Amino Acids (2007) 33: I–LXX
DOI 10.1007/s00726-007-0578-0
Printed in The Netherlands

Amino Acids

# 10th International Congress on Amino Acids and Proteins (ICAAP)

Kallithea, Greece
August 20–25, 2007

## Abstracts

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# 10th International Congress on Amino Acids and Proteins (ICAAP)

## Amino Acids Metabolism – Diseases

### Homocysteine and methylated arginines – novel endogenous factors of the chronic musculoskeletal pain?

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Recurrent character of chronic musculoskeletal pain suggests that there are endogenous chemical mediators sustaining local aseptic inflammation in the connective tissue involved. We propose that homocysteine (HCys) and methylated arginines – $N^G$,$N^G$-asymmetrical dimethylarginine (ADMA) and N-monomethylarginine (N-MMA) – are not only endogenous damaging factors to the cardiovascular system but also act as destabilisers of collagen and elastin in the joint cartilage, capsules, ligaments, and intramuscular connective tissue stroma. This, in turn, may result in their mechanical weakness, recurrent structural imbalance, and, therefore, facilitate pain.

With a qualitative standardised muscle test (a non-invasive diagnostic procedure accepted in functional neurology) for the excess of HCys, ADMA and MMA in the tissues, we have examined 110 randomly selected out-patients (men: 30, women: 80, age: 40–68) with chronic recurrent lower back/neck/joint pain of various origin. HCys was detected elevated in 23 patients (20.9%), ADMA – in 76 (69.1%), N-MMA – in 11 (10%). Specific nutritional supplementation protocols designed for the activation of the hydrolysis of HCys, ADMA, and N-MMA in the tissues, were introduced and have resulted in a substantial decrement of the intensity of pain and the frequency of its recurrence (within one year of follow-up). Beneficial effects of the nutritional supplementation were registered in all patients with the elevation of HCys, in 70 of 76 – with the elevation of ADMA, and in 10 of 11 – with N-MMA elevation. Mechanisms of destructive effects of HCys, ADMA, and N-MMA on connective tissue proteins and of the amending action of specific nutritional compositions are being discussed.

### Glutamine, branched-chain amino acids and protein metabolism

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Several studies have reported improvements in clinical outcome and in nitrogen balance when glutamine (Gln) itself or Gln-containing peptides were given to critically ill patients. However, the mechanism by which Gln administration affects protein balance is not clear. Because of a tight metabolic relationships between glutamine and branched-chain amino acids (BCAAs; valine, isoleucine and leucine), it can be hypothesised that favourable effect of glutamine administration on protein metabolism is related to its effect on BCAAs.

Using male Wistar rats two separate studies were performed in which the effect of glutamine on leucine oxidation, protein synthesis and proteolysis was estimated. At in vivo study, alanyl-glutamine or saline solution were infused to endotoxemic, whole body irradiated, or intact (control) rats. At in vitro study, *M. soleus* and *M. extensor* digitorum longus were incubated in medium containing 0, 500 or 2000 μmol glutamine/L. The parameters of protein metabolism and leucine oxidation were measured using L-[1-$^{14}$C]leucine and/or according to the rates of tyrosine release. Statistical comparisons were performed using ANOVA, Bonferroni test, and Student's *t*-test.

Infusion of glutamine (alone or as alanyl-glutamine) induced a decrease in plasma BCAA levels, in leucine oxidation and an improvement of protein balance related to the decrease in proteolysis both in intact, endotoxemic and irradiated rats. In an in vitro study, supplementation of incubation media with glutamine in concentration of 2000 μmol/l decreased leucine oxidation by isolated muscles.

It is concluded that there are at least two mechanisms by which glutamine administration may affect protein metabolism in stress illness. The first is related to inhibitory effect of glutamine on production of proinflammatory cytokines, the second is related to its effect on metabolism of BCAA. It is supposed that a negative feedback exists between GLN level and BCAA oxidation in muscle which by unknown mechanism affects proteolysis. GLN administration can block this feedback mechanism, decrease BCAA catabolism and improve protein balance.

### Is heme oxygenase-1 relevant to the anti-inflammatory activity of taurine chloramine?

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Fibroblast-like synoviocytes (FLS) are engaged in chronic synovitis and joint destruction, characteristic for rheumatoid arthritis (RA). We have previously reported that taurine chloramine (Tau-Cl) normalizes pathogenic functions of RA FLS by: (i) diminishing transcription factors (NFκB, AP-l) activities, (ii) down-regulation of pro-inflammatory gene transcription (IL-6, IL-8, COX-2, VEGF), and (iii) triggering growth arrest. Heme oxygenase-1 (HO-1), an inducible heme-degrading enzyme, has also been reported to exert potent anti-inflammatory effects in vitro and to restrict inflammation-associated tissue injury. Presently we have observed that similar to hemin (25–100 μM), a known potent inducer of HO-l expression, also Tau-Cl treatment (300–400 μM) up-regulates expression of HO-1 in RA FLS at both mRNA and protein levels. Therefore, we ask the question whether in these cells HO-1 activity mediates anti-inflammatory effect of Tau-Cl. We have found that hemin inhibits both IL-1β-triggered production of pro-inflammatory cytokines (IL-6, IL-8) by RA FLS and PDGF-triggercd proliferation of these cells with potency similar to Tau-Cl. However, pretreatment of the cells with HO-1 inhibitor (Zinc(II) Deuteroporphyrin

1X-2,4-bisethyleneglycol – ZnDP) reverses only cytokine secretion but has no effect on either intracellular cytokine content or cell proliferation. These observations suggest that either HO-1 activity is irrelevant to these RA FLS responses or that in our system ZnDP is not effective enough to inhibit activity of this enzyme. Thus, further studies are required to answer this question. On the other hand, in synovial fluid of RA patients we have found very high ($763 \pm 87$ ng/ml) concentration of ferritin, a by-product of HO-1. Ferritin is an iron-binding protein, reported to possess anti-oxidant properties. We observed that upon either hemin or Tau-Cl treatment RA FTS express only low intracellular level of ferritin light (FTL) and heavy (FTH) chains, Thus, RA FLS are not the major source of ferritin in rheumatoid joints. Moreover, neither exogenously added FTL nor FTH affected RA FLS responses (proliferation, cytokine synthesis). It is therefore likely, that the other product of HO-l activity (e.g. CO) is probably more relevant in regulating RA-FLS metabolism. In contrast, both FTL and FTH prolonged in vitro survival of leukocytes isolated from rheumatoid synovial fluid, suggesting that ferritin may support chronic inflammation.

## Creatine supplementation modulates arginine metabolism in alcoholic rats

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The purpose of this study was to determine the effect of creatine supplementation on blood parameters, urea and creatinine levels and tissue enzymes arginase and transamidinase in reflecting of liver and kidney function in alcoholic intoxication.

Male Sprague Dawley rats were treated for 3 weeks with 1-ethanol (as 15% solution), 2-creatine monohydrate (2 g/kg/daily), and 3-ethanol + creatine monohydrate in same doses). 4-control group.

Urea and cretonne levels were measured by standard biochemical analyses. Liver enzyme arginase and kidney enzyme transamidinase were measured on the basis of formed ornithine. Results of our study show elevation of arginase activity in the liver of rats treated with alcohol ($p < 0.05$). and increase of plasma urea and creatinine levels ($p < 0.05$) compared to control group. Creatine supplementation to alcoholic rats has more profound effect on plasma parameters, urea and creatinine ($p < 0.001$), and leads to suppression of kidney transamidinase activity ($p < 0.01$).

**Conclusion:** Creatine supplementation to alcoholic rats modulates metabolism of arginine. Suppression of creatine synthesis on the level of transamidinase reaction may result in upregulation of other metabolic fates of arginine, such as formation of urea, ornithine, and nitric oxide.

## Wiring and volume transmission in rat amygdala. Implications for fear and anxiety

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Considerable evidence indicates that in addition to classical synaptic transmission (wiring transmission, WT) within the mammalian central nervous system (CNS) neurotransmitters act via volume transmission (VT). Thus, they can diffuse through the extracellular space and reach from their terminal release sites extrasynaptic receptors located close by or in mismatch locations. Such a neurotransmission exerts a tonic modulatory role in several physiological and pathological conditions. The amygdala plays a key role in the modulation of anxiety and it is commonly accepted that emotionally relevant information reaches this structure where it is processed within the basolateral complex and relayed to the central nucleus where an appropriate anxious/fear output is implemented. The intercalated paracapsular islands and main intercalated island, clusters of dopamine D1-rich GABAergic cells, seem to control the impulse trafficking between the two nuclei. Several lines of evidence indicate that within the amygdala, GABA and glutamate, respectively have an overall anxiolytic and anxiogenic role and that a number of other neurotransmitters interact with them to modulate anxiety. Experimental evidence from our laboratories is presented which suggests that dopamine and glutamate activate, via WT and VT, dopamine D1 and metabotropic glutamate 5 receptors (mGluR5) located in the rostral and dorsal amygdala and facilitate an anxiogenic output from the central nucleus. Immunocytochemical evidence is also provided which indicates that CCK terminal/CCK2 matches and mismatches exist in the intercalated cell masses and the basolateral complex respectively. These observations suggest the CCK-2 receptor mediated CCK transmission operates both via WT and VT in the amygdala. CCK-4 and CCK-8S microinjected into the rostral amygdala produce an anxiogenic profile indicating that CCK transmission participates in anxiety. Our studies indicate that WT and VT signals specifically glutamate, dopamine and CCK-peptides become integrated in the intercalated islands illustrating their role as a major node in the anxiety circuits. In conclusion, by combining double immunolabeling immunocytochemistry and behavioural studies in models of anxiety indications have been obtained that the amygdaloid circuits involved in regulation of anxiety operate via WT and VT.

