiltration, reabsorption and secretion. In search of disease biomarkers, urine qualifies as an ideal biological fluid that can be easily collected in a non-invasive manner. Proteomics studies confirm urine as a rich source for the discovery of potential biomarkers, but few studies describe urine from a glycoproteomic perspective. Here, we present a structural characterization of urine glycoproteins from one healthy male individual. We employed solid phase hydrazide chemistry for the selective enrichment of sialoglycoproteins (Nilsson et al.). Captured glycoproteins were trypsin digested on solid phase and glycopeptides were selectively released by mild acid hydrolysis. Mass spectrometric analyses were conducted on a nanoLC-LTQ-FTICR system. Glycopeptides were fragmented with collision-induced dissociation (CID) from which glycan and peptide sequences were elucidated. *O*-linked glycopeptides were additionally characterized by electron capture dissociation (ECD) to define Ser or Thr specific attachment sites of *O*-linked glycans within the peptides. By this approach we were able to characterize 16 *N*- and 47 *O*-linked glycosylation sites on 41 urine glycoproteins. $Hex_5HexNAc_4$-*N*-Asn and HexHexNAc-*O*-Ser/Thr

glycans were typically observed, in agreement with known biantennary complex-type and core 1 structures, respectively. Glycopeptide macro- and microheterogeneities were also revealed. This report demonstrates the applicability of hydrazide capture for comprehensive glycoproteomic characterization of urine samples.

Reference:
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C3.20
Partial characterization of antimicrobial peptide produced by *Bacillus cereus* strain

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Antimicrobial peptides (AMPs) are synthesized by most - if not all - living organisms. The AMPs consist of less than 100 amino acid residues, nearly all are amphiphilic and positively charged in neutral pH, that enables them to interact with target cell membrane. These interactions disturb membrane integrity, either by disruption or pore formation, resulting in cell death. Nowadays, when pathogens became resistant to known conventional antibiotics, antimicrobial peptides have attracted much attention as a promising class of therapeutics and biological control agents. While the AMPs from eukaryotic cells show broad inhibitory spectra against both gram-positive and gram-negative bacteria as well as fungi, protozoa, viruses, their procaryotic counterparts, termed bacteriocins or bacteriocin-like substances, usually exhibit narrow activity range towards closely related strains. In this paper we describe bacteriocin-like substances produced by selected *Bacillus cereus* strain, belonging to food microflora, giving its preliminary characterization. The activity range of antimicrobial substance secreted by *B. cereus* is limited to gram-positive bacteria, with the highest activity against *Bacillus* spp. The inhibitory compound acts against microorganism phylogenetically related to the producer strain. *B. cereus* metabolite was classified as a bacteriocin-like inhibitory substance, without any lipid or carbohydrate moiety involved in the inhibitory activity. The activity was maintained after heating up to 90°C for 30 minute, but disappeared following treatment at 100°C. It was also stable in the pH range of 4.0–9.0 and relatively unaffected by organic chemicals.

C3.21
Optimization of cellulase production by newly isolated Bacillus sp

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Enzymes are widely used in various sectors as additives to improve the effectiveness of the processes they are involved in. These fields range from detergent- and textile-processing technologies to animal feed. Whereas treatment of fabrics with this enzyme helps to give a soft appearance, the use of this enzyme as an additive in animal feed improves the breakdown of raw materials for the animals to gain maximum benefit. The most significant bottleneck in the utilization of such enzymes is the dictated by the cost of their production. The aim of this work was to screen various newly isolated Bacillus strains for cellulose production and then improve the production of this enzyme by the selected microorganism. Among the isolates studied, Bacillus sp. 3 had the highest level of cellulase production with 1220 U/L at 45°C. Following the addition of different carbon and nitrogen sources to growth media for inducing enzyme production, cellulase production improved by almost four fold to a value of 4380 U/L. When the time rate of enzyme production was monitored under the same conditions, it was found that there was no enzyme synthesis before 10 hours of growth and the maximum enzyme amount was detected after 15 hours of growth as cells entered stationary phase. Changing the growth temperature showed no significant improvement on total amount of enzyme produced. This work has been supported by Marmara University Research Fund Project FEN-C-DRP-171108–0267.

C3.22
A lectin histochemical study of the skin treated with *Origanum hypericifolium* essential oil in mice

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Recently, herbal compounds such as phenolic acids, flavonoids, and high molecular weight polyphenols as botanical antioxidants have been extensively investigated in human health care. *Origanum hypericifolium*, the endemic species in the region of Denizli-Turkey, contains p-simene, γ-terpinene, cis- and trans-sabinene hydrate, borneole, terpinen-4-ol, α-terpineol, thymol and carvacrol. Monoterpens like carvacrol have antioxidant and antitumor activities. Glycoconjugates (glycoproteins, glycolipids, proteoglycans) in cell, cell membrane, basement membrane and intracellular spaces, play an important role in many biological events. Lectins have been used for the detections of specific carbohydrates. In this study, the staining patterns of glycoconjugates in skin were investigated in mices topically treated with *Origanum hypericifolium* essential oil by means of lectin histochemistry. Two lectins were studied: *Datura stramonium* agglutinin (DSA) and *Galanthus nivalis* agglutinin (GNA). GNA specific for terminal mannose residues and DSA specific for Galβ(1 → 4)GlcNAc. In comparison with control, dense dermal connective tissue fibres were intensively stained using both lectins. On the other hand, epidermis was weakly stained. However, the sebaceous glands weakly stained by GNA. The results indicated that *Origanum hypericifolium* essential oil application can alter the carbohydrate compositions of skin.

C3.23
Expression levels of matrix metalloproteinase-7 and in situ localization of caseinoliytic activity in colorectal cancer

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Recent years it is believed that MMP-7 can contribute to tumor invasion and metastasis. The aim of our study is to investigate expression levels and latent-active forms of MMP-7, to determine local caseinolytic activity in colorectal cancer. 31 colorectal tumor and paired normal tissues were taken from patients with colorectal carcinoma. mRNA and protein expressions of MMP-7 were detected by Real Time PCR and ELISA, MMP-7 localization with immunohistochemistry. Casein Zymography and In Situ Zymography methods were used to detect proteolytic activity of MMP-7 and local caseinolytic activity, respectively. The quantitative results were compared with clinicopathological variables. MMP-7 mRNA expressions in tumor tissues were signifi-

cantly higher than normal tissues. Furthermore mRNA expression levels were positively correlated with Tumor and Pathological staging and negatively correlated with age. In MMP-7 protein expressions, there were no significant differences between tumor and normal tissues. We recorded a significant correlation between levels of MMP-7 protein expression and perineural invasion. In the results of immunohistochemical analysis, positive staining was observed in cytoplasm and cell membrane of tumor cells whereas there was no MMP-7 staining in normal cells. In addition, it was found that caseinolytic activity, localize epithelium region, was higher in tumor tissues than normal tissues. Our results indicated that MMP-7 influences tumor invasion of colorectal cancer by degrading extracellular matrix and promoting signal transduction pathways. Therefore MMP-7 may be selected as a target molecule in the use of new chemopreventive therapy in colorectal cancer.

C3.24
Comparision of MUC2 O-glycosylation from the colon of core 1- and core 3-knockout mice

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The major protein component of the mucosal layer in the colon is the multimerising MUC2 mucin, which has densely O-glycosylated domains, contributing to approximately 80% of its weight. The highly diverse O-glycans are linked via a GalNAc in a α1 linkage to serine or threonine residues, and mainly extended by core 1 or core 3 structures[1]. The biosynthesis of these core structures are regulated by specific glycosyltransferases. Mice lacking core-1 and core 3-derived glycans in colon by deletion of colonic epithelial glycosyltransferase activity, displayed increased susceptabiliy to colitis and colorectal tumours[2]. In this study we have analyzed the glycans present on colonic MUC2 from both the wild type and the knock-out mice. Mucins were purified from mucosal scrapings and the glycans released with reductive beta elimination. The reduced glycans were sequenced and relative amounts estimated using negative ion mode liquid chromatography-mass spectrometry (LC/MS) and MSn. Preliminary results confirmed the lack of the deleted core structures from the knockout mice, however the mucins from the knockout-mice were still found to be densely glycosylated, and carrying oligosaccharides differing by both average size and terminal epitopes, compared to the wild type mouse.

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C3.25
Complete characterization of the MUC2 mucin C-terminal using a proteomics approach

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The highly glycosylated MUC2 mucin constitutes the major protein component of the mucus layer that covers and protects the epithelial surface of the intestinal tract. It was previously assumed that the mucus layers of the body served merely as a physical barrier, whereas today it is clear that the individual glycans and specific protein properties of the mucus layer are adapted to harbour the commensal bacterial flora, as well as suppressing and clearing pathogens. The aims of the current project are to characterize peptide modifications in the C-terminal part of MUC2, as well as obtaining complete sequence coverage in order to get a better insight in the protein function. Here we present a method to improve sequence coverage on a construct of the muc2 protein C-terminal (106 kDa). The approach is based on bottom-up proteomics using multiples enzymes for protein digestion prior to analysis by nanoLC-MS/MS on a hybrid linear ion trap/orbitrap mass spectrometer, using high resolution HCD fragmentation to obtain accurate peptide sequence information. Combined data from the differently digested samples resulted in a sequence coverage of approximately 60% as compared to a 30% coverage when using conventional trypsin digestion. The C-terminal is known to contain at least 9 potential N-glycosylation and multiple O-glycosylation sites. Using this approach 7 of these sites were characterized after manual interpretation of the fragmentation spectra. In addition, error tolerant protein database searches were used to gain further sequence coverage. By using the described method we managed to increase sequence coverage and characterized previously unknown protein modifications.

C4 – Membrane Transport

C4.01
The periplasmic protein YohN RcnB is involved in nickel and cobalt homeostasis in *Escherichia coli*

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Metals are ubiquitous on Earth and become, with the evolution of Life, necessary for living organisms like bacteria. Otherwise, there is a subtle balance between the life-required amount and toxicity resulting from a too high quantity of metal ions. A strategy often used by cells is to regulate import and efflux of metal ions by the dynamic expression of specific proteins involved in this transport. In *Escherichia coli*, nickel and cobalt toxicity is counterbalanced by action of the RcnA efflux pump that permits to exclude excess ions. Its expression is under the control of the nickel and cobalt inducible RcnR metalloregulator that represses *rcnA* transcription in absence of metals. Recently, we identify a new gene that we called *rcnB* (formerly *yohN*) involved in this system. As shown by RT PCR experiments, this gene is a part of the operon *rcnA-yohN* and its expression is nickel and cobalt dependant. It encodes a small monomeric protein of 10 kDa localized in the periplasm. Databases do not contain any described homologs. By reporter gene studies, we show that, unlike *rcnA*, a *rcnB* mutant accumulates less nickel and cobalt ions suggesting a role in the transport or storage of these ions. However, RcnB seems not to function with the specific nickel transporter *nik*. Furthermore, several methods were employed to show that RcnB does not interact directly with nickel ions *in vitro*. Further investigations are required to precise its role but it could be the first member of a new family of accessories proteins.

C4.02
Multidrug resistant P-glycoprotein positive cells are also cross-resistant to cisplatin

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P-glycoprotein (P-gp, a drug transporter in the plasma membrane) mediated multidrug resistance of neoplastic cells represents a real problem in the chemotherapeutic treatment. Our experimental model are mouse leukemia cells L1210/VCR in which the overexpression of P-gp was induced by vincristine (VCR). Cisplatin (cisPt) is a substance that is untransportable by P-gp. We found that L1210/VCR cells are also resistant to cisPt. However, resistance to this substance could not be reversed by addition of verapamil (P-gp inhibitor). CisPt induced more pronounced entry into apoptosis in P-gp negative sensitive L1210 cells than in L1210/VCR cells. While similar levels of Bax and Bcl-2 proteins were observed in sensitive and resistant cells, cisPt induced a more pronounced decrease of the Bcl-2 levels in L1210 cells than in L1210/VCR cells. Consistent with this observation, cisPt induced a larger decrease of the Bcl-2 content in the Bcl-2/Bax heterooligomer in L1210 cells than in L1210/VCR cells. Moreover, CisPt induced a similar apoptotic DNA fragmentation pattern in both resistant and sensitive cells. All observations indicated that multidrug resistant L1210/VCR cells are also resistant to cisPt (non-substrate of P-gp) and that this resistance is not connected with drug efflux activity of P-gp. This work was supported by: VVCE-0064-07, APVV-0084-07 a VEGA 2/7122, 2/0155/09.

C4.03
Overexpression of P-glycoprotein in L1210/VCR cells is associated with changes in several endoplasmic reticulum proteins

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Multidrug resistant cells L1210/VCR, which express membrane transporter, P-glycoprotein (P-gp, responsible to multidrug resistance), was found to be resistant to thapsigargin an inhibitor of SERCA, in contrast to parental sensitive cells L1210. L1210/VCR cells was obtained from P-gp negative cells L1210 by their gradual adaptation to vincristine (VCR). We have studied differences among L1210 and L1210/VCR cells in expression of endoplasmic reticulum proteins involved in the regulation of calcium homeostasis and calcium-dependent processes. Amounts of mRNA encoding RyR and IP3-receptor channels were found to be at similar levels in sensitive and resistant cells. However, mRNAs encoding IP3R1 or 2 were decreased in resistant cells cultivated in the presence of VCR, while mRNA encoding RyR remained unchanged. The amount of mRNA for SERCA2 was decreased in resistant cells when compared with sensitive cells. This decrease was more pronounced when resistant cells were cultivated in the presence of VCR. Calnexin was found to be associated with immature P-glycoprotein in resistant cells, but surprisingly, calnexin was found to be less expressed at the protein level in resistant as in sensitive cells. Thus, differences exist between sensitive and multidrug resistant cells in the expression of endoplasmic reticulum proteins involved in the control of intracellular calcium homeostasis or calcium-dependent processes. This work was supported by: VVCE-0064-07, APVV-0084-07 a VEGA 2/7122, 2/0155/09.

C4.04
Pyrenyl fluorescence to figure out bilayer polarity in diverse lipidic membranes and partitioning plus collisional quenching in fluid homogeneous bilayers

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As mixing the cholesterol into pure phospholipid bilayers, changes in the bilayer polarity occurs, depending on the chemical composition and on other thermodynamic parameters (e.g. T, P) [1]. Using the pyrene polarity scale, we characterized the polarity of pure POPC and egg-sphingomyelin, as well as of defined POPC/cholesterol and egg-sphingomyelin/cholesterol mixtures. When increasing cholesterol proportions, the polarity values for POPC decrease (as similarly for alcoholic solvents). For egg-sphingomyelin, an increased polarity occurs with augmenting cholesterol, and the results show no relation to the thermal behavior of homogeneous solvents (polar and apolar). We also quantified the partition of 1-pyrenesulfonate (PSA, anionic amphiphile), into bilayers composed by POPC and zwitterionic/anionic POPC/POPS mixtures. Comparing with POPC at 25°C, we found increasing Kp values in parallel with relatively low molar

proportions of anionic POPS (until 10 mol %) in mixed fluid and flexible bilayers [2]. We studied then the quenching of PSA at low concentrations of a DOXYL quencher group, in fluid POPC. Steady-state fluorescence of PSA decreased with increasing proportions of quencher. However, the results are not well described by a collisional kinetic formalism for reactions occurring in two-dimensional (2D) media [3]. Furthermore, we used the pyrene excimer formation process to test the 2D formalism in analyzing steady-state fluorescence self-quenching in lipid bilayers.

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C4.05
The potassium uptake transporters of the yeast *Hansenula polymorpha*

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Potassium is the most abundant intracellular cation in living cells, playing several important roles in vital physiological processes. Most of the information concerning K+ fluxes has been obtained in the usual model yeasts: the budding *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*. In both of these, *TRK1* and *TRK2* genes are present and code for the main K+ uptake systems. However, further studies using the yeast *Hansenula polymorpha*, a member of the *Saccharomycetaceae*, reveal the presence of two main K+ transporters belonging to the Hak and Trk families, respectively. Deletion mutants lacking *HAK1* grow poorly at low K+, while trk1 shows Na+, Li+ and hygromycin sensitivity. Test growth of trk1hak1 at low K+ suggests at least a third K+ transporter. An in-depth kinetic analysis revealed the high affinity of Hak1 for K+. Furthermore, *HAK1* expression is up-regulated under limited K+. Likewise, Hak1 levels are also postranslationally regulated by K+.

C4.06
Hak1 and Trk1, two different systems mediating potassium uptake in *Debaryomyces hansenii*

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Potassium is required for numerous cellular functions and cells have developed efficient systems to accumulate the cation against high concentration gradients. Two different systems for K^+ uptake have been identified in most yeast. While *Saccharomyces cerevisiae* or *Schyzosaccharomyces pombe* are endowed with two Trk type transporters (Trk1 and Trk2), other yeasts such as *Hansenula polymorpha* or the halotolerant *Debaryomyces hansenii* posses a Trk type plus a Hak (High Affinity K^+) type transporter (1,2) which may be related to the limiting K^+ amounts of the ecosystems where these yeasts can be found. We have studied the role of DhHak1 and DhTrk1 in K^+ transport. We have followed gene expression of *DhTRK1* and *DhHAK1* by qRT-PCR and, in addition, we have biochemically characterised K^+ (Rb^+) fluxes under conditions at which the genes coding for both transporters are differentially expressed. We conclude that whereas *DhTRK1* expression is poorly regulated by external factors such as external potassium, sodium or pH, *DhHAK1* expression is highly induced during potassium and sodium starvation. We propose that under potassium limiting conditions most, if not all, the potassium influx process observed in *D. hansenii* is mediated by DhHak1. Moreover, we found that the activity of the potassium transporters working in control cells (50 mM KCl grown cells) and in starved cells can be distinguished by the differential effect of potassium, sodium or pH on K^+ (Rb^+) transport.

C4.07
Lactococcus lactis, an alternative system for functional expression of peripheral and intrinsic plant membrane proteins

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Despite their functional and biotechnological importance, the study of membrane proteins remains difficult due to their hydrophobicity and low natural abundance in cells. Moreover, into well-known heterologous systems, these proteins are often produced at very low levels, toxic and mis- or unfolded. *Lactococcus lactis*, a Gram-positive lactic bacterium, traditionally used in food fermentations, is now widely used in biotechnology for large-scale production of bacterial and eukaryotic proteins. We tested the expression, in *L. lactis*, of six *Arabidopsis thaliana* membrane proteins that could not be produced in sufficient amount using classical expression systems. In an effort to easily transfer genes from Gateway-based *Arabidopsis* cDNA libraries to the *L. lactis* expression vector pNZ8148, we first established a cloning strategy compatible with Gateway entry vectors. Interestingly, all the *Arabidopsis* proteins could be produced, in *L. lactis,* at levels compatible with further biochemical analyses. We then successfully developed solubilization and purification processes for three of them. Finally, we demonstrated that two proteins (one peripheral and one intrinsic protein) were active in this system. Altogether, these data suggest that *L. lactis* might be an attractive system for the efficient and functional production of difficult plant membrane proteins.

C4.08
The presence of P-glycoprotein in L1210 cells directly induces down-regulation of cell surface saccharide-targets of Concanavalin A

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Overexpression of P-glycoprotein (P-gp), a plasma membrane drug transporter (an ABCB1 member of the ABC transporter family), is the most prevalent cause of multidrug resistance in cancer tissues. Lectin Concanavalin A (ConA) induces massive cell death of L1210 leukemia cells. Cell sublines of L1210 in which P-gp overexpression was induced by selection with vincristine (R) or by stable transfection with a plasmid encoding full-length human P-glycoprotein (T) were less sensitive to ConA.

Both P-glycoprotein-positive cell lines exhibited typical P-glycoprotein-mediated multidrug resistance. Resistance of R and T cells to ConA was associated with lower binding of ConA as compared to parental L1210 cells when analyzed by the following methods: (i) SDS PAGE and electrobloting of proteins in the crude membrane fraction followed by detection with biotinylated ConA and avidin-peroxidase; (ii) fluorescent cytometry or confocal microscopy of the intact cells with surfaces labeled by FITC-ConA. These data indicated that the presence of P-glycoprotein in L1210 cells independently on mode of its expression induced down-regulation of cell surface saccharide-targets of ConA. Therefore, this feature may be considered as a secondary cellular response on P-glycoprotein expression.

C4.09
Does any relationship exist between P-glycoprotein-mediated multidrug resistance and intracellular calcium homeostasis?

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Multidrug resistance (MDR) of neoplastic tissue represents a real obstacle to the effective chemotherapy of cancer. Several mechanisms of MDR were identified, from which the over-expression and efflux activity of P-glycoprotein (P-gp) – a plasma membrane ATPase (ABCB1 member of ABC transporter family) – represents the most commonly observed reason for neoplastic disease chemotherapy malfunction. The process of P-gp-mediated MDR seems to be related to intracellular calcium homeostasis, at least indirectly, for the following reasons: (i) substances blocking calcium influx through L-type of calcium channels like verapamil were often found to antagonize P-gp-mediated MDR; (ii) calcium signal abnormalities were observed in cells over-expressing P-gp; (iii) Cells with P-gp-mediated MDR were often resistant to thapsigargin; (iv) several differences in intracellular calcium localization were observed when P-gp-negative and P-gp-positive cells were compared; and (v) differences in the contents of several proteins of the endoplasmic reticulum involved in calcium homeostasis were observed to be associated with P-gp over-expression. This current study represents an attempt to summarize the knowledge about the possible relationship between P-gp-mediated MRD and intracellular calcium homeostasis.

C4.10
Yeast cells export glutathione as an extracellular defence mechanism

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Glutathione is a tripeptide important in stress responses and redox regulation in all eukaryotic cells. During exposure to the toxic metalloid arsenite, yeast cells (*Saccharomyces cerevisiae*) strongly increase the biosynthesis of glutathione (*Thorsen et al., Physiol Genomics, 2007*). In this work we present a novel role for glutathione as part of an extracellular defence mechanism. We show that prolonged arsenite exposure conveys a significant increase of glutathione levels in the extracellular environment. Extracellular glutathione sequesters arsenite as glutathione conjugates, As(III)-GS_3, thereby preventing the toxic compound to enter the cell. Arsenite-induced growth inhibition is alleviated by extracellular glutathione and mutants which cannot externalize glutathione efficiently, become sensitive to arsenite.

C4.11
Interaction of lectins with specific binding affinity to surface of P-gp negative and P-gp positive cells L1210

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Multidrug resistance (MDR) mouse leukemic cell line L1210 obtained by adaptation to vincristine is accompanied by expression of P-glycoprotein (P-gp). During adaptation give out to expression of P-gp and also to various kinds of metabolic changes. Our foregoing work shows decrease of UDP-saccharides in resistant cells, that are substrate of glycosylation reactions. We discovered changes in spectrum and levels of glycoproteins in resistant cells. Aim of this study was to observe changes in content of surface saccharides accompanied by expression of P-gp by lectins with affinity to different saccharides. Histochemical staining of negatively charged cell surface binding sites (mostly sialic acid) by ruthenium red (RR) revealed a compact layer of RR bound to the external coat of sensitive cells (S). In resistant cells cultivated in the absence (R) or presence of vincristine (V) the RR layer is either reduced or absent. Interaction of lectins to membrane proteins was observed by lectin blots. Application of some lectins caused changes in spectrum of marked proteins in membranes of sensitive and resistant cells. Lectins interact to sialic acid exerts almost non toxic effect to this cell lines. We can summarize that overexpression of P-gp caused also remodelation of another glycoproteins of membranes. This work was supported by: APVV-0084-07, VVCE-0064-07, VEGA-2/7122/27, VEGA-2/7028/27

C4.12
Melanosome transport in inner melanocytes – Regulation and possible functions of inner melanin

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In contrast to the thoroughly investigated cutaneous chromatophores which serve as widely used model systems for intracellular membrane transport, little is known about the possible functions of the scarcely investigated internal chromatophores. We here analyzed the capacity for internal colour change in eight species of fish, in order to investigate how common this phenomenon is. In all species, inner chromatophores, mostly melanocytes, were present. The capacity for regulatory control of these cells was common, but not ubiquitous. To reveal whether the capacity of regulatory control could be explained by the level of body transparency, we tested the potential correlation between body transparency and capacity of melanosome aggregation in peritoneal

melanocytes. We show that the capacity to respond in different species correlates strongly with the degree of body transparency. Thus, the capacity to regulate internal chromatophores was larger in species where the effect would potentially be visible. Our study is the first to show evidence for a possible adaptive role of internal colour change, and one of few providing a function of melanin in internal tissues, and of non-cutaneous chromatophores in general.

C4.13
Beyond the membrane: Characterization of the oligopeptide binding protein from *Escherichia coli*

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Escherichia coli is often cultured on media that are based on rich peptone or tryptone mixtures supplying it with nutritious peptides. Specialized binding proteins in the periplasm facilitate the transfer of these substrates to permease systems in the cytoplasmic membrane. One such binding protein is the oligopeptide binding protein OppA. Although OppA is one of the most abundant proteins in the periplasm of *E. coli*, its natural substrates have never been identified and our knowledge of its functioning is only limited. Therefore, we have initiated a comprehensive characterization of this peptide binding protein combining biochemical and biophysical approaches. So far, a crystal structure at 2.2 Å resolution of OppA was solved. In the binding cleft density fitting a tripeptide substrate with a Lysine at the second position was observed. Tripeptides appear to be the preferred substrates *in vivo*. To identify the natural substrates of OppA, we produced the protein in its native environment (the periplasm) where it could bind the natural ligands. The protein was purified and the bound peptides were analyzed using reverse-phase nano LC coupled to mass spectrometry. Finally, to understand the dynamics of the proposed "Venus-fly-trap" mechanism for ligand binding, we use multidimensional NMR spectroscopy complemented with CD studies. Eventually our studies will lead to insight in the structure of OppA, identification of its natural substrates and a detailed description of its *modus operandi*.

C4.14
Functional analysis of the *Ashbya gossypii* Fps1 homolog

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Saccharomyces cerevisiae aquaglyceroporin Fps1 plays a central role in yeast osmoregulation, controlling the intracellular level of the compatible solute glycerol. When a cell encounter hyperosmotic conditions, Fps1 closes rapidly to ensure retention and accumulation of glycerol. In adaptation to lower external osmolarity, Fps1 opens again to release glycerol and hence turgor pressure. Mutants lacking Fps1 cannot withstand a hypo-osmotic shock to the same extent as wild type cells. Fps1 has unusually long N- and C-terminal extensions, and amino acids that are crucial for the gating mechanism have been identified on both termini. Fps1 also facilitates passive uptake of other small molecules such as arsenite and acidic acid. The filamentous fungi *Ashbya gossypii* Fps1 homolog (AgFps1) has shorter termini than Fps1. The aim of this study is to determine the function of AgFps1 by heterologous expression in *S. cerevisiae fps1* deletion mutants, and to study the physiological role of AgFps1 by deletion analysis in *Ashbya gossypii*. We can show that heterologous expression of AgFps1 in *S. cerevisiae* can substitute for Fps1 by releasing excessive glycerol upon a hypo-osmotic shock. AgFps1 expressed in *S. cerevisiae* appears to be hyperactive under hyperosmotic conditions, and exchanging the N- and C-terminal extensions for the corresponding termini of Fps1 is not sufficient to generate a regulated channel. Further, successful deletion of AgFPS1 renders an *Ashbya gossypii* mutant more resistant to arsenite than wild type fungi, indicating that AgFps1 transports arsenite.

C4.15
Comparison of lipid and fatty acids composition of basolateral membrane from rat (*Rattus norvegicus*) and Atlantic cod (*Gadus morhua*) intestinal enterocytes: the question of homeoviscous adaptation

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The physical properties of biological membranes are sensitive to changes in temperature due to the phase behavior of the lipids. Therefore, it is important that membranes can adjust to different temperatures in order to fulfill their physiological role. Our aim was to compare the basolateral part from the intestinal endothelial cell membranes (BLMs) of a mesophilic species (rat, *Rattus norvegicus*) and a psychrophilic species (Atlantic cod, *Gadus morhua*) to see if it showed signs of homeoviscous adaptation in relation to cold-adaptation.

Major lipid classes were analyzed by thin layer chromatography (TLC) and GC/MS spectrometry. TLC analyses showed similarity between the two species for neutral lipids. However for phospholipids, less phosphatidylethanolamine (PE) and phosphatidylserine was present in cod than in rat and higher amounts of phosphatidylcholine (PC) and spingomyelin in the former. Results for fatty acids showed a lower amount of poly-unsaturated fatty acids (PUFAs) in the cod lipids, but higher amount of mono-unsaturated fatty acids (MUFAs). Furthermore, saturated fatty acids were fewer in the cod BLM fraction as expected, as such fatty acids decreases the viscosity of the membrane in the cold.

Our findings show some characteristics expected in the maintenance of proper cell membrane fluidity at low temperatures. However, lower PE/PC ratio in cod and lower amount of PUFAs was unexpected, although it has previously been observed in psychrophilic species. High amount of MUFAs is instead likely a factor contributing to cold-adaptation by preventing perfect side-by-side packing of fatty acids in the BLM.

C4.16
Interaction of sanguinarine with the Na^+/K^+-ATPase

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The balance between concentrations of Na+ and K+ ions across cell membranes is a fundamental property which is utilized in numerous physiological processes. The establishment of the concentration gradient is carried out by Na+/K+-ATPase. Sanguinarine (SG), the benzo[c]phenanthridine alkaloid, exhibits a plethora of biological effects. SG is used in dental hygiene preparations, veterinary medicine and feed additives. On the other hand, SG is linked to adverse effects on humans such as the leu-

koplakia and the epidemic dropsy syndrom. In solution, at physiological pH, there is an acido-basic equilibrium between the cation (SG+) and pseudobase (SGOH) forms of SG. In the mammalian gastrointestinal tract SG is converted to biologically inactive dihydrosanguinarine (DHSG). All three forms of SG exhibit fluorescence. Na+/K+-ATPase has been proposed as their possible cellular target. Our experiment, using a combination of steady-state and time-resolved techniques enabled separate the observation of individual SG forms present in the solution. It was shown that fluorescence spectra of SG or SGOH and to lesser extent also DHSG are sensitive to interactions that may be predicted from their binding to proteins. This study revealed that Na+/K+-ATPase was inhibited by SG+/SGOH. We did not find the evidence for DHSG/enzyme interaction. The fluorescence spectra analysis and fluorescence quenching experiments revealed that Na+/K+-ATPase binds SG+ and SGOH. For the former, the binding site on Na+/K+-ATPase was identified. <.

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C4.17
Interaction of fluorone dyes with Na^+, K^+-ATPase

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Na^+, K^+-ATPase is a transmembrane protein which is found in each animal cell. It translocates sodium and potassium ions across the plasma membrane in $3Na^+_{out}/2K^+_{in}$ ratio, using the energy from ATP hydrolysis. It is one of the most important enzymes which is essential for establishing and maintaining high K^+ and low Na^+ in cytoplasm, required for various cellular activities such as a regulation of cell volume, maintenance of membrane potential and secondary nutrient transport. Malfunction of the Na^+, K^+-ATPase is connected with several diseases, like diabetes, hypertension, cataract, asthma and others. We investigated the interaction of Na^+, K^+ATPase with fluorone dyes – eosin, erythrosine, bengal rose and fluorescein. These dyes are used in food industry or like a part of medicaments. The possible interaction between fluorone dyes and Na^+,K^+-ATPase could account some of adverse effects of drugs. Based on various steady-state fluorescence experiments we were able to observe binding of these fluorone dyes to Na^+,K^+-ATPase.

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C4.18

Abstract withdrawn

C4.19
Effects of dicyclohexylcarbodiimide (DCCD) treatment on H^+ permeability of plasma membrane from marine microalga *Dunaliella maritima*

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DCCD is a carboxyl group modifier, an inhibitor of various membrane ion-translocating proteins. The effects of DCCD on electric potential difference ($\Delta\psi$) across the vesicular plasma membranes isolated from marine microalga *D. maritima* were investigated. Transmembrane electric potential was created as Na^+- or K^+-diffusion potential. Na^+-diffusion potential was imposed by a combination of Na^+ and Na^+- ionophore ETH 157. K^+-diffusion potential was imposed by a combination of K^+ and K^+- ionophore valinomycin. Formation of $\Delta\psi$ across the vesicle membranes was monitored using optical $\Delta\psi$ -sensitive probe oxonol VI. The experiments revealed that DCCD induces proton permeability of the vesicle membrane in a Na^+- and voltage-dependent manner. It was indicated by the following findings. In the vesicles pre-incubated with DCCD Na^+-diffusion potential could be created upon Na^+ addition which rapidly and spontaneously dissipated immediately after establishement. DCCD-induced dissipation of Na^+-diffusion potential should be referred to proton leakage from the vesicle lumen since $\Delta\mu Na$ across the vesicle membranes equals zero in the presence of ETH157. In the absence of Na^+ the proton permeability of the membranes was not induced by DCCD. Thus, K^+-diffusion potential sustained permanently in the vesicles treated with DCCD. The key mechanism for the DCCD-facilitated and Na^+-dependent H^+ efflux from the vesicle lumen is likely to be Na^+/H^+ antiporter which operates in *D. maritima* PM (Popova et al., 2005). Na^+ protects the antiporter against interaction with DCCD since DCCD-induced dissipation of Na^+-diffusion potential could be completely prevented by Na^+ added before DCCD.

C4.20
Transport via cell membrane of the novel porphyrins enhanced by electroporation *in vitro* as anticancer therapy

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Porphyrins are a large class of fluorescent crystalline pigments, with natural or synthetic origin, having in common a substituted aromatic macrocyclic ring consisting of four pyrrole-type residues, linked together by four methine bridging groups. Porphyrins are verified as an ideal photosensitizers because they are non-toxic, selectively retained in tumor tissue in high concentrations, water soluble to a certain level, cleared in a reasonable time from the body and rapidly from the skin which avoid photosensitive reaction. In current study we determined the cells viability (MTT assay) after standard photodynamic treatment (PDT) and after PDT combined with electroporation (EP). We applied two novel porphyrins CoTPPS and MnTPPS. We examined two cell lines of human colon adenocarcinoma (LoVo and LoVo/DX). The EP parameters were: 500 V/cm, 50 us, 5imp. The intracellular distribution of both porphyrins was also performed. With standard PDT cells were incubated 1 and 4 hours before irradiation, however the results were not efficient. The cells viability reached for both dyes about 60% for LoVo cells and over 80% in case of LoVo/DX cells. The application of EP provided shorter time of incubation (only 20 minutes) and enhanced effect of applied therapy. For LoVo cells and irradiation with 435 nm CoTPPS reached 33% of control cells and for LoVo/DX 25,9%. For irradiation with filter 530–550 nm for

LoVo/DX CoTPPS reached 44,2% of cells viability. In case of the second porphyrin MnTPPS we obtained better results for 435 nm in both cell lines where cells viability was on the level of 50%.

C4.21

Abstract withdrawn

C4.22
Role of K^+ efflux in phosphatidylserine exposure in platelets

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Phospholipid reorganization with translocation of phosphatidylserine (PS) to the external leaflet of cell membranes occurs in cell apoptosis and in platelets during blood coagulation. The aim of this work was to determine if Ca^{2+}-stimulated PS exposure relies on cell shrinkage and K^+ efflux leading to cell dehydration and resulting in energetically favourable sites for phospholipid translocation. Immunoblotting studies demonstrated large-, small- and intermediate-conductance Ca^{2+}-activated K^+ channels (K_{Ca} channels) in platelets. Flow cytometry analyses with Annexin V showed that in a high (120 mM) [K^+] medium, PS exposure did not occur in platelets stimulated by thapsigargin, which releases Ca^{2+} from the endoplasmic reticulum and induces store-operated Ca^{2+} entry, or through receptors activation by a mixture of thrombin and convulxin. However, PS exposure induced by Ca^{2+}-ionophores occurred in this medium, and also in a low (2.7 mM) K^+ buffer in the presence of inhibitors of K_{Ca} channels (apamin, iberiotoxin, charybdotoxin and clotrimazole). Efflux of K^+ occurred in low but not in high [K^+] buffer in platelets stimulated by all agents, as measured with the fluorescent dye potassium-binding benzofuran isophthalate. Therefore, PS exposure is associated to K^+ efflux in platelets stimulated by inducers of capacitative Ca^{2+} influx, whereas it occurs without K^+ movement with Ca^{2+}-ionophores. Since PS exposure in EBV-immortalized B lymphocytes from Scott syndrome patients is defective under stimulation with Ca^{2+} ionophores despite normal Ca^{2+} influx, our results suggest that the founding element in this haemorrhagic pathology may not involve abnormal K^+ movements.

C4.23
Cation exclusion in aquaporin water channels

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Aquaporin (AQP) water channels conduct water, glycerol and other small uncharged molecules across the plasma membrane along the respective osmotic/chemical gradient. Selective permeability for water and glycerol is achieved via the ar/R constriction and the NPA motif regions inside the monomer pores. These structural determinants render the pores highly effective in conducting water (water-specific aquaporins) or water plus solutes (aquaglyceroporins). Simultaneously, protons and cations are strictly excluded to maintain ionic transmembrane gradients. To experimentally address the mechanism of proton/cation exclusion in aquaporins, we conducted point mutagenesis within both constriction regions in AQP1 and characterized their permeability for water and proton/cations in X. laevis oocytes and different yeast mutants. We showed that three point mutations turned the water-specific AQP1 into a proton/alkali cation channel with reduced water permeability and the permeability sequence: $H+ >> K+ > Rb+ > Na+ > Cs+ > Li+$. Contrary to theoretical models, our data indicated that electrostatic repulsion at the central NPA region does not suffice to exclude protons. Full proton exclusion was reached only in conjunction with the ar/R constriction at the pore mouth. In contrast, alkali cations were blocked by the NPA region but leaked through the ar/R constriction. Expression of alkali-leaking AQPs depolarized membrane potentials and compromised cell survival. Our results hint at the alkali-tight but solute-unselective NPA region as a feature of primordial channels and the proton-tight and solute selective ar/R constriction variants as later adaptations within the AQP superfamily.

C4.24
A cellular fractionation approach to characterize the apical expression of SGLT1 in $Caco_2$ and HT29 cells in response to glucose and insulin

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Diabetes is a metabolic disorder caused by the lack of insulin production and/or a deficient response of target tissue cells to insulin (defined as insulin resistance). In addition to increased blood glucose levels, diabetes is also characterized by a raise in intestinal expression of the sodium-glucose cotransporter (SGLT1), a high affinity glucose/galactose transporter that requires the inward $Na+$ gradient to drive sugar transport across the brush border membrane (BBM) of the enterocytes. $Caco_2$ and HT29 are cell lines often considered good *in vitro* models for the study of intestinal cellular adaptations. Here we report the effects of glucose and insulin on SGLT1 and GLUT2 protein expression in $Caco_2$ and HT29 cells, which were submitted to a cellular fractionation procedure. Our results show that apical SGLT1 expression decreases with increasing media glucose, a response that is in contrast to the *in vivo* adaptations to luminal glucose. Studies are underway to evaluate the effect of apical versus basolateral exposure to glucose, insulin and short-chain fatty acids in these cell lines. CMS is supported by the Foundation for Science and Technology, Portugal, grant – SFRH/BD/42566/2007.

C4.25
Towards the structure-function-specificity relationship of glucose transporters

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Glucose plays a central role in nutrient sensing and signalling and its uptake through facilitated diffusion is mediated by membrane associated glucose transporters, GLUT proteins in mammals and Hxt proteins in the yeast *S. cerevisiae*. These proteins belong to the Major Facilitator Superfamily (MFS), which is present in all studied organisms. They transport a wide range of solutes such as amino acids, sugars, nucleotides, drugs, peptides,

organic and inorganic anions, metabolites, neurotransmitters, polyols etc. All MFS proteins structurally investigated have been purified from their natural sources and successful heterologous production has not yet been reported. While there is a wealth of reports in the literature on mutagenesis studies, to analyze the structure-function relationship of glucose transporters, there are no three dimensional structures available. Our research goal is to achieve a more detailed understanding of the structure-function-specificity relationship of glucose transporters. To achieve this overall goal we have successfully expressed mammalian GLUTs and yeast Hxts in yeast. Initial solubilsation trials suggest that Brij35 solubilises GLUT4. Purification and crystallization analyses of the target proteins are ongoing.

C4.26
Morphological and biochemical changes in k562 lines at plasma membrane induced by TOM

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Determination of the relation between apoptosis and cellular process from the level of plasma membrane is important for *in vitro* evaluation of potentially chemotherapeutical agents. It represents an interesting trend for identification of events that can be used as indicators of tumor cell physiological disorders. The programmed cellular death is a fundamental physiological process observed during normal development but it is also involved in pathophysiological phenomena. Apoptosis is characterized by a variety of biochemical and morphological changes including chromatin condensation, DNA fragmentation and bleeding of the plasma membrane. Membrane potential is involved in the regulation of several immune functions developed in cells. The Na+/K+ gradient across the plasma membrane, mainly generated by the Na+/K+ pump plays a key role in the maintenance of membrane potential. This study is focused on the correlation between plasma membrane potential, apoptosis and membrane fluidity of a human chronic myelocytic leukemia cell line. After 24 hour of culture the K562 cells were exposed to different concentrations of TOM, a bio-compound isoleted in Biotehnos laboratories. The cells are fluorescent marked in order to establish the percent distribution of cellular events corresponding to different apoptosis stages, membrane potential and membrane fluidity. The results show that treatment of K562 cells with TOM caused an increase in membrane fluidity and cells became depolarized during apoptosis in a dose-dependent manner. The implementation of this correlative evaluation technique as current testing method will contribute to raising efficiency of *in vitro* pharmacological screening.

C4.27
CFTR anion channel modulates expression of human transmembrane mucin MUC3 via the PDZ protein GOPC

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Mucins play a major role in maintaining epithelial protection in luminal organs. They act as lubricants against mechanical stress and they protect the underlying epithelial cells against pathogens. They have a rigid and extended structure due to their large mucin domains, rich in proline, threonine and serine residues that allow O-linkage of glycans. Three membrane-associated mucins, MUC3, MUC12 and MUC17, are unique in harboring Class I PDZ motifs, making them suitable ligands for PDZ-containing proteins. We performed a screening of 123 different human PDZ domains for binding to MUC3 and identified a strong binding between MUC3 and a PDZ protein GOPC. We demonstrated that this interaction was mediated by the C-terminal PDZ motif of MUC3, binding to a single PDZ domain on GOPC. GOPC acts as a binding partner of CFTR, down-regulating mature CFTR by degradation in post-Golgi compartments. Here, we show that GOPC down-regulated total levels of MUC3 and that this effect was reversed by introducing CFTR. Hence, these results demonstrate for the first time a regulative role of CFTR anion channel on expression of membrane-associated mucins.

C4.28
Lpe10p alters the activity of the Mrs2p-based yeast mitochondrial Mg^{2+} channel

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The inner mitochondrial membrane forms a tight barrier for the passage of cations and protons. Mrs2p was found to be the first protein being involved in cation transport across the mitochondrial membrane. In previous studies we could show that it has the ability to form homo-oligomeric complexes in the inner mitochondrial membrane acting as a Mg^{2+}selective channel of high conductance. In this study we wanted to further characterize its homologue *LPE10* also being a distant relative to CorA, the major Mg^{2+}uptake system in bacteria. Deletion of either one or both genes results in a growth defect on non-fermentable carbon sources and the loss of rapid Mg^{2+}influx into mitochondria. Unexpectedly, only co-expression of both genes in a double deletion strain (*mrs2*Δ, *lpe10*Δ) leads to full complementation being the first indication ever that these two proteins cannot fully substitute for each other. In contrast to *MRS2*, deletion of *LPE10* causes a considerable loss of the mitochondrial membrane potential ($\Delta\psi$). Addition of the K^+/H^+ exchanger nigericin, which artificially increases the mitochondrial membrane potential, leads to restoration of Mg^{2+}influx into mitochondria from *lpe10*Δ, but not *mrs2*Δ, *lpe10*Δ cells. Thus, the loss of Mg^{2+}influx into *lpe10*Δ mitochondria is not due to the absence of a channel-forming protein, but rather because of the lack of a driving force essential for an active channel. Lpe10p therefore plays a dual role as constituent of a Mrs2p-Lpe10p Mg^{2+}selective channel and as an essential regulator of the membrane potential in yeast mitochondria.

C4.29
Aquaglyceroporins selective for arsenite as alternative detoxification channels in some actinobacteria

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Widespread inorganic arsenic compounds are potential toxins to almost all organisms. Pentavalent As (V) and trivalent As (III) represent the two major forms in the biosphere environments, however under aerobic conditions As (V) is the prevailing form. As (V), structurally mimicking phosphate ion, enters the cell via phosphate transporters and interferes with cellular metabolism involving phosphate; while As (III), with a pKa of 9.2, may pass through aquaglyceroporins in the form of neutral trihydroxide

As(OH)3 under normal pH. A major detoxification system common to most microbes is to rapidly reduce As (V) into As (III) by arsenate reductases ArsC or Acr2 and to excrete the reducted arsenite by exporters ArsB (or ArsBA complex) or Acr3 or even by aquaglyceroporins presumably. Although in recent years several aquaglyceroporins have been shown to conduct As (OH)3 in heterologous expression systems, only one example is reported for physiological involvement of an aquaglyceroporin homolog in arsenic detoxification. Genome sequences of some actinobacteria reveal special aquaglyceroporin genes located in the ars operon replacing ArsB, suggesting a direct function of these isoforms in arsenic detoxification. We will present data regarding the physiological and biochemical functions and the selectivity features of these arsenite-selective aquaglyceroporin.

C4.30
Novel components of an active mitochondrial K^+/H^+ exchange

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Defects of the mitochondrial K^+/H^+ exchanger (KHE) result in increased matrix K^+ content, swelling and autophagic decay of the organelle. We have identified the yeast Mdm38 and its human homologue LETM1, the candidate gene for seizures in Wolf-Hirschhorn syndrome, as essential components of the KHE. In a genome-wide screen for multi-copy suppressors of the *pet*$^-$ (reduced growth on non-fermentable substrate) phenotype of *mdm38*Δ mutants, we now characterized the mitochondrial carriers *PIC2* and *MRS3* as moderate, and *MRS7* and *YDL183c* as strong suppressors. Like Mdm38, Mrs7 and Ydl183c are mitochondrial inner-membrane proteins and constituents of ~ 500 kDa protein complexes. Triple mutant strains (*mdm38*Δ *mrs7*Δ *ydl183c*Δ) exhibit a remarkably stronger *pet*$^-$ phenotype than *mdm38*Δ and a general growth reduction. They totally lack KHE activity, show a dramatic drop of mitochondrial membrane potential and heavy fragmentation of mitochondria and vacuoles. Nigericin, an ionophore with KHE activity, fully restores growth of the triple mutant, indicating that loss of KHE activity is the underlying cause of its phenotype. Mdm38 or overexpression of Mrs7, Ydl183c or LETM1 in the triple mutant rescues growth and KHE activity. A LETM1 human homologue, HCCR-1/LETMD1, described as an oncogene, partially suppresses the yeast triple mutant phenotype. Based on these results, we propose that Ydl183c and the Mdm38 homologues Mrs7, LETM1 and HCCR-1 are involved in the formation of an active KHE system. Furthermore, we are currently analysing the composition of the high molecular weight complexes of Mdm38 and Mrs7.

C4.31
Identification of the glycerol transporter-like protein in the halophilic black yeast *Hortaea werneckii*

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A halophilic black yeast *Hortaea werneckii* (Dothideomycetes, Ascomycota) inhabits hypersaline waters of marine salterns. *H. werneckii* grows at a wide range of salinities (0 – 5.2 M NaCl) and shows optimal growth at 0.85 – 1.7 M NaCl. Its ability to grow at such a wide range of salinities is rare amongst eukaryotic microorganisms and *H. werneckii* therefore represents a good model to study osmoadaptation. We have found previously that *H. werneckii* adapts to high NaCl concentration by accumulating glycerol and erythritol. In this study, we have identified a putative glycerol transporter protein of *H. werneckii*. A partial gene sequence was obtained from the expression library of *H. werneckii* and extended by genome walking. The gene was cloned and sequenced. It encodes a putative protein of 543 aa, which belongs to Major Facilitator Superfamily of transporters. BlastP showed its similarity to a number of putative sugar transporters and also to Stl1p from *Saccharomyces cerevisiae*. Stl1p is glycerol/H^+ symporter of the plasma membrane with a role in osmoadaptation. The putative protein of *H. werneckii* was named HwStl1 and it was characterized *in silico*. The studies of heterologous expression of HwStl1 in *S. cerevisiae* Ä*stl1* mutant are currently under way. Additionally, we will evaluate the glycerol transporting ability of HwStl1 to conclude whether HwStl1 functions as a glycerol transporter in *H. werneckii*.

C4.32
The anterograde transport of the human Y2 receptor is regulated by the receptor C-terminus

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Neuropeptide Y receptors (YR) belong to the large superfamily of heptahelical G-protein coupled receptors. Four YR subtypes have been cloned from human tissue (Y1R, Y2R, Y4R and Y5R). These receptors are activated by the members of the NPY hormone family: neuropeptide Y (NPY), pancreatic polypeptide (PP) and peptide YY (PYY). Up to now the mechanism underlying intracellular trafficking processes, namely export, internalization, recycling and degradation, of the YRs is still unknown. In our work we investigated the cell surface transport of the Y2 receptor. We were able to identify a specific hydrophobic sequence motif in the proximal tail of the Y2 receptor C-terminus which is responsible for the export of the receptor from the endoplasmatic reticulum (ER) to the plasma membrane. Cell surface ELISA and fluorescence microscopy studies of alanine-substituted mutants revealed an accumulation and therefore retention of the receptor in the ER. Furthermore several C-terminal deletion mutants were generated by which we obtained a distinct accumulation of the receptor in the Golgi compartments. This implicates the presence of additional consensus sequences. These results demonstrate for the first time that a proximal C-terminal short sequence-motif is responsible for the export-regulation of the human Y2 receptor from intracellular compartments.

C4.33
Role of Rab GTPases in regulating neuronal membrane trafficking in *C. elegans*

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The Rab family of small GTPases are molecular switches that orchestrate intracellular membrane trafficking in eukaryotes. As

opposed to the 61 Rab family members encoded in the mammalian genome, C.elegans has only 28 members facilitating a systematic analysis. To characterize the rabome in C.elegans, we determined the expression patterns of all C.elegans Rabs. To our surprise many Rab proteins were found to be highly cell-type specific. Rab GTPases seem to be particularly important for neuronal function, since they were found to be highly enriched in neurons. To analyze their roles in neurons, we determined their sub-cellular localizations. In addition, we systematically characterized all Rab mutants using a combination of tests ranging from a behavioral and biochemical analyses to high resolution imaging using High Pressure Freeze Electron Microscopy. From these studies, we have identified a novel Rab cascade specifically involved in the regulation of Dense Core Vesicle trafficking. Despite having identified specific functions for several of the Rabs, results have shown that there is significant functional redundancy between them. To address the redundancy among the Rab family members, we conducted large-scale synthetic RNAi screens. This study will provide for the first time a complete systematic analysis of Rab function within a multi-cellular organism leading to new insights on how intracellular membrane transport is dynamically regulated.

C4.34
Novel method for inhibitor screening of *Plasmodium falciparum* aquaporin

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Malaria – causing millions of deaths every year worldwide – is caused by the protozoan parasite *Plasmodium falciparium*. As most forms of life, these contain proteins called aquaglyceroporins, which transport water and glycerol across the cell membrane. In particular, the transport of glycerol has been suggested to be essential to the parasite's survival during its blood stage. We have developed a novel method to screen for potential inhibitors to this protein based on Surface Plasmon Resonance (Biacore™). Lipid vesicles containing the protein are immobilized on a gold surface and subjected to an osmotic gradient of sugar alcohols, which can be transported by the aquaglyceroporin. The transport kinetics of the solute are subsequently monitored via SPR and the differences in protein activity (NB: NOT binding) upon addition of a potential inhibitor are determined. The assay has been automated to be used in a 96-well format and capable to screen up to 50 compounds/day at very low protein consumption. First screening results yielded a compound which showed an effect on live *Plasmodium falciparum* cells in preliminary studies. To our knowledge, this is the first method, which is able to study the transport of uncharged solutes across membranes directly. The method also has the potential to easily be adapted to examine the function of other transporter proteins.

C5 – Energy Transduction

C5.01
On the rotary mechanism and ion binding specificity of F1Fo-ATP synthases

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The mechanism of the F1Fo ATP synthase couples the downhill membrane translocation of H+ or Na+ to the rotation of an oligomeric ring of c-subunits (c-ring) in the Fo motor. The torque is transduced into the F1 motor, which causes sequential conformational changes in the catalytic centers, finally resulting in the generation of ATP. The design of the c-ring rotor provides the ion binding specificity and contributes to the translocation of the ions through the membrane during enzyme operation. The crystal structure of the c15 ring of the F1Fo-ATP synthase from Spirulina platensis has been solved at 2.1 Å resolution (Pogoryelov et al., Nat. Struct. Mol. Biol., 2009). The way the proton is bound to this c-ring proposes that all ion binding sites of the c-ring remain in the proton-locked conformation while exposed to the membrane, whereas exposure to a more hydrophilic environment can unlock the ion binding site and promote ion release. This model is supported by combined structural, biochemical and in silico generated data of the proton binding site. A comparative study of the H+-binding c15 ring with the Na+-binding c11 ring from Ilyobacter tartaricus furthermore supports the notion that H+-selectivity is a property that is, in fact, shared by both Fo rotors but the structural arrangement of the c11 ring binding site is more prone to bind sodium ions instead of protons under physiological settings.

C5.02

Abstract withdrawn

C5.03
Antiporter activity of the complex I subunits NuoL, NuoM and NuoN from Escherichia coli analyzed in an *in vivo* model system

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The complex I membrane spanning subunits NuoL, M and N are homologous to one particular class of Na+ or K+/H+ antiporters, encoded by the gene cluster denoted mrp/sha/pha/mnh in different bacteria. These subunits are prime candidates for harboring important parts of the proton pumping machinery of complex I. Deletion of mrpA or mrpD in Bacillus subtilis result in a Na+ and pH sensitive growth phenotype, making this a suitable model system to study antiporter function. Expression of MrpA in a B. subtilis ΔMrpD strain and vice versa did not result in any growth improvement under any condition tested. The expressed NuoL could rescue ΔMrpA to wild-type growth properties at pH 7.4, but enhanced the growth of ΔMrpD only to a lesser extent at this pH. The expressed NuoN could fully restore the wild type properties of ΔMrpD in the pH range from pH 6.5 to 7.5. In the ΔMrpA strain, expression of NuoN did not improve growth at pH 7.5 but resulted in some enhancement at pH 6.5. Cells expressing NuoM did not reach wild type growth levels in either deletion strain, but showed growth improvement under some of the conditions tested. At pH 8.5 no strain could be rescued by any complex I subunit. Taken together, this demonstrates that i.) the antiporter-like Nuo proteins can functionally replace real antiporters and ii.) each of the three complex I antiporter-like subunits have unique functional specializations and operate at different pH. The implications for the Complex I functional mechanism are discussed.

C5.04
Sodium interaction of the complex I antiporter-like subunits NuoL, M and N from *Escherichia coli* studied by ^{23}Na NMR

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The complex I (NADH:quinone oxidoreductase) proteins NuoL, NuoM and NuoN are homologous to one type of Na^+/H^+ antiporters. These subunits are prime candidates for harbouring important parts of the proton pumping machinery. If they also retain antiporter function and/or the ability to translocate sodium is a matter of debate. In this work the sodium binding properties of the NuoL, NuoM and NuoN subunits from *E. coli* complex I was investigated and compared to those of the bona fide antiporters MrpA and MrpD from *Bacillus subtilis* using ^{23}Na NMR spectroscopy. This technique is particularly suitable to monitor ion binding properties of macromolecules under conditions of very fast chemical exchange as expected from a transporter protein. High amounts of the individual proteins were obtained by expressing them as cytochrome c fusion proteins in E. coli. Purification was facilitated by a C-terminal histidine tag fused to the cytochrome c domain. The mobility of Na^+ in the presence of the antiporter proteins was measured at gradually increasing concentrations. Cytochrome c alone was used as negative control protein. The specific binding constant for Na+ was estimated for each of the five proteins. The Na^+ interaction was then assessed under different conditions and pH, in the presence of quinone and in the presence of the sodium-hydrogen exchange inhibitor 5-ethylisopropyl amiloride (EIPA). The sodium interaction was compared to the real antiporters MrpA and MrpD.

C5.05
Red complex I – using cytochrome c550 from Bacillus subtilis as a fusion domain to study NADH:quinone oxidoreductase

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Complex I (NADH:quinone oxidoreductase) contains four very large membrane spanning protein subunits that hitherto have

been difficult to express individually in any appreciable amounts in *Escherichia coli*. The polypeptides contain no prosthetic groups or visual redox pigments and are poorly antigenic. In this work we have constructed fusion proteins where the C-terminal end of complex I protein subunits NuoH, NuoL, NuoM and NuoN from *E. coli* were genetically fused to the cytochrome c domain of Bacillus subtilis cytochrome c550. A naturally occurring transmembrane helix anchor was removed from the cytochrome c550 and was substituted by the membrane spanning polypeptide to be tagged. To facilitate purification of the proteins, a C-terminal his-tag was added to the cytochrome domain. The fusion proteins were expressed in *E. coli*, together with genes encoding cytochrome c maturation proteins, enabling holo-cytochrome c synthesis under aerobic conditions. The heme in cytochrome c is covalently bound to the polypeptide, renders the proteins visible by optical spectroscopy, and can be used to monitor and quantify the proteins, and to determine the orientation of the polypeptides when reconstituted in liposomes. Particularly the three large antiporter-like subunits NuoL, NuoM and NuoN, that previously had been particularly cumbersome to produce in *E. coli*, could be made in unprecedented amounts when expressed with a fused cytochrome c domain. Finally, a gene fragment encoding the NuoN-cytochrome c fusion protein was reintroduced into the nuo operon on the *E. coli* chromosome, allowing the production and characterization of cytochrome-tagged whole Complex I.

C5.06
Low NAD(P)H fluorescence in rabbit skeletal myogenic stem cells increases upon differentiation

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Skeletal myoblast cells have high potential for cell therapy based repair of the injured myocardium. Stem cell differentiation markers are important for searching appropriate cellular state optimal for engraftment. We followed differentiation induced changes in cellular distribution of mitochondria, cellular respiration, adenine and pyridine nucleotide content, NAD(P)H fluorescence, cellular size distribution in skeletal myogenic stem cell populations derived from rabbit skeletal muscle. Increase in fraction of larger size cells during differentiation was characteristic for all cell lines, although lines were heterogeneous by size and differentiation rate. Mitochondrial distribution changed from perinuclear in undifferentiated cells to even distribution in cytoplasm in differentiated cells. Cellular respiration rate decreased by 42–54% after 7 days differentiation compared to undifferentiated cells. The most remarkable feature of undifferentiated myoblasts was very low NAD(P)H fluorescence, that augmented after initiation of cell differentiation, due to metabolic and physiological changes but that did not correlate with increase in cellular size. Chromatographically determined concentration of the reduced and oxidized forms of pyridine dinucleotides and NAD(P)H fluorescence markedly increased on the 7th day of differentiation. Thus, an increase in NAD(P)H fluorescence may be useful for non-invasive detection of the onset of stem cell differentiation. The total amount of adenine nucleotides (ATP, ADP, AMP) increased, but the ATP/ADP ratio decreased during differentiation. This work was supported by Lithuanian State Science and Studies Foundation, project No. B-26/2009.

C5.07
Binding oxygen to heme *d* in cytochrome *bd* from *Escherichia coli*

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Cytochrome *bd* is expressed under low oxygen tension and has high affinity for O_2. The enzyme as isolated is a mixture of two forms: (i) ferrous heme *d* bound to molecular oxygen (“state A”), and (ii) ferryl oxene heme *d* (“state F”), with the latter form contribution of 20–50%. Both forms can be reversibly destroyed either by oxidation (anaerobically or aerobically) or by depletion of oxygen at E_h where the enzyme remains in one-electron-reduced state. Binding of O_2 to heme *d* was studied using the quasi-equilibrium OTTLE spectroelectrochemistry, where oxygen was allowed to equilibrate with the enzyme at a given E_h. Under the anaerobic conditions heme *d* has E_m^{app} ~ +260 mV (vs. NHE, pH 7, 0.1% SML); at 1.2 mM O_2, the E_m^{app} value becomes +495 mV. The $[O_2]$ -dependence of E_m^{app} is linear at the concentrations above ~10 μM with the slope -60 mV/pO_2 and the effective dissociation constant K_D ~ 150 nM O_2. The latter value differs from the earlier data (K_D ~ 280 nM) where the oxygen affinity was directly measured in one-electron-reduced isolated enzyme. The difference is attributed to the fact that in the presence of O_2, the enzyme catalyzes a steady-state flux of electrons supplied by the working electrode. Modeling proves that in the steady-state, the K_D value of 280 nM is reached when the kinetics of the O → A and A → (F) → O transitions are the same and limited by the electron delivery from the working electrode, and the A → F transition is much faster. The O ↔ A and A ↔ F transitions show similar E_m^{app} values over the broad $[O_2]$ range. We propose that in the state F, heme *d* has an unusually low redox potential comparable to that of the state A-enzyme.

D1 – Secretory Pathways

D1.01
Functional analysis of the putative signal peptide of the PAR1 receptor

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Protease-activated receptor-1 (PAR1) belongs to the family of G protein-coupled receptors which can be activated by proteolytic cleavage of their N-terminal extracellular domain. Cleavage at the R41/S42 bond of human PAR1 by thrombin unveils a tethered ligand with the recognition sequence SFLLRN. This sequence binds to conserved regions in the second extracellular loop of the receptor resulting in receptor activation and initiation of signal transduction. The N terminus of the PAR1 contains a hydrophobic domain (residues 1–23) which may represent a cleavable signal peptide according to bioinformatic analyses. Signal peptides have been shown to be necessary for the integration of G protein-coupled receptors into the ER membrane, the first step of their intracellular transport. Usually, these signal peptides are cleaved-off from the mature receptor following ER integration. However, this may not always be the case, as it has been shown recently in the case of the uncleaved pseudo signal peptide of the CRF2(a) receptor (Rutz et al., 2006, J. Biol. Chem. 281, 24910-24921. In the present study we have investigated whether the hydrophobic sequence of PAR1 indeed represents a functional signal peptide. To address this question, we have constructed GFP marker protein fusions with the putative signal peptide and have expressed these constructs in HEK 293 cells. Signal peptide cleavage was assessed by GFP secretion analyses using confocal microscopy and immunoprecipitation experiments. Our results indicate that the hydrophobic sequence 1-23 of PAR1 represents a functional signal peptide.

D1.02
Regulation of the COPI coat dependent trafficking by ubiquitination and phosphorylation in yeast *S. cerevisiae*

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The trafficking of proteins between membrane limited organelles is mediated by vesicles which form from one membrane and fuse with the other. Formation of vesicles is mediated by coat proteins. One of the coat type is COPI that is involved in trafficking along the secretory and biosynthetic pathways. This coat consists of seven coatomer subunits: α-, β-, β'-, δ-, ε-, γ-, ε-COP. It was shown that β'COP is ubiquitylated and this ubiquitination has a regulatory role. Ligase responsible for β'COP ubiquitination is unknown. Rsp5, an ubiquitin-protein ligase is involved in several intracellular trafficking steps. Because *rsp5*mutations interact genetically with mutations in genes encoding β-COP and ε-COP and Rsp5 cooperates with the COPI proteins in Golgi-ER trafficking we tested if Rsp5 can be responsible for β'-COP ubiquitination. We also found additional form of β'-COP subunit, probably phosphorylated one and focus our study on identification of kinases and phosphatases responsible for modification of β'-COP and on determination of the role of this modification on Golgi to ER trafficking. The NetPhos server predicted that β'-COP could be a substrate for protein kinase A, kinase C and casein kinase II, consequently strains with mutations affecting function of these kinases were tested for the presence of phosphorylated form of β'-COP. Additionally, strains lacking some phosphatases were examined for the accumulation of phosphorylated form of β'-COP protein.

D1.03
Biochemical and functional diversity of the yolk granule organelle present in the sea urchin egg and embryo

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Using sucrose density gradient ultracentrifugation, we have identified two distinct populations of the yolk granule organelle resident in the sea urchin egg and embryo. Biochemical analyses of the higher and lower density granule populations revealed striking differences in their protein contents. Gelatin substrate gel analysis identified unique cleavage activities associated with each of the populations. Further characterization revealed that different forms of the Major Yolk Protein were housed in each of the granule populations. Structural differences between the Major Yolk Protein variants could be detected by their differing buoyant densities, susceptibilities to digestion with chymotrypsin and their interactions with the detergent, Triton X-100. Clearly, in the egg and developing embryo the yolk granule organelle exists in at least two biochemically distinct populations. The implications for the functional role(s) of the yolk granule during embryonic development will be discussed. Supported by a grant from the Natural Sciences and Engineering Research Council of Canada.

D1.04
The ultrastructure of the corpora allata of pimpla turionellae L. (Hymenoptera: Ichneumonidae)

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As a known endocrine gland, corpora allata (CA) of adult female Pimpla turionellae were examined ultrastructurally by Transmission Electron Microscopy (TEM). The gland was covered by thick fibrous connective tissue and it was dispersed in the gland as a stromal ramification. The ultrastructure of gland cells had the same characteristic ultrastructure of protein-secreting cells. These cells had a large and oval shape nucleus, numerous and little mixed together granules but occasionally had lysosome, mitochondria and vacuoles. There are electron-dense, electron-modarate, and electron-lucent type of these granules. These granules are actually membrane-limited secretory vesicles which are filled with the proteinaceous hormone produced by the corpora allata cells. Numerous secretory granules, microtubules and mitochondria were observed in the cytoplasm of axons of neurosecretory cells.

D1.05
The paraldehyde fuchsin positive material in corpora cardiaca of pimpla turionellae *L. (Hymenoptera: Ichneumonidae)* during egg maturation

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The purpose of this study is to examine the effects of the neurosecretory material in corpus cardiacum (CC) on reproduction of endoparasitic Pimpla turionellae L. For this purpose, the egg maturation and amount of the neurosecretory material in corpus cardiacum of the insects were examined by the serial cross sections. Egg maturation was determined by measuring the terminal oocyte length in the serial cross sections of the ovariole. The egg growth which has observed on the 3rd day, reached the maximum on 15th day and after that day the egg laying was observed. The sections of insect brains were stained by paraldehyde fuchsin. The amount of the paraldehyde fuchsin positive neurosecretory (PF + NS) material in CC was at a certain level in the first day of egg development. While terminal oocyte was reaching the maximum length, the amount of the PF + NS material in CC decreased the minimum level. During the egg laying phase, the amount of the PF + NS material in CC reached the maximum level. These observations give us the idea that the neurosecretory material in corpus cardiacum of this insect may be related to the egg development.

D1.06
Systems Biology of Protein Secretion in *Saccharomyces cerevisiae*: Mapping of Global Regulatory Structures

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The yeast *Saccharomyces cerevisiae* serves as an important eukaryotic model organism, both in the elucidation of molecular mechanisms underlying many human diseases and in the production of drugs, food products, industrial chemicals, and fuels. The secretory pathway, responsible for processing a third of all synthesized protein, has been studied extensively by reductionist approaches, but until recently, tools for systemic study of the pathway have been inadequate. Here we undertook a global analysis of the protein secretion pathway in *S. cerevisiae*. Using network-based analysis of transcriptome data, key transcription factors and biological processes were identified related to disturbances in the secretory pathway. The disturbances, (i) recombinant secretion of human insulin and α-amylase proteins, and (ii) deletion of the Unfolded Protein Response (UPR) transcription factor *HAC1*, caused significant physiological changes that could be mapped to (a) oxidative stress, (b) amino acid synthesis/salvage, and (c) overall transcriptional synthesis pathways. Through our analysis, we propose a holistic model for secretory pathway stress, linking endoplasmic reticulum folding to oxidative radical formation and NADPH consumption. This model should be useful as a scaffold for understanding protein secretion in higher eukaryotic cells and in the analysis of protein folding-related disease.

D1.07
Alpha amylase secretion from *S. cerevisiae*

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Being one of the best studied model organisms and armed with a vast array of molecular and biochemical technologies, *S. cerevisiae* has the potential to be an attractive cell factory for producing heterologous proteins. Here we evaluated α-amylase secretion using different expression strategies and aimed to gain a more detailed understanding of protein production mechanisms. The α-amylase from *A. oryzae* was directed by two kinds of leader sequence, the alpha factor leader and a synthetic leader and expressed separately in three different plasmids, POT plasmid with *POT1* marker from *S. pombe* and *TEF1* promoter for protein expression, CPOT plasmid with *POT1* marker and *TPI1* promoter, and p426GPD plasmid with *URA3* marker and *GADPH* promoter. The synthetic leader in plasmid POT and p426GPD, compared to the alpha factor leader, produced more amylase, whereas the combination of the alpha factor leader and the CPOT plasmid showed the highest titer and specific production rate. This study sheds light on the effect of plasmid, promoter, and secretion leader sequence on protein secretion.

D1.08
The Pex11-protein family members control peroxisome proliferation

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Peroxisomes are essential single membrane-bound organelles present in all eukaryotic cells. They enclose hydrogen peroxide-generating and degrading enzymes, their function is mainly associated with lipid metabolism and in yeasts, they are the exclusive site of fatty acid β-oxidation. These organelles quickly adapt their size, number and protein content to the cellular needs. Peroxisome biogenesis and proliferation is controlled by peroxins (PEX-proteins), more than 30 of these have been identified so far. *Saccharomyces cerevisiae* cells lacking *PEX11* display fewer and larger peroxisomes and reduced utilization of oleic acid compared to wild type cells. Together with Pex25p and Pex27p Pex11p represents the Pex11-protein family, orthologs of which have been identified in all eukaryotic organisms. Pex11-proteins participate in the proliferation of peroxisomes but their detailed molecular function is not known. Hence, we performed functional tests in *S. cerevisiae* wild type cells and deletion mutants lacking either one, two or all three PEX11-protein family members but expressing one of the yeast homologs from a plasmid. We recorded complementation of the deletion phenotypes, changes in size and number of peroxisomes and analysed mRNA levels using real-time PCR. Expression of homologs altered peroxisome number and/or re-established wild type levels of oleic acid consumption. Pex11p and Pex25p seem to play a crucial role regarding peroxisome abundance and function, while Pex27p might be a negative regulator. In summary our data suggest that these proteins influence each others expression thereby preparing the peroxisome for fission.

D1.09
Understanding SNARE regulation via SM proteins: Insights from Sly1p/Sed5p interaction

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Sec1/ Munc18 (SM) proteins are important regulators of intracellular membrane fusion. They exert their function through interacting with the SNARE machinery, primarily with syntaxins. Despite the high structural homology among SM proteins, different modes are proposed for their association with syntaxins. Neuronal Munc18a binds to a "closed conformation" formed by Syntaxin1, whereas several SM proteins interact only with the N-peptide motifs of their cognate syntaxins. On the other hand, recent findings suggest that all SM proteins might exert a regulatory role, acting on both the N-peptide motifs and the closed conformation of syntaxins. So far, yeast Sly1p, the SM protein of ER-Golgi trafficking, is considered to interact solely with the N-peptide portion of its cognate syntaxin Sed5p. We investigated the Sly1p/ Sed5p association in light of the proposed common binding model through biochemical and biophysical methods. The current analysis shows that N-peptide binding is indeed the major contributor to the high affinity, yet the remainder of Sed5p contributes as well. Interestingly, individual Sed5p seems to exist in a stable closed conformation, which slows the formation of a SNARE complex. Therefore, we are interested to know how Sed5p switches to a SNARE-accessible conformation and whether Sly1p has a role on this.

D1.10
Membrane-localized chaperone interactions in the folding of polytopic membrane proteins

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In eukaryotic cells polytopic membrane proteins fold to attain their native three-dimensional conformations within the lipid bilayer of the endoplasmic reticulum (ER). Folding requires specific interactions between the transmembrane segments. In analogy with the folding process of soluble proteins, chaperones might transiently associate with the transmembrane segments during synthesis and thereby facilitate folding by preventing non-productive interactions. Shr3 is an integral membrane protein of the ER of *Saccharomyces cerevisiae* that potentially functions as a specialized membrane-localized chaperone for members of the amino acid permease family. In *shr3*Δ cells the 12 membrane-spanning segments of amino acid permeases insert into the membrane resulting in the correct topology but the permeases do not exit from the ER and instead accumulate in large detergent-resistant aggregates. Currently, we are investigating the structural and temporal requirements for the interaction between Shr3 and the amino acid permeases. Specifically, we are implementing an *in vivo* cross-linking approach using unnatural photo-reactive amino acids incorporated into the transmembrane segments of Shr3. Moreover, we have set up conditional expression of Shr3 via fusions to regulated degrons that target the protein for proteasomal degradation. By employing these two methodologies we hope to define how and when Shr3 interacts with its substrates.

D1.11
A new putative intracellular route to the cell wall in *Arabidopsis thaliana* seeds

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Trafficking pathways to the cell wall are poorly studied since the default pathway was described in 1990. This pathway is based on the assumption that proteins entering the secretory pathway, and not having other sorting signals, are transported from the Golgi directly to the plasma membrane. However, in recent years, studies involving the endosome, suggested the involvement of the endocytic compartments in protein secretion and relaunched the discussion on the routes proteins follow to the cell wall. Cardosin B is an aspartic proteinase considered to be a valuable tool to study the trafficking pathways to the cell wall. In this work, we detected cardosin B in the cell wall of transgenic *Arabidopsis thaliana* seedlings from lines stably expressing this protein under the control of an inducible promoter. This system allows controlling the beginning of expression, and so to dissect the intermediate compartments of cardosin B route. To map the pathway that this protein follows to the cell wall, we performed immunolocalization at different time-points after induction. Its presence was detected in the Endoplasmatic Reticulum in the first stages and in dot-shaped compartments just before it reaches the cell wall. By co-localization with a prevacuolar marker, we demonstrate that cardosin B goes to the cell wall through the prevacuole, which has been reported to be analogous to the multivesicular body (belonging to the late plant endosomal system). These considerations lead to the propose of a new putative route to the cell wall, in which proteins leave the Endoplasmatic Reticulum, go through the Golgi and reach the cell wall via the prevacuolar compartment.

D1.12
Two vacuolar targeting signals in the same protein – two different pathways?

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Intracellular trafficking to the plant vacuole has been issue of many reports, but still far from being clear. Cardosin A has been studied for several years and has been considered a valuable tool for studying vacuolar trafficking in plants. This protein can accumulate in different types of vacuoles according to the organ where it is expressed and its expression is developmental regulated. In previous works, both the PSI and VGFAEAA peptide have been implicated in cardosin A sorting to the vacuole, not being clear whether these two domains act in cooperation or work separately. With this study we intend to clarify the role of cardosin A domains, PSI and VGFAEAA peptide, in the transport of a secreted protein to the vacuole. Constructions were obtained where each domain was cloned with the fluorescent protein mCherry, and it was demonstrated that each region per se is sufficient to redirect mCherry to the vacuole. The results obtained led to the hypothesis that the existence of two vacuolar signals in one protein could reflect the existence of different pathways depending on which signal is commanding the sorting. To confirm this theory, BFA assays and co-expression with a dominant negative RabD2a protein, revealed that vacuolar targeting mediated by the PSI was bypassing the Golgi Apparatus, while vacuolar targeting medi-

ated by the VGFAEAA peptide was not. Given the outcomes of this study we propose a model for the trafficking of cardosin A to the vacuole depending on each of the described domains. The validation of cardosin A vacuolar sorting determinants and of its trafficking pathways can provide helpful tools for the study of vacuolar transport of other proteins.

D2 – Mitochondria

D2.01
Human mitochondrial transcription and replication proteins are regulated via differential pathways

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The expression of many nuclear genes encoding mitochondrial proteins is regulated by several nuclear transcription factors. NRF-2 is a transcription factor involved in the control of cell cycle progression, protein synthesis and mitochondrial biogenesis. Concerning NRF-2 control on mtDNA transcription and replication proteins, the few available data indicate that it positively controls the transcription initiation factors TFAM and TFB2M. In the current study, we investigated the dependence on NRF-2 of the genes for other proteins of the minimal mitochondrial transcription and replication machineries: the transcription termination factor mTERF, the RNA polymerase (POLRMT), the transcription repressor MTERF3, the helicase TWINKLE, the single-stranded binding protein (mtSSB) and the catalytic (POL-γA) and accessory (POL-γB) subunits of DNA polymerase γ. By EMSA and ChIP assays, we demonstrated the functionality of *in silico* predicted binding sites for NRF-2 in the promoters of mTERF, POLRMT, TWINKLE, mtSSB and POL-γB genes. A putative site predicted in POL-γA promoter was unable to interact with NRF-2. No binding was predicted and experimentally observed for MTERF3 gene promoter. Moreover, knock-down and overexpression experiments definitely ruled out the NRF-2 control on MTERF3 and POL-γA genes, and confirmed the positive regulation on the expression of other proteins. In conclusion, the current study extends the collection of mitochondrial genes controlled by NRF-2. Interestingly, NRF-2 does not regulate the genes for POL-γA and for MTERF3; this evidence arouses interest in the study of differential pathways responsible for the regulation of these two proteins.

D2.02
Is mitochondrial genome chicken or egg of carcinogenesis?

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Objectives: Investigation of the role of mtDNA mutations and inherited polymorphisms in breast cancer development.
Methods: mtDNA NADH-dehydrogenase genes, D-loop sequence and haplogroup distribution analysis was performed in breast cancer patients, centenarians and woman with cancer free history cohorts. Subsequent comparison with general Polish population was performed.
Results: The mitochondrial haplogroup distribution in patients with breast cancer differs from a group of cancer-free controls and general population as haplogroup I is overrepresented. Breast cancer patients in comparison to cancer free control cohort abundantly carry 10398G ($p < 0.001$). This polymorphism is cancer development factor with OR = 9.510 and RR = 7.576. A10398G RFLP test has high sensitivity = 0.769 and specificity = 0.740. Moreover in breast cancer population A1811G and A4529T are overrepresented ($p = 0.001$) if compared with centenarians. Patients with biopsy-proven breast cancer carry specific sets of mtDNA polymorphisms including G3915A, A4727G, A4769G, T5082C, A8860G, T10034C, T10238C, A10398G, G10589A, T11233C, A11467G, G11719A and G12372A. Some of the polymorphism are very rare (i.e. T11233C) or not reported before (i.e.T5082C) in general population and may be classified as breast cancer bio-markers.
Conclusions: mtDNA polymorphisms establish a specific genetic background for cancer development and may enable selection of populations at high cancer risk and support the process of prevention and early diagnosis.

D2.03
Consequences of the activation of mitochondrial ATP-sensitive potassium channel on the physico-chemical properties of mitochondria from rat myometrium

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Mitochondrial ATP-sensitive potassium channel is a main factor of regulation of K^+ exchange in mitochondria. It is assumed, that activation of this structure leads to improvement of mitochondrial physiology. MitoKATP channel from cardiac muscle is relatively well described, but functioning of this channel in other tissue, in particular in smooth muscles, is not fully understood yet. Aim of this work was to determine the consequences of activation of mitoKATP on the functioning of mitochondria from the rat myometrium. Using photon correlation spectroscopy we measured an average diameter of mitochondrial particles. It was found that average size of mitochondria in KCl-containing medium was 470.6 ± 23 nm, while ATP decreased their size to 416.4 ± 21.7 nm. Addition of channel activator diazoxide restored mitochondrial size to 459 ± 32.3 nm, while blocking of the channel with selective blocker glybenclamide decreased an average mitochondrial size to 430 ± 28 nm. By means of Rhod-123 fluorescence we showed, that activation of mitoKATP caused partial depolarization of the inner mitochondrial membrane. While in the presence of glybenclamide mitochondrial potential was restored to control values. When we added valinomycin, we also detected partial depolarization, but in this case glibenclamide failed to restore mitochondrial potential. And none of the above mentioned effects were showed in medium which did not contain KCl. Thereby, using selective activator and blocker of mitochondrial ATP-sensitive potassium channel we showed that activation of this structure in mitochondria from myometrium increased their average diameter and leaded to partial depolarization of the inner membrane.

D2.04
Brain mitochondrial proteome changes in Streptozotocin-induced diabetic rats

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Diabetes mellitus may lead to a number of complications, which determine the clinical progression of the disease and affect various tissues. Streptozotocin (STZ) is widely used to induce experimental diabetes due to its ability to selectively target and destroy insulin producing pancreatic beta-cells. Several groups have investigated mitochondrial function in type 1 diabetes, reporting mitochondrial oxidative stress and impairment of mitochondrial respiration and OXPHOS complex activities. Inhere, protein expression profiles of rat brain mitochondria were compared between animal models of streptozotocin-induced diabetes mellitus (STZ) and age matched controls (AMC) at 3 weeks after induction of hyperglycemia. Protein samples from four STZ and four AMC rat brain mitochondria were analyzed with a quantitative 2D-gel electrophoresis, to assess statistically significant expression changes. First tests were conducted on a wide range of pH 3-10, and later ones using zoomed pH range, as 6–11 and 4–7, and only in the latter range proteins show a statistically significant variation. A total of four spots were differentially expressed between the two experimental conditions, and all were decreased in the diabetic condition. These spots were cut from the gel, digested with trypsin, and subsequently analyzed by HPLC-ESI-MS/MS. Among these identified proteins a decrease of more than a half of NDUFS3 expression is of particular interest, as this is a core subunit of complex I, fundamental for both its assembly and function. These results underline the involvement of complex I in type 1 diabetes. We are carrying out further studies in order to confirm the other identified proteins.