## Antiglutamatergic agent riluzole protects against pilocarpine-induced seziures in rats

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Pilocarpine-induced seizures is a model of intractable epilepsy commonly used to investigate the antiepileptic effect of different drugs and mechanisms of epileptogenesis.

In this study the influence of antiglutamatergic agent riluzole, which is used to slow the progress of amyotrophic lateral sclerosis, was investigated. Seizures and status epilepticus was induced by pilocarpine (400 mg/kg, i.p.) in male adult Wistar rats pretreated with N-methylscopolamine (1 mg/kg, s.c.). Riluzole (1–4 mg/kg, i.p.) was administered 15 min before pilocarpine. It was found that riluzole dose-dependently protects rats against seizures, status epilepticus, and mortality. Thus, it can be concluded that the glutamate release inhibitor riluzole possesses potent anticonvulsant activity in pilocarpine model of epilepsy.

## Hypotaurine biosynthesis in rat liver mitochondria

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Main final metabolites of L-cysteine in mammals are inorganic sulfate and taurine. Major pathway of taurine formation is believed to be that via hypotaurine. Hypotaurine is produced from L-cysteinesulfinate (CSA) by decarboxylation catalysed by CSA decarboxylase. It has been reported that, in mammalian liver, CSA is produced by cysteine dioxygenase in cytosol and that CSA decarboxylase is present also in cytosol, indicating hypotaurine formation occurs in cytosol in mammalian liver. Main pathway of sulfate formation is also that via CSA. CSA is converted to

sulfinopyruvate, which is decomposed to pyruvate and sulfite. Sulfite is then oxidized to sulfate by sulfite oxidase in mitochondria.

We have studied L-cysteine metabolism in rat liver and have reported that L-cysteine is partly metabolized through mercaptopyruvate pathway in mitochondria. In this pathway cysteine sulfur is finally metabolized to inorganic sulfate via 3-mercaptopyruvate and thiosulfate. The latter compound is suggested to be the source of iron-sulfur compounds in mitochondria, indicating mercaptopyruvate pathway functions for the formation of iron-sulfur cluster in mitochondria.

During the study of mitochondrial cysteine metabolism, we noticed that hypotaurine was produced when L-cysteinesulfinate was incubated with mitochondrial fraction from rat liver. The mitochondrial fraction was prepared by the method of Hogeboom and repeatedly wahed with 0.25 M sucrose solution. The incubation mixture consisted of 100 mM potassium phosphate buffer (pH 7.8), 20 mM CSA, 0.1 mM pyridoxal 5′-phosphate, 10 mM GSH and mitochondrial fraction prepared from 1 g of rat liver for 30 min. After deproteinization with perchloric acid (PCA), the neutralized PCA extract was treated with dabsyl chloride. By RP-HPLC of the dabsylated PCA extract, a peak corresponding to dabsyl hypotaurine was detected. It was confirmed that the peak was quantitatively converted to dabsyl-taurine upon treatment with hydrogen peroxide. Upon direct treatment of the PCA extract with hydrogen peroxide, the presence of taurine was confirmed by dabsylation and RP-HPLC. By sonication of mitochondrial fraction, hypotaurine-forming activity increased approximately by 60%. Tentative values are $K_m$ for CSA is 0.11 mM, optimum pH is 7.5–7.8, and specific activity is 6–8 nmol/mg of protein per 60 min. These results indicate that biosynthesis of hypotaurine takes place also in liver mitochondria.

### Differential effects of diallyl disulfide and N-acetyl-L-cysteine on the proliferation of prostate cancer cell line PC-3

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Prostate cancer is the most predominant cancer in men and related death rate increases every year. Till date, there is no effective therapy for androgen independent prostate cancer. Previous studies reported that diallyl disulfide (DADS), organosulfur compound of garlic, suppresses cell growth of prostate cancer cells in vitro. We confirmed the inhibition of PC3 cells proliferation for 200 and 400 μM DADS concentration in culture medium after 24 and 48 h of incubation, compared to the control. The similar effect was observed for 800 μM N-acetyl-L-cysteine (NAC) after 48 h of incubation. However, after 24 h the proliferation was increased what was accompanied by the increased ratio of GSH/GSSG, as NAC serves as L-cysteine precursor. DADS at both concentrations and in investigated time points caused a decrease in GSH concentration in cells what may lead to oxidative stress and the generation of reactive oxygen species. DADS and L-cysteine released from NAC are both precursors of sulfane sulfur which level can be correlated with cells proliferation. In our investigations the inhibition of the proliferation of PC3 cells was accompanied by increased level of sulfane sulfur and increased expression of gamma-cystathionse, enzyme involved in the production of sulfane sulfur-containing compounds. It seems that a better inhibitor of the proliferation of PC3 cells is DADS, upregulating gamma-cystathionase expression and inducing sulfane sulfur level, than NAC which antiproliferative activity is time/dose dependent.

# Amino Acids Racemization

### Glucocorticoid receptor phosphorylation in bipolar disorder

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**Introduction:** Bipolar disorder (BD) has been correlated with alterations in glucocorticoid receptor (GR) signaling. Due to the importance of GR phosphorylation in receptor function, we examined the role of total phosphorylation status of GR and phosphorylation of serine 211 (S211) in bipolar disorder.

**Methods:** In lymphocytes from 48 BD patients (normothymic $n = 24$, depressed $n = 13$, manic $n = 11$) under multiple anti-depressant therapy and 17 healthy individuals we measured total GR phosphorylation and phosphorylation of serine 211 (whole cell and nuclear extracts respectively) by using immunoprecipitation, phosphospecific antibody and Western-blot analysis. Whole cell extracts were immunoblotted to determine total GR levels in order to normalize total GR phosphorylation and S211. One-Way ANOVA statistical analysis was carried out.

**Results:** Total phosphorylated GR levels were lower in depressed ($P < 0.05$) or normothymic ($P < 0.05$), or manic patients ($P < 0.05$) as compared to healthy individuals. The GR S211 was higher in depressed ($P < 0.05$), normothymic ($P < 0.05$) and manic ($P < 0.05$) patients compared to healthy individuals ($P < 0.05$), the depressed patients showing the highest GR S211 levels among the groups.

**Conclusions:** We provide the first evidence of alterated GR phosphorylation in BD, characterized by reduced total GR phosphorylation and increased phosphorylation of GR serine 211, in particular in the depressed state of the disease. We speculate that phosphorylation status of GR might be trait-dependent and associated with the pathophysiology of bipolar disorder.

### Ultra-sensitive determination of multiple D-amino acids in mammals using two-dimensional micro-HPLC with fluorescence detection

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D-Amino acids, the enantiomers of L-amino acids, are the candidates of novel physiologically active substances and the biomarkers of diseases in mammals. Although much attention has been paid to the functions, origins and diagnostic values of D-Ser and D-Asp, the investigations on other D-amino acids are limited, because the amounts of these D-amino acids in mammalian tissues are extremely small in many cases. Therefore, reliable analytical methods with sufficient sensitivity and selectivity are needed. In the present work, we have designed and established ultra-sensitive

and selective two-dimensional micro-HPLC methods for the analysis of multiple D-amino acids in mammals. For the sensitive determination, the amino acids were derivatized with a fluorescence labeling reagent, 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), and determined by the HPLC system equipped with the columns of narrower internal diameters than conventional ones. For the selective determination, the two-dimensional separation was carried out using a microbore-reversed-phase column and a narrowbore-enantioselective column; each amino acid was isolated in the first dimension, and their enantiomers were separated in the second dimension. Using this two-dimensional procedure, we have established fully-automated HPLC systems for comprehensive enantiomer determination of (1) hydrophobic amino acids (Val, *allo*-Ile, Ile, Leu and Phe), (2) proline analogs (Pro, *trans*-hydroxyPro and *cis*-hydroxyPro) and (3) Ala and Ser. In all these 2D-HPLC systems designed, optimized enantioselective columns enable the rapid enantiomer separation of each amino acid within about 10 min, and the total analysis time for the two-dimensional determinations of target amino acids are around 60 min. The lower limits of determination of D-amino acids are 1 fmol or lower. By using the 2D-HPLC system for hydrophobic amino acid enantiomers, we demonstrated the presence of D-Val and D-*allo*-Ile in the urine of various mammalian species such as rats, mice, dogs and humans; their origins, physiological functions and diagnostic values are now being investigated.

## Physiological role of D-serine in the growth of silkworm *Bombyx mori*

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Silkworms are known to contain high concentrations of D-serine, an optical isomer of the common L-serine. In order to elucidate the physiological effects of the D-amino acid, we investigated the relationship between D-serine content and growth rate of the silkworm, *Bombyx mori.* The D-serine concentration gradually increased during the larval stage, and it rapidly increased in the spinning larvae, resulted in the highest D-serine level in the late pupal stage. The D-serine concentration decreased sharply in the imago. Activity of serine racemase, which synthesizes D-serine from L-serine, was completely lost in the spinning larvae, and then, it increased sharply until the late pupal stage. We examined the effects of O-phospho-L-serine (OPLS), an inhibitor of serine racemase, on the growth of the silkworms by adding it to the artificial diet, Silkmate 2S. The results showed clear effects of the OPLS on growth, which was delayed by 5 to 7 days with significant reductions in body weights. The effects of OPLS were somewhat ameliorated by simultaneous administration of D-serine, suggesting the importance of D-serine in the growth of silkworms. The results also suggested that D-serine was synthesized from L-serine by serine racemase in *B. mori*.

## D-aspartate and D-glutamate oxidases in yeast *Rhodotorula glutinis*

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D-Amino acid oxidase catalyzes the enantioselective oxidation of a variety of D-amino acids to the corresponding 2-oxo acids and ammonia with the concomitant reduction of molecular oxygen to hydrogen peroxide, but does not catalyze acidic amino acids such as D-aspartate and D-glutamate. We have screened some stock cultures of yeasts in our laboratory for the ability to utilize D-glutamate as the sole source of nitrogen. *Rhodotorula glutinis* JCM 5949 was found to grow well in the medium containing 0.1% D-aspartate or D-glutamate as the sole nitrogen source. In the cell-free extract, we found two amino acid oxidases that catalyze oxidative deamination of D-aspartate and D-glutamate, respectively. By several purification procedures such as fractionation with ammonium sulfate and column chromatographies, D-aspartate oxidase (DDO) and D-glutamate oxidase (DEO) were purified by 74 and 68 folds, respectively. The optimal temperature and pH were 16 °C and 7.8, respectively, for DDO, and 24 °C and 7.3, respectively, for DEO. The molecular weights of native DDO and DEO were estimated to be 52,000 and 33,000, respectively, with gel filtration through a TSK G3000SWXL column. Both DDO and DEO were highly specific to each D-amino acid. As the result, *R. glutinis* was found to be a unique yeast that had DDO and DEO induced by D-aspartate or D-glutamate in culture media.

## Purification and properties of serine racemase from a hyperthermophylum *Pyrobaculum islandicum*

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*Pyrobaculum islandicum* is an anaerobic hyperthermophilic archaeon that lives most actively at 100 °C. A pyridoxal 5′-phosphate dependent serine racemase was purified from cells. The serine racemase gene *srr* was cloned (accession No. AB244101) and the gene-product Srr was purified from the recombinant *Escherichia coli* cells. The sequence homology of the deduced primary structure with other serine racemases and threonine dehydratases is less than 32% except the value 87% with *P. aerophilum* IIvA. Srr was revealed to be a homotrimer based on SDS-PAGE and gel filtration analyses, of a molecular weight 44,051 subunits as calculated from the primary structure. The enzyme showed the highest racemase activity toward L-serine followed by L-threonine, D-serine and D-threonine. Srr was found to be a bifunctional enzyme possessing a high dehydratase activity toward L-serine and L-threonine. The optimal temperature both for racemase and dehydratase activities was 95 °C. The optimal pH for racemization and dehydration was pH 8.2 and 7.8, respectively. Both of the enzyme activities were affected neither by ADP, AMP, L-valine, or L-isoleukine, nor by metal ions except for $Fe^{2+}$ and $Cu^{2+}$ which showed 20–30% inhibition and 30–40% stimulation, respectively. The racemase activity was inhibited by ATP only by 10–20%. Srr is not an allosteric enzyme. $K_m$ and $V_{max}$ values of racemase activity of Srr for L-serine were obtained by Lineweaver-Burk plot to be 185 mM and 20.1 μmol/min/mg, respectively. The $K_m$ and $V_{max}$ values of dehydratase activity for L-serine were 2.16 mM and 80.4 μmol/min/mg, respectively.

## Purification of branched-chain amino acid aminotransferase from *Helicobacter pylori* NCTC11637

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*Helicobacter pylori* is a microaerophilic gram-negative bacterium that lives in the mucous layer of the human stomach and duodenum. Branched-chain amino acid aminotransferase [EC 2.6.1.42] (BCAT) is an enzyme that converts isoleucine, leucine and valine into their corresponding 2-oxo acids, with 2-oxoglutarate as the amino group acceptor. The enzyme was purified by several column chromatographies from *H. pylori* NCTC11637, and the N-terminal amino acid sequence was analyzed. We searched a protein of similar primary structure with the above N-terminus, in *H. pylori* 26695, and found *ilvE*, a putative branched-chain amino acid aminotransferase. The *H. pylori* NCTC11637 enzyme gene was sequenced (accession No. AB274528) based on *ilvE* of *H. pylori* 26695, and the whole amino acid sequence was deduced from the nucleotide sequence. The enzyme existed in a homodimer with a calculated subunit molecular weight of 37,539 and an isoelectric point of 6.47. The enzyme showed high affinity to 2-oxoglutarate ($K_m = 0.085$ mM) and L-isoleucine ($K_m = 0.34$ mM), and $V_{max}$ value was 27.3 μmol/min/mg. The best substrate was found to be L-isoleucine followed by L-leucine and L-valine. No activity was shown toward the D-enantiomers of these amino acids. The optimal pH and temperature were pH 8.0 and 37 °C, respectively. To the best of our knowledge, this is the first report on the purification of BCAT from organisms in the order *Helicobacter*.

## D-amino acid dependant electron transfer system in the hyperthermophile *Pyrobaculum islandicum*

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*Pyrobaculum islandicum* is a strictly anaerobic archaeon that grows optimally at 100 °C. The organism grows both organotrophically on complex organic compounds such as peptone, and lithotrophically using $H_2$ as an electron donor and sulfur as an electron acceptor. We previously reported on D-amino acid contents and D-amino acid dehydrogenase (DAD) activity of this organism. In the present study, we purified two b-type cytochromes, cytochrome *b*-559 and *b*-557, and DAD by using ammonium-sulfate fractionation and several column chromatographies. SDS-PAGE analysis of cytochrome *b*-559 indicated that the protein is a heterodimer composed of a 95 and a 58-kDa subunits. The purified DAD showed the highest activity toward D-proline and D-valine at 95 °C. The optimal temperature of DAD was 95 °C, and the enzyme showed only 3% activity at 30 °C of that at 80 °C. The possibility that D-valine provides hydrogen molecules as respiratory substrates in *P. islandicum* has been shown by an experiment where cytochrome *b*-559 was reduced with electrons upon addition of D-valine to the mixture of cytochrome *b*-559 and DAD.

## Respiration with D-amino acids in *Helicobacter pylori*

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*Helicobacter pylori* is a microaerophilic Gram-negative spiral bacterium that resides in the human stomach. *H. pylori* causes chronic inflammation and is a strong risk factor for development of peptic ulcer and gastric cancer. The bacterium cannot use sugars as respiratory substrates; instead, it utilizes organic acids as energy and carbon sources. Using the whole cells, we found D-proline to be a favorable respiratory substrate. In the present study, we confirmed the respiration with D-proline by a reconstituted electron transport system composed of purified proteins such as D-amino acid dehydrogenase (DAD), cytochrome $bc_1$, cytochrome *c*-553 and cytochrome $cbb_3$, as well as ubiquinone and D-proline. DAD was partially purified from *H. pylori* membrane by an anion exchange column chromatography with DEAE-Toyopearl (pH 8.0). D-Proline was found to be the best substrate of DAD when 2.6-dichlorophenolindophenol was used as an electron acceptor. Cytochrome *c*-553 was prepared from the soluble fraction of the cell-free extracts. Cytochrome $cbb_3$ was obtained from the membrane by using Q-Sepharose (pH 7.0) and Sephacryl S-200 (pH 7.0) columns after solubilization with 3% Triton X-100. Cytochrome $bc_1$ was partially purified from the fraction solubilized with 1% n-octyl-β-D-glucoside by DEAE-cellulose (pH 7.0) column chromatography.

## Simple and rapid genotyping of D-amino acid oxidase gene using microchip electrophoresis

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D-Amino acids are the novel physiologically active substances and the candidates for marker molecules of various diseases in mammals. The D-amino acids, especially the neutral ones, are metabolized by D-amino acid oxidase (DAO), and the amounts of D-amino acids are consequently altered depending on the activity of this enzyme. In inbred strains of mice, the DAO gene has coding SNPs and one SNP causes the complete loss of enzymatic activity. Therefore, the identification of the DAO genotype of this SNP in individual mouse is important to investigate the distribution, regulation and function of the D-amino acids, as well as to estimate the relationships between D-amino acids and patho-physiological conditions. The widely used DAO genotyping procedure includes two steps of polymerase chain reaction (PCR) and a subsequent restriction enzyme digestion, followed by agarose gel electrophoresis (AGE). However, this procedure needs skillful techniques and lengthy time to identify the genotype of DAO in distinguishing two DNA fragments of approximate 100 bp with the difference of 12 bp.

In the present study, we have developed a simple and rapid genotyping procedure which employs microchip electrophoresis (MCE). Genomic DNA was extracted from a mouse tail, and a DNA fragment including a region encoding DAO was amplified by one step PCR with optimized primers. The PCR products were digested with *Hpa*II which recognizes a single-base-pair substitution in the DAO gene, and applied to MCE. Two types of MCE system, Agilent 2100 bioanalyzer (Agilent Technologies, Waldbronn, Germany) and Hitachi SV1210 (Hitachi High-Technologies, Tokyo, Japan), were tested. In either MCE system, two DNA fragments used for genotyping were clearly separated within 3 min, and three genotypes of the DAO gene ($DAO^{+/+}$, $DAO^{+/-}$ and $DAO^{-/-}$) were definitely determined in the ddY mice. The accuracy of the present genotyping method was evaluated by comparing the results of the genotyping with the amount of D-proline (D-Pro) in the serum of these mice, because mice lacking DAO activity were reported to have larger amount of D-Pro than that in wild-type mice. D-Pro was determined using 2D-HPLC after deriv-

atization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). The results indicated that the amounts of D-Pro in these mice were consistent with their genotypes determined by the newly developed method. The present genotyping procedure is considered to be a versatile method for any strains of mice, and also applicable to establish novel strains of mice lacking DAO activity.

### Occurrence of free D-aspartate in microalgae

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We have previously reported the distribution of free D-aspartate in various species of seaweeds (macroalgae). However, the role of D-aspartate in seaweeds remains unclear. To clarify details of the physiological function of D-aspartate in algae, we studied this compound in microalgae that can be cultured under germfree and artificial conditions in the laboratory.