D2.05
Evaluation of direct antioxidative and pro-oxidative properties of some nucleotide, nucleoside and nucleobase analogues

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Many analogues of nucleic acid components are well-established as antiviral and anticancer agents. In some cases, their clinical use is limited by mitochondrial toxicity. Mitochondria represent a source of reactive oxygen species (ROS), whose overproduction might lead to injury to lipids, proteins and nucleic acids and contributes to cellular senescence or even apoptosis. On the other hand, several modified nucleotides, nucleosides and nucleobases have been described in literature to act as antioxidants. In this study, we evaluated antioxidative properties of selected acyclic nucleoside phosphonates, 8-aza-7,9-dideazaxanthines and xanthosines, poly-substituted pyrimidines and purines and carbocyclic nucleoside analogs synthesized in our Institute with the aim to determine relationships between their structure and antioxidant activity. To determine their direct radical scavenging properties, trolox equivalent antioxidant capacity assay (TEAC) was used. Consequently, we studied the effects of the analogues on Fe^{2+}/ascorbate-induced lipid peroxidation in rat liver microsomes. Although several compounds were active in a TEAC assay (mostly those possessing -SH groups) they largely failed to prevent microsomal peroxidation. This can be the result of their additional properties such as lipophilicity or iron-chelating ability. The results obtained from *in vitro* antioxidant assays will be compared with the effects of the compounds on ROS production in intact cells and discussed accordingly. This work was supported by the Research project of the IOCB OZ40550506 and the Project No. 1M0508 by the Ministry of Education, Youth and Sports of the Czech Republic.

D2.06
Interaction of mitochondria-targeted antioxidants with normal and tumor cells in culture and their protective effects during oxidative stress

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Production of reactive oxygen species (ROS) in mitochondria was studied using the novel mitochondria-targeted antioxidants (SkQ) in cultures of human cells. These molecules were designed as conjugate of antioxidant part (plastoquinol) and cationic part (triphenylphosphonium). It was shown that SkQ rapidly (1–2 hour) and selectively accumulated in mitochondria and prevented oxidation of mitochondrial components under oxidative stress induced by hydrogen peroxide. At nanomolar concentrations, SkQ inhibited oxidation of glutathione, fragmentation of mitochondria, and translocation of Bax from cytosol into mitochondria. Mitochondria-targeted antioxidants at nanomolar concentrations prevented accumulation of ROS and cell death under oxidative stress. These effects required 24 hour or more (depending on the cell type) preincubation, and this was not related to slow induction of endogenous antioxidant systems. It is suggested that SkQ slowly accumulates in a small subpopulation of mitochondria that have decreased membrane potential and produce the major part of ROS under oxidative stress. This population was visualized in the cells using potential-sensitive dye. This hypothesis explained slow development of antioxidant and protective effects of SkQ, which accumulated in mitochondria due to membrane potential. Antioxidant effect of SkQ in individual mitochondria with high membrane potential (the major mitochondrial population) developed much faster, in agreement with the rate of accumulation of SkQ in the cell. The possible role of the small fraction of ''bad'' mitochondria in cell physiology is discussed.

D2.07
Mitochondrial and nuclear relationship in apoptosis resistance. The importance of bcl-2 and redox environment

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The phenomenon of apoptosis resistance, at the root of malignant transformation and resistance to cancer therapy, has been associated with oncogen bcl-2 present at membranes of mitochondria, nucleus and endoplasmatic reticulum. The orchestrated response of nucleus and mitochondria in the process of apoptosis is induced by alterations of cellular redox state and $[Ca^{++}]$ homeostasis, mediated by bcl-2, by a mechanism still not completely elucidated. We have performed a time course analysis of

events in the cytoplasm, mitochondria and nucleus involved in the apoptosis resistance to cisplatin (10 μM and 50 μM) in MCF7 cells WT and MCF7 that overexpress bcl-2. The compartmentation of GSH, mitochondrial distribution, apoptosis detection and [Ca^{++}] were studied by confocal microscopy. The cell cycle, ROS level and the mitochondrial transmembrane potential (mmp) was studied by flow cytometry and the cellular level of reduced glutathione (GSH) by spectrophotometry. Mitochondrial perinuclear rearrangement was an early event, followed by the decrease in mmp. The doses dependent increase in [Ca^{++}] was followed by its perinuclear distribution, and release to the cytoplasm. The GSH level and its nuclear compartmentation were increased in the cells that resisted apoptosis. All these events were delayed or less significant in MCF7 bcl-2 cells. Bcl-2 overexpression conferred increased apoptosis resistance and was demonstrated to increase nuclear glutathione concentration, thus providing a protection at nuclear level, apart from its well documented effect on mitochondria. The mitochondrial morphology and distribution could represent a key factor in the nucleus-mitochondrial cross talk.

D2.08
cAMP dependent protein kinase regulates post-translational processing and expression of complex I subunits in mammalian cells

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Work is presented on the role of cAMP-dependent protein phosphorylation in post-translational processing and biosynthesis of complex I subunits in mammalian cell cultures [1,2]. PKA-mediated phosphorylation of the NDUFS4 subunit of complex I promotes in cell cultures *in vivo* import/maturation in mitochondria of the precursor of this protein [3]. The import promotion appears to be associated with the observed cAMP-dependent stimulation of the catalytic activity of complex I. These effects of PKA are counteracted by activation of protein phosphatase(s). PKA and the transcription factor CREB play a critical role in the biosynthesis of complex I subunits. CREB phosphorylation by PKA and/or caMKs activates, at nuclear and mitochondrial level a transcriptional regulatory cascade which promotes the concerted expression of nuclear and mitochondrial encoded subunits of complex I and other respiratory chain proteins [4].

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D2.09

Abstract withdrawn

D2.10
A new mitochondrial three-component pyridine nucleotide pathway, namely a NMN → NAD → NADP route, is functional in plant heterotrophic tissues

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The multiple roles of NAD(P) in bioenergetics and production of secondary messengers as well as in protein modifications and generation of free and protein-associated poly-ADP-ribose require a constant resynthesis of NAD(P) to avoid depletion of the intracellular NAD(P) pool. Nicotinamide mononucleotide adenylyltransferase (NMN-AT) (EC 2.7.7.1) is a central enzyme in NAD^+ biosynthesis. It catalyzes the reversible reaction $NMN + ATP \rightarrow NAD^+ + PPi$. $NADP^+$ is produced by NAD^+ kinase (EC 2.7.1.23): $NAD^+ + ATP \rightarrow NADP^+ + ADP$. The aim of this study was to investigate whether mitochondria isolated from plant heterotrophic tissues can synthesize nicotinamide adenine dinucleotides from externally added NMN. The experimental work was articulated in the following phases: first, plant mitochondria were checked with respect to their functional features (intactness of mitochondrial outer membrane, intactness of mitochondrial inner membrane, Respiratory Control, $\Delta\Psi$ generation). Next, total NAD(P) was extracted from freshly isolated mitochondria and quantified by HPLC analysis. Plant mitochondria were incubated with NMN and ATP and the amount of NAD(P) synthesized was determined. Our findings show mitochondrial permeability to externally added NMN and a significant increase of newly synthesized NAD(P) in plant heterotrophic tissues. Moreover, we determined the kinetic parameters of the NMN-AT in fresh slices of plant heterotrophic tissues. Dependence of enzyme activity on pH and temperature and sensitivity to different inhibitors were also investigated. Based on our biochemical results, we discuss the profiles and the function of pyridine biosynthesis in plant mitochondria.

D2.11
Saccharomyces cerevisiae mitochondria contain enzymes capable of hydrolyzing FMN and FAD to riboflavin: probably function in flavoprotein deflavination and reconstitution in cell grown under glucose limitation

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FMN (Flavin mononucleotide or riboflavin 5'-phosphate) and FAD (flavin adenine dinucleotide) are mainly located in mitochondria, where they act as redox cofactors of a number of dehydrogenases and oxidases that play a crucial function in both bioenergetics and cellular regulation. In this report, *Saccharomyces cerevisiae* mitochondria (SCM) competence to metabolise externally added and endogenous FAD and FMN was investigated. Because intact and coupled mitochondria are strictly required to carry out studies concerning mitochondrial metabolism, a series of preliminary experiments was performed using isolated SCM in order to ascertain structural and functional features (intactness of mitochondrial outer and inner mem-

branes, Respiratory Control, $\Delta\Psi$ generation). Then, the amounts of flavins in aliquots of neutralized perchloric extracts of both spheroplasts and mitochondria were measured by HPLC. SCM capability to metabolise externally added and endogenous FAD and FMN was investigated both spectroscopically and via HPLC. FAD deadenylation and FMN dephosphorylation were studied with respect to following features: dependence on substrate concentration, pH profile and inhibitor sensitivity. The existence of two novel mitochondrial FAD pyrophosphatase (diphosphatase) (EC 3.6.1.18) and FMN phosphohydrolase (EC 3.1.3.2) activities, which catalyse $FAD + H_2O \rightarrow FMN + AMP$ and $FMN + H_2O \rightarrow$ riboflavin + Pi conversion, respectively, is here shown. In the light of cytosolic riboflavin, FMN and FAD concentrations, probably mitochondria play a major role in regulating the flavin pool in yeast cell under glucose limitation and in relation to flavoprotein deflavination and reconstitution.

D2.12
The functions and mitochondrial targeting of NADH dehydrogenase (ubiquinone) flavoprotein 2 (NDUFV2) subunit in human mitochondrial complex I

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NADH dehydrogenase (ubiquinone) flavoprotein 2 (NDUFV2), containing one iron sulfur cluster ([2Fe-2S] binuclear cluster N1a), is one of the core nucleus-encoded subunits existing in human mitochondrial complex I. Defects in this subunit have been associated with Parkinson's disease, Alzheimer's disease, Bipolar disorder and Schizophrenia. In this study, we applied the RNA interference (RNAi) technology in human T-REx 293 cells to investigate the function of NDUFV2 subunit. The results showed that suppression of NDUFV2 expression in the cells would slow down the growth rate of cell in galactose medium, decrease the oxygen consumption rate, reduce mitochondrial membrane potential and increase reactive oxygen species (ROS) generation, but did not affect complex I assembly. These observations provided the evidences that NDUFV2 plays an essential role for energy transduction in cells. In addition, we also generated various truncated constructs and point-mutated mutants to investigate the mitochondrial targeting mechanism of NDUFV2. It was found that the cleavage site of NDUFV2 was located around amino acid residue 32 and the first 22 residues of NDUFV2 were enough to function as a mitochondrial targeting sequence (MTS) to carry the passenger protein into mitochondria. In a recent study, the patients with hypertrophic cardiomyopathy and encephalomyopathy were found to contain a 4 bp-deletion in the second intron of NDUFV2 gene (IVS2 + 5_ + 8delGTAA) thus cause the exon 2 losing. To dissect the pathogenetic mechanism of this mutation, we established the human disease model and found that lost of this exon 2 causes NDUFV2 to lose its mitochondrial targeting ability.

D2.13
Mitochondria are a biochemical target of the perfume chemical components: Lilial, Lyral and Hedione

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Fragrance chemicals are among the basic components of commercial perfumes formulation and are extensively used in cosmetics and food products. The majority of fragrance chemicals are lipophilic molecules that cross the plasma membrane and interact with intracellular or intra-organelle sites initiating thus, many events that may lead to cell death. In this study the biological effect of lilial, lyral and hedione, widely used chemicals in perfume industry, was investigated on several cell lines in culture including: MCF7, $Caco_2$, Hek293, NIH3T3, HepG2 and HaCat. Cells were treated for 24 hours with each chemical at concentration varying between 10–100 μM. Viability was evaluated using MTT assay. No significant effect was found with MCF7, Caco2, Hek293, HepG2 cells or NIH3T3. A decrease of 87%, 76% and 89% in the viability of Hacat (primary keratinocytes) cell was obtained with 50uM of each lilial, lyral and hedione and an estimated EC50 of 59 nM, 100 nM and 5 μM respectively. Further investigations have shown a significant decrease in ATP level of: 40%, 69%, and 50% and a significant increase in reactive oxygen species level of: 50%, 50%, 53% following HaCat treatment with lilial, lyral and hedione at their EC50 value respectively. The effect of these chemicals on the activity of NADH dehydrogenase and Succinate dehydrogenase (complexes I & II) and complex V (ATPase) of mitochondria isolated from HaCat cells is currently being investigated. Our preliminary data suggest mitochondria as a target of these chemicals

D2.14
Effect of minocycline and ATP on structure and function of non-native conformations of cytochrome c

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Cytochrome c (cyt c) is a peripheral membrane protein that is associated with the inner mitochondrial membrane via the interaction with the phospholipid cardiolipin (CL). Beyond the role as electron carrier in the respiratory chain, cyt c is involved in the triggering of apoptosis. Chronologically, the latest function attributed to cyt c has been associated to its ability to be activated and fulfill the task of a peroxidase. It's been demonstrated that post-translational modifications, such as oxidative or nitrative chemical modifications of the protein or its interaction with detergents, denaturant agents and lipids, can trigger peroxidase activation. The disruption of the M80-Fe ligation increases the peroxidise activity of cyt c by increasing the access of H_2O_2. We studied the structural features of a) cyt c bound to cardiolipin (CL)-containing liposomes, b) Y67H and H26Y cyt c mutants. In particular we have used the weak absorption band at 695 nm to assess the conformational transitions in cyt c associated with the disruption of Fe-S(M80) bond, that brings about the acquirement

of peroxidase activity. The kinetic parameters of such peroxidase activity were assessed also in the presence of minocycline, that behaves as an uncompetitive inhibitor. We have studied also the effect of the ATP on peroxidase activity and on the absorption band at 695 nm: the ATP acts as a regulatory effector in modulating structural transitions of cytochrome c. Finally, we have been discussing the implications of the ATP and minocycline on the apoptogenic activity of cyt c.

D2.15
Proanthocyanidins in acute dose affect mitochondrial respiration in muscle of Wistar rats

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Proanthocyanidins are some of the main polyphenols found in plant derived food and beverages. They have a protective effect against metabolic and cardiovascular diseases. Mitochondrion is the principal energy source of cell that converts nutrients into energy through cellular respiration. Compromised mitochondrial function has been linked to numerous diseases, including those of the metabolic and cardiovascular systems. To evaluate if the proanthocyanidins affect mitochondrial activity, we measured the levels of mitochondrial respiration *ex-vivo* from skeletal muscle of Wistar rats treated with an acute dose of 250 mg of GSPE (Grape Seed Proanthocyanidins extract)/ Kg. For this, male Wistar rats were fasted prior to oral gavage of GSPE using lard oil as a vehicle and after 5 hours rats were sacrificed and the *gastrocnemius* muscle was extracted for isolation of mitochondria. We used these mitochondria to measure their respiration using pyruvate as substrate in an oxygraph-2k (OROBOROS). Mitochondrial states were achieved adding pyruvate to get the state 2, state 3 was obtained with the addition of adenosine 5'-diphosphate (ADP), the state 4o was achieved by adding oligomycin and, finally, uncoupling state (Unc.) was evaluated with the addition of carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP). The results obtained indicate that the proanthocyanidins may effect mitochondrial respiration in *gastrocnemius* muscle, causing an increase of oxygen consumption statistically significant in all mitochondrial states. We may conclude that an acute dose of GSPE improve muscle mitochondrial functions. This work was supported by Grant AGL2008-00387 from the Spanish government.

D2.16
The ischemic tolerance signaling may be involved in nephroprotective effects of mitochondria-targeted antioxidant SkQR1

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Acute kidney injury (AKI) is a pathology, which apparently impairs the life of patients after surgery or kidney transplantation. The main cause of AKI is accepted to be an oxidative stress in kidney tissue, which can be observed in ischemia/reperfusion (IR) model. Mitochondria play important role in renal cell damaging as they produce large amount of reactive oxygen species (ROS). We explored the mechanism implied in the nephroprotective action of mitochondria-targeted antioxidant SkQR1 (10-(6'-plastoquinonyl)decylrodamin) after rat kidney IR. Single injection of SkQR1 before ischemia and four times in post-ischemic period resulted in significant restoration of renal function. Survival rate after IR rose up from 15% to 80%. SkQR1 also decreased ROS production (revealed by confocal mycroscopy of vital kidney slices) and the level of lipid peroxidation products in kidney tissue exposed to IR. We observed increased level of erythropoietin (EPO) and phosphorylated GSK-3b in kidney 3 hour and 24 hour after SkQR1 injection. The amount of EPO and p-GSK-3b were also higher in rats obtaining SkQR1 after IR. EPO and p-GSK3b are known to be crucial elements in ischemic tolerance mechanisms resulting in better cell survival. We observed that ishemic and hypoxic preconditioning of kidney also provided elevation of p-GSK3b and EPO and decreased oxidative damage of kidney. Thus we demonstrated the efficiency of mitochondria-targeted antioxidant SkQR1 as a new nephroprotector in ischemic AKI. The mechanism of SkQR1 action apparently includes induction of preconditioning signaling and abolishing of oxidative burst in mitochondria. Supported by RFBR 08-04-01667 and Rostok Group (Mr. A.V. Chikunov).

D2.17
Phosphatidylglycerol specific phospholipase C, Pgc1p, in *Saccharomyces cerevisiae*

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Cardiolipin (CL) is a unique phospholipid predominantly located in the inner mitochondrial membrane. It plays an important role in mitochondrial structure, function and dynamics. In eukaryotes, CL is synthesized from phosphatidylglycerol (PG) and cytidinediphosphate diacylglycerol (CDP-DAG). Under normal circumstances, PG is a very low abundance phospholipid in yeast. Under the conditions of CL depletion, PG can substitute for CL in most cellular functions. Importantly, in mammals, PG is a crucial component of pulmonary surfactant, an essential fluid that covers the surface of the lung. Considering the importance of this anionic phospholipid, little is known about the mechanisms controlling cellular amount of PG. Our recent results (Simockova et al., J Biol Chem 283, 17107) demonstrate the existence of a novel regulatory pathway which removes excess PG via a phospholipase C type degradation. Pgc1p is the key enzyme of this pathway that converts PG to diacylglycerol (DAG) and glycerol-3-phosphate. To further analyze the role of Pgc1p in cellular physiology we analyzed the response of the Pgc1 degradation pathway to factors affecting mitochondrial development and function, and to the factors regulating phospholipid biosynthesis. The physiological role of this degradation pathway will also be discussed. This work was supported by APVV LPP-0291-09 and VVCE-0064-07 grants.

D2.18
mtDNA and electron transport chain integrity in lifespan extention mediated by calorie restriction in *Saccharomyces cerevisiae*

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Aging involves a decline in metabolic function and efficiency of biological systems over time. Our group elected *S. cerevisiae* as a

model organism and calorie restriction (CR) as an energetic intervention to uncover relevant aging hallmarks in eukaryotic cells. Since oxidative metabolism plays a role in *S. cerevisiae* viability during stationary growth phase, we investigated the influence of the mitochondrial genome – mtDNA is responsible for the synthesis of a number of electron transport chain constituent proteins – and respiratory pathways on this phenotype. We determined lifespans and the respiratory growth capacity in abf2D (mutants harboring marked mitochondrial genome instability) and of rho-/0 (mutants in which mtDNA is partially or totally absent) and observed that neither of them responded to CR by extending lifespan, nor exhibited respiratory growth. CR was also unable to increase lifespan in cyt1D (mutants in which cytochrome c1 is absent and are, therefore, unable to grow in respiratory medium). Interestingly, the lpd1D mutant (which does not display dihydrolipoyl dehydrogenase activity, an important enzyme involved in oxidative metabolism and nucleoid assembling) showed not only a residual respiratory growth capacity, but also a response to CR. Moreover, we also found mtDNA is less stable in the absence of dihydrolipoyl dehydrogenase when in CR and aconitase – also a nucleoid protein – under standard conditions. Altogether, our data present evidence that mitochondrial electron transport chain functional integrity is required for lifespan extention mediated by CR and that glucose governs mtDNA-protein interactions in *S. cerevisiae*. Supported by FAPESP/CNPq.

D2.19
Malignant transformation of human bronchial epithelial cells (BEAS-2B) by Cr(VI) is associated with altered mitochondria and bioenergetic phenotype

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Mitochondria play important roles in cellular energy metabolism, free radical generation, cell signaling and apoptosis. Defects in mitochondrial function have long been suspected to contribute to the development and progression of cancer. Warburg's pioneering work hypothesized that a key event in carcinogenesis involved the respiratory machinery, with malignant cells satisfying their energy needs by producing a large portion of their ATP through glycolytic mechanisms, rather than through oxidative phosphorylation. Certain malignant cells have also been reported to have alterations in mitochondrial content as compared to normal cells of the same tissue. In lung fibroblasts, taken from a lung epidermoid carcinoma, low mitochondria content was associated with (to) decreased oxidative phosphorylation and increased glycolysis. Aiming to establish whether the malignant transformation of bronchial epithelial cells was paralleled by changes in cellular bioenergetic and mitochondrial phenotypes, we evaluated the energy metabolism, the mitochondria membrane potential and the mitochondria content in a normal bronchial epithelial cell line and in its malignant derivatives. To this end, the mitochondria membrane potential was evaluated by flow cytometry using the JC-1 fluorescent probe. Fluorescence microscopy was used to evaluate the mitocondria morphology and number, and 1H and 13C NMR spectroscopy was used to assess the cell's bioenergetic phenotype. Our results revealed that the more malignant phenotypes correlate with increased mitochondria biogenesis, decreased membrane potential and altered bioenergetic phenotype. This work was supported by CIMAGO (Grant 16/06).

D2.20
The mitochondrial response to photodynamic reaction mediated by Photofrin in A549 cells

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Photodynamic therapy (PDT) is an effective cancer treatment that induces cytotoxicity through intercellular generation of reactive oxygen species (ROS). Photofrin (Ph) mediated photodynamic reaction was applied to the human lung carcinoma cell line A549. There were studied colocalization of photosensitizer with Mito-Tracker Green. The mitochondrial cells' response to the PDT was examined by changes in mitochondrial superoxide dismutase (SOD2) activity and by measurement of mitochondrial oxidoreductive potential. The colocalization studies have shown that Ph was mainly localized in mitochondrial membranes and also in the intracellular compartments. Photodynamic reaction induced dynamic changes in SOD2 activity. Its level raised immediately after PDT reaction. Work of mitochondria was evaluated via measuring mitochondrial dehydrogenases in MTT assay. The A549 cells showed significant disorder in the mitochondria function immediately after PDT and it decreased with increasing time post treatement. These results demonstrated that A549 cells respond to PDT -Ph by involving the mitochondria.

D2.21
Effect of oxidized lipid containing hydroperoxide or carboxylic acid group on interaction of cytochrome *c* to model mitochondrial membrane

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In the inner mitochondrial membrane (IMM) lipid oxidation can affect the binding and activity of cytochrome *c* (cytc) with repercussions for the cell respiration and apoptosis. The binding of cyt*c* to IMM mimetic liposomes containing the oxidized phosphatidylcholine was investigated at pH 6.2 and 7.4, which are expected to be present in the intermembrane space of respiring and uncoupled mitochondria. The binding was assessed by fluorescence quenching promoted by resonance energy transfer from the PPDPC pyrene from membrane (donor) to heme group of cyt*c* (acceptor). The binding isotherms were treated using a model based in Hill plots presented two binding sites K_D^1 and K_D^2, representing two distinct states in the membrane, and a cooperativity coefficient n. The presence of PazePC (1-O-Hexadecyl-2-Azelaoyl-sn-Glycero-3-Phosphocholine) decreased K_D^1 value one thousand fold at pH 6.2 and promoted a decrease in K_D^2 at pH 7.4. We characterized a strong interaction of PazePC with cyt*c*, which may explain the decrease in the K_D^2 values. The presence of POPCox (POPC containing hydroperoxide) also diminished the K_D^1 value but caused a significant increased K_D^2 values in both pHs. The expressive in K_D^2 in the presence of POPCox can be related to detachment process of cyt*c* from IMM and consequently to trigger the apoptotic process. We have also

observed that POPCox reacts with heme iron of cyt*c* bound to membrane, generating hydroxyl radical, which may further damage the IMM facilitating the release of cyt*c*. Supported by FAPESP and CNPq.

D2.22
Self-loading of the mitochondrial replicative helicase TWINKLE onto circular ssDNA

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Helicases are motor proteins that catalyze the separation of nucleic acid duplexes using energy released by the hydrolysis of nucleoside triphosphate (NTP). This unwinding activity is crucial for many nucleic acid metabolic processes, especially for DNA replication. Replicative helicases assemble into ring-shaped hexamers that typically have a ring structure where the central channel accommodates one DNA strand while the second strand is excluded. One consequence of the ssDNA binding in the central channel is that replicative helicases have to be loaded onto the ssDNA during initiation of replication. Many helicases need accessory proteins, helicase loaders, to assemble the monomers into hexamers around ssDNA or to break up the hexameric ring structure. There are also examples of helicases that can load onto DNA without assistance of a loading factor. One of these helicases is the bacteriophage helicase T7 gene 4 protein (gp4). TWINKLE is the only known replicative helicase involved in replication of human mitochondrial DNA (mtDNA) and displays high sequence similarity to T7gp4. TWINKLE can unwind short stretches of dsDNA in a 5′to 3′ direction. Together with other mitochondrial replicative proteins TWINKLE form a processive replisome *in vitro* that can synthesize ssDNA molecules of 16 kb. Moreover, TWINKLE is a very stable hexameric helicase in solution which raises the question of how TWINKLE is loaded on ssDNA. We show that TWINKLE is able to load onto circular ssDNA *in vitro* without help from a loading factor and support DNA replication on a circular dsDNA substrate.

D2.23
Propolis reduces mitochondrial oxidative stress but not cell death induced by doxorubicin in melanoma cells

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Propolis is a natural honeybee product resinous and dark coloured, gathered by bees from leaf buds of various tree species. We recently demonstrated that propolis extract protected heart and liver tissues from oxidative stress by protecting the mitochondria. Besides, it has also been shown that propolis induced human melanoma cell death and that this effect is comparable to doxorubicin effect, a clinical anticancer drug. Then, we wondered whether propolis extract would modulate doxorubicin-induced toxicity by reducing oxidative stress in melanoma cells. In this study, we first confirmed that propolis extract inhibited significantly superoxide anion $O_2^{\cdot}$ production from isolated mitochondria. We then demonstrated that propolis extract had a strong capability to scavenge O_2^{-} production using the WST-1 test on human and murine cultured melanoma cells. Additionally, propolis extract at 10 μg/ml exhibited a cytotoxic effect on melanoma cells comparable to doxorubicin used at 3 μM. However, although the combined effects of propolis extract and doxorubicin reduced mitochondria O_2^{-} production, co-treatment of melanoma cells with propolis extract did not protect from doxorubicin-induced cell death. These results suggest that mechanisms not dependent on the production of ROS are involved in doxorubicin induced apoptosis in melanoma cells.

D2.24
Mitochondrial RNA polymerase is needed for activation of the origin of light-strand DNA replication

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Mitochondrial DNA is replicated by a unique enzymatic machinery, which is distinct from the replication apparatus used for copying the nuclear genome. We have examined the mechanisms of origin-specific initiation of lagging-strand DNA synthesis in human mitochondria. We demonstrate that the mitochondrial RNA polymerase (POLRMT) is the primase required for initiation of DNA synthesis from the light-strand origin of DNA replication (OriL). Using only purified POLRMT and DNA replication factors, we can faithfully reconstitute OriL dependent initiation *in vitro*. Leading strand DNA synthesis is initiated from the heavy-strand origin of DNA replication and passes OriL. The single stranded OriL is exposed and adopts a stem-loop structure. At this stage, POLRMT initiates primer synthesis from a poly-dT stretch in the single-stranded loop region. After about 25-nt, POLRMT is replaced by DNA polymerase γ and DNA synthesis commences. Our findings demonstrate that POLRMT can function as an origin specific primase in mammalian mitochondria.

D3 – Endocytosis

D3.01
Functional Caveolin-1 is required for Hepatitis B virus infection

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Investigation of the entry pathways of hepatitis B virus (HBV) has been hampered by the lack of versatile *in vitro* infectivity models. Most concepts on HBV infection come from the more robust duck HBV system; however, whether the two viruses use the same mechanisms to invade target cells is still a matter of controversy. In this study, we investigate the role of an important plasma membrane component, caveolin-1 (Cav-1), in HBV infection. Caveolins are the main structural components of caveolae, plasma membrane microdomains enriched in cholesterol and sphingolipids, which are involved in the endocytosis of numerous ligands and complex signaling pathways within the cell. We used the HepaRG cell line permissive for HBV infection to stably express dominant-negative Cav-1 and dynamin-2, a GTPase involved in vesicle formation at the plasma membrane and other organelles. The endocytic properties of the newly established cell lines, HepaRG(Cav-1), HepaRG(Cav-1Δ1-81), HepaRG(Dyn-2), and HepaRG(Dyn-2K44A), were validated using specific markers for different entry routes. The cells maintained their properties during cell culture, supported differentiation, and were permissive for HBV infection. The levels of both HBV transcripts and antigens were significantly decreased in cells expressing the mutant proteins, while viral replication was not directly affected. Chemical inhibitors that specifically inhibit clathrin-mediated endocytosis had no effect on HBV infection. We therefore concluded that functional Cav-1 is essential for HBV to initiate productive infection in HepaRG cells which strongly sugest that the virus is using Cav-1-mediated endocytosis to enter the host cells.

D3.02
Evaluation of hyperbranched polyester as a drug carrier system for intestinal cells

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Boltorn® H40 (BH-40) is a biodegradable, non-toxic polymer with 64 terminal hydroxyl groups (7,300 MW) that has a highly branched polyester structure. When this dendritic polymer functionalized with polyethylene (PEG) it becomes a water-soluble unimolecular micelle. This *in-vitro* study aimed to: (i) Evaluate the BH-40 aliphatic polyester as a nanocarrier of drugs in Caco-2/TC7 epithelial model cells. (ii) Explore the factors responsible for improved adsorption and uptake of these nanoparticles. As we assume that the structure and physiochemical properties of nanoparticles affect their pharmacodynamic and pharmacokinetic behavior, we have evaluated the uptake characteristics of four variants of FITC labeled BH-40 nanoparticles differed by their size (6730–24780 MW), charge (7–32 carboxylated groups), and PEG groups (0–3). All the four nanoparticles were found to be non-toxic to cells up to 500 μM at 37°C. FACS analysis and confocal microscope images showed a different extent of uptake levels that were time, charge and temperature dependent – in favor of the BH-40 bears the smallest amount of carboxylic groups. These nanoparticles are accumulated in the lysosomal compartments following exposure of 24 hours. When cells exposed to nanoparticles in presence of cyclosporin A (a non-specific multi drug resistance (MDR) blocker), the uptake was significantly elevated suggesting that BH-40 is effluxed by MDR transporters. BH-40-7C (with seven additional carboxylated groups) linked to artesunate (anti-cancer drug) was fivefold more toxic to cells than free artesunate. These results suggest that BH-40 based nanocarriers could potentially be used as an oral delivery vehicle of drugs.

D3.03
Characterization of murine macrophages eliminating different types of dying neutrophil granulocytes

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Macrophages can efficiently eliminate dying neutrophil granulocytes preventing development of inflammation. It is well known how macrophages can engulf apoptotic and necrotic cells separately; however, it is still unclear how they behave when different types of dead cells are present in their environment simultaneously. We have investigated both bone-marrow derived and peritoneal murine macrophages and have initiated different types of cell death forms: 1. Spontaneous apoptosis, 2. Spontaneous necrosis induced by serum deprivation; 2. Heat-induced necrosis; 3. Necroptosis induced by a pan-caspase inhibitor, the Z-VAD.fmk. We have characterized the death processes of neutrophils, and performed phagocytosis assays. It has been demonstrated that cells undergoing spontaneous necrosis are engulfed by macrophages most efficiently compared to other death types. We have found that different forms of dead cells have no influence on each other's uptake by macrophages when they were applied simultaneously in phagocytosis assays. Our results suggest that the engulfment of several phagocytosis substrates present together is a more complex molecular process than just a simple competition between the substrates and uptake could be inhibited by recombinant Annexin V. We have also investigated how production of pro-inflammatory cytokines (IL-6 and Il-1β) is changed during the phagocytosis processes in co-incubation assays. Our results may contribute to our better understanding on the development of some inflammatory and autoimmune diseases.

D3.04
Structural studies of the human tetraspan vesicle membrane proteins

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Integral membrane proteins of the tetraspan vesicle protein (TVP) family are highly abundant components of different vesicle types. They are believed to be involved in various aspects of the synaptic vesicle cycle, including vesicle biogenesis, exocytosis and endocytotic recycling. It is suggested that TVPs provide a core set of components required for a minimal vesicle that could serve as a matrix for assembly of specialized vesicular carriers in different cells. This class of proteins is characterized by four trans-

membrane segments with an M-shaped topology. They are grouped into three distinct families: (i) gyrins, (ii) physins and (iii) secretory carrier-associated membrane proteins (SCAMPs). Multiple interactions of TVPs with lipids (mainly cholesterol) and components of the recycling machinery like the SNAREs, dynamin, adaptor proteins and eps15 homology (EH)-domain proteins have been described. To succeed in structure determination of membrane proteins, a robust expression and purification protocol for TVPs was established to guarantee homogeneous high quality material for characterization and crystallization. New methods like automated analytical gelfiltration and static light scattering were implemented for that purpose, which allow the expression and purification of mg quantities of members of the gyrin and SCAMP family up to now. TVPs lack the presence of larger folded soluble domains, which otherwise might be favourable in crystallization. To increase the chance for crystal formation, antibody fragments were selected and complexed with TVPs for crystallization. Known interactions partners were cloned and used together with TVPs to improve their crystallizability.

D3.05

Abstract withdrawn

D3.06
ITSN2 adaptor protein: functional comparison and impact on early development

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Adaptors of intersectin (ITSN) family serve as scaffolds for multiprotein complex assembly during endocytosis. Whereas most analyses dealt with ITSN1, participating in cytoskeleton rearrangements, cell signaling and survival, little is known about ITSN2. Our research is focused on ITSN2. We demonstrated significant colocalization of ITSN2 and ITSN1, and showed that endogenous proteins coimmunoprecipitated but did not interact. Study of ITSN2 interactions revealed that most protein partners of ITSN1 are common – dynamin 1, SOS1, Synj1, N-WASP, c-CBL – but the specificities in binding are observed. Interaction with regulator of RTK signalling CIN85 was shown only for ITSN1. We found new ITSN partners – Sema6A implicated in axon guidance, Reps1/2 adaptors and ubiquitin ligase inhibitor Spr2. Thus, intersectins function in common protein complexes and provide different interfaces for partners from endocytosis, signaling and cytoskeleton rearrangements processes. Furthermore, we used Xenopus model and microinjection of mRNAs encoding different ITSN2 domains in embryos. The strongest effect of hyperpigmentation and gastrulation failure was observed in case of DH/PH domains expression. Actin cytoskeleton could be the target of DH/PH action, as both pigmentation and gastrulation movements require accurate regulation of cytoskeleton dynamics. DH/PH expression induced aberrant actin distribution arguing for this hypothesis. Moreover using Cdc42 mutants we showed that observed phenotype was Cdc42-dependent. Obtained results suggest a possible role of ITSN2, mediated through Cdc42, in the coordinated changes of actin cytoskeleton during early embryonic development of vertebrates.

D3.07
Functional characterization of adaptor protein ITSN1 isoforms

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Our research is focused on investigation of adaptor protein intersectin 1 (ITSN1) and plethora of its isoforms in cells of vertebrates. ITSN1 is engaged in clathrin-mediated endocytosis, mitogenic signaling, axonal growth, spine development and actin cytoskeleton rearrangements. We investigated neuron-specific isoform of ITSN1. It is expressed since the beginning of nervous system development and is conserved from fish to human. Neuron-specific inclusion of microexon changes binding specificity of the SH3A domain of ITSN1. We studied how neuron-specific alternative splicing controls specificity of the SH3 domains of ITSN1 and Src towards dynamin 1. Inclusions encoded by the microexons affect distribution of charged amino acids in the interface of interaction of the SH3 domains. Also the novel isoform ITSN1-22a with alternative C-terminus was characterized. Expression analysis of ITSN1-22a revealed its presence in all tissues tested, but in comparison to ITSN1-s levels of expression were significantly lower. The appearance of the ITSN1-22a was presumably due to mobile elements transpositions during early evolution of mammals. We showed that ITSN1-22a forms complex *in vivo* and codistributes with ITSN1-s isoform and is also engaged in clathrin-mediated endocytosis but possesses distinctive features. Alternative C-terminus of ITSN1-22a is engaged in isoform homodimerization via disulphide bonds. Moreover, it provides specific interactions with amphiphysin 1 and by intramolecular binding controls interactions of the SH3A domain of ITSN1-22a. Thus, neuron-specific isoform modifies binding of ITSN1 in brain whereas ITSN1-22a could be regulator of ITSN1-s function in mammals.

D3.08
Endocytosis and traffic of human lactoferrin in human promonocytic cell line THP-1

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Lactoferrin, an iron-binding glycoprotein of the transferrin family, is a critical component in mediation of immune response, especially for coordinated interactions between innate and adaptive components and associated responses. Different cell types have been reported to internalize lactoferrin by specific or non-specific receptors. Our studies focused on the endocytic pathway of human lactoferrin in macrophage like cells. Lactoferrin was found to bind specifically to human promonocytic cell line THP-1 treated with phorbol myristate acetate, as shown by flow cytometry and live fluorescence microscopy performed at 4°C for 1h of incubation. Kinetic studies performed at 37°C revealed that lactoferrin is rapidly internalized into cells, the maximum fluorescence being recorded after 2 hour of incubation. In an attempt to identify the mechanism responsible for lactoferrin internalization, endocytosis inhibitors were used. THP-1 cells treated with chlorpromazine and dansylcadaverine, inhibitors of clathrin-dependent endocytosis led to 50% inhibition of lactoferrin internalization compared with untreated cells. Endocytic uptake of human lacto-

ferrin was also cholesterol-dependent as resulted after methyl-beta-cyclodextrin treatment. Bafilomycin A1 treatment demonstrated that the internalization of lactoferrin might use of acidic endosome-like organelles. Following lactoferrin internalization, we observed partial co-localization of lactoferrin and early endosome antigen in vesicular/endosomal – like structures. Our results suggest that lactoferrin is internalized primarily via clathrin-dependent pathways in THP-1 cells, but need further studies to address this issue.

D3.09
Molecular studies and 3D electron microscopy to reveal components of phagosome maturation

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Our extensive bioinformatic analysis of evolution and phylogeny of Rab7 proteins indicate that they evolved before radiation of main eukaryotic supergroups and numerous Rab7 copies were identified in some unicellular eukaryotes suggesting both the functional specialization of these proteins and complexity of membrane trafficking in these species (Mackiewicz and Wyroba 2009). A model to study this problem is *Paramecium* in which paralogous genes encode 98% identical Rab7 copies. Mass spectroscopy, 2D gels, immunoprecipitation with specific antibodies (Abs) and 3D localization were used to study Rab7 role in phagolysosomal system. MS confirmed that each Ab precisely recognized only one of the two Rab7 proteins and their localization was distinct. RNAi of rab7a gene inhibited phagocytosis and impaired acidification due to the lack of proton pomp. Silencing of rab7b gene had no this effect and its protein product is modified postranslationally and seems to acquire a new function. Rab7a was detected in the phagosomal compartment where it co-localized with its putative effector RILP1-like protein in the primary and secondary lysosomes. The nature of these Rab7a positive vesicles was also confirmed by the presence of the lysosomal markers with Abs against LAMP2 and cathepsin L in double immunogold labeling that was proved by tomographic analysis in JEM1400 (Olympus iTEM software). Taken together these results show that tripartite machinery: Rab7, RILP and LAMP2 required for phagosome maturation in mammalian cells, as proposed by Huynh and coworkers (2007), seems to appear early in eukaryote evolution. Supported by grant N N301 256836 of the Ministry of Science and Higher Education

D4 – Nucleus

D4.01

Abstract withdrawn

D4.02
RNA polymerase I directed synthesis and expression of polycistronic viral RNA

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During last years we gathered enough evidence that RNA synthesized under control of different promoter types recognized by polymerase (Pol) types I, II and III in plants share different synthetic and export pathways. Contrary to this RNA synthesized by same Pol type tend to compete for processing and export, and this is particularly true with Pol II-directed RNAs (mRNA, miRNA). Here we put forward the question whether Pol I could provide synthesis of mRNA ($mRNA^{Pol\ I}$), would it be then properly processed, exported to cytoplasm and translated. To study this we designed Pol I promoter-based transient system for Tobacco mosaic virus RNA – a GFP encoding TMV-based viral vector that is delivered to a host plant by *A. tumefaciens*. Our studies demonstrated that Pol I can provide synthesis of significant amount of 6 kb $mRNA^{Pol\ I}$, and that it can be successfully transported to the cytoplasm, since GFP synthesis can be observed. TMV genomic $mRNA^{Pol\ I}$ neither shares $mRNA^{Pol\ II}$ processing pathway nor undergoes splicing and capping which is likely to explain low level of GPF accumulation. Yet joint expression of TMV genomic $mRNA^{Pol\ I}$ with Pol II-directed TMV 126k capping protein resulted in slight GFP production increase. However, co-expression of TMV 126K capping protein and silencing suppressor TBSV p19 stimulated virus-directed GFP production more than 100-fold. We concluded that despite the lack of capping for $mRNA^{Pol\ I}$ transcripts, the amount of this RNA exported to the cytoplasm was sufficient for the initiation of TMV replication and GFP production.

D4.03
Vinerolbine can affect the chromatin proteins of alveolar macrophages

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Vinorelbine is a semi synthetic vinca alkaloid antitumor drug, which is used in the treatment of some cancers such as advanced nonsmall cell lung cancer, breast cancer and ovarian epithelial cancers. Vinerolbine suppresses depolymerization of mitotic microtubules and destroys mitotic spindle so it can block division of cancer cells in mitosis. In the present study the effect of vinerolbine on chromatin components of alveolar macrophages was investigated using SDS and agarose gel electrophoresis and western blot techniques. Alveolar macrophage were obtained by lavage, and after preincubation, the cells were treated with various concentrations of vinerolbine for 6 hours. Histones and high mobility group non histone proteins (HMG) were then extracted and analyzed. The results show that increasing drug concentration reduced viability of the cells thus at 80 mic g/ml nearly 68% viability is obtained. SDS-PAGE demonstrates aggregation of histone proteins by drug action, when vinerolbine concentration exceeded higher than 20 mic g/ml. Western blot analysis against histones and HMG proteins antisera confirmed the result. DNA extracted from the drug treated and the controls also exhibited compaction of DNA in a dose dependent manner. From the results it is concluded that the effect of vinerolbine on chromatin proteins of alveolar macrophage is dose dependent and binding of drug to chromatin induces chromatin compaction thus the proteins are not extractable with usual procedures.

D4.04
Comparision of the effect of heavy metals lead, nickel and cobalt on chromatin ýproteins of hepatocyte nuclei

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Heavy metals are environmental pollutants and their application in developing technology and citizens have increased. In the present study we have compared the effects of lead, nickel and cobalt on chromatin proteins in hepatocytes nuclei employing SDS, agarose gel electrophoresis. and western blotting techniques. The results showed that upon addition of metals to hepatocytes nuclei turbidity is occurred which could be monitored at 400 nm. Turbidity is increased as metal concentrations is raised and lead and nickel were more potent compared to cobalt. Analysis of the histone proteins extracted from the metal treated samples and the control revealed that the content of core histones, H1 and H1 were decreased upon addition of lead and nickel whereas in the case of cobalt the histones pattern remained unchanged. Western blots were also confirmed the results. The metals also bind to high mobility group proteins, HMG B, but not to HMG N. In the presence of metals the DNA pattern of nuclei digested with MNase was decreased as metals concentration, were increased. From the results it is concluded that lead and nickel bind to chromatin proteins and reduced their extractability by common procedures, suggesting that their binding to chromatin may proceed the chromatin into condensation which inhibits DNA and RNA synthesis in the cell.

D4.05

Abstract withdrawn

D4.06
Induction of the cold shock protein YB-1 as a result of the hypoxic development in human brain cancer

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Y-box proteins belong to a family of DNA- and RNA-binding factors, also called cold shock proteins that are highly conserved

during evolution and have been shown to function as regulators of gene transcription and translation. A wide range of nucleic acid structures are reported to be specifically bound by Y-box proteins, most of which harbor an inverted CCAAT-box (ATTGG) as the core binding site. HIF-1α is the main the regulator of several hypoxia induced genes.

Materials and methods: YB-1 regulation level was examined in human glioblastoma cells lines like U373, U251, GaMG and U87-MG under extreme hxpoxic oxygenation conditions (0.1% oxygen), reoxygenation after hypoxia for 24 and 48 hour and oxygenated conditions (21% oxygen and 5% carbon dioxide) *in vitro*. Protein and mRNA level were detected via western blots and RT-PCR. Cells incubated for 24 hour with 100 μM DFO served positive control for hypoxia and β-actin served as loading control, respectively.

Results: YB-1 was over expressed via hypoxic oxygenation development in different glioblastoma cells micromilieu *in vitro* under extreme hypoxic conditions (0.1% oxygen or reoxygenation after hypoxia, both on protein and mRNA level. Further, there was an association between YB-1 expression and the hypoxia induced HIF-1α and the regulated genes in human glioblastoma tumor specimes examined, *in vitro*.

Conclusions: YB-1 is upregulated as an answer to hypoxic oxygenation conditions development in glioblastoma micromileu, both on protein and mRNA and can be considered as a coregulator of HIF-1α and as a consequence the genes that are regulated by the hypoxic HIF-1α pathway in cancer human cells.

D4.07
THAP11 and HP1BP3 proteins are component of the HB-2.8 enhancer-blocker located in the human c-Myc chromatin domain

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Insulators are DNA elements that block the extension of a condensed chromatin domain into a transcriptionally active region or prevent the interaction of a distal enhancer with a promoter when placed between the two. Human c-Myc and Pvt-1 genes map close each other and are separated by an intergenic region rich of DNAseI hypersensitivity sites. Starting from the observation that PVT1 expression is restricted to a low number of normal tissues compared to the wide distribution of c-Myc mRNA we focus our studies on the function and structure of the regions surrounding the DHs present between the two genes. Stable and transient transfection, indicate that one of the regions (HB-2.8) has enhancer blocking activity. Further evidences indicate that HB.2.8 blocks the activity of c-Myc distal enhancer (PA-A) on the Pvt-1 promoter, suggesting that c-Myc and Pvt-1 genes are insulated in two distinct chromatin. A Matrix-attachment region (MAR) has been mapped in close proximity of the HB-2.8 region and we found it is associated to the core element responsible for the HB-2.8 enhancer-blocking activity. By means of DNA affinity chromatography and proteomic analysis we characterized two proteins interacting with HB-2.8, from nuclear extract of Hela cells: THAP11 and HP1BP3. Overexpression of THAP11 inhibits growth of different cells types and its function is mediated by its ability to repress transcription of c-Myc. HP1BP3 is a component of heterochromatin and may be involved in chromatin structure. Functional characterization of these proteins may contribute to define MYC chromatin domain.

D4.08
The nuclear compartimentation of glutathione: effect on cell cycle progression and chromatin condensation

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One of many functions of GSH is control of cell proliferation, possibly by modulation of redox sensitive cell cycle regulatory proteins and telomerase activity. We have studied the importance of nuclear compartimentation of glutathione (GSH) in the proliferation of 3T3 fibroblasts for a period of 5 days. Cells were treated with diethyl maleate (DEM) that depletes GSH in the whole cell and buthionine sulfoximine (BSO) which preserves the nuclear pool of GSH. The cell cycle progression was delayed in the cells treated with DEM, when the nuclear GSH was depleted. We found lower level of proliferating cells, Id2 protein expression, and glutathionylated nuclear proteins and increase in level of oxidized nuclear proteins comparing to control and BSO treated cells. The cells with high nuclear GSH, in G1/S phase, showed sparse chromatin pattern, while the depletion of nuclear GSH caused chromatin condensation, which was reverted by GSH ester. Our results demonstrate for the first time that nuclear GSH levels plays a decisive role in cellular proliferation, possibly by favouring the chromatin decompactation during G1/S transition. The pattern and level of glutathiolation of nuclear proteins change both along the cell cycle and with the depletion of nuclear GSH, which points to the possible implication of this modification in the control of chromatin structure dynamics. Our report underscores the important role of nuclear glutathione in cell physiology and suggests that manipulation on nuclear GSH levels could be of paramount importance during development and cancer.

D5 – Chloroplasts

D5.01
Chloroplast thylakoid transporters

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The aim of this study is to identify and functionally characterize solute transporters from the chloroplast thylakoid membrane of *Arabidopsis thaliana*. As compared to chloroplast envelope transporters, much less information is available for transport processes across the thylakoid membrane, which is mostly studied as the site of light-driven photosynthetic reactions coupled to ATP synthesis. Although there are many reported examples of transport activities, only a few thylakoid transporters have been identified at the gene level. Using bioinformatics analyses, we have predicted the existence of approx. fifteen thylakoid transporters. For experimental validation, we have carried out immuno-localization studies used peptide-specific antibodies, functional analyses in heterologous system and validation using knockout mutants. We have recently identified one ATP/ADP carrier (Thuswaldner et al. JBC 2007) and one Na(+)-dependent phosphate transporter (Ruiz Pavon et al. JBC 2008). They are proposed to participate in the nucleotide metabolism in the thylakoid lumen (Spetea et al. PNAS 20004) as well as to balance the transthylakod proton electrochemical gradient storage. Based on phenotypic analyses of knockout mutants, we will present novel data about the key physiological role of the two transporters during the high-light-induced repair of photosystem II complex in the thylakoid membrane. Subsequently, we will make a survey on the outlook of thylakoid activities awaiting identification of responsible proteins. Such knowledge is necessary to understand the thylakoid network of transporters, and its role in photosynthesis and adaptation to environmental stress.

D5.02
Chloroplast protease AtDeg5 is involved in wounding-related degradation of PsbF apoprotein

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Deg5 is a serine type chloroplast protease found to be peripherally attached to the lumen side of thylakoid membrane. Knowledge of the enzyme's function is limited to its possible involvment in degradation of photodamaged PsbA in *Arabidopsis thaliana*. Given the lack of knowledge concerning potential AtDeg5 involvment in disposal of chloroplast proteins damaged during plant exposition to stresses other than elevated irradiance we have sought to determine the function of the enzyme in degradation of PsbA-F apoproteins mediated by brief (3 hour) treatment with high salt, wounding, desiccation, cold and heat. In order to reach this goal insertion line devoid of AtDeg5 was prepared. Immunotitration of PsbA-F in wild type plants showed that all the apoproteins except for PsbB underwent remarkable decrease in abundance in response to prevailing majority of stresses. When thylakoids isolated from leaves of wild type plants which had previously faced brief treatment with the five stresses were incubated in darkness for 6 hour further disappearance of PsbA and C-F took place. It was demonstrated that PsbA decline in thylakoids isolated from high salt, desiccation and wounding – treated leaves as well as PsbF decline in thylakoids isolated from wounding-exposed leaves was remarkably reduced in the presence of aprotinin, a typical inhibitor of serine-type proteases, including AtDeg5. In contrast to a pronounced decline in PsbF which took place *in vivo* in leaves of wide type plants exposed to wounding, the apoprotein resisted disappearance in deg5 mutant. Clearly, therefore, AtDeg5 is specifically involved in wounding-related hydrolysis of PsbF.

D5.03
Modeling and Mutational analysis of Anion transporter 1 protein of *Arabidopsis thaliana*

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The thylakoid anion transporter 1 (ANTR1) from *Arabidopsis thaliana*, has been characterized as a Na-dependent Pi transporter when expressed in *E. coli* (1), but no data is yet available for the protein structure and amino acids involved in transport of Pi. In this study a three-dimensional structural model of ANTR1 was constructed in silico using the crystal structure of glycerol-3-phosphate/phosphate antiporter from *E. coli* as a template. Based on Multiple Sequence Alignments (MSAs) with other plant ANTRs and mammalian SLC17 homologues, five highly conserved amino acids involved in Pi transport have been identified, namely Arg-120, Ser-124 and Arg-201 inside the putative translocation pathway, Arg-228 and Asp-382 exposed at the cytoplasmic surface of the protein. The activity of the protein as a Na-dependent Pi transporter in the wild type and mutants was analyzed by heterologous expression and uptake of radioactive Pi into *E. coli* cells. Substitution of the three Arg (120, 201 and 228) for Glu residues and of Asp-382 for an Asn residue resulted in an inactive ANTR1 transporter. All other mutants had sufficient activity to allow measurement of kinetic parameters, attesting that the mutated proteins were functional. Based on our results, we propose that Arg-201 is a critical residue for substrate binding and translocation, whereas Ser-124 may function as periplasmic gateway for Na^+ ions. Residue Arg-120 plays an important role in Pi binding and associated conformational changes, and finally that Arg-228 and Asp-382 only weakly participate in interactions allowing conformational changes to occur at the cytoplasmic surface of the transporter.

D5.04
The chloroplast protein CPSAR1, dually localized in the stroma and the inner envelope membrane, is involved in thylakoid biogenesis

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Thylakoid biogenesis is a crucial step for plant development involving the combined action of many cellular actors. CPSAR1 is shown here to be required for the normal organization of mature thylakoid stacks and ultimately for embryo development. CPSAR1 is a chloroplast protein that has a dual localization in

the stroma and the inner envelope membrane according to microscopy studies and subfractionation analysis. CPSAR1 is close to the Obg nucleotide binding protein subfamily and displays GTPase activity as demonstrated by *in vitro* assays. Disruption of CPSAR1 gene via T-DNA insertion results in an arrest of embryo development. In addition, transmission electron microscopy analysis indicates that mutant embryos are unable to develop thylakoid membranes and remain white. Unstacked membrane structures resembling single lamellae accumulate in the stroma and do not assemble into mature thylakoid stacks. CPSAR1 RNA interference induces partially developed thylakoids leading to pale green embryos. Altogether, the presented data demonstrate that CPSAR1 is a protein essential for the formation of normal thylakoid membranes and suggest a possible involvement in the initiation of vesicles from the inner envelope membrane for the transfer of lipids to the thylakoids.

D5.05
Characterization of CPARF1 and its potential role in vesicular membrane transport in chloroplasts

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The aim of the project is to better understand how the thylakoid membrane in initially formed and maintained. More specifically, we aim to further scrutinize the chloroplast localized vesicular transport system, similar to that responsible for membrane transport between the ER and the Golgi. The thylakoid membrane of higher plant chloroplast is made of membrane lipids synthesized in the chloroplast envelope. As the inner envelope membrane and the thylakoid are separated by the aqueous stroma, a system for transporting newly synthesized membrane material from the inner envelope membrane to the thylakoid is required. A study using the Arabidopsis genome and web-based subcellular localization prediction tools, reported several putative chloroplast-localized proteins with a high sequence similarity to components of cytosolic vesicular trafficking. After having established the predicted chloroplast- localization of the putative chloroplast vesicular trafficking components CPARF1, the major goal is to identify the molecular events that drive chloroplast localized vesicular transport. Both *in vivo* and *in vitro* experiments are to be used.

D5.06
Function and regulation of the protein import translocon of the inner envelope membrane of chloroplasts: translocation channel(s)?

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Most chloroplast proteins are encoded for on the nucleus and have to be transported into the organelle after translation in the cytoplasm. The Toc and Tic machineries (Translocon at the outer/inner envelope membrane of chloroplasts) mediate the import of these proteins across the chloroplast membranes. Tic110 is the central component of the Tic translocon. We were able to demonstrate that it constitutes a cation-selective channel that is sensitive to the presence of chloroplastic transit peptides. With the help of computational studies, limited proteolysis experiments coupled with mass spectrometry and cysteine modification analysis, we were furthermore able to establish a topological model for Tic110. Finally, we could identify Tic110 as a Trx target, as it was shown to possess a redox active disulfide bridge, which can be reduced by stromal thioredoxins *in vitro*. Another Tic component, Tic20 has also been proposed to form a channel in the inner envelope membrane of chloroplasts. Both Tic110 and Tic20 are clearly important for plant viability and preprotein translocation, but there are neither electrophysiological nor biochemical data supporting that Tic20 can form a channel. Here we demonstrate that we successfully established systems to express and purify full-length Tic20 and were able to determine its secondary structure and topology. Further experiments were designed to verify the notion that Tic110 and Tic20 function together in the import process.

D5.07
An interplay between chloroplastic N-terminal methionine excision and FtsH protease is essential for normal chloroplast development and function

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N terminal Met excision (NME) is the earliest proteolytic event affecting proteins whilst they are still bound to the ribosome. About two thirds of all proteins in any organism are subjected to this type of processing, and all cell compartments where protein synthesis occurs contain dedicated NME machinery. Recent studies have shown that developmental defects, induced in the model plant Arabidopsis thaliana by inhibition of cytoplasmic or chloroplastic NME, are accompanied by an increase in cellular proteolytic activity. Whereas there is increasing evidence to support a tight connection between NME and protein degradation, the identity of the proteases involved has been unknown. Here we report that chloroplastic NME acts upstream of the nuclear-encoded chloroplastic FtsH metalloprotease. The chloroplast developmental defects and their higher sensitivity to photoinhibition, observed in plants lacking the FtsH2 protease, are abolished when chloroplastic NME is inhibited, either genetically or chemically. Moreover, the characterization of accumulation of D1 and D2 proteins of PSII reaction centre in different genetic backgrounds reveals that their level is strongly dependent on the previous action of NME enzymes. Under standard light conditions, correctly NME-processed D1 and D2 are turned-over primarily by FtsH2 protease. Absence of FtsH2 induces accumulation of the correctly NME-processed D1 and D2. Inhibition of chloroplastic NME compromises the recognition of D1 and D2 by FtsH2 and the unprocessed forms seems to be mainly recognized by other chloroplast proteases. These results highlight the tight functional interplay between NME enzymes and the FtsH protease.

E1 – Synthetic Biology

E1.01

Abstract withdrawn

E1.02

Lactic acid bacteria with surface-displayed binding proteins can bind antibodies and tumor necrosis factor α

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Lactic acid bacteria (LAB) are widely used in the food industry for the fermentation of vegetable, dairy and meat products. LAB also have beneficial health effects, which mainly include alleviation of intestinal diseases and are widely used as probiotics. Their health effects can be strengthened by the introduction of new properties via genetic modification. We have designed LAB which display binding proteins (affibodies) on their surface. Affibodies can be directed against various targets in gastrointestinal tract. We have used B domain of staphylococcal protein A, which is capable of binding Fc region of antibodies, as a model protein. We have prepared and compared several gene constructs for displaying the B domain on the surface of the model lactic acid bacterium *Lactococcus lactis*. Its surface display and functionality were confirmed by flow cytometry and fluorescence microscopy of recombinant lactococcal cells stained with specific or nonspecific antibody. The maximum capacity of the recombinant lactococci for antibody binding was estimated at 0.146 μg of antibody per 10^8 cells. The stability of the displayed B domain was tested against simulated gastric juice and a maximum 14% decrease in binding of antibody was observed after treatment. A TNFα-binding affibody was also displayed, in the same manner. Its ability to bind fluorescently labelled TNFα was confirmed by flow cytometry and fluorescence microscopy. *L. lactis* with TNFα binding capability could be used in amelioration of chronic intestinal inflammations such as ulcerative colitis or Crohns disease, which have a high long-term risk of colon cancer development.

E1.03

Genotoxicity and cytotoxicity effect of cadmium chloride on male mice kidney

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Introduction: Cadmium is one of the most toxic heavy metals in our environments having a very strong ability to accumulate in body organs especially in kidney. Our aim of present study was to determine the Genotoxicity and Cytotoxicity in mice kidneys exposed to cadmium.
Material and method: We sacrificed 30 male mice in this study that randomly divided into three different groups (1control& 3tratment). Every five mice in one cage and remain in standard conditions. After 1 week, we applied peritoneal injection to exposed mice with Cd (300 μm/Kg.b.wt) on 0, 6, 12, 24, 48 hours. Twenty hour after latest injection the mice were killed and their kidney obtained. Then Oxidative stress markers (Malondialdehide (MDA), Glutathione (GSH) and Superoxide Dismutase (SOD)) were assayed on homogenized kidney for studying cytotoxicity effect, and for Genotoxicity& DNA damage studies, we used Comet assay on separated kidney cells. Also for cytotoxicity affect we employed histological tests. Finally, to analyze statistical data we used SPSS15 software.
Results: Present results shown that MDA and GSH concentration and SOD activity in treated group have a significantly increase in compare with control group ($p < 0.01$). The comet assay results obviously showed DNA breakage in treated group that was stimulated by Cd where this is not seen in the control group. Even in histological experiments, we were able to see cellular damage obviously.

E1.04

Oxidative stress and DNA damage in mice brain exposure to Cadmium Chloride

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Introduction: Cadmium is one of the most toxic heavy metals in our environments having a very strong ability to accumulate in body organs especially in kidney. Our aim of present study was to determine the Genotoxicity and cytotoxicity in mice kidneys exposed to cadmium.
Material and method: We sacrificed 30 male mice in this study that randomly divided into three different groups (1control& 3tratment). Every five mice in one cage and remain in standard conditions. After 1 week, we applied peritoneal injection to exposed mice with Cd (300 μm/Kg.b.wt) on 0, 6, 12, 24, 48 hours. Twenty-four hour after latest injection the mice were killed and their brain removed. Then Oxidative stress markers (Malondialdehide (MDA), Glutathione (GSH) and Superoxide Dismutase (SOD)) were assayed on homogenized kidney for studying cytotoxicity effect, and for Genotoxicity& DNA damage studies, we used Comet assay on separated kidney cells. Also for cytotoxicity affect we employed histological tests. Finally, to analyze statistical data we used SPSS15 software.
Results: Present results shown that MDA and GSH concentration and SOD activity in treated group have a significantly increase in compare with control group ($p < 0.01$). The comet assay results obviously showed DNA breakage in treated group that was stimulated by Cd where this is not seen in the control group. Even in histological experiments, we were able to see cellular damage obviously.

E1.05

Endo-α-1,4-polygalactosaminidase structure and evolution

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Family GH114 of glycoside hydrolases (or COG3868) includes endo-α-1,4-polygalactosaminidase [EC 3.2.1.109] from *Pseudomo-*

nas sp. 881 and several dozens of its homologues (http://www.cazy.org/). This family is closely related to functionally uncharacterized COG2342. The latter includes proteins with TIM-barrel type of the three-dimensional structure (PDB, 2AAM). We have revealed 132 non-identical protein sequences of GH114 domains from GenPept database using the blast algorithm. They include representatives of several phyla of Bacteria (Actinobacteria, Aquificae, Chloroflexi, Deferribacteres, Deinococcus, and Proteobacteria), as well as some Eukaryota (Alveolata, Ascomycota, Basidiomycota, Chlorophyta, and Oomycetes). The majority of the proteins contain only one domain (GH114) and have similar length (251–375 amino acid residues). COG2342 was used as outgroup. Three main clusters can be recognized on the GH114 phylogenetic tree. Two of them have high bootstrap support and include mainly representatives of Actinobacteria. The third cluster is formed by proteins from Ascomycota, but they are not very well separated from some bacterial proteins. The tree topology supports the important role of horizontal transfer in the evolution of GH114 proteins. Two very conserved residues (Asp and Glu) most probably are the nucleophile and proton donor in the active center, respectively. Iterative screening of the protein database by PSI-BLAST and the following analysis of the results by PSI Protein Classifier allowed us to reveal relationship of GH114 and COG2342 with GH5, GH13, GH18, GH20, GH27, GH29, GH31, GH35, GH36, GH42, GH66, GH97, GH101, COG1306, COG1649, GHL3, and GHL4 families.

E1.06
Yeast perfume factory: metabolic engineering of *Saccharomyces cerevisiae* for plant isoprenoid biosynthesis

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The production of plant isoprenoids in microbial systems is an environmentally friendly and attractive alternative to the commonly used chemical synthesis and plant extraction. This class of compounds is mainly used by the chemical industry for production of fragrances and aroma chemicals. Isoprenoids represent a diverse class of secondary metabolites, natively present in the metabolic network of *Saccharomyces cerevisiae* and used to satisfy macromolecule precursor demand for biomass production. Isoprenoids are naturally produced in *S. cerevisae* through the mevalonate pathway (MVA). Aim of this project is the de-regulation of the MVA pathway in *S. cerevisiae* to increase the precursor pool for isoprenoid synthesis resulting in an efficient *S. cerevisiae* cell factory for biosynthesis of target compounds. Yeast is metabolically engineered for isoprenoids accumulation by introducing genetic modifications that enable to channel the flux through the isoprenoid pathway thus increasing the levels of the pathway products. Throughout the project different approaches for optimal isoprenoid production modulating the expression of key metabolic steps are investigated and the production capacity of the engineered strains is evaluated through a fermentation process coupling biochemical production of these compounds to biomass formation.

E1.07
Metabolic engineering of *Saccharomyces cerevisiae* for overproduction of plant isoprenoids

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Isoprenoids are becoming industrially important compounds because of their potent applications as anticancer, antitumor, antiviral, antibiotic, nutraceuticals, feed supplements as well as their characteristic flavors and aromas. Because of limitation in synthetic means or low amounts and complex mixtures of natural sources, heterologous microbial production of isoprenoids is therefore considered as a suitable alternative to extraction from plants or chemical synthesis. Here we engineered the yeast *Saccharomyces cerevisiae* as a microbial host for heterologous biosynthesis of two different plant isopreniods, namely santalene and patchoulol. By (i) introducing genetic modifications of the mevanolate pathway that enable the yeast to accumulate high levels of the key intermediate farnesyl diphosphate, (ii) enhancing the sesquiterpene synthase expression level by using codon optimized gene and changing promoter, and (iii) optimized fed-batch fermentation, titers in the g/L range were achieved. The results revealed the potential of yeast as a cell factory for production of plant isoprenoids.

E1.08
Evaluating the heterologous MEP pathway in *Saccharomyces cerevisiae*

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Isoprenoids, which are one of the most frequent natural and chemical compounds with varying roles in organisms, are produced via two different metabolic pathways, the well-known mevalonate pathway and the recently discovered MEP pathway. The 2-C-methyl-D-erythritol 4-phosphate or MEP pathway is found in eubacteria and plants (in plastids). This pathway involves eight enzymatic steps and it is initiated by combining one molecule of each pyruvate and D-glyceraldehyde 3-phosphate to produce IPP or DMAPP as the precursors of all types of isoprenoids. In contrast to the eukaryotic MVA pathway, which has been extensively studied, the MEP pathway is less investigated especially in heterologous hosts. In order to construct a yeast platform for producing varying types of isoprenoids, we introduced all genes encoding the bacterial MEP pathway enzymes including *dsx*, *dxr*, *ispD*, *ispE*, *ispF*, *ispG*, *ispH* and *idi* into the *S. cerevisiae* genome and evaluated the functionality of this heterologous pathway.

E1.09
DNA strand exchange on liposome surfaces – Towards an artificial recombination enzyme

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With the ultimate goal of constructing an artificial recombination enzyme we are interested in the mechanism of DNA strand exchange. This process, *in vivo* performed by proteins in the RecA family, is despite extensive studies not understood in full detail. We therefore want to study the molecular parameters involved in the exchange using an *in vitro* platform. Positively charged liposomes have been widely used as non-viral gene-carriers, where the positive charges attract the negatively charged DNA. We here show that DNA strand exchange is significantly enhanced, both in rate and yield, on the surface of cationic liposomes. The fastest exchange is seen for liposomes containing 35% cationic lipid and when the positive charges on the outer leaflet of the liposomes equal the amount of negative charges on the added DNA. Both increasing and decreasing the amount of DNA slows down the exchange rate, either due to competition for the binding sites or due to that the single-strands on average are further away from destabilized duplexes. By studying the exchange of mismatched DNA we conclude that the DNA opens in a ''zipper-like'' manner on the liposome surface, since a mismatched base-pair in the end of the sequence affects the exchange rate much more than a mismatched base-pair in the middle of the sequence. The liposome surface can be easily functionalized with lipids bearing specific functionalities and we intend to modify the surface to study the effects of hydrogen bonding and/or hydrophobic interactions.

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E1.10
Engineering of the fungal phosphoketolase pathway in the yeast *Saccharomyces cerevisiae,* effects on glycerol overproduction and flux distributions

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As the science of metabolic engineering becomes more and more a reliable tool for reprogramming microbial cellular machinery, we attempted to force the boundaries of a novel approach to increase intracellular levels of cytosolic acetate by expressing the fungal genes from the phosphoketolase pathway (*phk*). This pathway is used by the species *Aspergillus* to deviate the PP fluxes towards the production of the intermediates AcetylCoA or Acetate. Through an elegant approach, whose efficacy was partially evaluated with simulation based on a genome scale metabolic model, we expressed the genes from the phosphoketolase pathway (*xpK* and *acK*), together with other enzymatic activities, in the yeast *S. cerevisiae* and physiologically characterized the recombinant strain. To our knowledge, this is the first time that this pathway is expressed in yeast. The effect of this pathway on the yeast's metabolism was studied both in a wild type *S. cerevisiae* CEN.PK 113-5D strain and in a strain deleted in the glycerol-phosphomutase gene (gpm1). As previously shown, such a deletion mutant, is not capable of utilizing glucose as carbon source. In the work we evaluated whereas the expression of the *phk* genes could restore this growth defect. This has the potential to lead to the development of a first generation strain able to produce glycerol with a theoretical maximum yield from glucose higher as those reported before.

E1.11
A simple, robust and highly efficient transient expression system for production of secreted proteins

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We have developed an optimized mammalian transient expression system, capable of producing up to 700 mg/l of native secreted (glyco)proteins in less than a week. The system is composed of commercially available components and is based on transient expression in the fast growing suspension cell line, HEK-293F. The method depends on an optimal combination of a gene transfer method, an efficient expression vector and cotransfection with expression enhancing plasmids. Optimization of all components of the expression system by Experimental Design Techniques, yielded expression levels of up to 400 mg/l for antibodies (including IgG, IgA, IgM and Fab fragments of various species) and of up to 700 mg/l for over 100 other (glyco)proteins. Expression volumes were scalable from 0.1 ml up to 1.2 l in a simple shaker flask system in animal component free, low protein, medium enabling straightforward purification of relatively large amounts of native protein in a short, 4–5 days, production time. Details of the production method and extensive protein analysis in comparison to material produced from stably transfected CHO cell lines will be presented.

E1.12
Function-Spacer-Lipid (FSL) constructs enable inkjet printing of blood group antigens

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A limitation of inkjet printing antigens to solid surfaces is that the molecule to be printed must be dispersible in solution, retain antigenicity, and remain on the printed surface when exposed to biological solutions. FSL (function-spacer-lipid) constructs were designed to be dispersible in water and to attach a range of synthetic molecules to cell membranes. FSL constructs currently include carbohydrates, peptides, fluorophores, ligands and biotin and are being used in the manufacture of modified cells called ''kodecytes'' for use in blood group typing laboratories and for research purposes (1–2). FSL constructs have now been used to create prototype diagnostic assay kits, using standard desktop inkjet printers, by simply replacing the ink in a spent cartridge with a solution of FSL construct. The FSL constructs can then be printed onto a substrate in much the same way one might print a letter or drawing on a sheet of paper. Blood group FSLs have been printed onto silica and nitrocellulose surfaces, and used to identify blood group antibodies in diagnostic and research samples. Combining FSL technology with existing inkjet printing technology provides a cost effective alternative to existing methods of manufacturing diagnostic test kits, including microarrays which, for the most part, use robotics. In addition to cost savings the versatility of this manufacturing method provides additional benefits for research laboratories and micro-analyses.

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E1.13
Antiradical activity in hydroxylated betalains

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Betalains are natural water soluble pigments present in most plant families belonging to the order Caryophyllales. They can be classified as either betacyanins or betaxanthins. Betaxanthins contain different amino acid or amine side chains, and exhibit yellow-orange coloration. Selected betaxanthins were designed to explore the effect of the presence of phenolic hydroxyl groups in the free radical scavenging activity of betalains under the ABTS + radical assay. Activity shown by betaxanthins was compared with that of Trolox. Effect of pH on the free radical scavenging activity of betalains was studied. The antioxidant activity of betaxanthins was characterized under the FRAP assay. Betaxanthins present high antioxidant and free radical scavenging activities not linked to the presence of phenolic hydroxyls. This may be general to all betalains, which contain a similar electronic resonance system. In addition to their intrinsic activity, a clear enhancing effect on the scavenging of the free radical ABTS + and in the antioxidant power of betaxanthins has been demonstrated to be linked to the presence of one or two phenolic hydroxyl groups in their structure. This work was supported by the Project AGL2007-65907, and by Programa de ayudas a Grupos de Excelencia de la Región de Murcia, de la Fundación Séneca, Agencia de Ciencia y Tecnologia de la Región de Murcia (Plan Regional de Ciencia y Tecnologia 2007/2010) (GERM 2007-2010). F. Gandia-Herrero holds a contract with the ''Programa Ramón y Cajal'' (MICINN, FEDER, Spain).

E1.14
Stability analysis of the main pigment in cactus pears

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Betalains are nitrogenous plant pigments which are present in plants belonging to the order Caryophyllales. Betalains replace anthocyanins and exhibit yellow (betaxanthins) or violet (betacyanins) coloration. Interest in these molecules has grown since their antiradical activity was characterized. In cactus pears, the characteristic betalain is indicaxanthin, a proline derived betaxanthin. Two different lines of prickly pear fruits of deep yellow and violet colors were used to confirm and quantify the presence of indicaxanthin in this edible fruit. In this work, yellow solutions of pure indicaxanthin were obtained and its stability was analysed at 20°C and at different pH values, either in the presence or in the absence of light. The presence of light increased the degradation of indicaxanthin for all the pH values assayed. The free radical scavenging activity of indicaxanthin was evaluated by its effect on stable colored solutions of radical ABTS + and the pH effect on this activity was also evaluated. This pigment exhibited a high free radical scavenging capacity at much lower concentrations than Trolox. The antioxidant activity of indicaxanthin was also characterized using the FRAP assay. This work was supported by the Project AGL2007-65907, and by Programa de ayudas a Grupos de Excelencia de la Región de Murcia, de la Fundación Séneca, Agencia de Ciencia y Tecnologia de la Región de Murcia (Plan Regional de Ciencia y Tecnologia 2007/2010) (GERM 2007-2010). F. Gandia-Herrero holds a contract with the ''Programa Ramón y Cajal'' (MICINN, FEDER, Spain).

E1.15
Stability and antioxidant activity of a maltodextrin encapsulated betalain

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Betalains are water-soluble nitrogen-containing secondary metabolites which are present in plants belonging to the order Caryophyllales. They are pigments widely used as additives in the food industry and are classified in betacyanins (violet) or betaxanthins (yellow). In cactus pears, the characteristic betalain is indicaxanthin, a proline derived betaxanthin first isolated from Opuntia ficus indica fruits. In the present work, indicaxanthin has been obtained pure and its stability has been highly promoted by its encapsulation into a maltodextrin matrix. A suitable spray-drying procedure for encapsulation has been described, obtaining a bright yellow powder. Stability of the encapsulated pigment has been analysed under different conditions. In the absence of light, pure encapsulated pigment can be stored at 20°C for months without appreciable loss of the bioactive substance and colour variation. Free radical scavenging and antioxidant properties of the encapsulated pigment have been studied under the ABTS + radical and FRAP assays. These results demonstrate the high degree of stability of betalains under encapsulation and the maintenance of the antiradical properties. This work was supported by MEC (Spain) and FEDER (Project AGL2007-65907), and by Programa de Ayudas a Grupos de Excelencia de la Región de Murcia, de la Fundación Séneca, Agencia de Ciencia y Tecnología de la Región de Murcia (Plan Regional de Ciencia y Tecnología 2007/2010). F. Gandía-Herrero holds a contract with the ''Programa Ramón y Cajal'' (MICINN, FEDER, Spain).

E1.16
Synthesis and physicochemical characterisation of hemoglobin anionic polyelectrolyte conjugates

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Blood is an important biologic fluid with many diverse properties and functions. One important and unique function of blood is to transport oxygen to tissues [1]. Hemoglobin (Hb), a tetrameric protein consisting of ab dimers, is responsible for the transport of O_2 and has been evaluated as an ideal blood substitute. However, stroma-free Hb solutions suffer from disadvantages of high O_2 affinity, tetrameric dissociation, short intravascular retention time and high colloid oncotic pressure (COP) [2–3]. To circumvent these disadvantages, recent attentions were focused on increasing the molecular dimension of Hb through chemical modification [3–4]. Polymer conjugated Hb is, therefore, one strategy to enlarge Hb molecules [4]. The aim of synthesis of Hemoglobin-Polymer conjugates is to produce oxygen carriers with a high molecular weight and low oxygen affinity. In this study, we conjugated Hemoglobin (Hg) to different anionic polymers. Polymer-Hemoglobin conjugate reaction was prepared with the water-soluble 1-ethyl-3-(3-dimethylaminopropyl) cardobodimide hydrochloride (EDC). We investigated the mechanism for the formation of Hemoglobin-Polymer conjugates different ratio of components by physicochemical analyses methods (High Performance Chromatography, Furier Transfor Infrared Spectroscopy, Ultra-viole Visible Spectroscopy, Refractive Index, Light Scattering, Viscosity Quadruple Detector System 'VISCOTEK).

E1.17
Plasma membrane vesicles as a cell-like model system for studying cell-penetrating peptide – membrane interactions

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Efficient delivery of gene-targeted drugs faces difficult problems due to the hydrophobic and impermeable nature of the cell's plasma membrane. This creates the need for delivery vectors that can promote uptake at the cell surface. Cell-penetrating peptides (CPPs) have emerged as promising vector candidates, due to their ability to deliver macromolecular cargo into cells in a non-toxic manner. The uptake mechanisms of these peptides are despite extensive studies not well-described, but today endocytosis is considered a major pathway. However, it remains to be established which interactions on the cell surface that are responsible for triggering endocytosis. In this study, we have investigated the membrane interactions of the basic and amphipathic CPP penetratin, using plasma membrane vesicles (PMVs) derived directly from live mammalian cells. This is a new model system in CPP research and is attractive because of its cell-representative membrane composition, including cell surface-attached proteoglycans, which are believed to be crucial for CPP uptake. Specifically this study addresses the influence of basic residues by comparing penetratin to its arginine- and lysine-enriched analogues PenArg and PenLys. We show, using confocal microscopy imaging and a quantitative assay to determine binding affinity, that these peptides bind to PMVs in an arginine-dependent manner that correlate directly with uptake levels in live cells. Such correlation has not been obtained in simpler model systems such as lipid vesicles or solubilized proteoglycans. The present results confirm, for the first time, that membrane affinity is indeed a key property for efficient CPP internalization.

E1.18
Thermal Biosynthesis of Homophenylalanine by *Thermus thermophilus* aspartate aminotransferase

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Aspartate aminotransferases (AspAT) can catalyze the reversible transamination reaction from aspartate to α-ketoglutarate to form glutamate and oxaloacetate. AspAT plays an important role in the physiological metabolism of amino acids. It presents a substrate preference of amino acids with carboxyl group and is also found to possess significant activity against aromatic amino acids. Therefore, AspAT can be used to produce L-aspartate for synthesizing sweetener aspartame, and also to produce L-aromatic amino acids for nutrition addition in foods or medicinal purpose. *Thermus thermophilus* is a well-known thermophilic bacterium from hot spring. *T. thermophilus* AspAT (TtAspAT) was cloned and expressed in *E. coli*. We found that TtAspAT possesses high activity (1.64 U/mg at 37°C and 7.66 U/mg at 70°C) against homophenylpyruvate (HPP) to form homophenylalanine (HPA), an important compound in synthesizing some anti-hypertensive drugs, the ACE inhibitors. Enzyme characterization showed that TtAspAT had the highest activity at 70°C and the highest stability at 50°C. A compromised condition was set at 60°C to have better activity and stability, and a thermal biosynthesis method of HPA was developed, including preparing thermostable TtAspAT, optimizing conditions for the amino-transferring reaction in producing HPA, precipitating the product and recycling the used TtAspAT. Kinetic analyses showed that the Km and kcat of TtAspAT against HPP was 13.8 mM and 10.9 s-1, respectively. TtAspAT is of advantage in high thermostabilty and better activity against HPP. TtAspAT may be another good biocatalyst suitable for the production of unnatural amino acids in biopharmaceuticals.