The biologically rare D-aspartate was found in several species of freshwater and marine microalgae. In particular, the marine diatom *Asterionella japonica* showed a considerably higher content than the other microalgae. The aspartate of *Asterionella japonica* was consisted of equal amounts of D- and L-isomers.

All species of microalgae were collected after one week when the population had reached the stationary phase. Microalgal cells were collected by centrifugation, and the resulting cell pellet was then homogenized with 3 vol (w/v) of 80% ethanol by an ultrasonic homogenizer. After centrifugation, the resulting supernatant was evaporated to dryness under reduced pressure. The residue was dissolved in a small amount of milli-Q water and stored at $-20\,^{\circ}C$ until used for the HPLC analysis. D-Aspartate content was determined by reversed-phase HPLC analysis after it was derivatized with a chiral adduct of *o*-phthalaldehyde and *N*-acetyl-L-cysteine. Chromatography was performed by the method of Nimura and Kinoshita.

The occurrence of free D-aspartate was verified in 20 species of microalgae by the same method (reversed-phase HPLC). Generally, Chlorophyta and Heterokontophyta contained a high concentration of free D-aspartate in contrast with the low concentration in Cryptophyta, Dinophyta and Haptophyta.

## Bioinformatics and Biomedical

### A protein interaction atlas for the nuclear hormone receptors: properties and quality of a hub-based dimerisation network

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The nuclear hormone receptors are a large family of eukaryotic transcription factors that constitute major pharmacological targets. They exert their combinatorial control through homotypic heterodimerisation. Elucidation of this dimerisation network is vital in order to understand the complex dynamics and potential cross-talk involved. Phylogeny, protein–protein interactions, protein–DNA interactions and gene expression data have been integrated to provide a comprehensive and up-to-date description of the topology and properties of the nuclear receptor interaction network in humans. We discriminate between DNA-binding and non-DNA-binding dimers, and provide a comprehensive interaction map, that identifies potential cross-talk between the various pathways of nuclear hormone receptors. We infer that the topology of this network is hub-based, and much more connected than previously thought. Furthermore, a significant number of negative feedback loops is present, with the hub protein SHP [NR0B2] playing a major role. Finally, we compare the evolution, topology and properties of the nuclear hormone receptor network with the hub-based dimerisation network of the bHLH transcription factors in order to identify both unique themes and ubiquitous properties in gene regulation.

### Assembly and mechanism of the Type I multi-drug efflux pump AcrAB-TolC from *Escherichia coli*

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Type I multi-drug efflux pumps are ubiquitous in Gram-negative bacteria and expel a wide range of organic compounds such as detergents and antibiotics and play an important role in multidrug resistance and infectivity. Type I multidrug efflux pumps invariably consist of three components: an Outer membrane protein, an inner membrane translocase, comprising a proton antiporter or an ATPase and an adaptor protein that links the IM and OM components, thus forming a tripartite assembly. One example of a Type I multidrug efflux system is TolC-AcrAB from *E. coli.* The structures of TolC, AcrA and AcrB, the OM, adaptor and IM component respectively, have been elucidated by X-ray crystallography. Isothermal calorimetry studies on the same system have shown that the adaptor protein interacts both with the IM and OM component. The IM and OM do not bind each other with a high affinity if at all.

Although the Structural and ITC studies deepened the insight in the mechanism of Type I multidrug efflux pumps, it does not tell how the components form a functional assembly. Hypothetical models of pump assembly have been published, but there has been no substantive study experimental studies of intermolecular contacts between the Type I efflux components. In this study we carried out systematic in vivo site-specific cross-linking by introducing cysteine residues into the periplasmic domain of TolC and the α-helical hairpin of AcrA. Cross-linking of TolC cysteine mutants to WT AcrA using cysteine to amine specific cross-linkers, identified residues on the lower α-helical barrel near the entrance duct of TolC. Reciprocally, Cross-linking of AcrA cysteine mutants identified the interaction surface on the N-terminal α-helix of the Acra coiled-coil domain. The experimental data allowed us to build a model of the AcrA-TolC interaction surface. The lowest energy model satisfying

all the distance constraints places AcrA at the intramolecular groove of TolC, aligning the Acra coiled coil with the exposed TolC outer helix. A key feature of this positioning is that it allows space for the proposed movement of the inner coil of TolC during transition to its open state.

Because co-crystallization of protein complexes is often problematic, the in vivo cross-linking approach combined with docking described here is a good alternative method of obtaining information about interaction surfaces in protein complexes.

## Anthrax lethal factor (ALF)–MEK peptide interaction through molecular dynamics simulations

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Anthrax is a disease of animals and humans, caused by the bacterium *Bacillus anthracis*. Anthrax toxin (AT) consists of three proteins, one of which is the anthrax lethal factor (ALF). ALF is a *gluzincin* Zn-dependent highly specific metalloprotease (~90,000 kDa), which belongs to the M34 family of the MA clan of zinc metalloproteases. ALF cleaves most isoforms of mitogen-activated protein kinase (MAPK)-kinases (MEKs) close to their amino termini, leading to the inhibition of one or more signaling pathways. No data are available on the enzyme-substrate interaction at the molecular level.

Therefore, we performed classical molecular dynamics simulations on the ALF-MKK/MEK complexes in order to probe protein-substrate interactions. The simulations pinpointed specific hydrophobic as well as electrostatic ALF-peptide substrate interactions and these data were exploited in the building of virtual combinatorial libraries of di- and tri-peptides using the twenty native aminoacids. By applying docking simulations to Anthrax Zn-metalloprotease around 1,000 peptide substrates were virtually screened according to their binding affinity. Data suggest that complexes of ALF with peptides substrates bearing Arg, Trp, Lys and Phe aminoacids, exhibit the highest binding affinity providing evidence for electrostatic interactions between negatively charged residues of ALF's active site and positively charged side-chains of di/tri-peptides. New libraries of substrates were built incorporating non-protein residues, organic moieties and chelating groups. ALF-substrate complexes with the best score (in terms of binding energy) are further analysed.

## Short amino acid stretches from sequences of silkmoth chorion proteins are amyloid fibril formers

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Peptide-analogues of the A and B families of silkmoth chorion proteins form amyloid fibrils under a variety of conditions, which led us to propose that silkmoth chorion is a natural protective amyloid.

In this work, in our attempts to determine the shortest possible element from the sequences of silkmoth chorion proteins that folds and self-assembles forming fibrils similar to those appearing in vivo, in the structure of silkmoth chorion, we designed and synthesized a 12-residue peptide-analogue of silkmoth chorion proteins and studied its folding and assembly mechanisms. This 12-residue peptide, folds and self-assembles, forming amyloid-like fibrils very similar in properties and structure to the fibrils formed by the 51-residue cA peptide representing the entire central domain of the A family chorion proteins, and the 24-residue cA_m1 peptide. Therefore, the results of this study, further confirm that the amyloidogenic properties of silkmoth chorion peptides are evolutionarily encoded into the tandemly repeating hexapeptides comprising the central domains of silkmoth chorion proteins.

## Prediction of protein folding properties based on interresidue average distance statistics

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The information on the 3D structure and function of a protein must be coded on its amino acid sequence and how we decode a protein sequence is the main problem to predict 3D structural information. We usually use sequence alignment of sequentially homologous proteins to solve this problem. The prediction of folding properties of a protein is significant for 3D structure prediction. However, it is rather difficult to extract the information of folding process by sequence alignment or other standard bioinformatics techniques. For this purpose, we introduce a predicted contact map constructed with the interresidue average distance statistics for prediction of folding properties of a protein. We refer this map as average distance map (ADM). ADM was originally used to predict compact areas such as domains in the sequence of a protein. We can show that the information of folding processes of a protein is reflected on its ADM, i.e., the location of subdomains predicted by its ADM is related to early folding segments. In this report, we present the results of analyses of ADMs for several proteins of which folding properties have been experimentally investigated well. In particular, we focus on the sequentially homologous proteins with different folding mechanisms. It can be demonstrated that the ADMs of those proteins reflects the differences of folding properties. ADM is a conversion of an amino acid sequence and ADM analysis can be regarded as a kind of sequence analysis of a protein.

Furthermore, the statistics of interresidue distances can be converted to the effective interresidue potential. We calculated the contact frequency of each residue of a protein with random conformations, and then we obtain values similar to $\phi$ values. Of course, these values, we refer as $p$ values, is not exactly same with $\phi$ values. However, the comparison of $p$ values to $\phi$ values for a protein suggests that $p$ values can reveal the information on folding initiation site well. The several folding properties can be predicted with the $p$ value analysis in the combination with the ADM analysis. We show the results of the present method for the several proteins of which the $\phi$ value analyses have been made. We also discuss the possibility of the present method to protein 3D structure prediction.

## Molecular insights of H5N1 avian influenza and implication for inhibitor design

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In order to simulate new strategies for designing drugs against bird flu, based on database search, studies of docking some drug (AG7088) to the crystal structure H5N1 avian influenza neuraminidase (PBD code: 2HTY) were conducted. It has been observed that the above compound interacting with the active site of the H5N1 influenza virus neuraminidase has better docking energy than current anti-influenza drug, which target the neuraminidase enzyme of the virus, zanamivir (Relenza) and oseltamivir (Tamiflu). Also, a clear definition of the binding site for the

drug has been presented. Furthermore, an in-dept study into the binding conformation was conducted by means of molecular dynamics (MD) simulation. The binding profile and energy landscape were explored to complement the docking results. These findings could be very helpful for designing new inhibitors against H5N1. Starting from the compare between AG7088 and current anti-H5N1 drugs, we performed a similarity search of about 400,000 druggable compounds to narrow down the scope of docking studies. The template molecule is AG7088. 13 compounds were found that may be promising candidates for further investigation. The main feature shared by these 13 most favorable potential inhibitors as well as the information of involved of other inhibitors might provide useful insights for the development of potent inhibitors against H5N1.