E1.19
Cell-free protein synthesis for NMR spectroscopy applications

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Cell-free protein synthesis is a convenient method for producing preparative amounts of protein for structural biology. Highly specific isotope incorporation for NMR spectroscopy is especially efficient as the absence of an active metabolism decreases the amount of isotopic scrambling. For certain amino acids, however, scrambling as well as unproductive consumption in the cell-free reaction is significant. Only about 15% of the supplied amino acids are currently incorporated into the target protein even in the best reported systems, suggesting a significant potential for improvement by optimization of the existing system as well as by introducing new additives. This is important as the amino acids account for more than half of the costs associated with producing a $^{15}N,^{13}C$-labelled sample for NMR. Deleting genes responsible for these processes enhances the labelling specificity. Here, we show that by adding exogenous inhibitors it is possible to increase expression yield as well as promote selective labelling of aminos acids.

E1.20
Lysozyme microbubbles with new properties for smart applications

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Microbubbles (MB) are small gas-filled colloidal particles that have specific acoustic properties that make them useful as a contrast agent in ultrasound imaging. The use of the MBs in clinical practice led to the development of more sensitive imaging techniques both in cardiology and radiology, including subharmonic and multipulse imaging, pulse inversion and harmonic power Doppler. Protein-based microbubbles are typically obtained by dispersing of gas phase in the protein solution. The protein deposited/cross-linked on the gas/liquid interface stabilizes the gas core. Innovative applications of protein-MBs prompt the investigation on the properties of MBs obtained using different proteins that are able to confer them specific properties and functionality. Recently, growth factor-releasing scaffolds have been also fabricated by incorporating growth factor-releasing BSA-MBs in the scaffold-manufacturing processes. BSA-MBs have been used as a new porogen to produce BSA-coated cell-friendly surfaces in preserving the bioactivity of loaded growth factor. In a previous work, we have synthesized stable lysozyme microbubbles (Lys-MB) using high intensity ultrasound-induced emulsification of a partly reduced lysozyme in aqueous solutions. Both the

hydrophobic nature of the enzyme, to provide foaming properties, and the formation of disulfide bonds are requirements for the formation of stable protein-microbubbles. In the present work a better characterization of Lys-MB obtained was performed demonstrating their biodegradability property. We also present evidence that Lys-MBs can be functionalised by protein, DNA coating and drugs, as carriers for therapeutic applications.

E1.21
Environmental factors enhancing *in vitro* catalysis of DNA strand exchange

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Strand exchange involving single and double stranded DNA is a well-known biological phenomenon and the basis for DNA repair and maintenance of genetic diversity as performed by recombination proteins like RecA and Rad51 *in vivo*. Artificial systems, including lipid vesicles and polypeptides, have also been reported to catalyze strand exchange. However, the basic mechanisms behind strand exchange biocatalysis are not understood despite intense structural and functional studies of both recombination proteins and simple model systems. In order to get a better understanding of the role of environment and water activity at active catalytic sites, we here study the strand exchange kinetics of matched and mismatched fluorescently labeled DNA oligonucleotides, using FRET and CD spectroscopy. Taking advantage of critical effects in crowded media, we have systematically investigated the significance of various factors for catalysis, including balancing of water activity, volume of exclusion, ionic strength and temperature. We report the discovery of specific conditions at which the DNA strand exchange rate is increased by several orders of magnitude, while maintaining B-DNA conformation. Our results are discussed in terms of fundamental principles governing strand exchange, and relationship between stability and activity of the DNA double helix. They may also find practical applications in DNA nanotechnology and gene therapy.

E1.22

Abstract withdrawn

E1.23
Effects of PEGylation and acetylation of PAMAM dendrimers on DNA binding, cytotoxicity and *in vitro* transfection efficiency

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Poly(amidoamine) (PAMAM) dendrimers are promising multipotent gene delivery vectors, providing favourable DNA condensation properties and also the possibility of conjugation of different targeting ligands to their surface. They have been used for transfection both *in vitro* and *in vivo*, but their application is limited due to cytotoxicity. In this work we investigate two kinds of surface modifications, acetylation and PEGylation, and how they affect the DNA binding characteristics, the cytotoxicity and the in vitro transfection efficiency of generation 4 and 5 PAMAM dendrimers. Further, we evaluate how the morphology of DNA-dendrimer complexes changes upon dilution in cell growth medium, which occurs before the complexes reach the cell surface. We find that the surface-modified dendrimers have excellent biocompatibility profiles. However, their transfection efficiency is very low, which is explained by our observation that DNA is not sufficiently condensed by these dendrimers. Although small particles are obtained, the availability for ethidium intercalation and nuclease degradation is significantly higher in the modified dendrimer-DNA complexes than in unmodified ones. Dilution in cell growth medium has a dramatic effect on these electrostatically assembled complexes. Therefore we want to emphasize the importance of performing biophysical characterization under conditions as close to the transfection situation as possible, to enable conclusions regarding structure- activity relations of gene delivery vectors.

E1.24
Uptake mechanisms of cell penetrating peptides: the role of tryptophans and arginines in cell internalization efficiency

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Cell-penetrating Peptides (CPPs) are naturally derived peptides that have been revealed as promising candidates for intracellular delivery of proteins and other macromolecules such as DNA and RNA. Endocytosis is recognized as a major entry pathway for many CPPs, but the cell surface interactions leading to efficient internalization via this route have still not been fully characterized. Our goal is to deepen the understanding of how CPPs enter cells. Many studies suggest that arginines and tryptophans are important for CPP function. We are studying the influence these amino acids have on cell internalization, by comparing a series of new peptides, consisting of arginines and tryptophans in different combinations, with the well studied CPP penetratin in CHO-KI cells by use of confocal microscopy as well as by cytotoxicity measurements. Our results propose that tryptophan content has influence on internalization and cytotoxicity, but also that the patterning of the tryptophans and arginines is of great importance.

E1.25
ClChE: Exploiting stabilized tandem repeats for heterologous expression

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Episomal plasmids have been used to acquire high copy expression of many valuable products and have been widely used in biotechnology industries. Plasmid expression systems require a selective pressure such as antibiotics or auxotrophic markers in order to maintain a plasmid in the cell for consistent production of target products. Here, we present progress in developing a novel, stable, high copy expression system for heterologous genes in *Saccharomyces cerevisiae*. Chemically Induced Chromosomal Evolution (CIChE) achieves high copy number through tandem gene duplication in the genome. By virtue of being on the genome, no selective pressure is required and genetic stability is significantly improved. Homologous recombination facilitates both integration of the CIChE construct into the genome and evolu-

tion process of the CIChE construct via subculturing in increasing concentration of antibiotic. This evolution process offers the advantage to select the daughter cell with higher copy of CIChE construct. Here, we characterize the evolution process of CIChE construct in order to increase the yield of recombinant product compare to high copy plasmid. Productivity and genetic stability of CIChE constructs compared to analogous high copy number plasmids will be determined. The accomplishment of CIChE strategy in yeast will make a great impact on many industrial applications.

E1.26
In vitro studies regarding the interactions of some ruthenium (III) fluoroquinolones complexes with double stranded calf thymus DNA

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Cancer is the second major cause of death after cardiovascular diseases. Therefore developing new antitumor therapies represents a target for the pharmaceutical research worldwide. Numerous biological experiments have demonstrated that DNA is the primary intracellular target of anticancer drugs, due to the interaction between small molecules and DNA, which cause DNA damage in tumor cells, blocking their division and resulting in neoplasic cell death. Cisplatin success in cancer treatment, but also its important toxicity, drove to new transitional metal research, like ruthenium (III). The present paper presents the DNA-binding properties of some ruthenium (III) complexes with fluoroquinolones, namely ciprofloxacin, ofloxacin and norfloxacin. In this regard we investigated *in vitro* the interactions of the ligands studied with double stranded calf thymus DNA through fluorescence emission spectrophotometry. Our results showed that there are interactions between the studied complexes and the double stranded calf thymus DNA, expressed as linear variations of fluorescence intensity for Ru-cpx, exponential variations for Ru-nf and logarithmic for Ru-oflo.

E1.27

Abstract withdrawn

E1.28
Cell-free production of integral membrane proteins for incorporation into nanodiscs and micelles

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One bottleneck in obtaining structures by NMR or X-ray crystallography is that the protein needs to be purified in high concentration. This problem often occurs when the protein is expressed in low concentration in the natural source or causing toxicological problems when overexpressed. Cell-free protein synthesis is not subject to cell toxicity and offers the possibility of manipulating the reaction conditions to stabilize the produced protein. We have produced and purified wide range of integral membrane proteins, including both α-helical and β-barrel integral membrane proteins. By supplying detergents or lipids directly to the reaction mixture the environment can be fine tuned allowing the protein to be synthesised in a solubilized and functionally folded state. Hence, avoiding the need for refolding or extraction from membranes. To find the best condition for a protein one needs to evaluate different lipids and detergents to see which maximizes expression levels of solubilized. An attractive alternative to lipids and detergents are nanodiscs. Nanodisc allows for easy purification and allows access to both sides of the membrane. We have found that nanodiscs can be added to the cell-free reaction allowing proteins to be incorporated as they are synthesized without inhibiting the protein synthesis. Cell-free protein synthesis also provides the possibility of selective incorporation of isotope labelled amino acids. This makes it possible to only label one or a few types of amino acid, greatly reducing the amounts of peaks in a NMR spectra, thus minimizing the amount of overlapping signals and simplifying sequential assignment.

E1.29
[FeFe]-hydrogenases for hydrogen gas production: Evolving natural bio-catalysts for artificial devices

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A recombinant expression system for [FeFe]-hydrogenase was used to produce and characterize *Chlamydomonas reinhardtii* hydrogenase (CrHydA1) and *Clostridium acetobutylicum* hydrogenase (CaHydA). The suitability of these enzymes as bio-catalysts in artificial devices was tested. In such devices, electrons and protons are supplied to the hydrogenase through nanostructured electrodes. Stability and optimized interaction/electron transfer with electrodes are the key elements to exploit the highly evolved ability of [FeFe]-hydrogenases to catalyze hydrogen production without the need for expensive noble metals. CrHydA1 and CaHydA were expressed in the active form in *E. coli* and affinity purified with a yield of milligrams of pure protein per litre of culture. The whole process was performed under strict anaerobic conditions to avoid inactivation of the enzymes. Functional characterization confirmed that recombinant CrHydA1 and CaHydA are highly thermo stable: activity is conserved up to 60–70°C with a half-life of several hours at high temperatures. These features will be particularly desirable in artificial devices and other future applications. Preliminary trials of immobilization on different types of electrodes are under study to identify the best targets for the construction of the cathodic hydrogen evolving electrode. Directed evolution techniques are being applied to engineer new variants of the recombinant [FeFe]-hydrogenases with improved catalytic properties and enhanced stability to oxygen.

E2 – Molecular Recognition

E2.01
Canonical serine protease inhibition: last stand of the lock-and-key hypothesis?

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Canonical serine protease inhibitors interact with their target enzyme in a substrate-like manner. Their efficiency is conventionally attributed to the rigidity of their protease binding loop with no conformational change upon enzyme binding, yielding an example of the rigid lock-and-key model for biomolecular interactions. However, solution-state structural studies reveal that the protease binding loop displays considerable flexibility in the free state and structural differences between the free and protease-bound states are observable. We resolve this apparent contradiction by showing that the ps-ns timescale dynamics of two related small, 35-residue inhibitors map a relatively large conformational space that includes conformers close to the enzyme-bound ones [1]. This type of fast time-scale dynamics enables the association rate to be solely diffusion-controlled just like in the rigid-body model. Eliminating the necessity for an induced fit means that enzyme binding requires no energetic costs related to conformational rearrangements of the inhibitor backbone. Our results suggest that conformer selection is a general phenomenon that might occur on multiple time scales depending on the specific dynamical properties of the partners. This reinforces the importance of internal dynamics as a key factor in fine-tuned biomolecular interactions, which must be considered in inhibitor design.

Reference:

1. Gaspari Z, Varnai P, Szappanos B, Perczel A. Reconciling the lock-and-key and dynamic views of canonical serine protease inhibitor action. *FEBS Lett*. 2010, **584,** 203–206.

E2.02
Human galectin-3 interacts with two anticancer drugs

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Human galectin-3 (hGal-3) is a mammalian lectin involved in regulation of RNA splicing, apoptosis, cell differentiation, and proliferation. Multimerized extracellular hGal-3 is thought to crosslink cells by binding to glycoproteins and glycosylated cancer antigens on the cell surface or extracellular matrix. Fluorescence spectroscopy and circular dichroism (CD) were used to study the interaction of hGal-3 with two anticancer agents: bohemine and Zn porphyrin (ZnTPPS4). The dissociation constant for binding of bohemine with hGal-3 was kD 0.23 ± 0.05 μM. The hyperbolic titration curve indicated the presence of a single bohemine binding site. The binding of ZnTPPS4 to hGal-3 (with and without lactose) is of high affinity having kD = 0.18–0.20 μM and not inhibited by lactose, indicating that the ZnTPPS4 binding site and the carbohydrate site are different. CD spectra of the complexes with hGal-3 suggested that the binding of the hydrophobic compounds changed the hGal-3 secondary structure. In summary, we show that two compounds with anticancer activity, bohemine and ZnTPPS4, have high affinity for hGal-3 at a site that is distinct from its carbohydrate site. Since hGal-3 binds to several carbohydrate cancer antigens, the results suggest that it may have utility in the targeted delivery of drugs for cancer.

E2.03
Crystal structure of the C-terminal carbohydrate recognition domain of human galectin-9

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Galectin-9 (Gal-9) is a member of the family of β-galactoside-binding lectins and belongs to the tandem-repeat-type galectins having two carbohydrate recognition domains (CRDs) joined by amino acid linker peptide. Gal-9 is associated with various biological functions and known to be an eosinophil chemoattractant, T-cell immunoglobulin domain and mucin domain3 ligand, and inducer of T helper 1 cell apoptosis. The sequence identity between N- and C-terminal CRD (N-CRD, 1-148; C-CRD, 210-355) is 39%. The C-CRD has higher similarity with CRD of chimera-type Galectin-3 with 42%. To elucidate the relationship between the structure and biological activity of Gal-9, each structure of N-CRD and C-CRD is important. So far, the crystal structure of N-CRD in complexes with various ligands have been reported, however, the structure of C-CRD was not available. Here we report the crystal structure of C-CRD of Gal-9. The structure of C-CRD was solved by a molecular replacement using the structure of Gal-3 (PDB ID, 1KJL) and refined at 1.7 Å resolution. A comparison of monomeric structure of C-CRD with that of N-CRD does not show a major difference but reveals a significant difference in ligand binding sites which would be responsible for a difference of carbohydrate binding activity. Unexpectedly, N-terminal region (210–219) of C-CRD was invisible due to not only poor electron density but also insufficient space, since the three monomeric structure forms a trimer. Though it is not clear whether C-CRD forms timer only in crystal structure or also *in vivo*, it might be related in any biological activity of Gal-9.

E2.04
The HIV-1 gp120 interaction with Nt-CCR5 – New insights from NMR

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Human immunodeficiency virus type 1 (HIV-1) is the retrovirus that causes the *acquired immunodeficiency syndrome* (AIDS). HIV infection in humans is a pandemic affecting millions of people around the world. HIV-1 entry into target cells is mediated by the successive interaction of the viral envelope glycoprotein gp120 with the cellular receptor CD4 and with a G protein-cou-

pled chemokine co-receptor, mainly CCR5 or CXCR4. Interaction of CCR5 with the HIV-1 gp120-CD4 complex involves the amino-terminal domain of the CCR5 receptor (Nt-CCR5) and requires posttranslational sulfation of its tyrosine residues. We studied a 27-residue peptide corresponding in sequence to Nt-CCR5 (residues 1-27) and containing two sulfated tyrosine residues at positions Y10, Y14, in complex with a truncated, homogenously glycosilated, gp120 JR-FL (residues 88–492, ΔV1, ΔV2) and a CD4-mimic miniprotein. $T_{1\text{-rho}}$-filtered NOE experiments revealed evidence for a helical conformation in the center of the peptide, induced upon gp120 binding. Saturation Transfer Difference (STD) experiments allowed identification of the Nt-CCR5 residues that participate in gp120 binding and highlight the importance of the sulfated tyrosine residues.

E2.05

Abstract withdrawn

E2.06
cAbVIM4, a nanobody inhibiting the metallo-β-lactamase VIM-4

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The introduction of β-lactam antibiotics like penicillins in medical practice is one of the biggest clinical successes. Unfortunately, bacteria evolved rapidly to develop resistances. The most common resistance mechanism is the bacterial production of β-lactamase enzymes which cleave the β-lactam ring. Besides serine β-lactamases of classes A, C and D, metallo-β-lactamases (MBL) of class B are emerging as a problematic group of enzymes challenging the efficiency of modern antibacterial therapy. Notably, MBLs of the VIM family hydrolyse a broad range of β-lactam substrates including the last generation carbapenems. These enzymes can also be observed during nosocomial infections caused by multiresistant pathogens. A successful strategy to counter β-lactamases mediated resistance is the use of β-lactamase inhibitors which restore bacterial susceptibility to the antibiotic. To date, there is no clinically useful inhibitor of class B MBL. In the field of drug discovery, camel heavy chain antibodies are of particular interest. These antibodies are devoid of light chains so that their heavy chain's variable domains (VHH or nanobody) are actually the smallest intact antigen-binding fragments derived from functional immunoglobulin. Here we present cAbVIM4, a dromedary VHH inhibiting the MBL VIM-4 which was isolated by phage display from an immune library. Its μM level inhibitory ability spans substrates of different types. In an effort to identify binding ''hot spots'' of this VHH, alanine scanning mutagenesis has been realised. Measurements of the binding potency and the inhibition percentage of those mutants reveal insights into the interaction and the inhibition mechanisms.

E2.07

Abstract withdrawn

E2.08
Construction of a tethered lipid bilayer for membrane protein research

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Membrane proteins have a wide range of important functions in cell and deficiencies related to their function cause a number of diseases. They could not be studied in aqueous solutions due to their high hydrophobic content; hence it is necessary to develop cell membrane mimicking systems (artificial lipid membranes) to study them. In our work, we developed an artificial lipid membrane for the integration of a multi-drug resistance protein called P-glycoprotein (P-gp). To form the first layer, different concentrations of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino polyethylene glycol)-2000] (DSPE-PEG) were incubated with 3,3-dithiodipropionic acid di(N-hydroxy succinimide ester) (DTSP) self-assembled gold slides. Liposomes were then spread to the surface for the formation of the second layer. For the formation of P-gp incorporated membranes, liposomes with different P-gp content (Tris-EGTA, pH 7.0) were used. According to surface plasmon resonance and quartz crystal microbalance analysis, liposome spreading was significantly affected by DSPE-PEG amount on the surface and 0.03 and 0.04 mg/ml of DSPE-PEG concentrations were observed to be optimum for bilayer construction. Preliminary studies showed that 1–10 μl P-gp addition gave better results when compared to higher concentrations.

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E2.09
The epitope on monomeric beta-actin recognized by anti – phosducin antibodies

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The peptide SQSLEEDFEGQATHTGPK (p33) originates from regulatory proteins – phosducin (Phd, MW 28 kDa) involved in heterotrimeric G protein beta subunit sequestration and related protein phosducin-like protein 1 (PhLP1, MW 32 kDa) taking part in protein chaperoning. This peptide linked to Keyhole limpet hemocyanin was used for rabbits immunization. It was found that ap33 antibodies obtained recognize apart from Phd and PhLP1 also 43 kDa protein. Mass spectroscopy analysis of the 43 kDa protein, purified from human thyroid tissue homogenate, identified it as cytoplasmic beta-actin, the cell cytoskeleton element different in respect of sequence from the phosducin family proteins. The aim of the present study was to characterize epitope common for phosducin and beta-actin. Analysis of beta-actin sequence performed by conformational epitope prediction servers let to choose amino acids L D F E Q A T K as those which build the epitope. Most of them belong to the 221–241 peptide (p37) located on the actin – actin interface of the fibrillar form of actin. The p37 peptide was synthesized and used in reactions with ap33. Good affinity of the soluble p37 to ap33 confirmed participation of this region of actin molecule in formation of immunological complex with ap33. In polymeric actin the p37 localization site is not accessible for antibodies, so ap33 recognizes only monomeric form of this molecule. This observation was confirmed by immunostaining of thyroid cells by ap33 and anti actin antibodies. The existence of the epitope common for actin and phosducin may reflect a topological similarity of small region of their surfaces.

E2.10
Interaction of α-crystallin with argininosuccinate lyase

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α-Crystallin is the member of the small heat shock protein family and present ubiquitously in all vertebrate to maintain the transparency of lens. Argininosuccinate lyase (ASL) is a urea cycle enzyme that catalyzes the reversible conversion of argininosuccinate into arginine and fumarate. In the present study, the protein-protein interaction behaviors of α-crystallin and ASL were investigated. Incubation with different weight ratio of α-crystallin led to ˜30% increase in the catalytic efficiency of ASL. α-Crystallin protected the ASL from low and high temperature induced inactivation. An apparent conformational change and extensive exposure of hydrophobic patches was observed when ASL was incubated with α-crystallin at room temperature. Real-time measurement of protein-protein interaction by using surface plasmon resonance showed that the association of α-crystallin with ASL at different weight ratio did not perform a simple interaction mechanism.

E2.11
The lactococcin G immunity protein recognizes specific regions in both peptides constituting the two-peptide bacteriocin lactococcin G

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Ribosomally synthesized antimicrobial peptides produced by lactic acid bacteria are often termed bacteriocins. Bacteriocins are secreted by the producer cell and kill target cells by permeabilizing the membrane. To avoid being killed by its own bacteriocin the producer strain also produces an immunity protein. Lactococcin G and enterocin 1071 are two so-called two-peptide bacteriocins, i.e. they both consist of two different peptides and both peptides need to be present to obtain optimal antimicrobial activity. The two lactococcin G peptides (LcnG-α and LcnG-β) are thought to form a transmembrane helix-helix structure in target membranes, thereby causing membrane leakage. Lactococcin G and enterocin 1071 show about 55% amino acid sequence identity, whereas their immunity proteins show 38% amino acid sequence identity. Despite their sequence similarity, lactococcin G and enterocin 1071 recognize and kill different target bacteria. Hybrid peptides consisting of the N-terminal part of lactococcin G and the C-terminal part of enterocin 1071 were constructed and combinations of these hybrid peptides and wild type lactococcin G and enterocin 1071 peptides were assayed against two strains expressing the lactococcin G immunity protein. The results indicate that the N-terminal end of LcnG-α (residues 1–13) and the mid region of LcnG-β (residues 14–24) are important for the recognition between lactococcin G and its immunity protein. Both regions are positioned adjacent to each other in the proposed transmembrane helix-helix structure. Moreover, the results indicate that the recognition between the immunity protein and the cognate bacteriocin depends on a cellular component.

E2.12
The cytochrome c-cardiolipin interaction: looking for a model of protein binding to the mitochondrial membrane

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Several models have been proposed to explain the interaction of cytochrome c (cyt c) with cardiolipin (CL), a component of the mitochondrial membrane. One hypothesis proposes that an acyl chain of the phospholipid accommodates into a hydrophobic channel of the protein located close the N52 residue. An alternative model considers the insertion of the acyl chain in the region of the M80-containing loop, in which the K72 and K73 residues seem to play a primary role. Recent studies indicated that two distinct transitions take place in the cyt c-CL binding reaction and data obtained on the N52I variant of the protein provided support to a model in which two (instead of one) close acyl chains of the CL liposome are inserted into the same cyt c molecule, one at each of the hydrophobic sites of the protein, to form the cyt c-CL complex. To clarify better this still controversial point, we have undertaken a study on the K72N, K73N and the K7273N double mutant of cyt c to establish the role played by lysines K72 and K73 in the cyt c-CL binding reaction. Preliminary results show that these variants bind to CL by a two-state transition and that M80, i.e. the axial ligand of the heme-iron in the native protein, is replaced by an intrinsic residue, likely a histidine (lysine) residue upon complex formation for the K7273N (K73N) mutant. Conversely, binding of CL to K72N has relatively little effect on the heme pocket structure, suggesting that the lysine residue replacing M80 upon complex formation is primarily K72.

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E2.13
Nanofluidics for single DNA molecule manipulation and analysis

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During the last decades the advances in nanotechnology has enabled us to fabricate artificial structures and fluidic channels that are on the order of the natural size scales for DNA (˜1 nm – 100 nm). When placing a DNA molecule into a nanofluidic channel with a diameter smaller than the natural coiled up state of the molecule it stretches out along the channel. Positions along one of these linearized DNA molecules directly correspond to positions along the sequence of the DNA. This has enabled investigations and visualizations of DNA-protein interactions as well as genomic information within single DNA molecules. In addition, it has revealed many new and important insights into the conformational changes of DNA molecules in crowded and confined environments. The advantages of using nanofluidics over other means of stretching DNA, for example molecular combing, shear flow or optical/magnetic tweezers, is that it supports massive parallelization, is compatible with Lab-On-a-Chip technology and that the DNA is in equilibrium during measurements, ensuring reproducibility. We will present an overview of new techniques and appli-

cations based on nanofluidics for DNA analysis to provide a brief introduction to the field and its potential use in biology.

E2.14
Detection of the ligand binding/release state of membrane protein bacteriorhodopsin by the change of solubility in Dodecyl-β-D-maltoside

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A seven transmembrane protein bacteriorhodopsin (bR) exists on the biological membrane of *Halobacterium salinarum*, and forms two-dimensional lattice structure called purple membrane (PM) by specific interaction between bR molecules or bR and lipids. bR that functions as light-driven proton pump shows a large shift of λmax and a subtle tertiary structural change accompanied by the binding/release of a ligand, retinal. In this study, we investigated the correlation between the ligand binding/release and the solubility in a non-ionic detergent, Dodecyl-β-D-maltoside (DDM) on bR. When PM was treated by 20 mM-DDM at 25°C for 24 hour, only about 20% of bR was solubilized. Contrary to this, all protein molecules were solubilized from the completely photo-bleached, that is, retinal-released membrane by hydroxylamine. In addition, the plots of solubilization ratio of the protein by DDM versus bleaching ratio of PM were well fitted by a simple exponential curve, indicating that the ratio of apo-protein in PM can be calculated from the solubilization ratio. Interestingly, the solubility of the bleached membrane in DDM was decreased by re-addition of the retinal to the level with that of intact PM. These results suggest that the change of solubility of bR in DDM is attributed to the tertiary or quaternary structural changes accompanied by the ligand binding/release.

E2.15
Evolutionarily conserved furofuran region of aflatoxin B_1 and related micotoxins is electrostatically preorganized to bind DNA

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Aflatoxin B_1 and related furofuran micotoxins produced by the common molds of *Aspergillus* are of great interest due to their role in the etiology of human liver cancer. After intake they get epoxidized by cytochrome P450 to corresponding ultimate carcinogens which alkylate DNA, evidently after intercalation. Aflatoxin B_1 *exo*-8,9-epoxide intercalation free energy of -4.1 and -4.9 kcal/mol was calculated using Molecular Dynamics (MD) simulations in conjunction with Linear Interaction Energy (LIE) and Linear Response Approximation (LRA) methods, respectively, and compares favorably with the range of -3.9 to -4.9 kcal/mol obtained experimentally using various techniques and nucleobase sequences. Moreover, to provide an insight into interactions responsible for intercalation, the electrostatic, dispersion, preorganization as well as various group contributions to the calculated free energy were explicitly determined. The furofuran region is electrostatically preorganized to bind DNA, which has the potential to explain, why this structural element was evolutionarily conserved in a variety of micotoxins. On the other hand, the large planar body enhances intercalation solely through dispersion interaction, which again explains, why this region of related micotoxins could have been evolutionarily optimized in a different way. Finally, comparing generation of Near Attack Configurations (NAC) during the course of MD simulations involving charged and uncharged aflatoxin B_1 *exo*-8,9-epoxide, respectively, revealed that preorganized electrostatics plays a vital role also in the subsequent chemical step and speeds up the corresponding alkylation by a factor of 4.5.

E2.16
New designs of protein binding aptamers with LNA and UNA

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Synthetic nucleic acid aptamers can recognize a wide variety of targets, from simple ions to whole cells. Even so, the ''classical'' aptamer targets are proteins. Aptamers typically bind with a dissociation constant in the low nanomolar range and with high specificity comparable to those from antibodies. New nucleic acid aptamers are often created by selection from a large random sequence pool. The final aptamer can be synthesized in a reproducible manner in short time and modified to improve thermodynamic stability, decrease nuclease sensitivity or contain reporter groups. We are investigating advantages of incorporation of LNA (locked nucleic acid) and UNA (unlocked nucleic acid) in aptamers. LNA is an RNA-like structure with a bridge in the ribose ring providing a stiffness that significantly increases melting temperatures of short duplexes and confers nuclease resistance. UNA has an opened ribose ring and therefore work the opposite way of LNA thus providing flexibility and decreasing melting temperatures of duplexes. Some of our studies benefit from already known aptamers that we redesign and modify to investigate changes in various features and we study in the molecules modifications are allowed and how advantageous they are. Furthermore, we have developed template-directed enzymatic methods to incorporate LNA in nucleic acid strands using LNA triphosphates with DNA polymerases. In combination with other methodologies this allow us to make *in vitro* selection of protein binding aptamers from LNA- and UNA modified nucleic acid libraries.

E2.17
In vitro selection of LNA-containing aptamers

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Aptamers are structured RNA or DNA molecules with high affinity for various molecular targets, and they can be obtained through a process known as *in vitro* selection. In many cases aptamer stability and chemistry needs further refinement to achieve optimal properties, but *ad hoc* modifications frequently interfere with the desired aptamer structure. Inclusion of modified nucleotides in the selection scheme yields aptamers that are tailored for the desired nucleotide derivatives. The use of modified nucleotides is limited, however, by the specificity of the enzymes used in the *in vitro* selection process and occasionally also by specific properties of the nucleotide derivative in question. Locked nucleic acid (LNA) is a nucleoside analogue with a 2'-4' linker that locks the ribose in the 3'-endo form. LNA is known to confer nucleolytic resistance as well as excellent thermal stability to secondary structures. LNA is therefore well suited for application in the aptamer field. We present an outline of *in vitro* selection of DNA aptamers in the presence of LNA triphosphates and how we have managed to overcome the challenges associated with each step in the selection scheme.

E2.18

Abstract withdrawn

E2.19

Molecular recognition mechanism of p63 by itch-e3 ligase: advances and effects of a p63 mutation related to ectodermal dysplasias

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Recently, it has been shown that Itch mediates the degradation of TAp63 and ΔNp63 proteins[1]. Itch E3-ligase contains four WW domains important in the recognized process. Several signalling complexes, that these domains mediate, have been implicated in human diseases (Muscular Dystrophy, Alzheimer's Disease, Huntington Disease etc.). WW domains are highly compact protein-protein binding modules that interact with short proline-rich sequences. Based on their ligand-binding specificity they have been categorized into four groups. WW domains fold into stable three-stranded antiparallel β-sheet structures, and their primary sequence share two conserved tryptophan residues spaced 20–22 amino acids apart. The four WW domains of Itch are considered belonging to the Group I, which binds polypeptides with a PY motif characterized by a PPXY consensus sequence, where X can be any residue. It is likely that the Itch-p63 interaction results from a direct interaction of Itch-WW2 domain with the PY motif of p63. Here, we present a structural characterization of the interaction by fluorescence, CD and NMR spectroscopy of the Itch-WW2 domain. Interaction studies *in vitro* between Itch-WW2 domain and pep63, which correspond to the fragment of the p63 protein including the PY motif, were performed. Moreover, the effects of a site specific mutation of p63, that has been reported in both Hay-Wells syndrome and Rapp–Hodgkin syndrome, was also evaluated both on the conformation of pep63 and on the WW-pep63 interaction.

Reference:

1. Rossi M, Aqeilan I, Neale M, Candi E, Salomoni P, Knight RA, Croce CM, Melino G. *PNAS* 2006, **103,** 12753–58.

E2.20

Trapping conformational states along ligand binding dynamics: The impact of induced-fit on enzyme catalysis

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It is now accepted that the functions of proteins are governed by their dynamic nature, especially in relation to catalysis or their interactions with other proteins. Protein-ligand induced-fit is one example of such a dynamic mechanism that mediates molecular recognition. However, a detailed molecular description and demonstration of this biochemical phenomenon have remained largely obscure, primarily due to the difficulty of capturing the steps of this transient process. To identify the structural and dynamic bases governing this conformational change, we took advantage of the "slow tight-binding mechanism" induced by the potent antibiotic actinonin, which is a pseudopeptide inhibitor, on its specific target enzyme peptide deformylase. We solved more than six distinct crystal structures showing the kinetics of the conformational change from the initial open state, including the encounter complex, to the final closed state of the enzyme. These structural determinations included intermediates that were trapped during the process, allowing us to monitor the action of an enzyme in real time at atomic resolution. Our data along with further biochemical and biophysical analyses will enable a coherent causal reconstruction of the sequence of events leading to inhibition of enzyme activity. Moreover, we provide new insight on how enzymes achieve a catalytically competent conformation in which the reactive groups are brought into close proximity, resulting in catalysis.

E2.21

Separation of monomer, multimer and complex forms of IGFBP-1 from human serum using lectin-affinity chromatography

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Insulin-like growth factor-binding protein 1 (IGFBP-1) is a phosphoprotein which regulates the action of the insulin-like growth factors and, thus, the metabolism. The majority of IGFBP-1 in the circulation is bound to a glycoprotein α2-macroglobulin (α2M) which protects IGFBP-1 from proteolysis, and a minor portion is found in a monomer or multimer form. In order to separate these molecular species, a panel of eleven agarose-immobilised lectins was used. Electrophoresis and immunoblotting were employed for identification of the IGFBP-1 forms. IGFBP-1/α2M complexes bound selectively to agarose immobilised Con A, SNA, RCA I, PHA-E, WGA and LCA lectin, whereas IGFBP-1 multimers (having similar electrophoretic mobility as IGFBP-1/α2M complexes) remained unbound. Monomer IGFBP-1 was not expected to interact with lectins, however it bound to Con A and was eluted only with the pH 3.0 buffer. It is known that phosphoproteins interact with metal ions and the presence of Ca (II), Mn (II) and Mg (II) is needed for the Con A activation. In this study, it was shown that the presence of all three ions was necessary to obtain such a strong bond between IGFBP-1 and Con A. The absence of Mg (II) reduced the binding to some extent, whereas the absence of either Ca (II) or Mn (II) abolished the interaction. Therefore, chromatography with the immobilised SNA, RCA I, PHA-E, WGA or LCA (or a mixture of these lectins) in the first step may be used to separate IGFBP-1/α2M complexes from the serum, chromatography with Con A in the second step may be used to isolate IGFBP-1 monomers, whereas IGFBP-1 multimers would remain among the unbound proteins.

E2.22

New avenues to malaria diagnosis – nucleic acid aptamers against *P. falciparum* histidine rich protein 2 and lactate dehydrogenase

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Malaria diagnosis is an ongoing challenge, particularly in many malaria-endemic regions that have inadequate access to traditional microscopic diagnostic approaches. Self-diagnosis and misdiagnosis are causing overuse of antimalarial drugs leading to increased drug resistance and other problems. Non-microscopic

tests have been developed including rapid dipstick immunoassays and polymerase chain reaction, but immunoassays have issues with antibody stability and PCR requires equipment, electricity and technical expertise. We are developing nucleic acid aptamers against plasmodium proteins histidine rich protein 2 (HRP2) and lactate dehydrogenase (LDH) with a view to developing such aptamers for incorporation into a more robust rapid diagnostic test. We present the cloning and purification of these targets, and demonstrate specific selection of nucleic acid aptamers against these targets. This work lays a foundation for further development of applications of nucleic acid aptamers for malaria diagnosis.

E2.23
Do intramolecular CH...O hydrogen bonds really contribute to the DNA shaping? Quantum chemical and AIM analysis

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The aim of this work is to cast some light on the H-bonds in double stranded DNA in AI and BI forms. For this purpose, there were performed the MP2 and DFT quantum chemical calculations of the canonical nucleoside conformers, relative to the AI and BI DNA forms, and their Watson-Crick pairs, which were regarded as the simplest models of DNA. Based on the AIM analysis, five types of the CH...O hydrogen bonds, involving bases and sugar, were detected numerically from 1 to 3 per a conformer: C2'H2...O5', C1'H...O2, C6H...O5', C8H...O5', and C6H...O4'. Strengths of H-bonds occupy the range of 2.3 – 5.6 kcal/mol, i.e. surely exceeding the kT value (0.62 kcal/mol) at the room temperature 298.15 K. The nucleoside CH...O bonds appeared to "survive" changes in mutual turns of bases and sugar, determined by angle chi, in rather large ranges, admissible for a given conformation. That points out to the source of the DNA lability, lowing it to adapt necessary conformations in processes of its functioning. The calculation of the interaction in the dA T nucleoside pair gives evidence that between the bases adenine and thymine, additionally to the N6H...O4 and N1...N3H canonical H-bonds, the C2H...O2 third one is formed, which, being rather weak (about 1 kcal/mol), satisfies the AIM criteria of H-bonding and should be classified as a true H-bond. Interesting, total energy of all the CH...O nontraditional intramolecular H-bonds in nucleoside pairs proved to be commensurable (from 0.5 up to 0.8) with the energy of H- bonds between the bases in Watson-Crick pairs, which implies their significant role in the DNA shaping and in processes of protein-nucleic acid recognition.

E2.24
Relationship between apoptosis and total sialic acid level in regressing and remodeling organs during amphibian metamorphosis

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Amphibian metamorphosis contains several cellular processes such as proliferation and cell death. Thus, it is accepted as a significant model organism for study of vertebrate development and different cellular events. In this study, we aim to investigate changes of total sialic acid level and apoptosis during metamorphosis of Rana ridibunda larvae. For this purpose, it was composed of four groups namely premetamorphosis, prometamorphosis, metamorphic climax and postmetamorphosis in terms of larval developmental stages of the frog. In these groups, it was spectrophotometrically estimated sialic acid level in regressing (tail) and remodeling (intestine/liver) organs. Also, apoptotic cells were stained by TUNEL technique and the apoptotic index was determined in metamorphosing organs. Our findings showed that apoptotic cell death increased during metamorphic climax and decreased after metamorphosis in the larval organs. Sialic acid content of intestine and liver did not show any changes during developmental stages but increased after metamorphosis completed. However, there was a decline in sialic acid content of the tail in climax. Frog tail contains extensively cell death process and undergoes complete degeneration. In spite of increased apoptotic index, decreased sialic acid level in tail at this stage can be an indication of enhanced cell death. At the same time, this stiuation may be related to elimination of sialic acid's antigenic blockage during apoptosis. Intestine and liver contain both cell death and proliferative process. They remodel into their adult forms. Consequently, their sialic acid profiles during metamorphosis were different from the tail's profile.

E2.25
Computational studies on the interaction of ABO-active saccharides with the norovirus VA387 capsid protein can explain experimental binding data

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Norovirus strains are known to cause recurring epidemics of winter vomiting disease. The crystal structure of the capsid protein of VA387, a representative of the clinically important GII.4 genocluster, was recently solved in complex with histo-blood group A- and B-trisaccharides (2OBS and 2OBT). However, the VA387 strain is known to bind also to other natural carbohydrates for which detailed structural information of the complexes is not available. In this study, we have computationally explored the fit of the VA387 with a set of naturally occurring carbohydrate ligands containing a terminal α1,2-linked fucose. Molecular dynamics (MD) simulations show that type 1 and 3 extensions of the ABO-determinant including ALe^b and BLe^b pentasaccharides can be well accommodated in the site. Energetic scoring of the MD results indicate that the downstream extensions of the ABO-determinants give an increase in binding strength, although the α1,2-linked fucose is the single strongest interacting residue. An error was discovered in the geometry of the GalNAc-Gal moiety of the published crystal structure of the A-trisaccharide/VA387 complex. The A- structure modelled in carbohydrate binding site of VA387 shows a favorable conformation of the GalNAc-Gal moiety. The present modelling of the complexes with histo-blood group active structures shows contacts which provide insights into mutational data, especially I389A and Q331A. Our results may be applicable in structure-based design of adhesion inhibitors of noroviruses.