## A systems biology study and inhibitor design for 17beta-hydroxysteroid dehydrogenases involved in breast cancer

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Human 17beta-hydroxysteroid dehydrogenases are critical enzymes involved in the last step synthesis of active estrogens and androgens, thus being closely involved in hormone-dependant cancers. Systematic studies of the representative 17beta-HSDs in structural biology, enzyme mechanism and drug design have been pursued, yielding interesting knowledge of the enzyme structure-function and lead compounds for the enzyme inhibition. This is the case for the types 1 and 5 17beta-HSDs, for example. To obtain further insight of the enzyme behavior in cells, to develop truly efficient drugs, a study based on a systems biology approach is necessary, that involves research at both molecular and cellular levels, as well as combined fundamental study and medical application, facilitated by bioinformatics, indicating a new height of bio-medical study.

## Computational analysis of sequences and structures of conotoxins

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*Conus* peptides (conopeptides), the main components of *Conus* venom, represent a unique arsenal of neuropharmacologically active molecules that have been evolutionarily tailored to afford unprecedented and exquisite selectivity for a wide variety of ion-channel subtypes and neuronal receptors. From the point of view of protein sequence and structure analysis, conopeptides can serve as attractive systems for the studies in sequence comparison, pattern extraction, structure-function correlations, protein–protein interactions and evolutionary analysis. Despite their importance and extensive experimental investigations on them, they have been hardly explored through *in silico* methods. The work carried out in our group is perhaps the first attempt at deploying multi-pronged bioinformatics approaches for studies in the burgeoning field of conopeptides. Our contributions to the *in silico* analysis include, creation of protein sequence patterns and information highlighting the importance of the patterns as gleaned from the literatures for family classification; profile Hidden Markov Model (HMM) and multi-class support vector machines (MC-SVMs) for conotoxin superfamily classification; *in silico* characterization of $I_1$ and $I_2$ conotoxin superfamilies; studies of interaction with Kv1 channels of typical members of $I_2$ and J conotoxin superfamilies. The sequence patterns and associated documentation files created by us should be useful in protein family classification and functional annotation. Even though patterns might be useful at the family level, they may not always be adequate at the superfamily level due to hypervariability of mature toxins. In order to overcome this problem, we have demonstrated the application of MC-SVMs using pseudo-amino acid composition for the successful *in silico* classification of the mature conotoxins into their superfamilies. We have analyzed the I- and J-conotoxin-superfamily members in greater detail. The present work provides rationale for the high specificity of J-superfamily conotoxin pl14a to Kv1.6 channel relative to other Kv1 channel subtypes (Kv1.1–Kv1.5).

## Angiotensin-I converting enzyme (ACE)–peptide interaction through molecular dynamics simulations

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Angiotensin-I converting enzyme (ACE) belongs to the M2 family of the MA clan of zinc metallopeptidases and can act either as a dipeptidyl carboxypeptidase, which catalyses the proteolytic cleavage of dipeptides from the carboxy terminus of a wide variety of peptides, or as an endopeptidase, which hydrolyses peptides bearing amidated C-termini. Among the former category of ACE peptide substrates, the most distinguished are those involved in blood pressure regulation, such as angiotensin I (AngI) and bradykinin (BK). In the latter category falls the Gonadotropin-releasing hormone (GnRH).

In an attempt to analyze molecular interactions at atomic level we simulated the ACE-substrate complexes, using the recently determined 3D crystal structure of ACE *testis isoform* and a knowledge-based docking method in order to insert the peptide substrate (AngI, BK and GnRH) of ACE into its catalytic cleft. In order to introduce the effect of protein mobility and gain information about enzyme-substrate recognition and interaction we have sampled the conformational space of these complexes via molecular dynamics simulations with explicit solvent representation. We have also performed molecular dynamics calculations with tACE-inhibitor complexes, such as lisinopril, as well as with tACE mutated at specific sites, such as the ligands of the two buried chloride ions that have been shown to affect substrate activity. Our results provide new insights into the role of specific domains of tACE and their implication in the enzyme activity, which is not readily apparent from the available crystal structures.

## Prediction of subcellular localization of proteins using machine learning techniques and evolutionary information

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The sequencing of entire genomes and the possibility to access gene's co-expression patterns has moved the attention from the study of single proteins or small complexes to that of the entire proteome. The determination of protein functions is one of the most challenging problems of the post-genomic era. In this context, the search for reliable methods for proteins' function assignment is of uttermost importance. Despite lots of efforts there is limited success in developing direct method for predicting function of proteins. In order to overcome this problem number of indirect methods have been developed in past that includes subcellular localization of proteins. The determination of cellular localization of protein can provide important clue to elucidate the function of protein. In the past, numerous methods have been developed to predict subcellular location of proteins in the cells using different approaches. The similarity search is commonly used technique to assign function to a protein including subcellular location, where sequence is searched against experimentally annotated database. The approach based on similarity search fails in absence of significant similarity between query and target proteins. Another way to

predict subcellular localization of proteins is to identify sequence motifs such as signal peptide or nuclear localization signal. The main limitation of motif-based methods is that all proteins residing in a compartment do not have universal motifs. In order to overcome these limitations, researcher is using residue composition of proteins that includes amino acid, pseudo amino acid and dipeptides composition. Recently, evolutionary information in form of PSSM profiles has been used to predict subcellular localization of proteins. One of the major advantages of composition-based approach is that it provides fixed number of features irrespective of length of protein. This allows researchers to use machine-learning techniques like artificial neural network (ANN), support vector machines (SVM), nearest neighbors' method (KNN), etc.

## Polymorphic proteins and natural selection in wood mice (*Apodemus*): hypotheses from bioinformatics and population models

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Gene and protein databases allow testing of hypotheses about the factors which maintain or eliminate protein polymorphisms. Enhanced data banks for structural and functional bioinformatics have stimulated reexamination of organism performance associated with protein variation. Persistence of polymorphisms, differential fitness among genotypes, and the selective advantage(s) of allozyme heterozygosity are being investigated from the expanded perspectives of single nucleotide polymorphisms, gene-expression breadths, and catalytic geometries. Species' histories and current environments make wood mice from the Balkan region ideal for investigations of natural selection and protein functional structure. Four of the five species of wood mice (genus *Apodemus*) in Europe are in the Serbian province of Vojvodina, east and north of the Sava and Danube rivers, where original biotopes of steppes and woodlands are now subdivided by canals and agriculture into a mosaic of disturbed areas and enlarged ecotones. Northern Serbia was along the permafrost limit during the Quaternary glaciations, and two closely related, resident species of *Apodemus* had different (Iberian vs. Balkan) refugia. To assess population subdivision and potential introgression, we collected 260 specimens from 5 localities across 3 years, surveyed variation in 3 structural and 15 enzymatic (both housekeeping and tissue-specific) protein loci. In spite of operative factors that should reduce variation, there is greater protein variation in Serbian *Apodemus* than in other examined populations and geographic patterns of variation in allelomorph frequencies and heterozygosity, yet lack of isolation by distance among populations. To test for selection on candidate genes, we used our data and previously published data on *Apodemus* allozymes, plus mitochondrial and nuclear gene sequences, to test hypotheses that the majority of the variation in heterozygosity and the uniform array of polymorphic systems are explained by nonsynonomous-to-synonomous ($K_a/K_s$) amino acid substitutions, protein quaternary structure, and expression breadth.

## Multi-layered network structure of amino acid (AA) metabolism characterized by each essential AA-deficient condition

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The concentrations of free amino acids in plasma change coordinately and their profiles show distinctive features in various physiological condition, however, some of the changes observed in response to physiological status can not always be explained by the conventional flow-based metabolic pathway network. In this study, we have inferred the network structure of plasma amino acids with threshold-test analysis and multi-level-digraph analysis methods, without including the prior knowledge of metabolic pathway, using the plasma samples of rats which are fed diet deficient in single essential amino acid.

In the inferred network, we could draw some interesting interrelations between plasma amino acids as follows: 1) Lysine is located at the top control level and has effects on almost all of the other plasma amino acids. 2) Threonine plays a role in a hub in the network, which has direct links to the most number of other amino acids. 3) Threonine and methionine are interrelated to each other and form a loop structure.

Based on the inferred network structure, the dynamic analysis based on S-system is now being performed and recent advances will be discussed.

## Structural bioinformatics and computer aided design of novel drugs and functional proteins

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Genome projects are yielding protein sequences for which there is no knowledge about function or conformation. The draft human genome has now been available and the exploitation of this unique source of knowledge is a major challenge for biology. In parallel with these sequencing projects, there are structural genomics initiatives involving the determination of the conformations of uncharacterized proteins in the genomes with the particular aim of determining function. In addition, gene expression arrays are providing a mass of data that require analysis to relate protein sequences to activity under different conditions and in different cellular locations. Knowledge of the structure and function of the relevant proteins, revealed by crystallography and NMR, is central to the interpretation and exploitation of this pool of biological information. Three-dimensional structure can guide further experiments to probe activity and direct the systematic design of therapeutic agents for diseases. The experimental determination of protein structure remains difficult. There are large disparities between the numbers of protein sequences. Protein modeling provides a valuable approach to maximize the biological knowledge that can be obtained given these disparities. Accordingly, the task of the Structural Bioinformatics is the development of protein modeling algorithms and the application of the technology to systems of interests. Methodologies based on the known protein structures from experiments have been developed to predict 3D structures from a 1D sequence information, which is known as the homology modeling. It has become a reliable tool as long as the homologue with experimental 3D structure of significant similarity (usually greater than 35%) could be found. Other methods, for example, ab initio molecular dynamics, is still too slow to predict structure of a real protein. Often the structures of many drug targets are not available. The structural bioinformatics tools are needed to generate them readily to initiate drug design processes. We have predicted many protein structures using homology modeling to facilitate the computer aided drug design, which include database screening, pharmacophore search, docking and molecular dynamics conformation search. To join the worldwide efforts against H5N1 viruses which experience rapid mutation and become increasingly drug-resistant. A homology model of the H5N1-NA from the highly pathogenic chicken H5N1 A viruses isolated during the 2003–2004 influenza outbreaks in Japan was built based on the crystal structure of N9-NA complexed with DANA (PDB code: 1F8B). It was found that the traditional constituent residues around the active site of NA family are highly conserved in the H5N1-NA. However, a partially lipophilic pocket composed by Ala248

and Thr249 in N9-NA becomes a hydrophilic pocket because the two residues in the H5N1-NA are replaced by hydrophilic residues Ser227 and Asn228, respectively. On the other hand, two hydrophilic residues Asn347 and Asn348 in the N9-NA are replaced by two lipophilic residues Ala323 and Tyr324 in the H5N1-NA, respectively, leading to the formation of a new lipophilic pocket. This kind of subtle variation not only destroys the original lipophilic environment but also changes the complement interaction between the H5N1-NA and DANA. Such a finding might provide insights into the secret why some of H5N1 strains bear high resistance for existing NA inhibitors, and stimulate new strategies for designing new drugs against these viruses. Cytochrome P450 2C19 (CYP2C19) is a member of the cytochrome P-450 enzyme superfamily and plays an important role in the metabolism of drugs. In order to gain insights for developing personalized drugs, the 3D (dimensional) structure of CYP2C19 has been developed based on the crystal structure of CYP2C9 (PDB code 1R90), and its structure-activity relationship with the ligands of CEC, Fluvoxamine, Lescol, and Ticlopidine investigated through the structure-activity relationship approach. By means of a series of docking studies, the binding pockets of CYP2C19 for the four compounds are explicitly defined that will be very useful for conducting mutagenesis studies, providing insights into personalization of drug treatments and stimulating novel strategies for finding desired personalized drugs. NAD(P)H-dependent D-xylose reductase is a homodimeric oxidoreductase that belongs to the aldo-keto reductase superfamily. The enzyme has the special function to catalyze the first step in the assimilation of xylose into yeast metabolic pathways. Performing this function via reducing the open chain xylose to xylitol, the xylose reductase of Pichia stipitis is one of the most important enzymes that can be used to construct recombinant Saccharomyces cerevisiae strain for utilizing xylose and producing alcohol. To investigate into the interaction mechanism of the enzyme with its ligand NAD and NADP, the 3D structure was developed for the NAD(P)H-dependent D-xylose reductase from *P. stipitis.* With the 3D structure, the molecular docking operations were conducted to find the most stable bindings of the enzyme with NAD and NADP, respectively. Based on these results, the binding pockets of the enzyme for NAD and NADP have been explicitly defined. It has been found that the residues in forming the binding pockets for both NAD and NADP are almost the same and mainly hydrophilic. These findings may be used to guide mutagenesis studies, providing useful clues to modify the enzyme to improve the utilization of xylose for producing alcohol. Also, because human aldose reductases have the function to reduce the open chain form of glucose to sorbitol, a process physiologically significant for diabetic patients at the time that their blood glucose levels are elevated, the information gained through this study may also stimulate the development of new strategies for therapeutic treatment of diabetes. Eventually quantum chemical tools, such as, QM/MM studies will be extremely useful to study details of biological catalysis involved. Good efforts have been made by Prof. Hong Guo's group in elucidating the catalysis mechanism of many important enzymatic reactions using QM/MM approach. It is expected that it will become an accurate and predictive tool in the process of designing novel drugs and functional proteins.

### Peptide machines for data mining protein peptides

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Palmitoylation is one of the most important post-translational modifications involving molecular signalling activities. Two simple methods have been developed very recently for predicting palmitoylation sites, but the sensitivity (the prediction accuracy of palmitoylation sites) of both methods is very low (<65%) meaning that many true palmitoylation sites could be missed. This talk will review a number of machine learning algorithms implemented for peptide classification including a newly developed regularised bio-basis function neural network for palmitoylation site prediction. The aim is to improve the sensitivity. A set of protein sequences with experimentally determined palmitoylation sites are downloaded from NCBI for the study. The protein-oriented cross-validation strategy is used for proper model construction. The experiments show that the regularised bio-basis function neural network significantly outperforms the other methods. Specifically the sensitivity has been significantly improved with a slightly improved specificity (the prediction accuracy of non-palmitoylation sites).

## Biotechnology – Enzyme Technology – Food Science

### Effect of total germ number of raw milk on free amino acid and free D-amino acid content of various dairy products

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In the course of our researches we have examined free amino acid and free D-amino acid contents of milk samples with different germ numbers and composition of dairy products produced from them. Total germ number of milk samples examined varied from $1.25 \times 10^6$ to $2.95 \times 10^6$. It was established that with an increase in germ number concentration of both free D-amino acids and free L-amino acids increased, however, increase in D-amino acid contents was bigger considering its proportion. There was a particularly significant growth in the germ number range of $1.5 \times 10^6$ to $2.9 \times 10^6$. In the course of analysis of curds and cheese samples produced using different technologies we have come to the conclusion that for fresh dairy products and for those matured over a short time there was a close relation between total germ number and free D-amino acid and free L-amino acid contents, ratio of the enantiomers was not affected by the total germ number, however. For dairy products, however, where amino acid production capability of the microbial cultures considerably exceeds production of microorganisms originally present in the milk raw material, free amino acid contents of the milk product (both D- and L-enantiomers) seem to be independent of the composition of milk raw material.

### Generation of meat-like process flavouring from thermally treated hydrolyzed vegetable protein and amino acids

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Thermal process flavourings are mainly used to obtain the flavour characteristics of thermally treated food stuffs such as meat. Knowledge

of identities of the volatile aroma components of model systems that simulate the cooking of the meat is important for developing process flavors with authentic meat-like qualities, especially for species specific flavors such as beef, chicken.

Chicken broth flavour was generated by thermal treatment of model system solution containing ribose with various amino acids. The odour sensory evaluation of the sample revealed its high quality attributes. The volatile compounds in the sample headspace were isolated and subjected to qualitative and quantitative analysis by using gas chromatography-mass spectrometry. Only the most potent odorants compounds for chicken aroma were reported. The key odorant of chicken broth; 2-methyl-3-furfurylthiol, 3-mercapto-2-pentanone and 2-furfurylthiol were the main identified compounds.

A model mixture containing hydrolyzed soybean protein with xylose and cysteine was used as precursor for generation of beef-like process flavour via Maillard reaction. The sensory evaluation was carried out and the headspace volatiles of the sample that showed the best results were subjected to GC-MS analysis. The results revealed a quite agreement between the sensory attributes and identified compounds.

## α-Amylases and glucoamylases free or immobilized in calcium alginate gel capsules for synergistic hydrolysis of crude starches

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The hydrolysis of starch is usually carried out in two steps, consisting in a first stage of simultaneous gelatinization and liquefaction with a thermostable α-amylase and a second one of saccharification with a glucoamylase. However, when liquefaction and saccharification are separated processes, relatively long reaction times are needed. Reduction of hydrolysis time has been attempted by the use of a mixture of α-amylase and glucoamylase in a single step process. For this purpose a *Bacillus subtilis* thermostable α-amylase developed by our laboratory, as well as three commercial α-amylases and two commercial glucoamylases, in 8 combinations, were employed, free or immobilized in calcium alginate gel capsules, in the synergistic hydrolysis of crude starches. Enzyme immobilization in capsules prepared from 2% (w/v) sodium alginate and 5% (w/v) $CaCl_2$ was attempted in order to assess the activity retained upon immobilization in comparison to the free enzymes. Although the immobilized enzymes appeared to hydrolyze crude starches satisfactorily, lower hydrolysis rates were achieved in comparison to free enzymes.

The synergism of α-amylase and glucoamylase was observed by the higher reaction rates attained in the mixed-enzyme systems in comparison to the corresponding rates of sole glucoamylase and sole α-amylase systems. Generally the combinations containing *Aspergillus niger* glucoamylase were more efficient than the combinations employing *Rhizopus* mold glucoamylase. The enzyme system comprising *B. subtilis* thermostable α-amylase developed by our laboratory and commercial *A. niger* glucoamylase led to the highest degradation of all the crude starches tested.

In an attempt to further improve the performance of the laboratory co-enzyme system the effect of total enzyme concentration and the ratio of α-amylase/glucoamylase in the reaction mixture was studied. In the assayed range, higher reducing sugars yield was observed with increasing glucoamylase activity in the co-enzyme system. The α-amylase/glucoamylase ratio of 1:2 was the most effective for starch hydrolysis since more than 90 and 73% of substrate degradation was achieved in the case of free and immobilized co-enzyme system, respectively. Finally the immobilization of the co-enzyme system in calcium alginate gel capsules enabled its repeated use in 10 successive and efficient starch hydrolysis operations and the production of more than 3800 mg of reducing sugars during that period.