E2.26
Lectin staining patterns in metamorphosing organs of the frog (Rana ridibunda)

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Carbohydrate chains conjugated with proteins or lipids on cell surface and/or cytoplasm have an important role in various cellular functions such as cell interaction, signal transduction, cell adhesion and proliferation. In this present study, localizations and developmental variations of specific sugar moieties in the terminal carbohydrate chains of glycoconjugates were investigated during metamorphosis of Rana ridibunda larvae. For this purpose, it was composed of four groups so called premetamorphosis, prometamorphosis, metamorphic climax and postmetamorphosis in terms of larval developmental stages of the frog. In order to determinate the composition of terminal sugar residues such as L-fucose, N-acetyl-β-D-glucosamine, β-galactose, α-N-acetyl-D-galactosamine and α-D-mannose in metamorphosing organs, five horseradish peroxidase conjugated lectins were employed. It was found that Con A and PNA lectins gave no significant reaction in larval tissues. WGA, DBA and UEA-I lectins exhibited strong affinity to white blood cells in the liver. There were also an affinity in intestinal mucosal cells and tail epidermal cells. The binding activity of lectins showed some changes during distinct metamorphic developmental stages. Especially, it was decreased in climax stage and postmetamorphic period. These results suggest that the changes of lectin binding in metamorposis can be indicate the presence of some cellular events in larval metamorphic differentiation such as cell differentiation and damages of cell adhesion between death and differentiating cell. Also they can be useful model for detection of white blood cell in amphibian hematopoietic argans and metamorphic changes.

E2.27
cpSRP43 is a LHCP specific chaperone and targeting factor

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The chloroplast signal recognition particle (cpSRP) differs significantly from the canonical SRPs. It can act both co- and posttranslationally in the insertion of membrane proteins into the thylakoid membrane. The cpSRP consists of cpSRP54 a homolog of cytosolic SRP54, however instead of the SRP RNA a new protein component, cpSRP43, is present. cpSRP43 is involved in the posttranslational targeting of light harvesting chlorophyll a/b binding proteins (LHCPs) to the thylakoid membrane. LHCPs serve as antenna systems in photosynthesis and contain three transmembrane helices (TM1 to 3). After synthesis in the cytosol LHCPs are imported into the stroma where they form a soluble transit complex with cpSRP. LHCP recognition involves a highly conserved internal signal sequence consisting of the so-called L18 region and TM3. Subsequently the transit complex is targeted to the thylakoid membrane where the LHCPs are inserted in an Alb3 dependent process. However, it is not clear yet how the transit complex recognizes Alb3 and how the substrate transfer to Alb3 is regulated. We show that cpSRP43 is as a highly specific chaperone for LHCPs and cpSRP43 alone is sufficient to form a soluble complex with its membrane protein cargo. Furthermore, Alb3 recruits cpSRP43 to the thylakoid membrane using its intrinsically disordered stromal C-terminus (A3CT) in a coupled binding and folding mechanism. We identify two positively charged motifs in A3CT that mediate the interaction with cpSRP43. These motifs are absent in the C-terminal domain of the paralog Alb4, which therefore does not interact with cpSRP43.

E2.28

Abstract withdrawn

E2.29
Structural characterization of the SRP system

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Cotranslational targeting of proteins destined for membrane integration or secretion is mediated by the universally conserved signal recognition particle (SRP) and its receptor (SR). SRP is a ribonucleoprotein complex and binds to hydrophobic signal sequences of nascent polypeptide chains as they emerge from ribosomal exit tunnel. The SRP/RNC (ribosome nascent chain) complex interacts with the membrane associated SR. The delivery of the RNC to the translocation channel in the membrane leads to dissociation of the SRP/SR complex. In order to coordinate the presence of a signal sequence at the ribosome with the availability of a vacant translocation channel, two GTPases present in SRP and SR form a unique complex in which GTP hydrolysis is activated in a composite active site. We present recent progress in the structure and function analysis of the SRP systems from the crenarchaeon Sulfolobus solfataricus and the gram-negative bacterium *Escherichia coli*.

E2.30
Aza-β^3-amino acid containing peptidomimetics as cAMP-dependent protein kinase substrates

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Peptidomimetic analogs of RRASVA, known as the "minimal substrate" for the catalytic subunit of cAMP dependent protein kinase (PKA), were synthesized by consecutive replacement of natural amino acids by their azaβ^3-analogs. These pseudopeptides were tested as PKA substrates and kinetic parameters of phosphorylation reaction were determined. It was found that binding of these peptidomimetics with the enzyme active center was sensitive to location of the backbone modification, while the maximal rate of the reaction was practically not affected by the position of azaβ^3-analog residue in pseudopeptide. The kinetic parameters of phosphorylation reaction were in correlation with calculated data of structure modeling and computational studies of peptide docking in enzyme active center. It was concluded that certain structural fragments, modification of which influences binding kinetics can be distinguished for consensus sequence azaβ^3-analogs. Defining these structural features might open new perspectives for pharmacophore design of peptide and peptide-like PKA ligands.

E2.31
Puchellin, a RIP class lectin: elucidation of isoforms toxicity using biomembrane models

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Pulchellin, a heterodimeric lectin of Ribossome Inactivating Protein class, formed by two chain, toxic A-chain and a D-galactose-binding B-chain. Recently, our group described four pulchellin isoforms (PI, PII, PII, PIV) that presented different toxicity degree. PI and PII are more toxic than PIII and PIV, spite high identity between them. This difference in toxicity was investigate using biomembrane models systems content different glycolipids in their compositions. Langmuir Monolayers results indicated that PII and PIV bind to DPPC monolayers in the same extension, but, with addition of 5% of GalCer, this interaction was very different. PII remains strongly interacting with the mixed monolayer, but PIV seems to be inhibited by the presence of the glycolipids. To better evaluate this possible competition between specific interaction to the sugar and hydrophobic interactions we study the proteins using fluorescence spectroscopy with the ANS dye in presence of different sugars. Interestingly, PII was very dependent on the sugar type, since a blue shift was observed in the following sequence: lactose (4 nm), D-Gal (10 nm) and NAc-Gal (25 nm). In other hand, PIV present a high exposure of hydrophobic regions, which was not dependent of the sugar, since for both carbohydrates, the blue shift was equal to 55 nm. In conclusion we attribute the differences in pulchellin isoforms toxicity to possibility of PII isoform to strongly interact to the membrane, by sugar and hydrophobic interaction, differently of PIV, which seems to be more selective to one of the possible interaction with the membrane. Supported by Brazilian Agencies: FAPESP, CNPq.

E2.32

Abstract withdrawn

E2.33

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E3 – Design of Macromolecules

E3.01
Optimalization and designing of biosensors for wine analysis

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Biosensors are a type of sensors in which the recognition system utilizes a biochemical mechanism. The biological recognition system translates information from the biochemical domain, usually an analyte concentration, into a chemical or physical output signal with a defined sensitivity. A biosensor consists of two components: a bioreceptor and a transducer. The bioreceptor is a biomolecule that recognizes the target analyte whereas the transducer converts the recognition event into a measurable signal. The main areas of application of biosensors are health care, food industry and environment. In our laboratory, we are developing amperometric sensors which have immobilized oxidoreductase enzymes on thin-layer gold planar electrodes. During amperometric transduction, a constant potential was maintained with respect to a reference electrode. The current generated by the oxidation or reduction of molecules at the surfaces of the working electrode was measured. Amperometric signals were linearly dependent on analyte concentration. Present biosensors serve for an analysis of wine. We were able to set the concentration of lactic acid, malic acid, glucose + fructose, alcohol and some other components in wine samples. Our goal is selling our devices to wine-makers. To be commercially successful, biosensors have to meet specific requirements. Our biosensors showed suitable high sensitivity and resolution, specificity, dynamic range and rapid response. This work was supported by Slovak research and development agency APVV-VMSP-P-0073-09.

E3.02
Dissociation and re-complementation of Alpha Keratin and Melanin from hair to build a novel biopolymer that can be used as different biocompatible prostheses at wide polymerization ratios with a spectrum of tensile and impact strength

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Objectives: Alpha-Keratin is a ubiquitous polymer of low tensile and impact strength found in many tissues with different functions. Melanin is also a ubiquitous macromolecule whose function in organs like brain and adrenals is not well understood but it is the most effective UB absorbing agents in nature. During treatment of patients with alopecia universalis, we encountered patients who regained only white hair upon treatment and never relapsed. Besides all patients show regrowth of white hair at beginning and the disease never attacks white hair. We postulated that interaction of Melanin and Keratin is not only a physical co-impaction as microscopic studies of the hair show but a chemical co-polymerization that gives rise to new antigenic determinants.
Materials and Methods: In two different chemical environments, detergent treated black hair without fat was treated in a way that once Keratin and once Melanin could be extracted. After drying, the two were let to interact in a very delicate basic microenvironment that enabled growth of the crystals. Different non stochiometric proportions were used to evaluate chemical and physical properties.
Results: Co-polymerization of the two compounds is a real event with birth of compounds distinct from the parents. The largest product is at present 30 mm × 20 mm × 7 mm. Some of them are very resistant to tension and/or impact.
Conclusion: As the condition of co-polymerization is not optimum shape and physical properties cannot be controlled yet but as they are biocompatible they open a new horizon for prostheses in plastic surgery.

E3.03
In-silico evidence of a pAO1 encoded tagatose-derivate catabolic pathway in *Arthrobacter nicotinovorans*

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Based on similarity searches, two putative pathways were previously described as being encoded by the pAO1 megaplasmid of *Arthrobacter nicotinovorans*: an almost fully established nicotine-degrading pathway and a yet unknown putative sugar-catabolic pathway. The general organization of the latter indicated three possible targets for docking experiments: a periplasmic binding protein part of an ABC-type sugar transport system, a transcriptional control factor and an putative sugar dehydrogenase. In an effort to investigate a possible substrate of sugar-catabolic pathway, the tridimensional structures of the three target proteins were build by homology modeling using 3D-JIGSAW. *In silico* blind docking experiments using a database of 90 sugar as flexible ligands and the three structures as receptors were performed using Chimera/Dock6 suite. The results were ranked according to their potential binding place and grid score. Surprisingly 1,6-di-O-phosphono-D-tagatose proved to be the preferred ligand for all three proteins, indicating that the second pathway coded by the pAO1 megaplasmid is a tagatose-derivate catabolic pathway.

E3.04

Abstract withdrawn

E3.05
Characterization of water-soluble conjugates of enzymes with dextran

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Introduction: Protein–polysaccharide complexes are also important in industrial applications such as micro- and nano-encapsulation processes, the design of multi-layers structures, the formation and stabilization of food emulsions, the formation of new food gels and the recovery of proteins from industrial by-products. In biotechnology, stabilities of the enzymes *in vitro* are still being one of the most important issues. Storage and operational stabilities are both important for the usage of enzymes. The modification of the enzyme surface with a hydrophilic polymer could be a good strategy to form a shell around the enzyme molecule. In general, these modifications increase the stability of

the enzymes in high temperature, different pH and organic media.

Methods: The covalently-formed conjugates of HRP (Horseradish Peroxidase) and GOD (Glucose oxidase) with dextran has been proposed as an alternative modified enzyme towards industrial application and storage stability. The contribution of conjugate formations to the thermal stability of enzymes were evaluated with the activities determined. Interactions of industrial enzymes (HRP and GOD) with dextran in different molar ratios of components were systematically investigated by Fourier Transform Infrared (FT-IR) spectrophotometer, fluorescence spectrometer, gel-permeation chromatography (GPC), and Atomic force microscope (AFM) methods.

Results and Conclusions: Multipoint covalent bonding of dextran to horseradish peroxidase caused the formation of a conjugate whose thermal stability was increased and long storage lifetimes for these conjugates were obtained.

E3.06
Polysaccharides and glycolipids from probiotic bacteria

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Extensive investigations of biological active compounds from probiotic bacteria are of the interest for the preparation of therapeutic products. Among these substances are bacterial polysaccharides and polar lipids. Therefore, it is important to identify and characterise the biologically active compounds. A branched glucogalactan with a heptasaccharide repeating unit was found to be specific for the strain B. bifidum BIM B-465. Polar lipids were effectively extracted by supercritical carbon dioxide. One of the major components of interest was glycolipids. The highest glycolipids yield was obtained from dry bacterial biomass at process parameters 250 bar, 40°C, CO2 flow rate 10 g/min and hydromethanolic co-solvent 9.3%v/v (methanol-water 9:1 v/v). The thin layer chromatography revealed presence of two major glycolipid fractions. The fractions were identical to those obtained with classical organic solvents extraction. Fatty acids identification of glycolipids subjected to acid methanolysis and analyzed by gas liquid chromatography showed presence of palmitic acid, myristic acid and erucic acid. The sugar analysis after hydrolysis of carbohydrate moiety and acetylation was determinate by gas liquid chromatography. Glucose and galactose were the major sugar components of the isolated glycolipids. Glycolipids with high immunoreactivity were identified by enzyme linked immunosorbent assay. The results indicate that supercritical carbon dioxide does not alter native structure of glycolipids. In prospect the supercritical carbon dioxide technology may be combined with metabolic engineering and immunological studies in manufacturing of highly effective therapeutic products.

E3.07
A bioinformatics study of the human phosphofructokinase-1

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Phosphofructokinase-1 (PFK-1) is a kinase enzyme involved in glycolysis, a process that requires 12 enzymatic reactions for the breakdown of glycogen to lactic acid. The glycolytic system works predominantly within skeletal muscle tissue. The inhibition of PFK-1 by ascorbate in resting muscle of mouse was already shown. When muscle is in activity, this enzyme combines with muscle proteins being protected by inhibition and glycolysis is possible. The Protein Data Bank contains only a few structures for PFK-1 for invertebrate's organisms. The aim of this work is to perform a bioinformatics study to reveal if there are sequential and/or structural similarities between vertebrates and invertebrates PFK-1 in order to determine the active site for ascorbate binding and to perform computational drug design for the PFK-1 inhibition.

E3.08
The thermodynamic investigation of T5 bacteriophage ejected DNA

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The calorimetric measurements were used to investigate the thermal stability of T5 phage DNA under the physiological environmental condition and to check the existence of DNA-associated protein, which is thought to play protective role of virus genome against bacterial intracellular barrier (restriction enzymes). From the experimental data it have been observed that calorimetric curves of T5 phage ejected DNA contains two peaks, with melting temperature Tm ˜850C and Tm ˜97.5°C. Judging by the shape, the first one is typical for ds-DNA denaturation. As for the second thermal meting peak, supposedly it should belong to a protein. In order to check the presence of protein in T5 phage DNA solution, it was previous treated with trypsin at 37°C, after which it has been shown that only one heat absorption peak (Tm˜85°C) was remained on the thermal denaturation curve. It has revealed that second melting peak corresponds to the protein. It was interested to investigate if the thermal denaturation of phage DNA is reversible, in case of associated or without protein, i.e. to check the ability of heat-induced melted phage DNA to restore its double structure (at least partially) and the possible role of DNA-associated proteins in this process. As it was seen from experimental data thermal denaturation of T5 phage DNA is partially reversible (˜90%), when it is associated with protein and irreversible - without it.

E3.09
Monomerization of the viral entry inhibitor griffithsin yields insights into the relationship between multivalent binding to high mannose oligosaccharides and antiviral activity

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Mutations were introduced to the domain-swapped homodimer of the antiviral lectin griffithsin (GRFT) with the aim of preventing dimerization and thus making the molecule smaller and better suited to potential applications as an anti-HIV agent. Whereas several single and double mutants remained dimeric, insertion of either two or four amino acids at the dimerization interface resulted in a monomeric form of the protein (mGRFT). Several additional mutated forms of mGRFT were designed, expressed, and purified, with the goal of improving purification and eliminating monomer-monomer interactions. The monomeric character of the modified proteins was confirmed by sedimentation equilibrium ultracentrifugation and by their high resolution X-ray crystal structures, whereas their binding to carbohydrates

was assessed by isothermal titration calorimetry. Cell-based antiviral activity assays utilizing different variants of mGRFT indicated that the monomeric form of the lectin is essentially inactive against HIV-1, suggesting that the antiviral activity of GRFT stems from crosslinking and aggregation of viral particles via multivalent interactions between GRFT and oligosaccharides present on HIV envelope glycoproteins. Atomic resolution crystal structure of a complex between mGRFT and nonamannoside revealed that a single mGRFT molecule binds to two different nonamannoside molecules through all three carbohydrate-binding sites present on the monomer. Fusion proteins containing mGRFT and other antiviral lectins were also prepared.

E3.10
Camel beta-casein, a novel nano emulsifier for dissolving curcumin in pharmaceutical and nutritional industries

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Beta casein is an amphifilic protein with self-assembling behavior that can form micellar nanostructures and could be exploited as a safe vehicle for hydrophobic therapeutic agent such as curcumin. Curcumin is a potent anticancer and antioxidant natural polyphenol, which is almost insoluble at water. Critical association concentration of camel beta casein was detemined at 25, 30 and 37°C using pyrene fluorescence and the solubility of curcumn was evaluated according to the solvent-evaporation technique. Beta-casein can improve the solubilty of curcumin at least 2570-folds. Analysis of flourescence emmission of curcumin reveald that hydrophobic intractions are mainly countributed. We claim that these observations provide promise in the design of the suitable GRAS carrier for curcumin in nutritional industries. Curcumin encapsulated in camel β-casein micelles can be a novel vehicle for hydrophobic drugs.

E3.11
CoNSEnsX: an ensemble view of protein and peptide structures and NMR-derived experimental data

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Representation of the dynamical features of proteins becomes more and more important with the recognition of the role of internal motions in biological function. One of the most common way for such representation is the use of structural ensembles instead of individual conformers. However, the evaluating the relevance of such ensembles is far from being standardized. We developed a tool named CoNSEnsX (Consistency of NMR-derived Structural Ensembles with eXperimental data) that allows fast, simple and convenient assessment of the accuracy of protein structural ensembles. By analyzing the correspondence of the ensemble to chemical shift, NOE, S^2 order parameter, RDC and scalar coupling data together, the ability of the conformer set to represent the structure and dynamics of the protein can easily be judged. We provide thorough evaluation of various ensembles of human ubiquitin available publicly or generated in this study and show that most of them are in good agreement with the majority of the NMR data used, but there are parameters not matched by either of the conformer sets. This reveals that no current method is able to reflect all available NMR parameters.

E3.12
Genetic engineering of allosteric β-lactamases

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In nature, enzymes activities can be regulated by many ways, one of them being the allostery. This phenomenom received attention from protein engineering scientists since many years. The design of an allosteric site de novo within the scaffold of enzymes may be of different interests. From a fundamental point of view, this kind of engineering allows to better understand the molecular basis of signal transmission through the protein as well as the evolution mechanisms underlying the apparition and adaptation of allosteric regulation systems. In the applied field, the design of proteins regulated by molecules of interest could lead to the development of biosensors based on the measurement of activity modulation. In the lab, we chose the TEM1 β-lactamase as a scaffold protein to create a binding site by random peptides insertion within three loops. Those three loops are in close contact and not far from the active site. Taken together they might mimic the binding site found within the camel monochain antibodies. Using phage diplay, this strategy has already leaded to the selection of enzymes regulated by various ligands. Nevertheless, the strategy suffered from different disadvantages and a new collection of mutated beta-lactamases was rebuilt. By modifying the construction strategy and by stabilizing the beta-lactamase, we obtained a library of better quality and diversity. The libray is of 6.10^8 clones, with at least 30% presenting a β-lactamase activity. The next step will be the selection by phage display of enzymes that bind to various targets (proteins, small molecules).

E3.13
Delta-sleep inducing peptide entrapped in polymer matrices for biomedical applications

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Polymer scaffolds providing enhanced cell attachment and proliferation can be used for tissue engineering. Bioactive molecules entrapped in the scaffolds could stimulate cell growth and secreting biomolecules for tissue repair. The aim of the study was to develop peptide entrapment in various matrices and to estimate its *in vitro* release kinetics. Delta-sleep inducing peptide (DSIP, WAGGDASGE, pI 4.7) which possesses neuroprotective properties was used as a model peptide. A series of polymer matrices differed by their composition and charge were proposed, namely positively charged poly(dimethylaminoethyl methacrylate) (PDMAEMA),

negatively charged polyacrylic acid (PAA), noncharged poly(vinyl alcohol) (PVA) and poly(vinyl alcohol-co-glycidyl methacrylate) (PVA-GMA). Two techniques were developed: 1) peptide adsorption onto the preprepared macroporous matrix (PDMAEMA, PAA, PVA-GMA) from the aqueous solution and 2) adding DSIP solution to preformed matrix (PVA) or monomer solution before polymerization. The amount of DSIP released from the matrices was estimated by reverse phase HPLC. DSIP entrapment was 100% in case of PDMAEMA, PVA, PVA-GMA matrices while the peptide amount in PAA matrix didn't exceed 6% of initial quantity. Peptide release in MilliQ water and aqueous solutions (PBS, pH 7.4, 0.9% NaCl) from PDMAEMA matrix increased with increasing solution ionic strength and pH from acidic to neutral values. DSIP release kinetics from PVA matrix didn't depend on ionic strength while from PVA-GMA matrix no release was observed. Thus, DSIP release depended upon composition, structure and charge of the matrix as well as on the solution used for release study.

E3.14
The effect of deleting a putative salt bridge on the properties of the thermostable subtilisin-like proteinase, aqualysin I

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Aqualysin I is a subtilisin-like serine proteinase, from the thermophilic bacterium *Thermus aquaticus*. The enzyme is predicted to contain a salt bridge between Asp17 and Arg259, connecting the N- and C-terminal regions of the enzyme. We have previously reported on the stabilizing effect of incorporating a putative salt bridge at a corresponding site into the structure of VPR, a homologous cold adapted enzyme from a marine *Vibrio* sp [1]. Here we describe the effect of the reverse change on the thermophilic homolog, i.e. the elimination of the salt bridge by a single mutation; D17N. Deletion of the putative salt bridge in the D17N mutant of the enzyme destabilized the enzyme by 8–9°C in terms of $T_{50\%}$, determined by thermal inactivation, and more than 4°C in terms of T_m, as measured by melting curves of the inhibited enzyme. The mutation, however, had no significant effect on the kinetic parameters of the enzyme under standard assay conditions.

Reference:

1. Sigurdardottir AG, Arnorsdottir J, Thorbjarnardottir SH, Eggertsson G, Suhre K, Kristjansson MM. Characteristics of mutants designed to incorporate a new ion pair into the structure of a cold adapted subtilisin-like serine proteinase. *Biochim. Biophys. Acta*, 2009, **1794,** 512–518.

E3.15
Detection of the binding site of apolipoprotein E

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Apolipoprotein E is low-density lipoprotein and together with its receptor LDLR plays important role in the development of various cardiovascular diseases. The aim of our study was to detect binding site of Apo-E and to give a forecast of its peptide's low-molecular analogs. It is known that the specific protein binding sites usually are placed in the cavities on protein surfaces and form hydrophobic and hydrophilic clusters. Therefore assigned task we solved with the help of designed by us program complex which allows: to build the molecular protein surfaces, to detect cavities on the protein surfaces and to carry out the analysis of distribution hydrophobic and hydrophilic areas on protein surfaces. At the first step we imported the 3D structure of Apo-E with PDB ID 1GS9 from Protein Data Bank (PDB; http://www.rcsb.org/pdb/). Then using our program complex we built Apo-E surface and defined two cavities with rank one which are adjacent and form highly charged regions. One of the cavities is formed by residues Arg150, Asp151, Asp153 and Asp154; another cavity is formed by residues Gln156, Lys157 and Arg158. Both defined cavities are placed on a critical for Apo-E binding region (region 140–160, [Wilson C. et.al. 1991. Science. Vol. 252. P. 1817–1822]). We think that the defined cavities form receptor-binding site of Apo-E. Now we are synthesizing their peptide's analogues and investigating their ability to LDLR binding.

E3.16
Chimeric allene oxide synthase-lipoxygenase fusion proteins

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In plants, the conversion of fatty acids into allene oxides is catalyzed by two distinct enzymes: a lipoxygenase (LOX) and a cytochrome P450 type allene oxide synthase (AOS). In corals, the formation of hydroperoxide (HpETE) and its further conversion into the allene oxide is catalyzed by a unique fusion protein where 8*R*-LOX is fused to a catalase-related AOS. While crystal structure of AOS-LOX of the Caribbean coral *Plexaura homomalla* is available, it is not certain how two domains of the fusion protein work together. Another naturally occurring AOS/8*R*-LOX fusion protein has been cloned from the arctic coral *Gersemia fruticosa*. The other LOX present is arachidonate 11*R*-lipoxygenase (11R-LOX). By using domain swapping between two coral enzymes we created several chimeras and characterized their catalytic properties. Chimeric constructs of sequences encoding for *P. homomalla* AOS plus *G. fruticosa* 8*R*- or 11*R*-LOX were prepared by PCR cloning, sequenced and expressed in *E. coli*. The incubation products of arachidonic acid with recombinant fusion proteins were identified by RP-HPLC and mass-spectrometry. Our results show that the fusion of the *P. homomalla* AOS with *G. fruticosa* 8*R*-LOX or 11*R*-LOX does not alter the expression level of the co-expressed enzymes. The LOX domains of the chimeras convert arachidonic acid into 8*R*-HpETE and 11*R*-HpETE, respectively. In addition to its natural substrate 8*R*-HpETE, the AOS domain of the fusion protein converts 11*R*-HpETE into the corresponding allene oxide. In conclusion, results obtained indicate that at least *in vitro* conditions AOS and LOX domains operate independently and AOS tolerates different LOX partners.

E3.17
Influence of Cd707 Ile → Leu substitution in N-terminal deletional variant of *Taq* DNA polymerase on stability and activity of the enzyme

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The success, yield and specificity of PCR are affected by the DNA polymerase activity at room temperature. One way of pre-

venting this activity is by genetic modifications of the DNA polymerase. For *Taq* DNA polymerase, mutations in the gene (Glu626Lys, Trp706Arg, Ile707Leu and Glu708Asp), were found to contribute to the significant decrease of the enzyme activity at room temperature. The aim of this study was to evaluate the usefulness of Ile707Leu cold-sensitive mutation in the N-terminal deletional variant of *Taq* DNA polymerase (Klentaq278) in PCR reaction and the influence of the mutation on overall stability of the enzyme. The Ile(707) to Leu substitution was introduced to Klentaq278 by site-directed mutagenesis. Normal and mutant DNA polymerases were expressed under *tac* promoter and purified to homogeneity. The mutant polymerase showed reduced polymerase activity at room temperature for 12 times and approximately 88% of Klentaq278 thermostability. This substitution in the enzyme reduced twice the maximum tolerance of K and Mg ions, ten times the tolerance of Na ions and ten times the enzyme tolerance of blood and phenol as PCR inhibitors. The major effect of the amino acid substitution was the reduction of the amplification capacity of the polymerase. Mutant polymerase is suitable for amplification of single or multiple fragments without hot start but only up to 1 kb length. The mutation Ile707Leu in Klentaq278 DNA polymerase reduces the overall processivity and stability of the enzyme and limits the application of this DNA polymerase in PCR only to small fragments.

E3.18
When is an amino acid residue buried? Why should we care?

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Determining "when an amino acid is buried" is important to our understanding of protein function, protein-protein interactions and protein evolution. This may appear a trivial question, as solvent accessibility has been discussed in literature for almost 40 years. However there is no universally accepted definition or value of solvent accessibility that is used to decide if a residue is buried in the molecular structure of a protein, or exposed to solvent. To quantify this a better statistical and physical understanding of this property is needed. Here we developed a method to analyse the amino acid distribution in individual proteins and their preferred solvent accessibilities. This is in contrast to the more common method of performing a statistical analysis of the solvent accessibilities of all amino acids in an entire data-set of proteins. We show that each amino acid residue has a statistical preference for a range of solvent-accessibilities in a sample of approximately 25,000 structures from the Protein Data Bank. Our data gives information about how exposed different residue types should ordinarily be, which is important for a statistical analysis of the role amino acids play in molecular recognition and in the design of optimal protein structures.

E3.19

Abstract withdrawn

E4 – Folding

E4.01
Absence of peculiarities in far UV circular spectra of two potexviruses

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We have measured circular dichroism (CD) spectra of virions and coat proteins (CPs) of two members of the potexvirus group, Alternanthera mosaic virus, strain Russian (AltMV-Ru) and Potato Aucuba mosaic virus (PAMV). We obtained the purified preparations of AltMV-Ru and PAMV from their host plants and measured their far UV 190 to 250 nm CD spectra. It turned out that the two intact viruses principally differ in their CD spectra from that of potato virus X (PVX). Both CPs and the virions of AltMV-Ru and PAMV have absolutely ''normal'' CD spectra with λ0 at about 200 nm, λ-max at 208 nm and typical shoulder at ~ 220 nm. No strong decrease in the negative peak at 208 nm and no appearance of the peak at 228 nm (typical for intact PVX) was observed in the spectra of these two viruses. Absence of any anomalies in CD spectra in 200 to 250 nm region was also observed by Tremblay et al. (2006) for one more potexvirus, papaya mosaic virus. Thus, from the four potexviruses studied in this respect, peculiar nature of 200 to 250 nm CD spectrum in characteristic only for PVX, which was always considered a typical representative of this group.

E4.02
Nonlinear analysis of glycolytic enzymes sequences

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Glycolysis is an ancient, universal pathway of obtaining metabolic energy. During glycolysis glucose is broken down in ten steps to two molecules of pyruvate, which then enters the mitochondria where it is oxidised through the tricarboxylic acid cycle to carbon dioxide and water. The different enzymes involved in glycolysis act as kinases, mutases, and dehydrogenases, cleaving enzymes, isomerases or enolases. Within this study we apply the linear and non-linear analysis methods, such as spectral analysis (SA), detrended fluctuations analysis (DFA) and Hurst exponent calculation to reveal if there are non-linear aspects concerning the sequences of enzymes involved in glycolysis process. Our study shows that even if the enzymes do not share sequence or structural homology with one another, and are therefore found in different protein families, they reveal long-range correlation concerning the physicochemical properties of the amino acids within their sequences. This result sustains other published data revealing non-randomness of protein sequences with direct consequences on protein folding.

E4.03
Chemical cross-linking and H/D exchange combined with mass spectrometry: a tool to validate and refine 3-D protein X-ray model

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Chemical cross-linking and Hydrogen/Deuterium exchange combined with mass spectrometry are powerful tools for elucidating protein conformation in solution. We applied both methods to study structural details of an important activating lymphocyte receptor NKR-P1A and to distinguish between structural characteristics in crystal and under physiological conditions. The determination of crystal structure of NKR-P1A evokes questions about the unique loop which is in crystal directed towards symmetrically related molecule. Chemical cross-linking and H/D exchange combined with high resolution mass spectrometry have been employed to investigate the flexibility and conformation of the flap region. In order to study the flexibility and conformation of the flap region in the NKR-P1A protein, we have prepared recombinant NKR-P1A protein and its mutant NKR-P1A-NF in which the loop region containing residues Pro161-Asp187 was deleted and replaced by two alanines. While the main structure of NKR-P1A obtained by mass spectrometry techniques was consistent with the previously known crystal data, a difference was found in the flap region. In the crystal structure the flap region extends from the compact core region. On the contrary, analysis of the peptic fragments showed decreased local H/D exchange in the NKR-P1A protein in region containing residues 115–131, 138–143 and 190–206 in comparison with NKR-P1A-NF. This indicates a reorganization of the flap region and its association with the compact core region. The mass spectrometry results were further confirmed by the NMR structural analysis.
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E4.04
A yeast model for LRRK2: biological studies and screening for small-molecule inhibitors

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Mutations in the PARK8 locus, leucine-rich repeat kinase 2 (LRRK2), are the most common monogenetic cause of Parkinson's Disease (PD), responsible for 5–10% of the familial PD and also for a significant proportion of apparently sporadic cases. The most frequent LRRK2 mutation associated with PD is the G2019S mutation found in the kinase domain. Little is known about LRRK2 regulation and its physiological substrates. Additionally, no pharmacological inhibitors of these proteins have been found to date (Paisan-Ruiz, et al. (2004) Neuron. 44, 595–600) Yeast is a simple eukaryotic cell system that has been successfully used in the study and high-throughput screening of small-molecule modulators of human proteins (Barberis, et al. (2005) Drug Discov. Today Tech., 2,187–192). In order to under-

stand the biology of wt and mutant (G2019S) LRRK2 and to search for selective small-molecule inhibitors, human wt and mutant LRRK2 were individually expressed in the yeast Saccharomyces cerevisiae. High copy number expression of wt or mutant LRRK2 in yeast caused significant growth inhibition, which was more pronounced for the PD-causing mutant G2019S. This work represents the first attempt to express wt and mutant LRRK2 in yeast. This system may help uncovering basic aspects of both normal and pathogenic LRRK2 biology and may be further used as a first-line drug screening approach for selective pharmacological inhibitors with promising therapeutic applications in neurodegenerative disorders, such as PD. We thank REQUIMTE/CEQUP, FCT (I&D/No8/94) and U.Porto/Santander Totta for financial support. C. Pereira is recipient of a FCT fellowship (SFRH/BPD/44209/2008).

E4.05
Role of aggregation conditions in structure, and toxicity of Yeast Hexokinase aggregates

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Formation of well-ordered fibrillar protein deposits is common to a large group of amyloid-associated disorders, including Alzheimer's, Parkinson's, type II diabetes and prion diseases. Disease-unrelated proteins are also able to form amyloid assemblies *in vitro* displaying comparable structures and cytotoxicity. The nature of the pathogenic species and the mechanism by which the aggregation process results in cell damage are, however, the subject of intense debate. In the present study, we have investigated the propensity of hexokinase type B from Saccharomyces cerevisiae (YHKB) to form amyloid-like and amorphous aggregates under various conditions and determined their cytotoxicity. Amyloid-like aggregation was induced under conditions known to be most effective for globular proteins (i.e. low pH in the presence of salts or TFE). Under both conditions; the resulted products had β- structure and weak, yet significant, ability to bind ThT and Congo red. Atomic force microscopy indicated morphologies distinct from typical amyloid. At low pH and in the presence of salts, they were either globular or amorphous, while in the presence of TFE, an irregular morphology was observed. Thermal aggregation of the enzyme was also investigated in 50 mM phosphate buffer, pH 7 at 60°C. The aggregates were not found toxic to human neuroblastoma cells, as indicated by the MTT reduction assay while those formed at acidic pH and in the presence of TFE indicated cytotoxicity. The differences in cytotoxicity appear to arise from variations in the nature and morphology of the aggregates in the conditions tested.

E4.06
Importance of leucine 134 to the co-chaperone activity of *Bacillus licheniformis* nucleotide exchange factor GrpE

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GrpE proteins function as a nucleotide exchange factor of the eubacterium DnaK molecular chaperone system. The co-chaperone GrpE accelerates ADP dissociation from DnaK and cooperatively facilitates the DnaK chaperone cycle with another co-chaperone, DnaJ. To elucidate the role of leucine 134 of *Bacillus licheniformis* nucleotide exchange factor (BlGrpE), site-saturation mutagenesis was employed to generate all possible replacements for this residue. Wild-type and mutant proteins were purified by nickel-chelated chromatography and had a molecular mass of approximately 30 kDa. BlGrpE and 9 mutant proteins synergistically stimulated the ATPase activity of *B. licheniformis* DnaK (BlDnaK), whereas L134H, L134K, L134D, L134E, L134N, L134Q, L134S, and L134P had no co-chaperone activity. *In vitro* binding assay revealed that the functional BlGrpE variants mainly interacted with the monomer of BlDnaK, but non-specific interaction was observed for the remaining mutant proteins. Comparative analyses of the far-UV CD spectra revealed that the α-helical content of the inactive mutant BlGrpEs was reduced significantly with respect to wild-type protein. Moreover, the inactive mutant proteins also exhibited a more sensitivity towards the temperature-induced denaturation. These results suggest that Leu134 plays an important role in the structural integrity of BlGrpE.

E4.07
Production and refolding of soluble forms of mouse NK cell receptors NKR-P1F and Clrg

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Mouse NKR-P1F: Clr-g receptor:ligand pair is important component of the receptor ''zipper'' that occurs at the contact between natural killer cell and its target cell, and represent a recently discovered example of lectin-lectin interactions important for recognition of immune cell subsets. Previously, immunologists have brought evidence for the involvement of the NKR-P1F:Clr-g pair in cellular activation such as T cell proliferation. In future we want to study structure of these proteins and interactions between them, but the first task was expression and refolding. We have amplified the individual cDNA clones for the receptors by RT-PCR from spleens of C57BL/6 mice, and transferred DNA fragments coding for the extracellular ligand binding domains of the two receptors into pET-30 bacterial expression vectors. After induction of protein production with IPTG, the protein precipitated into inclusion bodies, from which they could be refolded *in vitro*. NKR-P1F construct produced a small amount of soluble protein using the standard protocol, but we have experienced difficulties with the reproducibility of the refolding protocol. In the case of Clrg the standard protocols for protein refolding were not sufficient. In order for the Clrg to fold properly, the fourth cysteine which does not fit into the pattern usual for this family of receptors was substituted for serine and resulting C64S construct was shown to be more useful. Supported by grants from Ministry of Education of Czech Republic (MSM 21620808 and 1M0505), and from The Grant Agency of Czech Rep. (GACR 305/09/H008 and 303/09/0477).

E4.08
Interaction of iron ion with camel alpha-lactalbumin

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Enrichment of foods with iron is a common vehicle for delivering iron to the consumer. However, when food products are fortified with minerals some reactions of minerals with food components may influence their structure. In the present study, the interaction between iron ion and alpha-lactalbumin (α-La), which is one of the major whey proteins in milk, has been studied by spectroscopic techniques. By the analysis of fluorescence spectrum, it was observed that the iron ion has an ability to quench the intrinsic fluorescence of α-La. The association equilibrium constant of iron with camel holo α-La is about 104 M-1 based on the fluorescence quenching results. Experimental results showed that the binding of iron to α-La induced conformational changes in α-La. Circular dichroism spectra showed that the alpha-helix of α-La decreased upon binding of iron ion. The alpha-helix content of the protein decreased about 8% when the molar ration of iron ion to α-La was ten. Hence, the secondary structure of the protein is altered by iron ion. The conformational change of α-La in the presence of iron ion was also confirmed by UV absorption spectra.

E4.09

Abstract withdrawn

E4.10
Amyloid-like aggregation of α-chymotrypsin in different aqueous-organic solvents

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The amyloidoses are a group of protein misfolding disorders characterized by the accumulation of insoluble fibrillar protein. The mechanism by which amyloidogenic proteins undergo conversion from a soluble globular form to the cross-β conformation has not yet been elucidated. Several observations suggest that amyloid represents a generic form of polypeptide conformation, and most proteins have the potential to form amyloid-like structures. It has recently been demonstrated that α-chymotrypsin is driven toward amyloid aggregation by the addition of 2,2,2-trifluoroethanol and at high temperature. The aim of this work was to study the aggregation of α-chymotrypsin in ethanol, methanol, *tert*-butanol and dimethylformamide (DMF). The formation of aggregates was followed via the binding of thioflavin T (ThT) and 1-anilino-8-naphthalenesulfonic acid (ANS), with intrinsic fluorescence and turbidity measurements. The role of the surface charges of the enzyme in aggregation was studied through the use of chemically modified enzyme forms. α-Chymotrypsin underwent amyloid-like fibril formation in all of the studied organic solvents. The ThT binding and turbidity measurements revealed that the aggregation was maximal after incubation in 55% ethanol for 5 hour at 24°C. The highest fibril formation after incubation for 24 hour at 24°C occured in methanol and in DMF at 60–70%, and in *tert*-butanol at 60–80%. The ThT and ANS binding and intrinsic fluorescence results showed that the hydrophobic residues are more solvent-exposed in the aggregated form of α-chymotrypsin. The chemical modifications indicated that the surface charges of the enzyme play an important role in the aggregation process.

E4.11
The effect of calcium ions and phosphorylation on the Starmaker conformation – FRET studies

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Biomineralization is the process by which living organisms produce biominerals such bones, shells, teeth and otoliths. Starmaker (Stm) is involved in biomineralization of otoliths in *Danio rerio*.Stm belongs to a class of intrinsically disordered proteins (IDPs). Stm demonstrates a low content of ordered secondary structure and no tightly packed hydrophobic core. Typical IDPs are very flexible, but may adopt rigid conformations in the presence of ligands or other proteins. Stm has a significantly extended rod-shaped conformation, however calcium ions, which are putative ligands of Stm, induce compaction of the extended conformation of Stm. We have applied Forster resonance energy transfer (FRET) to examine the flexibility of Stm. FRET D-A pairs spanning the protein molecule were created by inserting tryptophan (donor) and cysteine residues (labeled with IAEDANS probe as an acceptor) by site-directed mutagenesis. The Stm conformational changes detected by the distances measurements between chosen D-A pairs were monitored in the presence of calcium ions. Phosphorylation of Stm by PKA and GSK-3 kinases have been shown. We have examined the effect of phosphate groups on the Stm conformation.

E4.12
A native-like fold of region 4 of initially unfolded σ^{70} subunit of *E. coli* RNA polymerase induced by TFE

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Recombinant protein rEC$\sigma^{70}{}_4$ comprises domain 4 of *E. coli* RNA polymerase σ^{70} subunit, which is responsible for recognition of DNA promoter -35 region. It was found sufficiently soluble only unfolded in low-pH solution. Its folding, accompanying addition of 2,2,2-trifluoroethanol (TFE), was monitored by TFE-induced migration of signals in a series of ^{15}N HSQC spectra. A common trend of uniform upfield shift of resonances brakes down for some residues at 10–15% TFE, evidencing the buildup of non-helical regions separating initially induced helices. The cooperative reorganization of non-helical regions suggests that rEC$\sigma^{70}{}_4$ somehow folds into a structure compatible with canonical DNA-recognizing HLHTH motif existing in homologous domains of thermophilic bacteria. The sequential distribution of TFE-induced secondary chemical shifts (CSI) clearly indicates that TFE acts not only as a canonical helix inducer, but also tends to stabilize a spatial arrangement of the initially induced secondary structure elements. Analysis of ^{15}N relaxation data at 10 and 30% of TFE content demonstrated that the whole HLHTH motif displays increased values of the order parameter, indicating reduced backbone flexibility in the nanosecond time scale and some contributions from slow motional process on the micro- to millisecond time scale. For protein in 30% TFE solution, 23 structural cross-peaks unequivocally assigned in ^{15}N- and ^{13}C-edited NOESY spectra were used to build low-resolution model of protein structure.

E4.13
Crystal structure of an intramolecular chaperone mediating triple-β-helix folding

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Protein folding is often mediated by molecular chaperones. Recently, a novel class of intramolecular chaperones has been identified in tailspike proteins of evolutionarily distant viruses, which require a C-terminal chaperone for correct folding. The highly homologous chaperone domains are interchangeable between pre-proteins and release themselves after protein folding. We solved the crystal structures of two intramolecular chaperone domains in either the released or the pre-cleaved form, revealing the role of the chaperone domain in the formation of a triple-β-helix fold. Tentacle-like protrusions enclose the polypeptide chains of the pre-protein during the folding process. After the assembly, a sensory mechanism for correctly folded triple β-helices triggers a serine-lysine catalytic dyad to autoproteolytically release the mature protein. Sequence analysis shows a conservation of the intramolecular chaperones in functionally unrelated proteins presumably sharing triple β-helices as a common structural motif.

E4.14
Production of single chain antibodies against prion protein in *Escherichia coli* and *Bacillus subtilis*

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Prion diseases or transmissible spongiform encephalopathies are fatal neurodegenerative diseases that affect humans but also several mammalian species. Monoclonal antibodies with high affinity are used as immunodignostic and therapeutic reagents against prion diseases. Antibodies direct against PrPC, when added to scrapie–infected cell culture or delivered *in vivo*, can result in elimination of PrPSc or prevent its replication. Here we reported the expression and purification from *E. coli* and from Bacillus subtilis of a single chain antibody derived from 8H4 monoclonal antibody. The two systems were evaluated in order to obtain corrected and folded antibodies since 8H4 antibodies contain three disulphide bridge necessary for its functional activity. The expression of the only single chain 8H4 protein was very low and insoluble in *E. coli* and therefore we have produced it as fusion protein to thioreodoxin or chaperone Dnak. Thioredoxin fusion protein when expressed in Origami *E. coli* strain has shown the formation of correct disulfide bonds in cytoplasm and enhances the solubility of fusion protein. The fusion protein is able to recognized prion protein *in vitro* in western blot and also it is internalized by the cells via PrpC in bovine prion protein CHO expressing cells. The chaperone fusion DnaK although gives large amount of fusion protein it is largely insoluble and refolding system will be reported. *B. subtilis* is a gram positive bacteria most popular in the field of biotechnology for its high capacity of production and secretion of recombinant protein at low cost. Here will reported the expression of secreted protein via shuttle *E. coli*/*B. subtilis* expression plasmid pHT43.

E4.15
Assembly capacity of major capsid protein VP1 of hamster polyomavirus are influenced by size and position of truncations at its carboxy-terminal region

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The VP1 protein of polyomaviruses is composed of a central globular tightly packed domain (about 250 amino acids in length) and of more loosely packed domains at both ends. The carboxy-terminal region of VP1 is responsible for the interaction of pentameric subunits in the capsids of polyomaviruses. Therefore, it was considered that the carboxy-terminal region cannot be deleted or substituted by foreign sequences without disrupting the assembly process. The objective of this study was to investigate the potential influence of the carboxy-terminal region of hamster polyomavirus (HaPyV) VP1 protein on the assembly of virus-like particles (VLPs) using carboxy-terminally truncated or modified VP1 proteins expressed in yeast. The HaPyV-VP1 was modified at its carboxy-terminal region by consecutive truncations and single amino acid exchanges. The ability of of yeast-expressed VP1 variants to form VLPs strongly depended on size and position of truncations. VP1 variants lacking 21, 69 and 79 amino acid (aa) residues at its carboxy-terminal region efficiently formed VLPs similar to those formed by the unmodified VP1 (diameter 40–45 nm). On the contrast, VP1 derivatives with carboxy-terminal truncations of 35 to 56 aa residues failed to form VLPs. VP1 mutants with a single A336G aa exchange or internal deletions of aa 335 to aa 346 and aa 335 to aa 363 resulted in the formation of VLPs of a smaller size (diameter 20 nm). These data indicates that certain parts of the carboxy-terminal region of VP1 are not essential for pentamer-pentamer interactions in the capsid, at least in the yeast expression system used.

E4.16
Zn(II) ions inhibit fibrillization of monomeric insulin

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Insulin, a 51-residue peptide hormone is an intrinsically amyloidogenic peptide, forming amyloid fibrils *in vitro*. In the secretory granules insulin is densely packed into Zn2Insulin6 crystals that assures osmotic stability of vesicles and prevents the peptide fibrillization. However, after release from the pancreatic β-cells insulin dissociates into active monomers, which tend to fibrillize not only at acidic but also at physiological pH values. We studied the effect of Zn(II) that are co-secreted with the peptide on the fibrillization of insulin by monitoring the increase in the fluorescence of the fibril reactive dye Thioflavin T. Zn(II) inhibited the fibrillization of monomeric insulin at physiological pH values. At pH 7.3 the inhibitory effect of Zn(II) was very strong (IC50 = 3.5 μM), whereas Zn(II) ions decreased the fibrillization rate constant and increased the lag phase of the process. At pH 5.5 the inhibitory effect of Zn(II) was much weaker since 20 μM Zn(II) caused only a slight increase in the lag period. The pH dependence points towards participation of His residue(s) in the Zn(II)-induced inhibition of insulin fibrillization. It is known that His residue at position B10 participates in binding of Zn(II) ions. The results indicate that Zn(II) may be physiologically relevant suppressor of the fibrillization of insulin at its release sites and in circulation. It is hypothesized that misfolded oligomeric intermediates occurring in insulin fibrillization pathway, especially under

the conditions of zinc deficit, may induce autoantibodies against insulin that may lead to β-cell damage and autoimmune type I diabetes.

E4.17
Interrelationships between coiled-coils, intrinsically disordered sequences and charged single α-helices

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Both intrinsically disordered proteins and coiled-coil sequences are predicted to constitute a significant fraction of both prokaryotic and eukaryotic proteomes. Using a number of prediction algorithms, we present a detailed analysis of cross-predictions of such sequences. Our results show that coiled-coils are usually predicted to be unstructured, which is in agreement with the fact that they form dimers or multimers and are not folded as a monomer. Reverse cross-predictions are less frequent presumably due to the (usually) heptad repeat signature characteristic of coiled-coils. To achieve acceptable prediction accuracy while minimizing cross-predictions, we suggest the use of the programs Coils and IUPred. In addition, we have developed a web server for the prediction of charged single α-helices based on the consensus of two methods. We suggest that in some cases, cross-predictions might mark evolutionary transitions along the complex coiled-coil – charged single α-helix[1] – functionally disordered route[2].

Keywords: coiled-coil, intrinsically disordered protein, structure prediction, protein evolution

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E4.18
The tube model in Gromacs: a platform for the study of protein folding and aggregation

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Protein aggregation occurs on timescales that are extremely difficult to explore by using full atomistic simulations. In the last 20 years, however, it has been realized that many essential features of protein behaviour, including for example the importance of the initial hydrophobic collapse in protein folding and aggregation, emerge in a very simple and elegant fashion by using simplified models. Here we take advantage of this approach by adopting the so-called ''tube model'', which includes excluded volume and self-attractive interactions that capture very effectively hydrophobic effects and hydrogen bonding. The tube model has been already used to model successfully the essential features of folding landscapes and of the process of amyloid formation. In this study we present an implementation of the tube model in the Gromacs simulation package, which is one of the most used molecular dynamics software suites. Our specific objectives are: (i) To create a tool based on a commonly used software platform to facilitate the use of the tube model; (ii) To combine the computational advantages of the tube model with the integration of experimental data (including e.g. NMR chemical shifts) to obtain structural and dynamical information on systems with large time and size scales, such as those involved in protein aggregation; (iii) To augment the tube model with residue-specific interactions, including electrostatic and van der Waals interactions. We finally present an initial application of the current implementation to the modeling of formation of oligomeric assemblies.

E4.19
An algorithm and experimental protocol to obtain the kinetic parameters of fibrillar growth from a first-principles general model of breakable filament assembly

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Recently we presented an analytical solution to describe the kinetics of self-assembly of breakable filaments. This result is particularly interesting since it provides a powerful tool to study protein amyloid aggregation. Here we present an effective protocol to obtain, using the model, the kinetic parameters of amyloid aggregation from experimental data, including the rate of filament breakage, the rate of growth, and the nucleation parameters. The initial theoretical framework has been here extended to allow a general and accurate analytical solution that can be applied to almost all cases of interest. Furthermore a simple but detailed experimental protocol is laid down and validated to guide experiment design, so to obtain the best possible estimates of the parameters. The fitting algorithm is implemented on a publicly-accessible web server for use by experimentalists wordwide. Numerical and experimental validation of the procedure is presented.

E4.20
Different mechanisms behind low enzyme activity *in vivo* of two different variants of Thiopurine S-methyltransferase, TPMT

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In treatment of acute lymphoblastic leukemia and inflammatory bowel disease (IBD) thiopurines such as azathioprine and 6-mercaptopurine are used. All of these drugs are prodrugs and are, in the cell, converted to 6-thioguanines (6-TGNs) and incorporated into DNA or inhibiting purine synthesis. A key enzyme for this regulation is the cytosolic enzyme thiopurine S-methyltransferase (TPMT). This enzyme degrades azathioprine and 6-mercaptopurine to methylmercapto-purine and thereby reduces the bioavailability of the 6-TGNs incorporated into DNA. TPMT is a polymorphic enzyme with at least 29 different allelic variants known today and is one of the more classical examples of pharmacogenetics where the TPMT enzyme activity of the allelic variants is directly correlated to the clinical dosages of the

thiopurines, with a 10–15 fold dosage reduction for an allelic variant with low TPMT enzyme activity. Even though TPMT is a well studied protein. Many studies have been performed in yeast "suspensions" and not on pure protein solutions. It has been speculated and in a few cases shown that the reason for the low activity for most of the allelic variants is mainly due to the low stability and/or tendency to aggregate. The mutations in this study TPMT *2 (A80P) and TPMT * 5 (L49S) are both situated at a distance far from the active site, however the enzyme activities are severely affected at 37°C. Preliminary results, using a repertoire of techniques such as CD, fluorescence and limited proteolysis experiments suggest two different mechanisms for the low enzyme activity at a temperature corresponding to *in vivo* conditions.

E4.21
Oligomerization and conformation of amyloidogenic protein human stefin B. Insight from ESI MS

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Human stefin B, an 11 kDa intracellular protein, is a member of the cystatin family of cysteine protease inhibitors. Cystatins have a tendency to form domain-swapped dimers, higher oligomers and amyloid fibrils. Misfolding, aggregation and fibril formation of peptides and proteins is a common feature of amyloidogenic diseases including several major neurodegenerative diseases. The aggregation pathway of amyloidogenic proteins into the fibrils is thought to pass through transitional oligomeric forms, which are considered to be the toxic entities and their structure is in focus of intensive research. In the current study we demonstrate that electrospray ionisation mass spectrometry (ESI MS) with preliminary separation by size exclusion chromatography (SEC) is well applicable to study oligomerization and conformational states of human stefin B. We demonstrate that interpretation of ESI MS spectra allows clear determination of distinct stefin B oligomeric forms, starting from dimers up to dodecamers. Application of charge-state distribution analysis of ESI MS spectra indicated opening of compact conformational state of stefin B monomer in TFE solutions. We think that conformational changes of monomeric stefin B, which was shown to form molten globular intermediate before, might be crucial for oligomerization and fibrillization of this protein. We also show the applicability of ESI MS for determination a complex between amyloid β (Aβ) peptide implied in Alzheimer's disease and the dimers of stefin B. We propose that the ESI MS approach can be used for the study of aggregation of certain type of amyloidogenic proteins, especially those with rather stable oligomers.

E4.22
Characterization of the aggregation competent state of the acylphosphatase from Sulfolobus solfataricus

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Aggregation of proteins into amyloid-like aggregates is linked to the onset of several human diseases, ranging from neurodegenerative diseases to systemic amyloidoses. The first step of this process is the conversion of proteins from their functional state into an aggregation competent conformation. Understanding the properties of this conformation is fundamental in order to develop therapies aimed to combat these tremendous diseases. The acylphosphatase from Sulfolobus solfataricus (Sso AcP) is a globular protein, commonly used as a model for aggregation studies, characterized by the presence of an 11 residue long unfolded N-terminal segment. The protein aggregates starting from an ensemble of native-like conformations formed with no need of unfolding events across the major folding energy barrier. The crucial event in the process is the establishment of an interaction between the N-terminal segment and the globular unit of two different Sso AcP molecules. When separated, neither the N-terminal segment nor the globular unit of the molecule are able to aggregate. These features make this system particularly suitable to study amyloidogenic states. Using H/D exchange experiments monitored through NMR we are characterizing the aggregation competent conformational ensemble populated by the globular unit of the Sso AcP molecule. This approach will allow the determination of the regions that become more flexible under aggregation promoting conditions. The comparison between data measured in the presence/absence of peptides corresponding to the N-terminal segment will lead us to the determination of the regions implied in the establishment of the amyloidogenic interaction.

E4.23
An orientational preference of a transmembrane helix in a multispanning membrane protein can influence the membrane insertion efficiency of a marginally hydrophobic neighbour helix

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The efficiency with which a segment of a nascent polypeptide chain is incorporated into the ER membrane via the Sec61 translocon is to a great extent dependent on its overall hydrophobicity. Nevertheless, previous studies of natural and engineered membrane proteins have clearly shown that also features extrinsic to a specific polypeptide segment may influence its membrane insertion efficiency. In the current study, we analyze the effect of one such feature. In a transmembrane polypeptide, segments rich in positively charged amino acid residues show a strong tendency to be located on the cytosolic side of the membrane. We investigate how a lysine-induced orientational preference of a transmembrane helix influences the membrane insertion efficiency of a preceding marginally hydrophobic segment. For this purpose, we analyze the topology of leader peptidase variants synthesized in the presence of rough microsomal membrane. We found that the level of hydrophobicity required for efficient membrane incorporation of a polypeptide segment was altered by lysines inserted into the C-terminal flank of the subsequent transmembrane helix. The hydrophobicity threshold decreased when the N-terminal end of the segment oriented towards the cytosolic side of the membrane and increased when the N-terminal end oriented towards the lumenal side. In summary, this study constitutes a systematic characterization of how a transmembrane helix may influence the insertion efficiency of a neighbouring one. Additionally, it exemplifies a long-range effect of positively charged amino acids on the topology of a membrane-spanning protein.

E4.24
Structure studies of human frataxin and its higher order structures: implication in Friedreich's ataxia

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Iron is an essential part of prosthetic groups such as heme and iron sulphur clusters, which are involved in various redox reactions of central importance for life. Iron is not easily available to organisms, mainly due to the low solubility of its most common form Fe^{3+}. The Fe^{2+} variant has higher solubility however it is easily oxidized by atmospheric oxygen to Fe^{3+} as well as being toxic to organisms, since it promotes the generation of highly reactive oxygen species (ROS), capable of causing severe damage to proteins and nucleic acids. Therefore, cells require mechanisms to overcome the limited bioavailability and potential toxicity of this metal. Some severe human diseases are related to irregularities in iron metabolism and one of them is the neurodegenerative disease Friedreich's ataxia. Sufferers of this disease have extensive GAA repeats in the first intron of the gene encoding the protein frataxin which disrupts and reduces the production of frataxin. Frataxin is involved in iron detoxification, storage and delivery in the mitochondrial matrix and when being recombinantly expressed monomers as well as oligomers are produced. Only the 3D structure of the monomer has been resolved, this by X-ray crystallography to a resolution of 1.8 Å. The oligomers are well suited for studies with Transmission Electron Microscopy (TEM) and in combination with negative staining; single particle reconstruction can be performed. We have been working on optimising conditions and stains needed in order to perform TEM and do single particle reconstruction of frataxin, which presently is being carried out.

E4.25
Integration of chemical information with protein sequences and 3D structures from PDB

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PDBeMotif is an extremely fast and powerful search tool that facilitates exploration of the Protein Data Bank (PDB) by combining protein sequence, chemical structure and 3D data in a single search. Currently it is the only tool that offers this kind of integration at this speed. PDBeMotif can be used to examine the characteristics of the binding sites of single proteins or classes of proteins such as Kinases and the conserved structural features of their immediate environments either within the same specie or across different species. For example, it can highlight a conserved activation loop common to protein kinases, which is important in regulating activity and is marked by conserved DFG and APE motifs at the start and end of the loop, respectively. The prediction of the effect of modifications to small molecules that bind to the active and/or regulatory sites of proteins on their efficacy can be based on the outcome of analytic work done using PDBeMotif. It can be ported to all major operating system platforms such as MS Windows, LINUX, Apple Mac and Solaris as it is written in Java and uses Oracle and the free source PostGreSQL database server. PDBeMotif can be used online or downloaded and installed locally where public and private PDB files (including libraries of theoretically derived 3D structures) can be loaded and analyzed. There is also the capability to load protein site annotations, families and domains from Distributed Annotation System (DAS) servers.

E4.26
Reactive Oxygen Species stimulate phosphorylation of eIF2α independent of protein folding in ER-stressed cells

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The eukaryotic initiation factor-2 (eIF2) is an oligomeric protein that recruits the methionyl initiator transfer RNA (met-tRNA) to the 40S small ribosomal subunit during translational initiation. The α-subunit of eIF2 (eIF2α) can be phosphorylated by several eIF2α kinases during cellular stress. EIF2α kinases such as PKR and GCN2 could be activated by oxidative stress, suggesting that reactive-oxygen-species (ROS) produced within cells may lead to phosphorylation of eIF2α. However, it is currently uncertain if protein folding had any role to play in ROS-induced phosphorylation of eIF2α. We had addressed this issue by studying the effect of ROS on eIF2α phosphorylation in HeLa cells treated with tunicamycin (TUN) that interrupts protein folding in the endoplasmic reticulum. Incubation of HeLa cells with TUN resulted in a significant increase in the intracellular level of ROS, phosphorylation of eIF2α, activation of PERK, and splicing of XBP-1 mRNA. In the presence of cycloheximide (CHX), the intracellular level of ROS was still elevated and the phosphorylation of eIF2α was still observed in response to TUN. However, there was no activation of PERK or the splicing of XBP-1, suggesting that protein misfolding is not the only cause for TUN-induced production of ROS and phosphorylation of eIF2α. The addition of dithiothreitol (DTT) to cells treated with both CHX and TUN resulted in a marked reduction of eIF2α phosphorylation. Our data suggested that protein folding does not have to be involved in ROS-mediated phosphorylation of eIF2α.

E4.27
A potassium switch of ATP-induced GroEL conformational changes

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Bacterial chaperonin GroEL utilizes the co-chaperone GroES to assist folding of cellular proteins in an ATP-dependent manner. An ATP-induced allosteric communication through GroEL double rings is prerequisite for the overall chaperonin cycle. It has been shown by Horovitz et.al. (1995) that ATP-induced allosteric transitions of GroEL consist of two phases with one at relatively low ATP concentrations (≤ 100 μM) and the second at higher concentrations of ATP with a midpoint in the range of 16 to 160 μM. Here we studied the effect of essential metal cofactor, potassium ion, on the ATP-driven allosteric transitions of GroEL by using a tryptophan mutated GroEL. Within the test range of potassium concentration, two distinct patterns of ATP-induced allosteric transitions were observed. We showed that at 50 mM potassium concentration, a remarkable increasing second-phase of ATP-induced allosteric transition was observed which is contrary to the decreasing phase at 10 mM potassium concentration.

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E5 – Catalytic Mechanisms

E5.01

Abstract withdrawn

E5.02
Quantification of the primary antioxidant capacity of different molecules

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We have developed a kinetic method for determining the antioxidant capacity of a variety of molecules. In this method, denominated the enzymatic kinetic method, the free radical of ABTS is generated continuously in the reaction medium by the peroxidase/ABTS/H2O2 system. The presence of an antioxidant in the solution provokes a lag period in the accumulation of the free radical in the medium, and by studying this lag period it is possible to calculate the antioxidant capacity of the molecule in question. This antioxidant capacity will be quantified by n, the number of electrons donated per molecule of antioxidant in the primary antioxidant capacity, efficient concentration, EC50, ratio of the antioxidant concentration necessary to decrease the initial concentration of ABTS radical to 50% [EC50 = 1/(2n)] and primary antioxidant or antiradical power (ARP), (ARP = 1/EC50 = 2n). This work has been partially supported by grants from several Spanish organizations. Ministerio de Educación y Ciencia (Madrid, Spain) Project BIO2009–12956, from the Fundación Séneca (CARM, Murcia, Spain) Projects 08856/PI/08 and 08595/PI/08, from the Consejería de Educación (CARM, Murcia, Spain) BIO-BMC 06/01-0004 and from the Consejería de Salud y Bienestar Social de la Junta de Comunidades de Castilla La Mancha, Project FISCAM PI-2007/53. JLMM has a fellowship from the Ministerio de Educación y Ciencia (Madrid, Spain) Reference AP2005-4721. FGM has a fellowship from Fundación Caja Murcia (Murcia, Spain).

E5.03
Alteration of catalytic mechanism of tomato allene oxide synthase LeAOS3 and alfalfa hydroperoxide lyase MtHPL by site-directed mutagenesis

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Crucial enzymes of lipoxygenase pathway of fatty acids metabolism are cytochromes P450 of the CYP74 family comprised of allene oxide synthases (AOS), hydroperoxide lyases (HPL) and divinyl ether synthases (DES). For substitution with site-directed mutagenesis we have chosen amino acids forming conservative for all cytochromes P450 oxygen-binding domain and ERR-triad. Substitutions K302S,F295I,S297A and T366Y in LeAOS3 sequence led to changes in catalysis. Along with product characteristic for LeAOS3 reaction – α-ketol – there are product of HPL reaction, particularly 9-oxononanoic acid. Substitutions of K302S,S297A and T366Y possess both activities of AOS and HPL. The α-ketol and 9-oxononanoic acid are detected in different ratios. In the case of F295I substitution activity of AOS is absent. Along with 12-oxododecenoic acid – product characteristic for reaction of wild type enzyme – MtHPL mutant forms F284I,G288I,N285A,N285T and F287V catalyze formation of novel products 13-oxotridecadienoic and 11-oxoundecenoic acids due to catalysis disruption. Results of site-directed mutagenesis prove the CYP74 family origin from one ancestor with HPL activity. Amino acid substitution in any significant for catalysis site of LeAOS3 led to conversion it into HPL, but not DES as one could expect in some cases. Substitution in the same sites of MtHPL led to appearance of novel products due to catalysis disruption, but not to exchange of catalysis type.

E5.04
Quantification of the secondary antioxidant capacity of different molecules and their kinetic antioxidant efficiency

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When we study the antioxidant capacity of a compound and any deviations appears in the kinetic, it will reflect chemical reactions of the reaction products, in which more free radical molecules are consumed, reflecting a secondary antioxidant capacity. To compare the secondary antioxidant capacity of different antioxidants, we propose a non-enzymatic kinetic test consisting of the kinetic analysis of free radical consumption at fixed initial antioxidant concentration and excess free radical concentration. This excess of free radical would be the result of the initial concentration minus the concentration consumed in its reaction with the antioxidant. The secondary antioxidant capacity is characterized by n, the number of electrons donated per molecule of antioxidant in the chemical reactions of the reaction products, efficient concentration, ECS50, ratio of the antioxidant concentration necessary to decrease the initial concentration of ABTS radical to 50% in the secondary antioxidant capacity [ECS50 = 1/(2n)] and secondary antioxidant or antiradical power (ARPS), (ARPS = 1/EC50 = 2n). This work has been partially supported by grants from several Spanish organizations. Ministerio de Educación y Ciencia (Madrid, Spain) Project BIO2009-12956, from the Fundación Séneca (CARM, Murcia, Spain) Projects 08856/PI/08 and 08595/PI/08, from the Consejería de Educación (CARM, Murcia, Spain) BIO-BMC 06/01-0004 and from the Consejería de Salud y Bienestar Social de la Junta de Comunidades de Castilla La Mancha, Project FISCAM PI-2007/53.

E5.05
New links between protein biosynthesis and nonribosomal peptide synthesis

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Aminoacyl-tRNA synthetases (aaRS) catalyze ATP-dependent attachment of amino acids to tRNA in protein synthesis. Similar role play adenylation domains in template independent nonribosomal peptide synthesis (NRPS), by attachment of activated pre-

cursors to dedicated carrier proteins. Although they catalyze analogous reactions in mechanistically similar manner, using aminoacyl-adenylates as intermediates, adenylation domains and aaRS are structurally and evolutionary unrelated. The genomes of many bacteria are sprinkled with putative ORFs encoding the proteins homologous to class II aaRS catalytic core, but lacking tRNA binding domain. We have cloned and characterized three such aaRS truncated homologs. Sequence analysis shows clear homology, while crystal structure determination confirmed remarkable structural similarity to seryl-tRNA synthetases (SerRS) from methanogenic archaea. In spite of close resemblance to SerRS, these enzymes have different amino acid specificity. Even more surprising, the SerRS homologs do not aminoacylate tRNA, but instead they transfer amino acids to thiol group of phosphopantetheinyl prosthetic arm of carrier proteins found in their genomic vicinity. Thus, the reaction they catalyze is essentially the same as the reaction catalyzed by adenylation domains in NRPS. It has been long speculated about possible evolutionary relations between programmed ribosomal protein synthesis and template independent NRPS, based on weak thiol acylation activity of contemporary aaRS. Our findings of SerRS homologs engaged exclusively in carrier protein acylation and thioester bond formation provide direct experimental evidence that further supports this hypothesis.

E5.06
A QM/MM study of mechanism of antidote action in reactivation of tabun inhibited AChE

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The world food production is closely related with growing use of pesticides in agriculture. Pesticides based on neurotoxic organophosphorous compounds (OPC) and related chemical warfare agents have long attracted attention of the scientists to the development of efficient antidotes for the poisoning with OPC. OPC inhibit irreversibly acetylcholinesterase (AChE), a key enzyme in cholinergic neurotransmission, by phosphylation. Oximes are in use for reactivation of AChE and regeneration of its catalytic activity as antidotes. To design new reactivators a better understanding of the reactivation process is needed. Our aim was to study the reaction mechanism of reactivation using a QM/MM methodology. We selected the AChE-tabun conjugate since tabun is one of the most potent nerve agent, and very resistant to reactivation. We selected oxime reactivator K027 (4-carbamoyl-1-(3-{4-[(E)-(hydroxyimino)methyl]pyridinium-1-yl}propyl)pyridinium bromide) due to its effectiveness in reactivation of tabun or pesticide inhibited AChE. QM/MM approach allows computation of the reaction energy profile for the active site atoms trapped inside the native protein environment. First we modelled reactivation reaction on a truncated system using a quantum mechanical (QM) calculation at the BP86/6-311 + G(d,p) level of theory. For the molecular mechanical (MM) part, the CHARMM force field was used. We showed that during reactivation oxime needs to change its position inside active site of AChE to form transition state with AChE-tabun conjugate. Analysis of AChE-tabun-K027 transition state structure identified the critical residues not only for binding of oximes but also for reactivation of enzyme.

E5.07
Investigation of inhibition effect of human placental fructose 1,6 bisphosphate aldolase with bivalent ion

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Fructose-1,6-bisphosphate aldolase (FBPA) is a major glycolytic enzyme found in most cells. FBPA, is a homotetramer with a molecular mass of 160 kDa. Fructose-1,6-bisphosphate aldolase reversibly catalyses the cleavage of fructose 1,6 bisphosphate into the triose phosphates: glyceraldehyde phosphate and dihydroxyacetone phosphate. In this study, we carried out inhibition effects of bivalent ion on Fructose-1,6-bisphosphate aldolase. We have investigated the interaction of human placental aldolase with bivalent Mg + 2 metal. It was defined that, Mg + 2 is competitive inhibitor of healthy human placental FBPA. Ki values of healthy human placental aldolase for Mg + 2 ion was confirmed 14.678 ± 2.702 mM

E5.08
Oxidative protein folding catalyzed by a selenocysteine variant of DsbA

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The *Escherichia coli* dithiol oxidase DsbA, one of the most oxidizing thioredoxin-like disulfide oxidoreductases characterized to date (E°' = -122 mV), catalyzes formation of structural disulfide bonds in the bacterial periplasm. Previous studies with the redox enzymes *E. coli* thioredoxin (E°' = -270 mV) and glutaredoxin 3 (E°' = −198 mV) in which the catalytic cysteine was replaced with selenocysteine showed a marked decrease in redox potential and, in the case of glutaredoxin 3, an increase in the rate of reoxidation by an oxidized substrate. To extend the natural properties of DsbA, we replaced the nucleophilic Cys30 from the active-site with selenocysteine using cotranslational selenocysteine insertion via a designed SECIS element in the dsbA gene and coexpression of the selA, selB, and selC genes. The resulting enzyme (C30U DsbA*) shows remarkably little change in structure, redox potential, or kinetics of *in vitro* substrate oxidation and reoxidation by DsbB compared to the control variant harboring Cys30 (DsbA*) and wild type DsbA. Nevertheless, C30U DsbA* only partially complements DsbA in a *dsbA* deletion strain, most likely due to its low expression level. This new selenoenzyme provides a framework for investigating the differing reactivities of selenol and thiol groups in a protein context, and for evolving tailored enzymes for studying oxidative protein folding *in vivo*.

E5.09
Kinetic and dynamic properties of interface mutants of alkaline phosphatase from *E. coli*

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Although alkaline phosphatase (APase) from *E. coli* crystallizes as a symmetric dimer, it displays deviations from Michaelis-Menten kinetics, supported by a model describing a dimeric enzyme with unequal subunits. The proposed model, describing the mechanism of substrate hydrolysis, encompasses a conformational change mediated by subunit interactions. Role and mechanism of

possible communication between subunits of this dimmeric enzyme are not resolved. Rigid segments in the polypeptide structure supposedly accomplish transfer of conformational information, between the active sites, across the subunit interface. The importance of the subunit interface and the β-pleated sheet, stretching from underneath an active site to the subunit surface, in the catalytic mechanism has been probed by site-directed mutagenesis. The mutation replacing Thr-81 with alanine and Gln-83 with leucine were introduced into the APase gene. Amino acid residues Thr-81 and Gln-83 are located within the β-pleated sheet at the contact surface between the subunits, and form hydrogen bonds with analogous amino acids from the adjacent subunit. Two kinds of mutant proteins were prepared, a single T81A and double T81A/Q83L mutant. Dynamic properties and rigidity of Trp-109 environment of WT APase and mutant enzymes were assessed by acrylamide fluorescence quenching and by measuring phosphorescence lifetime of Trp-109 located in the vicinity of the mutation site and β pleated sheet. Influence of mutations on the kinetic properties determined in 1 M Tris/HCl, pH 8, and in 0.35 M 2A2M1P, pH 10.5 was compared with the effects that mutations had on rigidity of a protein structure.

E5.10
Nano-sized horseradish peroxidase cross-linked aggregates

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Horseradish peroxidase has been considered as a useful enzyme toward applied purposes such as remove of oil contamination. However various criteria limit soluble enzyme applications which can be relieved in immobilized preparations. In this study carrier free immobilization of horseradish peroxidase (HRP EC 1.11.1.7) is used and cross-linked enzyme aggregates (CLEAs) were prepared. Nano-CLEAs products were characterized for size and shape using dynamic light scattering (DLS) and scanning electron microscopy, respectively. Optimization of nano-scaled CLEAs preparation was carried out upon stoichiometric considerations on safe enzyme cross-linking using glutaraldehyde as a bifunctional cross-linking agent. The products of immobilization have resulted with improved kinetic features and stability. For example catalytic performance of horseradish peroxidase improved and thermal stability of nano-sized CLEAs was increased. However, the pH profile of cross-linked enzyme aggregates did not show observable difference in comparison with the free one.

E5.11
Immobilization of horseradish peroxidase aggregates into porous silica

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Enzyme stabilization is one of the major challenges in the industrial biocatalytic processes. In this investigation cross linked horseradish peroxidase aggregates (EC 1.11.1.7) were immobilized into porous silica with wide range pore size diameters. To reach this goal, cross-linked enzyme aggregates were prepared and then effective parameters on particle size including enzyme concentration, time of aggregation and pH were investigated. The particle size of CLEAs became bigger with increasing the concentration of enzyme. Hence, the loaded amount of CLEAs into porous silica decreased. CLEAs formed at different pH also immobilized into porous silica. The immobilized amount was much larger in acidic environment due to the fact that particle size of CLEAs was smaller in acidic condition. Time of aggregation that is the interval between adding the precipitant and the bifunctional cross linker had an direct influence on particle size, increasing this time led to formation of big particles and consequently low small loading into porous silica. The size of CLEAs was determined by dynamic light scattering.

E5.12

Abstract withdrawn

E5.13
Engineering of *Bacillus* sp. strain TS-23 α-amylase for improving its thermal and oxidative stabilities

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BAC£GNC/£GRS is a thermostable variant derived from the truncated α-amylase (BAC£GNC) of alkaliphilic *Bacillus* sp. strain TS-23. With the aim of enhancing its resistance towards chemical oxidation, Met231 of BAC£GNC/£GRS was replaced by leucine to create BAC£GNC/£GRS/M231L. The functional significance of the 31 C-terminal residues of BAC£GNC/£GRS/M231L was also explored by site-directed mutagenesis of the 483th codon in the gene to stop codon (TAA), thereon the engineered enzyme was named BAC£GNC/£GRS/M231L/£GC31. BAC£GNC/£GRS/M231L and BAC£GNC/£GRS/M231L/£GC31 were very similar to BAC£GNC in terms of specific activity, kinetic parameters, pH ¡V activity profile, and the hydrolysis of raw starch; however, the engineered enzymes showed an increased half-life at 70¢XC. The intrinsic fluorescence and circular dichroism spectra were nearly identical for wild-type and engineered enzymes, but they exhibited a different sensitivity towards GdnHCl-induced denaturation. Performance of the engineered enzymes was evaluated in the presence of commonly used detergent compounds and some detergents from the local markets. A high compatibility and performance of both BAC£GNC/£GRS/M231L and BAC£GNC/£GRS/M231L/£GC31 may be desirable for their practical uses in the detergent industry.

E5.14
Catalysis of immobilised human flavin-containing monooxygenase

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Human flavin-containing monooxygenase isoform 3 (hFMO3) is a hepatic enzyme that catalyses the oxygenation of a large number of structurally diverse drugs and xenobiotics transforming them into benign and readily excretible products. Since the design and development of new therapeutic drugs may take advantage of these detoxifying properties, much interest has been focused on the development of novel techniques suitable for pharmacological research applied to hFMO3 catalytic properties. With this aim, an hFMO3 electrochemical sensor, developed by modifying a glassy carbon electrode with an entrapping gel obtained by glutaraldehyde co-crosslinking of hFMO3 with bovine serum albumin, is reported in this work. Redox properties of hFMO3 sensor have been compared with those of FAD entrapped gel electrode by cyclic voltammetry, revealing for the protein bound FAD, a significant shift of redox peaks towards negative potentials and a total reversibility of the redox reaction. The redox potential measured for the entrapped protein was −411 ± 10 mV

(versus Ag/AgCl). The responsiveness of the sensor was investigated with four different substrates, trimethylamine, ammonia, triethylamine and benzydamine (nonsteroidal anti-inflammatory drug) by calculating kinetic parameters including the apparent Michaelis-Menten constant (82.3 ± 4.3; 94.1 ± 6.4; 120.7 ± 11.2 and 115.9 ± 6.8 μM, respectively), sensitivity (39–45 mAM-1cm-2) and response linearity from 2–80 μM. The data obtained confirm that the hFMO3 sensor has good characteristics in terms of substrate detection, reproducibility and stability, therefore can be employed for catalytic activity measurements of new chemical entities turned over by hFMO3.

E5.15
Least-motion mechanism in enzyme catalysis

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Enzymes are dynamic catalysts that lower the activation barrier compared to the corresponding reaction in solution by utilizing a set of catalytic strategies. These include proximity effects, electrostatic stabilization of the transition state (TS), symmetrical hydrogen bonds and tunneling. An additional possibility is that enzymes have evolved to use an economy in atomic motion upon the enzyme-substrate complex (ES) to TS transition, as has been shown for example for benzoyl formate decarboxylase (EC 4.1.1.7) as well as for serine proteases. Here we present data consistent with a least-motion mechanism for lipase catalysis based on experimentally determined k_{cat} values, molecular dynamics simulations and ab initio B3LYP calculations. Lipases are of particular industrial interest due to their high stability in organic media, substrate tolerance and stereoselectivity. It could be beneficial to take the least-motion mechanism into consideration in protein engineering and rational design projects since certain transition state conformations violate the least-motion mechanism and can thus be disregarded as unproductive.

E5.16
The effects of selected flavonoids on the adenosine deaminase activity

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Adenosine Deaminase (ADA, EC 3.5.4.4) was purchased from Sigma-Aldrich (A-1155, 1.000 Units) and its kinetic properties were appraised in this study. ADA, catalyses the irreversible deamination of adenosine and 2'-deoxyadenosine to inosine and 2'-deoxyinosine, respectively. Quercetin, apigenin, kaempferol, naringenin, rutin, luteolin, kaffeic acid as selected flavonoids, and chloroform, carrot extract were evaluated whether they have inhibitory potencies on the kinetic properties of ADA or not. Km and Vm values of the enzyme against adenosine substrate was found to be 105μM and 89.4 IU/l respectively by using Linewever-Burk graphics. The effects of above-mentioned flavonoids, chloroform, carrot extract and some factors like temperature and pH on the enzyme kinetics were observed. Quercetin, caffeic acid and naringenin were exhibited most potent inhibitor for ADA, and IC50 values were found 6.1, 6.06 and 6.15 μM respectively. Kinetic analysis showed a mixed type inhibition for quercetin, caffeic acid and naringenin. Ki values were found 314.2, 429.6 and 435.7 μM respectively. The other flavonoids and chloroform had no important inhibitory potencies on the ADA activity. Interestingly, carrot extract were exhibited strong inhibitory effect on the ADA activity, and IC50 values was found 105 μg/ml. The ranges of optimal pH and temperature were found 6.5–8.0 and 25–50°C respectively. As a result, inhibitory potencies of flavonoids seem to be structurally related (hydroxylation pattern), and this effect may be due to structural/pharmacological properties.

E5.17
Further evolution of β-lactamase activity from glyoxalase II to different quasi-species

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The global use of antibiotics is dominated by β-lactam derivatives, like penicillins and cephalosporins. However, widespread use of β-lactam antibiotics leads to the explosion of antibiotic resistance. Moreover, resistance is emerging rapidly after the introduction of new antibiotics into clinical practice. A major mechanism of β-lactam resistance involves the rapid evolution of β-lactamases, which hydrolyze the β-lactam ring, thus sabotaging antibacterial properties. In a previous study, human glyoxalase II (GlyII) was converted into a metallo-β-lactamase (MBL) by a combined approach involving rational design and directed evolution. The engineered enzyme (evMBL8) exhibited enhanced resistance with cefotaxime (a 3rd-generation cephalosporin antibiotic). However, catalytic efficiency with cefotaxime compared to IMP-1, the natural counterpart of evMBL8, was low and evMBL8 did not show any observable activity with other β-lactam antibiotics. Based on sequence homology analysis and homology modeling, a triple mutant of evMBL8 (evMBL9) was constructed. A preliminary screening in *Salmonella typhimurium* indicated that evMBL9 conferred resistance to different β-lactam scaffolds, including penicillins, cephalosporins and carbapenems. A combined approach to multiple site-directed mutagenesis and random mutagenesis by error-prone PCR was performed to further evolve evMBL9 to different quasi-species. And a systematic study of enzyme activity, enzyme stability and expression is being conducted.