## Bioprocessing of food and related wastes for production of biosurfactants

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One existing option for bioconversion of food waste to useful products is production of biosurfactants. The biosurfactants have several advantages over chemical surfactants including lower toxicity and higher biodegradability, and effectiveness at extreme temperatures or pH values. Several biosurfactants have strong antibacterial, antifungal and antiviral activity. Biosurfactants can only replace synthetic surfactants if the cost of the raw material and the process is minimal. In this work a feasibility study will be undertaken in order to elucidate the preliminary data on biosurfactant production from low-cost renewable-resources in order to choose the most economically appropriate for industrial application. Three substrates will be evaluated: cheese whey, molasses and potato substrates, which will be treated by probiotic bacteria (*Lactobacillus casei*) and/or thermophilic mixed culture (*Bacillus* sp.). Effect of C-source, N-source, and environmental factors on the efficiency of biosurfactant production will be studied. Analysis of the surface activity of the biosurfactants produced will be performed via surface tension measurements. At the same time further plans for joint project between UCD and Aristotle University of Thessaloniki will be discussed.

## Fluid dynamics simulation of micropipette-based chemotaxis assays

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Using FEMLAB software, we developed a mathematical model for simulation of micropipette-based assay and studied chemotaxis of *Dictyostelium discoideum* cells. The model took into account diffusion of the chemoattractant, and the breakdown of cyclic AMP by phosphodiesterase secreted by the cells. As a result concentration curves of cAMP surrounding the cell were simulated. It was shown that when the delivery of chemoattractant was limited by diffusion, there was a strong chemoattractant gradient along the cell surface. Michaelis-Menten kinetics was selected for description of the enzymatic degradation of cAMP. Concentrations of cAMP in the leading edge as well as in the rear of the cell were evaluated and compared. The initial concentration of cAMP, injected by needle into the experimental field, was $1^{-6}$ M. The chemoattractant concentration difference simulated at the front and rear of the cell varied from $5.10^{-9}$ to $4.5.10^{-8}$ M. This was mainly affected by the kinetics and mechanism of enzyme delivery as well as the distance of the cell from the needle orifice. The results obtained also showed that the concentration difference was slightly affected by changes in Reynolds number and variation of phosphodiesterase concentration. Finally, verification of the model was achieved by comparison the sensitivity of the cell receptors

(around $10^{-8}$ M), obtained experimentally, with the results simulated by the model.

### Nature's catalysts for biosynthesis of natural products

**J. Littlechild**

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Several enzymes of commercial interest for Biocatalysis have been isolated from a variety of archaeal and algal species. These enzymes are thermostable or thermo-tolerant making them more suitable for commercial biocatalysis. They are also more stable to organic solvents. In most cases the enzymes have been cloned and over-expressed in *Escherichia coli* in a soluble form and in high yield.

The archaeal enzymes to be discussed include a novel alcohol dehydrogenase from *Aeropyrum pernix* which is of interest for chiral alcohol synthesis. A L-aminoacylase from *Thermococcus litoralis* is used for the resolution of amino acids and aminoacid analogues. A gamma lactamase enzyme from the archaeon *Sulfolobus* species has been developed for the production of carbocyclic nucleotides for antiviral compounds.

The enzyme from algal species to be discussed is thermo-tolerant and is isolated from *Corallina* species. This enzyme is unusual in that it contains vanadium which is essential to its activity. The enzyme can carry out bromination of a variety of substrates and is thought to be involved with the synthesis of complex brominated cyclised terpenes. Many brominated compounds have antibacterial and anti-cancer properties. In addition the enzyme will carry out sulfoxidation reactions. When over-expressed in *E. coli* the protein is found in inclusion bodies which can be refolded to active dodecameric enzyme. Mutant enzymes have been constructed that also have chloroperoxidase activity. A truncated dimeric version of the protein can be produced by removing 212 amino acids from the N-terminal of the enzyme which can be over-expressed in *E. coli* in a soluble form.

The applications, substrate specificities and thermostability of several of these enzymes will be discussed in this presentation in relation to their 3D structure and mechanism of action.

### Effect of microorganisms on D-amino acid contents of milk

**G. Pohn**[2], **Cs. Albert**[1], **Sz. Salamon**[1], **P. Sára**[2], **B. Albert**[1], **Zs. Csapó**[2], and **J. Csapó**[1,2]

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In the course of our experiments it was established that certain microbe species causing mastitis (inflammation of the udder) (*Streptococcus dysgalactiae*, *Streptococcus uberis*, *Escherichia coli*, *Staphylococcus aureus*, *Pasteurella multocida*, *Corynebacterium bovis*, *Arcanobacter pyogenes* and *Pseudomonas aeruginosa*) contributed to D-aspartic acid, D-glutamic acid and D-alanine contents of milk to a different extent, however, examination of amino acids was only partially suitable for identification of pathogen microbe species causing mastitis. Out of D-amino acids of peptidoglycan D-glutamic acid contents provides the possibility of identifying the microbes. Based on D-aspartic acid contents only Mastitest-negative milk sample and the species *Staphylococcus aureus* can be identified. On the basis of D-alanine contents microbes examined by us with the exception of the species *Escherichia coli*, *Streptococcus aureus* and *Pseudomonas aeruginosa* can be identified. Free amino acid contents of milk derived from mastitic udder with mastitis caused by the individual bacterial species do not differ significantly from each other, there are, however, such free amino acids whose proportion is characteristic of the given microbe species. The species *Streptococcus uberis* produces the least glycine, for the *Escherichia coli* is typical the very high phenylalanine contents. Milk derived from mastitic udder with mastitis caused by *Pseudomonas aeruginosa* contains the most of free lysine.

## Drug Design

### Cyclic peptidio: Calix[4]arenes and pyridines as novel molecular architectures of versatile perspectives

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Calixarenes present a unique class of molecular architectures having a three dimensional cavity that can host other atoms or molecules. The structure has a wide rim with hydrocarbon functionalities and a narrow rim with phenolic groups. Synthetic modifications at these rims are carried out to introduce additional desired molecular properties. Macrocyclic peptido-calixarenes have been synthesized and investigated as analogues of Vancomycin-type antibiotics.

We have, previously, demonstrated the significance of 2,6-di-substituted pyridine derivatives as biologically active congeners. Herein, some bridged peptido-calix[4]arenes [A] and 2,6-di-substituted peptido-pyridines [B&C], are synthesized as potential molecular architectures of versatile biological and analytical perspectives.

While conventional azide or mixed anhydride peptide couplings served well for assembling the L-tyrosine or L-ornithine peptide backbones, the acid chloride of 2,6-pyridin-dicaroboxlic acid (dipicolinic acid) was manipulated as the acylating agent.

The structure assignments of the new compounds are based on chemical and multiple spectroscopic and chemical evidences.

### Development of new synthetic strategies for synthesis of α-conotoxin-MII

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Conotoxins form a large family of peptide toxins from cone snail venoms that act on a broad spectrum of ion channels and receptors. The subgroup α-conotoxins specifically and selectively bind to subtypes of nicotinic acetylcholine receptors (nAchRs), which are targets for treatment of several neurological disorders. Until now, conotoxins have been mainly prepared by Solid Phase Peptide Synthesis (SPPS) combined with formation of the disulfide bridges after releasing the linear conotoxin precursor from the solid support. We have developed a solid-phase microwave assisted synthesis strategy for the preparation of α-conotoxins. α-Conotoxin MII (αCtxMII) was used as a model system. The application of microwave heating to solid-phase peptide synthesis is particularly advantageous as the acceleration of coupling and deprotection reactions should lead to shorter cycle times, higher repetitive yields, and ultimately purer peptides. We present herein a comparison of the results of the synthesis by the classical SPPS and the MW-assisted synthesis. Furthermore, a comparison of several protocols for on-bead or in solu-

tion preparation of the two αCtxMII disulfide bridges has been performed. The arsenal of Cys protecting groups commercially available allows us to investigate and optimize several disulfide bond formation methods in order to increase the total yield of the synthesis and the purity of the final product.

The aim of this work is to develop an improved method able to generate conotoxins in high yield and purity. This will overcome a key barrier currently preventing the efficient synthesis of small focused libraries in order to investigate the structure-activity relationship (SARs) of those peptides. The development of general synthetic strategies for the preparation of conotoxins and analogues are essential to efficiently approach important questions within the area of neurobiology and for the development of novel drugs for treatment of various neurological diseases.

## Synthesis of hydrazino-peptoide and peptoid-peptide mimetics based on C-terminal fragments of substance P

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It has been reported that the peptide hormone Substance P (SP) alone or in a synergetic fashion with lipopolysaccharides, as well as many small synthetic peptides, analogs of Substance P C-terminal fragments, increase the secretion of tumor necrosis factor α (TNF-α) and of cytokines (IL-6, IL-10) from monocytes and macrophages and prevent the proliferation of several cancer cell lines. In the past we have shown the antiproliferative activity in vitro of tri- and tetra-peptides, against the breast (T47D, SK-BR-3) and prostate (PC-3) cancer cell lines. The synthesized peptides were containing N-MePhe and D-Trp in their sequence.

We are currently studying the biological role of SP C-terminal fragment analogs. Thus in the present study we report the synthesis of a series of hydrazino-peptoide analogs of SP C-terminal hexapeptide, containing the residues -NH-N(Bzl)-$CH_2$CO-, D-Trp and Tic and the N-substituted glycine residues NPhe and NAla. The latter are residues having their side chains shifted from the chiral α-carbon atom of an amino acid onto the anchiral nitrogen.

$Glp^1$-$Nphe^2$-$Gly^3$-[NH-N(Bzl)-$CH_2$-CO]$^4$-D-$Trp^5$-$Leu^6$-OH

$Glp^1$-$NAla^2$-$Gly^3$-[NH-N(Bzl)-$CH_2$-CO]$^4$-D-$Trp^5$-$Leu^6$-OH

$Glp^1$-$Tic^2$-$Gly^3$-[NH-N(Bzl)-$CH_2$-CO]$^4$-D-$Trp^5$-$Leu^6$-OH

Also the octa-peptide C-terminal fragment analogs incorporating the residues NPhe, NAla and D-Trp, instead of natural amino acids in there sequence, have been synthesized. All the syntheses were carried out stepwise in SPPS, using the Fmoc/$Bu^t$ methodology on the solid support of 2-chlorotrityl chloride resin and DIC/HOBt as coupling reagent.