E5.18
Evolvability of a molten globular chorismate mutase and its thermostable counterpart

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It has been hypothesized that many modern enzymes evolved from molten globular precursors. The diverse conformations that intrinsically disordered proteins can adopt could be advantageous in the search for novel function. To address this hypothesis, we are comparing the evolvability of crippled variants of an engineered molten globular chorismate mutase and its thermostable counterpart from *Methanococcus jannaschii*. The enzymes were diversified by error-prone PCR and DNA shuffling, and functional variants were selected in an *in vivo* complementation system whose stringency is controlled by a tetracycline-inducible promoter and a protein degradation tag. The first rounds of mutagenesis and selection have yielded improved catalysts in both cases. For the molten globule, favorable mutations also appear to increase stability. Detailed kinetic and biophysical characterization of the evolved variants will be used to examine the role that structural preorganization plays in enzyme catalysis and evolution.

E5.19
Glutathione reductase immobilized on chitin particles

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Glutathione reductase (GR) from baker's yeast (EC 1.6.4.2) catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) by NADPH. We covalently bound GR to chitin particles (CP) Tyler 35 prepared from crab carapace. The aim of this work is to compare the properties of free and immobilized GR and to check whether there is any evidence of altered catalytic mechanism after immobilization. Furthermore, this is the first step of a new method for specific quantification of glutathione. The immobilization method includes chitin activation with hexamethylenediamine (HEMDA) and glutaraldehyde. CP (1g) were activated with 12.8 ml 2% (w/v) HEMDA at 40°C for 2 hour. The excess of HEMDA was removed, and then 3 ml of 3% (v/v) glutaraldehyde were added. After 30 minutes at 30°C, active CP were washed five times with 10 ml of absolute ethanol. Subsequently, the material was washed three times with 10 ml of 100 mM phosphate buffer pH 7.0. For immobilization, GR (0.22 U) was added to 250 mg of active CP and incubated at 4°C, overnight. We show that GR was successfully immobilized on chitin with retention of its catalytic activity and could be reused. Its optimal pH and temperature were 6.5 and 35°C respectively. For the unbound GR, optimal pH and temperature were 7.0 and 25°C respectively. One factor that contributes to the shift in pH optimum value is the modification imposed on the ionizing groups of the enzyme caused by the immobilization reaction. If the reaction involves amino groups near the catalytic center, this may change the kinetic behavior of the enzyme, in particular in relation to pH.

E5.20
An insight in active obelin complex formation

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The Ca^{2+}-regulated photoprotein obelin is a bioluminescent protein emitting light on calcium binding. Apo-obelin can be converted into active photoprotein by incubation with coelenterazine in the presence of oxygen and reducing reagents. Concentration dependent quenching of the apo-obelin intrinsic fluorescence by coelenterazine has been applied to determine the apparent dissociation constant for coelenterazine binding with apo-photoprotein. Coelenterazine binding is found to be a millisecond-scale process, in contrast to the formation of active photoprotein complex taking dozens of minutes. A model describing active obelin formation is suggested. The model allows estimating the activation energy and rate constants of the process. According to the obelin spatial structure Tyr138, His175, Trp179 and Tyr190 side chains form hydrogen bonds with the peroxy and carbonyl groups of bound coelenterazine. Almost all mutants with substitution of these residues exhibit luciferase-like bioluminescence activities, very low photoprotein activities and extremely slow active complex formation. It is proposed that His175 participates not only in stabilizing the 2-hydroperoxycoelenterazine, needed for light production, but also in active obelin complex formation as a proton carrier. The Tyr138 function is supposed to provide the positioning of oxygen near the C2-carbon of coelenterazine followed by 2-hydroperoxycoelenterazine formation. Supported by: Wageningen University Sandwich PhD-Fellowship program, RFBR grant 09-04-12202.

E5.21

Abstract withdrawn

E5.22
Ca^{2+}-triggered coelenterazine-binding protein from *Renilla muelleri*: spatial structure and properties

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The luciferase from *Renilla* catalyzes an oxidative decarboxylation of coelenterazine with the generation of the coelenteramide in an excited state. The excited coelenteramide relaxes to its ground state with the production of blue light. At present *Renilla* luciferase is one of the widely used bioluminescent reporters for *in vivo* imaging. Bioluminescence of marine coelenterates is initiated by increase in intracellular Ca^{2+}. In sea pansy *Renilla*, Ca^{2+}-induced bioluminescence involves two distinct proteins, Ca^{2+}-triggered coelenterazine-binding protein (CBP) and luciferase. CBP contains one molecule of a tightly bound coelenterazine which is released for reaction with luciferase and O_2 only subsequent to Ca^{2+} binding. The stabilization of coelenterazine and its analogs within CBP against autooxidation and Ca^{2+}-triggered substrate release has a remarkably simple explanation from the spatial structures: CBP with bound coelenterazine and Ca^{2+}-loaded apo-CBP. We also demonstrate that CBP with bound coelenterazine and its analogs reveals greater thermostability and at least two times higher light output in reaction with both wild type luciferase and its mutants than that with free coelenterazine. Thus, in prospect CBP might be used as a carrier and protector of coelenterazine (or its analogs) in imaging experiments *in vivo*. Supported by RFBR grant 09-04-12202 and Lavrentiev grant of SB RAS.

E5.23
Is 2′-hydroxy group on the A76 of the peptidyl tRNA necessary for peptide bond formation?

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The peptide bond formation and peptide release reaction by hydrolysis of the last peptide bond takes place in the peptidyl transferase center of the ribosome. Despite remarkable progress in the last decade in the elucidation of ribosome structures, the catalytic mechanism of peptide bond formation and peptide release remains unclear. Peptide bond formation occurs through nucleophilic attack of the α-amino group of aminoacyl-tRNA at the carbonyl group of peptidyl-tRNA and formation of a tetragonal transition state. In this process, a proton is transferred from theα-amino group of aminoacyl-tRNA to the 3'-OH group (A76) of the peptidyl-tRNA. Recent investigations suggested that the (A76) 2'-OH of the peptidyl tRNA plays a role in this proton shuttle. Esterase 2 from Alicyclobacillus acidocaldarius (Est2) was used as a reporter to monitor translation in the *E. coli in vitro* translation system. Codons on different positions of mRNA were substituted by UAG, an RF1-dependent stop

codon. These constructs were used to test peptide bond formation by tRNASer(CUA)-2'dA as compared to tRNASer(CUA)-A in a coupled *in vitro* transcription/translation system. The results indicate that 2'-OH of both, peptidyl-tRNA and aminoacyl tRNA can be omitted in the peptide bond formation reaction. The former observation that tRNASer(CUA)-2'dA is inactive in elongation is probably related to applied experimental system and low processivity during translation initiation, before the nascent peptide chain is being localized in the ribosomal exit channel.

E5.24
Kinetic characterization of the *Drosophila lebanonensis* ADH T114V mutant

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Drosophila lebanonensis alcohol dehydrogenase (DlADH) belongs to the short-chain dehydrogenase/reductase (SDR) superfamily. DADH contains three conserved amino acid residues Ser138, Tyr151 and Lys155 in the active site and an eight-membered water chain that connects the buried active site with the bulk solvent. The OH-group in the side-chain of Thr114 is a part of the water chain. It has been proposed that this water chain functions as a proton relay system during catalysis. We have therefore produced a T114V mutant in DlADH and investigated the kinetic properties of the mutant. The results were compared with the results of the wild type DlADH. Kinetic studies revealed that the reaction mechanism for the mutant enzyme like the wild type enzyme was consistent with a compulsory ordered mechanism where the coenzyme binds to the free enzyme. As for the wild type DlADH, propan-2-ol is a better substrate than ethanol. For the mutant enzyme, the hydride transfer step in the oxidation of ethanol was reduced approximately four times compared to the wild type enzyme, while the dissociation rate of the binary enzyme-NADH product complex was unaffected. The k_{on} velocity as well as the binding strength for NAD^+ was reduced for the mutant compared to the wild type enzyme. Furthermore, the pK_a value for the binding of ethanol to the binary enzyme-NAD^+ complex was 7.9 compared to 7.1 for the wild type enzyme. These results will be discussed in view of the water chain as a proton relay during catalysis.

E5.25
Novel Inhibitors of Matrix Metalloprotease-9 and -14

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Matrix metalloproteinases (MMPs) have the capability to cleave any component of the extracellular matrix (ECM) and process a large number of ''non-ECM'' proteins. MMPs have complicated biological functions playing a role in several normal and pathological conditions. Cleavage of a substrate by one MMP may promote disease while cleavage of another substrate by the same or another MMP may prevent disease. As sites outside the catalytic region are involved in cleavage of macromolecular substrates, it is pertinent to develop compounds that bind either to these sites or to sites in the catalytic domain. In the present work, various compounds have been tested for their capacity to inhibit MMP-9 and MMP-14 activity. The compounds varied in size, structure and putative zinc chelating properties. Enzyme kinetic studies were performed with a fluorogenic substrate to determine both IC50 values and the type of inhibition caused by the compounds. Molecular modeling was done by docking the compounds into the X-ray crystal structures of MMP-9 and MMP-14 using the Glide docking program. The results revealed that the strongest inhibitors showed IC50 values in the lower μM region, while those with the weakest binding had IC50 values > 1000 μM. There were no striking differences between the two enzymes with respect to obtained IC50 values. Surprisingly, some of the compounds gave a mixed type of inhibition while others gave a competitive inhibition pattern against the fluorogenic substrate. When the compounds were docked into the free enzyme, they interacted with the catalytic zinc ion of both enzymes.

E5.26
Enzymatic modification of manno-glycans

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The interest of using hemicellulose as a renewable resource for novel oligomeric and polymeric products is currently increasing. With this in mind we combine molecular biology, protein crystallography, enzyme kinetics and mass spectrometry in our studies of enzymes active against the major softwood hemicellulose: *O*-acetyl galactoglucomannan (GGM). We have recently developed an enzyme triggered drug delivery gel on the basis of GGM. The hydrolytic properties of hemicellulose degrading enzymes from Aspergilli are being studied in detail. α-Galactosidases from family 27 and family 36 are compared in terms of substrate specificity and efficiency in removal of galactose side groups. β-Mannanases are interesting not only because of backbone cleavage of GGM. As retaining glycoside hydrolases they are theoretically able to catalyze transglycosylation reactions, useful for the biosynthesis of new hemicellulose-based glyco conjugates. Results on the enzyme substrate interactions important for transglycosylation capacity, based on comparison of kinetic properties, hydrolysis pattern and 3D structures of wild-type and mutated active site variants of a family 26 β-mannanase (CfMan26A) will be discussed. Several family 5 β-mannanases have strong aglycone (acceptor) binding and we have generated a range of mutants in this region to study differences in tolerance of various acceptors, ranging from sugar- to non-sugar acceptors such as alcohols. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry and high performance anion exchange chromatography (HPAEC) is used to analyze the transfer capacity of the enzymes being studied.

E5.27
Application of reverse-phase HPLC to quantify oligopeptide acetylation eliminates interference from unspecific acetyl CoA hydrolysis

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Protein acetylation is among the most common covalent modification in eukaryotic cells and is performed by different acety-

ltransferases. The typical protein substrates for these enzymes are either the ε-amino group of specific lysine sidechains or the α-amino group on protein N-termini. The lysine acetylation is performed by KATs while the N-terminal modification is performed by NATs. The most widely used methods to study the catalytic activity of these enzymes are based on two different principles. One alternative is to quantify the amount of radioactively labelled oligopetide products formed when radioactive acetyl-CoA is used as substrate. The other principle uses an enzyme-coupled reaction assay measuring the conversion of acetyl CoA to the product CoASH. Due to several disadvantages using these methods, we designed a new strategy to study oligopeptide acetylation. Using reverse phase HPLC, we show that the kinetic parameters of hNaa30p, the catalytic subunit of the human NAT C complex, can be precisely determined. The reported method quantifies the consumption of both substrates (acetyl-CoA and oligopeptides) and the formation of both reaction products (acetylated oligopeptides and CoASH). Further, this method is also fully compatible with subsequent product analysis, by e.g. mass spectroscopy, allowing for identification and verification of the acetylation site(s). The reverse phase HPLC method is highly reproducible and therefore well-suited to determine the kinetic parameters of protein acetyltransferases.

E5.28
Mutagenetic and crystallographic characterization of *r*Hint1, the phosphoramidates hydrolase with the P-S cleavage activity

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The histidine triad nucleotide binding protein1 (Hint 1) is an enzyme of the first branch of the HIT superfamily that catalyses the hydrolysis of P-N bond in AMP-N-ε-lysine, AMP-N-alanine, AMP-N-morpholidate substrates with the rate of ca.˜ 1 nmol/min/μg and adenosine- 5'- monophosphoramidate (AMP-NH2) with the rate of 1.71 nmol/min/μg [1]. Recently we reported that in the cleavage reaction of the P-diastereoisomers of 5'-O-[N-(tryptophanylamide)phosphoramidothioate catalyzed by the *wt* *r*Hint1, the hydrolysis of P-N bond was accompanied by a parallel loss of the sulfur from the resulting AMPS [2]. In an independent experiment we have demonstrated that the *wt* Hint1 hydrolyzes the AMPS with the rate of ca. 0.2 nmol/min/μg resulting in formation of AMP and H2S as final products. We have also noticed that other ribonucleoside phosphorothioates and corresponding deoxyribonucleosides are also substrates for Hint1 hydrolase. In our extended studies on desulfuration process and P-S cleavage ability of Hint1 enzyme, we have used mutagenetic approach to generate Hint1 cystein mutants. Since one of the product of desulfuration reaction is H2S, it was of great interest to measure the activity of three Hint1 mutants : C38A, C84A, C38/84A towards AMPS and AMP-Lys substrates in order to exclude that H2S (formed in the reaction with AMPS) is the product from cystein residues present in polypeptide chain of Hint1. We have also crystallized the cystein mutants and compared their structure with native Hint1 protein. The trials to co-crystallize the Hint1 enzyme with AMPS are in progress.

E5.29
Substrate binding mechanism of type I extradiol dioxygenase from *Rhodococcus* sp. strain DK17

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Rhodococcus sp. strain DK17 is known to employ a single extradiol dioxygenase in alkylbenzene metabolism. The crystal structures of AkbC and the enzyme-substrate (3-methylcatechol) complex were determined. AkbC was found to be composed of eight identical subunits, each containing one ferrous ion. A monomeric AkbC molecule has two structural elements, N-domain (residues M1 – G138) and C-domain (K139 – G300) including the C-terminal tail (H285 – G300), which covers its active site. In each monomer, the ferrous iron is coordinated by the typical 2-His-1-carboxylate facial triad motif plus an additional amino acid residue Y253 in the C-domain. In the substrate bound AkbC structures, 3-MC interacts the iron with single hydroxyl group representing an intermediate state in the substrate binding process. Structure-based mutagenesis revealed that the C-terminal tail and the β-hairpin structure (β11-β12) form parts of the substrate-binding pocket that is responsible for correct positioning of the substrate at the active site for subsequent cleavage and also for its substrate specificity. AkbC's longer C-terminal region compared to dihydroxybiphenyl dioxygenases restricts the substrate range. Widening the opening of the active site results in an increase of the enzyme activity against dihydroxybiphenyl. We propose a substrate binding mechanism which includes a sequential interaction of two hydroxyl groups of 3-methylcatechol for bidentate binding to the iron and a rearrangement of the substrate bound position in the octagonal iron coordination to be deprotonated and open the site for an oxygen.

E5.30
Mechanism of photoprotein bioluminescence

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Ca^{2+}-regulated photoproteins are responsible for the light emission of a variety of marine coelenterates. Direct information concerning the bioluminescence mechanism derives from X-ray structures of ligand-dependent conformational states of photoproteins. The bioluminescence results from the Ca^{2+}-triggered decomposition of a high energy intermediate, the 2-peroxycoelenterazine observed to reside in the binding cavity and stabilized in place by a H-bond network. We have proposed that movement of residues occurring as a result of accommodating the Ca^{2+} in the nearby Ca^{2+}-binding loops, would perturb these H-bonds, destabilizing this intermediate and allowing the decarboxylation reaction that generates the product coelenteramide in its excited state. In the cavity structure of the Ca^{2+}-discharged photoprotein, a water molecule nearby the coelenteramide amide is so positioned that, if it were in this same place in the intermediate conformation, it could donate a proton to the first formed dioxetanone anion, prior to its decomposition to the excited state product. Thus the primary product would be the neutral excited coelenteramide (λ_{max} = 390–405 nm). It is proposed that the lower energy excited state giving rise to the blue bioluminescence (λ_{max} = 460–495 nm), results from excited state proton transfer from the OH bound to the 6-phenyl substituent, to a nearby His residue as a proton acceptor. Supported by: ONR, NIH, Georgia

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E5.31
Antioxidant properties of new novel synthetic molecules; N-Methyl Nitrones

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Nitrones have the general chemical formula X-CH = NO-Y. They were first used to trap free radicals in chemical systems and then subsequently in biochemical systems. The nitrone-based free radical traps have significant potential in the treatment of many harmfull disease such as neurodegenerative complaint, cancer, ischaemia/reperfusion injury, endotoxaemia. It has been focused on free radical scavenging activity of new novel synthetic molecules named as Nitron 5.1.1 and 5.1.2.For this aim, N- Methyl Nitrones free radical trapping activities tested with based on the reduction of 1,1-diphenyl-2-picrylhydrazyl (DPPH). Due to the presence of an odd electron DPPH gives a strong absorption maximum at 517 nm. As this electron becomes paired off in the presence of a hydrogen donor, i.e. a free radical scavenging antioxidant, the absorption strength is decreased, and the resulting decolorization is stoichiometric with respect to the number of electrons captured. Result showed that nitrons 5.1.1 and 5.1.2 have more powerful free radical trapping activity compare with butylated hydroxytoluene (BHT) accepting as potent antioxidant molecule. Percentage inhibition of Nitron 5.1.1, Nitron 5.1.2 and BHT were found as 62.3, 62.2 and 23.6 respectively at 33.3 μM for each molecules. In the N- Methyl Nitrones studied on, methyl groups, which are found on the carbon adjacent to sulphur atom on the thiophene ring, are thought to end a second group DPPH radicals. This is also confirmed by the results of the study. In two of the most active experimented nitrones, the radical is delocalized on the carbon bound to methyl groups and this leads to using up of a second DPPH.

E5.32
Catalatic and peroxidatic mechanisms of *Mycobacterium tuberculosis* catalase-peroxidase (KatG)

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Catalase-peroxidases (KatG haem enzymes) share the catalytic characteristics of classical catalases and peroxidases, but function by different mechanisms. *M. tuberculosis* KatG, in its catalatic' reaction with peroxide, undergoes the reaction cycle of Eqs. (1) & (2): (1) KatGFe(III) + HOOH -> I* -> I (2) I + HOOH -> IS -> KatGFe(III) + dioxygen. The overall catalatic reaction involves at least three intermediate species, a transient form "I*", a persistent form "I", and the rate-limiting "Michaelis" complex "IS". Complex IS is populated only at high peroxide levels and decays as peroxide concentration falls to zero, but the compound I-like species "I" persists, as both formation and removal require reaction with peroxide. It decays slowly by secondary reactions with endogenous or added hydrogen donors. The three species have been partially characterised using stopped flow spectrophotometry and rapid freeze electron paramagnetic resonance (EPR). Their UV-visible spectra are unique, and their EPR signals indicate involvement of at least two distinct protein radicals. None of these intermediates seems to be chemically closely analogous to the corresponding compounds involved in catalysis by classical unitary catalases and peroxidases. Reaction of KatG with peracetic acid produces a somewhat different population of compound I–like species. Both peracetate-induced and peroxide-induced forms can also react peroxidatically with hydrogen donors such as guaiacol. The three catalatic intermediates however show different reactivities towards such donors. A model mechanism will be proposed for the roles of haem iron compounds and protein radicals in both types of catalysis.

E5.33

Abstract withdrawn

E5.34
Enzymatic mechanism of bacterial NAD kinases

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We determined the crystal structures of NADK1 from Listeria monocytogenes (Lm) in free state and bound to NAD and NADP. The conserved GGDGT motif of NADKs has been suggested to be catalytically important and to be involved in nucleotide binding or in phosphate transfer. Our analysis revealed that the Aspartate in the conserved GGDGT motif has a different role in Phospho Fructose Kinases (PFKs) and NADKs. In PFKs this conserved residue is involved in ATP activation, whereas in NADK it is involved in NAD activation. In the LmNADK1-NAD the side chain of Asp-45 becomes hydrogen-bonded to 2'-hydroxyl group of NAD. To investigate the role of Asp-45, we determined the crystal structure of LmNADK1-D45N mutant. The active site of this mutant was similar of the wild-type enzyme with the ligand present in the same conformation. The Asparagine adopted the same buried conformation as Aspartate but did not form any hydrogen bond with NAD. The mutation D45N resulted in a 10-fold decrease in activity. Thus Asp-45 appears as a key residue for catalytic activity of LmNADK1.

E5.35
Effects of various inhibitors on α-Amylase in *Anoxybacillus sp.* AH1 isolated from hotspring

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α-Amylase in *Anoxybacillus sp.* AH1 was partially purified and some properties such as optimum temperature, pH and thermal stability were studied. The optimum temperature and pH for the enzyme were found as 7.0 and 60°C, respectively, and found to be sensitive to temperature. The thermal stability were enhanced by 30% glycerol. Km and Vmax values were calculated from Lineweaver-Burk plot as 0.102 and 0.929, respectively. The enzyme activity was increased in the presence of various detergents, while significantly inhibited by the concentrations of urea. α-Amylase was significantly activated by Mg^{2+} (41% at 8 mM) and Ca^{2+} (70% at 8 mM), while greatly inhibited by Zn^{2+} (85% at 0.5 mM and 93% at 1 mM) and Cu^{2+} (76% at 0.5 mM and 100% at 1 mM) as well as by the metal ion chelators EDTA (63% at 10 mM) and 1,10-phenanthroline (22% at 10 mM), indi-

cating that this enzyme is a metalloenzyme. 2-Mercaptoethanol (64% at 10 mM) and DTT (more than 100% at 10 mM) were found to enhance α-amylase activity to a great extent, while p-chloromercuribenzoic acid (PCMB) inhibited the enzymatic activity (52% at 4 mM), indicating the presence of at least one essential Cys residue modified by the reagents in the active site of the enzyme. Iodoacetamide and N-ethylmaleimide had a little effect on the enzyme. Phenylmethylsulfonyl fluoride (PMSF) inhibited the enzyme strongly (60% at 4 mM), also showing the participation of serine at the enzyme activity. This study is one of the first studies to report for the characterisation of an α amylase in *Anoxybacillus* species.

E5.36
Structural analysis of active site of cytokinin oxidase/dehydrogenase

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The flavoenzyme cytokinin oxidase/dehydrogenase (CKO) irreversibly deactivates plant hormones cytokinins and thus participates in controlling their metabolic level. CKOs catalyze the oxidative breakdown of isoprenoid cytokinins to adenine/adenosine and the corresponding unsaturated aldehydes. Oxygen as well as 2,6-dichlorophenolindophenol (DCPIP) or para-quinone derivatives function as electron acceptors. An extracellular CKO from *Zea mays* (ZmCKO1) was expressed in the yeast *Yarrowia lipolytica*. Subsequently, several active-site mutants were constructed and their kinetic properties were analyzed to clarify the importance for catalysis of amino acid residues like H105, D169, E288, E381, P427, V378, L492, W397 and others located at the entrance or inside the active site. Certain mutations led to variations in substrate specificity but also influenced the sensitivity to inhibitions by *N*-phenyl- and *N*-benzyl-*N'*-pyridylurea derivatives. Reaction rates and affinity of several mutants to electron acceptors like DCPIP or coenzyme Q_0 also changed. The mutant H105A containing a noncovalently bound FAD was active and showed both reduced reaction rates and affinity to natural substrates. Several mutants were crystallized and the crystals infiltrated with substrates, inhibitors or coenzymes. Then X-ray data were collected and processed. Refinement of the structures is underway. *Supported by grants No. 522/08/P113 from the Czech Science Foundation and No. MSM 6198959215 from the Ministry of Education, Youth and Sports of the Czech Republic.*

E5.37
Structural analysis of plant aminoaldehyde dehydrogenases

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Aminoaldehyde dehydrogenases (AMADH, EC 1.2.1.19) catalyze the terminal step in polyamine catabolism. They oxidize various ω-aminoaldehydes like 4-aminobutyraldehyde (ABAL) and 3-aminopropionaldehyde (APAL) to the corresponding ω-amino acids that individually participate in processes of defense against drought, salinity and other stress events and biosynthesis of carnitine and secondary metabolites (fragrance compounds, alkaloids). Based on their amino acid sequence, the enzymes belong to the same group as stress-related betaine aldehyde dehydrogenases (BADHs, EC 1.2.1.8). There are commonly two AMADH isoenzymes present in plants. To understand their function, we expressed two AMADHs from pea (*Pisum sativum*) and performed an X-ray crystallographic study together with kinetic analysis using a large set of natural and synthetic aminoaldehydes. The crystal structures of pea AMADHs in complex with NAD^+ coenzyme were refined at 2.4 and 2.15 Å resolution, respectively. They revealed a dimeric status of the enzymes and provided a detailed description of the coenzyme and substrate binding site. Likewise, we analyzed kinetic properties of two AMADHs from tomato (*Lycopersicon esculentum*) and two isoenzymes from maize (*Zea mays*). Our results show that all six studied enzymes preferred APAL and ABAL to betaine aldehyde as substrates. Although substrate preference can vary among species, in terms of wider substrate specificity the above-mentioned enzymes should be considered as AMADHs and not BADHs. *This work was supported by grant 522/08/0555 from the Czech Science Foundation and grant MSM 6198959215 from the Ministry of Education, Youth and Sports of the Czech Republic.*

E5.38
Assay of insect polyphenol oxidase-educational aspect

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Polyphenol oxidase, PPO catalyses the hydroxylation of monophenols and the oxidation of o-diphenols to o-quinones. PPO is named phenolase, catecholase, tyrosinase still, although the EC assigns two names, monophenol monooxygenase and o-diphenol oxidase. Principally, PPO is involved in: synthesis of melanins, biogenic amines, betalains; detoxification and toxicaction of xenobioics; protein-phenol binding; carcinogenesis, ROS production; plant food processing. PPO analysis is applicable to biochemistry; clinical diagnostics; chemical resistance of plants; food biochemistry/technology and other fields. PPO assay appears significant practice for science and medicine students. Usually, students study potato PPO with pyrogallol/quaiacol. I propound staining procedure for aphid PPO (EC 1.10.3.1), with L-and D-DOPA, catechol, catechin, quercetin, caffeic, chlorogenic, gallic, protocatechuic acids. For detection of salivary PPO aphids (other Homoptera or Heteroptera) puncture agarose (1.25%)–sucrose (30%) gels incorporating 0.1% phenolics. Colouring of the saliva inside gels demonstrates PPO reaction. For PPO of salivary glands, digestive system, cuticle and hemolymph (1–10μl) these are freshly dissected/collected and incubated with 0.1% L-DOPA, in 0.1 M Na-phosphate buffer, pH 7.4 for 1hour at 30°C. Darkening is visible at the light microscope. Controls incorporate 0.005 M phenylthiourea. In my opinion this practice is appropriate to explain/discuss theoretical and analytical subjects on insect PPO i.e. exogenous/endogenous, extracellular/intracellular occurrence; lack of specificity; melanin synthesis pathway; microscopic visualization; analysis *in vitro*, *in vivo* and *in situ*.

E5.39
Structural parameters of *Zoanthus* yellow fluorescent protein chromophore from molecular dynamics in gas phase and condensed phase

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Fluorescent proteins are very useful for tracking the localization and dynamics of proteins in living cells and as reporters for gene expression studies. They are commonly used in resonance energy-transfer applications and in multicolour labelling. For this reason, it is important to understand how the protein functions and its chromophore on the atomic scale, explaining the role of particular atoms, residues and hydrogen bonds. Green fluorescent protein GFP (PDB entry code 1EMB), purified from the jellyfish *Aequorea victoria*, is the best known and most well described fluorescent protein. The properties of the protein, e.g. a structure, chromophore geometry, absorption and emission spectra have been widely investigated for years. In recent years, there has been an increase in published results from quantum mechanical (*ab initio* and DFT) analyses of the GFP chromophore *in vacuo* and condensed phase. Another GFP like protein is *Zoanthus* yellow fluorescent protein (zFP538). zFP538 (PDB entry code 2OGR), purified from coral, is less known and examined. It is believed, however, that the zFP538 chromophore comprises an additional six membered ring, causing a shift in the absorption and emission maxima to longer wave lengths compared to GFP chromophore. The zFP538 chromophore was investigated by molecular mechanics (MM), using the CHARMM27 force field. MM energy minimisations and molecular dynamics (MD) simulations were applied. Analyses were carried out in the gas phase, water and a protein environment. The calculated structural parameters were compared to *ab initio* and DFT data available from the literature.

E5.40
Towards the structural understanding of the catalytic activity acquisition Application to autoimmune diseases

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Since the catalytic antibody discovery, they are often found in an autoimmune disease background. Today, the catalytic antibody production and their role are still unclear. This work has two goals: To define the amino acids which confer a catalytic function to an antibody and to understand more precisely the relation catalytic antibody-autoimmune disease. For this purpose, a phage display system is used as a selection tool of single chain fragment variables (scFv) expressed in fusion with gIIIp. Four phage banks have been synthesized from spleen-cell of four various background mice (Healthy and nonimmunized; autoimmune and non-immunized; healthy and immunized; autoimmune and immunized). First of all, scFv banks derived from spleen-cell repertoires of mice were constructed. The heavy chain variable regions (VH) and light chain variable regions (VL) of antibodies were amplified by specific primer mix. The VH and VL sequences have been randomly associated by a sequence homology. The scFv banks were cloned into a phagemid. The phages will be produced after bacterial transformation. The hypothetical catalytic scFvs will be selected against a specific compound and tested to characterize a catalytic activity. The comparison of catalytic scFv sequences between themselves will provide insights to define the amino acids which confer a catalytic function. The comparison of catalytic scFv repertories from nonimmunized mice would give us information about the presence of catalytic sequence in the antibody germ line and the comparison between nonimmunized and immunized mice would bring insights about the role of somatic mutations for the acquisition of catalytic function by antibody.

E5.41
Substrate specificity and catalytic mechanism of the *Escherichia coli* FrlB amadoriase

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Amadoriases are a class of enzymes catalyzing the decomposition of Amadori products. The latter are formed in a spontaneous chemical reaction known as non-enzymatic glycosylation (glycation) of the Maillard reaction. Recently, an amadoriase enzyme, the FrlB amadoriase, has been identified in Eschrichia coli (Wiame et al. JBC 2002; 277 42523-42529). This enzyme has been shown to catalyze the hydrolysis of fructoselysine 6-phosphate to lysine and glucose 6-phosphate. In the reverse reaction the enzyme has failed to catalyze the attachment of six other amino acids (glycine, valine, glutamine, methionine, arginine and isoleucine) to glucose 6-phoshpate. Accordingly, the enzyme has been considered to contribute to bacterial utilization of fructoselysine in the intestine. In the present study we provide evidence for broader substrate specificity of the enzyme. In both the forward and the reverse reaction the enzyme was found to use as substrates predominantly charged and polar amino acids. Importantly, high molecular mass polypeptides such as Poly-L-lysine also served as substrates for the FrlB amadoriase. Thus the FrlB amadoriase appears the first enzyme capable of deglycating polypeptides by acting directly on the bond between the sugar moiety and the amino group. In addition, we obtained data arguing for an oxidative rather than hydrolytic mode of action of the enzyme. Based on these result we suggest a different physiological role for the *E. coli* FrlB amadoriase.

E5.42
Analysis of self-catalytic maturation capability of galactose oxidase by site-directed mutagenesis

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In this study, self-processing mechanism of galactose oxidase was investigated in *E. coli* by altering some amino acids which were supposed to play a crucial role in pro-peptide removal. Galactose oxidase from *F. graminearum* (NRRL 2903) is a monomeric, copper- containing 68 kDa enzyme with an unusual thioether bond. It has a 41 aa leader peptide, including a signal peptide, an 8 aa long putative pre-peptide, and a 17-aa N-terminal pro-peptide which is autocatalytically cleaved by the aerobic addition of Cu^{2+} to yield a 639 amino acid mature protein. Propeptide cleavage is followed by the formation of a thioether

bond at the Cu^{2+}-containing active center. Following the expression of mutant GOase constructs, mutated at the junction of the 25 aa long pre-pro peptide region and the mature protein region, protein samples were purified and analyzed. Results have shown that all mutant proteins were slightly above 70 kDa, which is above the size of the mature enzyme. N-terminal sequencing has shown that all constructs retained the 25 aa long pre-pro-peptide region, suggesting absence of cleavage. However, these mutant pre-pro-GOases were all active. Accordingly, it was shown for the first time that the cleavage of the pro-peptide is not essential for the formation of the thioether bond, hence, activity of the enzyme. Comparison of the activities of the pre-pro and mature forms of GOase showed that there is not any significant difference between them. It is suggested that the 8 aa long putative pre-peptide is responsible from the lack of cleavage, thus if deleted, autocatalytic cleavage of the pro-peptide is expected to take place. This hypothesis is now under investigation.

E5.43
Multiscale QM/MM modelling and enzyme catalysis

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Enzymes are catalysts with a very peculiar structure. They are macromolecules with usually tens of thousands of atoms, which have a small active site that participates directly in the reaction and a large scaffold that, among other functions, may create an electrostatic field that promotes catalysis. The way computational chemists deal with these systems is to build up a hybrid QM/MM model [1]. However, beyond the different size scales of the two regions, the enzyme structure changes within large time scales, which can not be efficiently explored with a QM/MM model that used a high accuracy hamiltonean in the quantum region. Here we review recent work in enzymatic catalysis, using coupled and decoupled methodologies, emphasizing the strategies that decouple the time-scales and size-scales of the system [2, 3]. The decoupling approximation may be very rewarding as it allows for the efficient exploration of all aspects of catalysis. It consists in the use of pure classical mechanics, pure quantum mechanics and hybrid methods, each one in a different model of the same enzyme, to study the same catalytic mechanism, and make use of the information of a given size scale to model/calibrate the adjacent size scale.

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E5.44
Cloning, expression and purification of two yeast genes (ADH, FDH) in *E. coli* and determination of their catalytic properties

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Natural flavoring additives of plant origin such as six carbon aldehydes and alcohols are currently used as additives in foods, beverages and cosmetics. In recent decades, enzymes are used for their processing, especially oxidoreductases. The most preferred enzymes of the group oxidoreductases is alcohol dehydrogenase (ADH), which in the presence of cofactors $NADH/NAD^+$ catalyzes the interconversion of C6-aldehydes and C6-alcohols. *In vitro* conditions were successfully used to reduce the trans-2-hexenal to trans-2-hexenol ADH from *Saccharomyces cerevisiae*. ADH, as most of the enzymes of the group oxidoreductases is dependent on nicotinmide cofactor β-1,4-nicotinamide adenine dinucleotide (NADH) or β-1,4-nicotinamide adenine dinucleotide phosphate (NADPH). Unlike enzymes, cofactors are consumed in stoichiometric amounts together with the substrates. Because of the high price of these compounds there is considerable interest in developing effective regeneration methods. Presently in enzyme bioreactors for the *in vitro* regeneration of NADH from NAD^+ intensively used enzyme is NAD^+ dependent formiat dehydrogenase (FDH) from methylotroph bacterial species *Candida boidinii* is used. In this work we have cloned the two above mentioned yeast enzymes in various expression vectors. After the optimization of the expression in *E. coli* cells, initial fermentation runs were carried out, followed by purification in preparative amounts using different affinity tags. The determination of enzyme activity and stability was carried out and the possibility of their subsequent application in bioconversion of C-6-aldehyde in the form of free or immobilized enzymes was verified.

E5.45
Conserved regions among azathioprine-active glutathione transferase chimeras show high sensitivity to mutations and give a 10-fold activation when inserted into low-active wild type enzymes

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Upon conjugation of azathioprine with glutathione, 6-mercaptopurine is released, which induces DNA damage. GSTs with improved azathioprine activity have potential uses as selectable markers in gene therapy and in antibody-directed enzyme prodrug therapy. Chimeric glutathione transferases (GSTs) were previously generated by DNA shuffling and two recombined segments were identified as primary determinants of azathioprine activity. These segments correspond to part of the substrate-binding H-site and they were examined with respect to sufficiency for azathioprine activity and tolerance to point mutations. Insertion of these two small regions from the active human GST A2-2 into the low-active human GST A3-3 and rat GST A3-3 generated stable chimeras with 10-fold enhanced azathioprine activity. Two potential active site residues in these regions were mutated to produce a library with all possible residue combinations. 1920 colonies were screened and 20 purified mutants were characterized and all of them showed decreased azathioprine activity, further emphasizing the importance of the region and its susceptibility to mutations with respect to azathioprine activity. Homologous recombination keeps conserved regions intact which the results show is not only beneficial for the stability of the chimeras but also for maintaining the regions with low tolerance to mutations, regarding the targeted property, and to acquire more variation in other parts of the sequence into the pool of active enzymes. Further modifications in directed evolution should thus be focused to regions with more variation present among the most active mutants. (Supported by the Swedish Cancer Society).

E5.46
Perspectives of application of Armenian highland plants in cancer treatment

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At cancer, the purine metabolism is accelerated, and the level of key enzyme of purine nucleotide pathway, adenosine deaminase (ADA), is enhanced in serum and tissues. The regulation of ADA activity seems prospective for cancer treatment. For this purpose the specific plant extracts might be a promising approach in the quest of remedy against cancer. We studied the influence on ADA activity of aqueous extracts of 25 plants, traditionally used in folk medicine and/or as food. The numbers of total and alive cells of mice Erlich Ascites Carcinoma (EAC), cultivated in the presence and absence of plant extracts significantly inhibiting ADA, were daily monitored. The kinetics of cells growth evidenced the decreasing of total and alive cells number in the presence of plant extracts down to 40–60% of control. To understand if the cells growth inhibition is consequence of enzyme inhibition by the extracts, the experiments in the presence of known ADA inhibitor, Erythro-9-(2-hydroxy-3-nonyl) adenine, EHNA, were performed. The influence of EHNA on the cell samples had been amplified by addition of cytotoxic substrate of ADA, 2'-deoxyadenosine (dAdo). The observed analogous kinetics of inhibition of EAC cells growth by EHNA + dAdo and by oregano (Origanum vulgare) + dAdo allow us suggesting that ADA inhibiting ability of oregano is a cause of inhibition of EAC cells growth. Hence, the plant extracts inhibiting ADA activity might be recommended for clinical testing as new, mild, and non-expensive remedy against cancer.

E5.47
Characterization of cytochrome c-554 from *Methylosinus trichosporium* OB3b, a heme protein exhibiting HALS EPR signals

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A novel low-spin cytochrome c-554 has been purified from the methane oxidizing bacteria *Methylosinus trichosporium* OB3b. This cytochrome exhibits a ferric HALS (highly axial low-spin) low-temperature EPR signal, with a typical HALS lineshape and a g-max value of 3.41. The EPR spectrum changes slightly with pH. MALDI-TOF-MS has shown that this cytochrome has a molecular weight of 12 230 Da, and that it only contains one heme group. The CD spectrum of the oxidized form of this cytochrome shows a peak at around 730 nm. Together with light absorption experiments this indicates a His/Met coordination of the heme iron. The sequence of the first 39 N-terminal amino acids and 27 C-terminal amino acids has been determined and sequence alignments performed. The most similar hits represent c-type cytochromes from bacteria belonging to the phylum Bradyrhizobiaceae, in which *Methylosinus trichosporium* OB3b is included. C-type cytochromes have similar His/Met coordination of the heme iron they can exhibit different EPR signals. The correlation between different EPR signals and structure is not known for His/Met coordinated hemes. Characterization of this protein aims to provide a broader basis for understanding this correlation. As EPR signals reflect the immediate surroundings of the unpaired electron, the HALS signal could indicate a difference in the electron transport pathway to and from the heme group.