$Glp^1$-$Nphe^2$-$Gln^3$-$Phe^4$-$Gly^5$-D-$Trp^6$-$Nphe^7$-D-$Trp^8$-OH

$Glp^1$-$Nphe^2$-$Gln^3$-$Phe^4$-$Pro^5$-D-$Trp^6$-$Nphe^7$-D-$Trp^8$-OH

$Glp^1$-$NAla^2$-$Gln^3$-$Phe^4$-$Gly^5$-D-$Trp^6$-$Nphe^7$-D-$Trp^8$-OH

$Glp^1$-$NAla^2$-$Gln^3$-$Phe^4$-$Pro^5$-D-$Trp^6$-$Nphe^7$-D-$Trp^8$-OH

According to the literature data the incorporation of N-substituted glycine in peptide chains as well as D-Trp have been proved to improve their stability against proteases. The design and preparation of these oligomeric peptidomimetics is expected to afford potential antagonist of cancer cells proliferation.

All the above analogs were purified (HPLC) and identified (ESI-MS). They are under investigation for their activity against cancer cells proliferation and these results will be presented.

## Impressive structural features from the interactions of α-aminoisobutyric acid (Aib) and Aib-based peptides with Zn(II)

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The coordination chemistry of aminoacids and small peptides is currently a research area of great interest for many reasons. Among them, we can mention the probing of metal-ion/protein interactions in biological systems, the ability of metal/aminoacid (peptide) complexes to develop 1D-, 2D- or 3D-polymeric structures via bridging coordination of the ligands and/or through hydrogen bonding, and the possibility that these complexes exhibit a variety of biological/pharmacological properties.

In continuation of our project concerning the interactions of α-aminoisobutyric acid (Aib) and Aib-based di-and tripeptides with different metal ions, we describe here the synthesis, the structural characterization and the spectroscopic properties of Zn(II) complexes with Aib, Aib-L-Leu and Aib-Aib-Aib.

The above interactions have led to interesting/novel structural features.

## Conformational restriction as a design concept for amino acids and peptidomimetics

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Restriction of conformational mobility is a widely used tool in the design of novel amino acids and peptidomimetics based on them. The restriction "freezes" the intramolecular motions, thereby leading to the decrease in the entropy barrier of the intermolecular noncovalent interaction, e.g. formation of ligand-enzyme complexes. This might lead to enhancement of the efficiency and selectivity of biologically relevant interactions, increasing the chance of finding promising drug candidates among the conformationally restricted peptidomimetics. We report on the synthesis of a library of conformationally restricted amino acids – analogues of L-proline and L-glutamic acid, both described in the literature and novel (compounds **1**–**15**). For synthesis of the known amino acids we used improved scaled-up synthetic procedures. For the novel compounds, namely, for **8**, **10**, **13**–**15** we developed original stereoselective synthetic approaches based on domino cyclopropane opening-Strecker reaction-intramolecular cyclization sequence.

In order to study conformational properties of the amino acids, we synthesized simple model derivatives, *N*-acetyl-*N*′-methylamides, and investigated their structure by X-ray and NMR spectroscopy.

## Molecular dynamics study of amyloid formation of two Abl-SH3 domain peptides: single and multiple β-sheets

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Short-peptide sequences drive protein aggregation in amyloid fibrils. Based on a homology search we have identified an aggregation-prone re-

gion in the Abl-SH3 domain of Drosophila with sequence DLSFMKGE (MK), and less amyloidogenic human homologous region with sequence DLSFKKGE (KK).

The antiparallel flat β-sheets consisting of two and ten strands of MK and KK were constructed. We created four single sheet systems: (1) two strands of MK ($2 \times$ MK), (2) two strands of KK ($2 \times$ KK), (3) ten strands of MK ($10 \times$ MK), and (4) ten strands of KK ($10 \times$ KK). Each of these β-sheet systems was surrounded by a 10 Å layer of water molecules over the solute and subjected to molecular dynamics (MD) simulations with the Amber 8.0 force field in the NPT (constant number of molecules, pressure, and temperature) scheme.

From the results of single sheet simulations we can conclude that the $2 \times$ MK β-sheet is more stable than the $2 \times$ KK β-sheet, and the $10 \times$ MK β-sheet is more stable than the $10 \times$ KK β-sheet; this suggests that the MK peptide is more prone to fibril formation than the KK peptide. The increased stability of the β-sheet formed from the $10 \times$ MK system arises because of the interactions between the methionine and the phenylalanine residues of the neighboring strands. Replacement of Met by Lys removes that stability factor.

Neither the two- nor the ten-strand β-sheet systems are not sufficiently stable at 300 K, which means that they should be stabilized by other parallely placed β-sheets to form fibrils. To prove this hypothesis some parallely placed multisheet systems of MK and KK were simulated by MD.

The MD simulation of multisheet sistems revealed that:

- $10 \times 6 \times$ MK beta sheet stack is stable, but $10 \times 6 \times$ KK beta-sheet stack is not. $10 \times 6 \times$ MK beta-sheet is stable because of hydrophobic interactions of metionine-phenilalanine and leucine of the neighbouring sheets. Met, Phe, Leu make a hydrophobic core for the stack of beta-sheets.
- During MD run the Met, Phe, Leu of neighbouring sheets act as conformational switch moving beta sheets by two amino acid step towards each other.
- Replacement of Met by Lys destroys the hydrophobic core, which is the stability factor of the beta sheets stack. $10 \times 6 \times$ KK system maintains beta sheets, but loses interactions between beta sheets.
- The calculacions of six beta sheets confirm the conclusion drawn for single sheet systems: parallelly placed beta-sheets stabilize each other.

## Structure-based design of inhibitors of glycogenolysis, as a promising therapeutic strategy in the treatment of type 2 diabetes

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Efforts towards improving glycaemic control in type 2 diabetes have been directed towards developing inhibitors of glycogen phosphorylase a (GPa) in collaboration with industry (Bayer, Novo Nordisk, Astra Zeneca Pharmaceuticals, and Sanofi-Aventis Pharma). Inhibitors of hepatic GPa have the potential to be effective therapeutic agents for the treatment of type 2 diabetes, as evidenced by studies showing glucose-lowering effects of these compounds. Acyl ureas were recently discovered as novel inhibitors of the enzyme. The X-ray structure of the screening hit 1 ($IC_{50} = 2\,\mu M$) revealed that 1 binds at the allosteric site, and induces conformational changes characteristic of a T-state conformation. Two cycles of chemical optimization supported by X-ray structural data of 21 ($IC_{50}$ of $23 \pm 1$ nM) in complex with human liver GPa (hlGPa) and by a 3D pharmacophore model derived from a training set of 24 compounds led to 42 with improved cellular activity (hlGPa $IC_{50} = 53 \pm 1$ nM; hepatocyte $IC_{50} = 380$ nM) and a significant reduction of the glucagon-induced hyperglycemic peak when administered to anaesthetized Wistar rats. Clinical studies to evaluate the use of acyl ureas for the treatment of type 2 diabetes are currently under way. Exposure of hepatocytes to acyl ureas promotes conversion of GPa to inactive GPb because the T-conformation is a better substrate for dephosphorylation by protein phosphatase-1. Since GPa is an allosteric inhibitor of glycogen synthase phosphatase, depletion of GPa by these compounds leads to sequential activation of glycogen synthase and stimulation of glycogen synthesis. Iminosugars (5, 8, 9), in contrast to acyl ureas inhibitors, promote conversion of GPb to GPa in hepatocytes, with consequent inactivation of glycogen synthase and inhibition of glycogen synthesis. The X-ray structures of 5, 8 and 9 in complex with muscle GPb show that iminosugars bind tightly at the catalytic site in the presence of the substrate phosphate, and induce conformational changes that characterise the R state conformation of the enzyme. Our results suggest that these compounds ($IC_{50} = 0.4$–$1.2\,\mu M$) function as oxocarbenium ion transition-state analogues: the charged nitrogen N1 is within hydrogen-bonding distance with the carbonyl oxygen of His377 (5) and in ionic contact with the substrate phosphate oxygen (8 and 9). The present evidence from X-ray crystallography that acyl ureas and iminosugars cause the R to T transition and vice versa, respectively, provides the explanation for the metabolic studies in hepatocytes.

## Canavanine in the design of the biologically active molecules

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The guanidine group is a common structural key fragment in a variety of natural and synthetic compounds, which have found various applications in medical, bioorganic and supramolecular chemistry, as potentially useful drugs and pesticides, flame retardants, super potent sweeteners, in positron emission tomographic imaging, in sugar, nucleotide and peptide mimetics. Among these compounds are antimicrobials, antifungals, antivirals, neurotoxins, hormones, and agents that act as transmitters of biological signals. Guanidine-containing bioactive molecules, particularly the analogs or derivatives of the natural products, are now significant targets for drug design.

L-Canavanine is a guanidine group-containing nonprotein amino acid and is involved in many important physiological and pathophysiological processes. Various canavanine derivatives have been synthesized and incorporated into peptides and peptidomimetics to study the structure-activity relationships of canavanine-containing molecules.

## Identification and characterization of peptides with high affinity against recombinant human prion protein

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Transmissible Spongiform encephalopathies (TSEs) are a group of neurodegenerative diseases that affect. humans and animals. The infectious agent of TSEs is believed to be an abnormal isoform of a naturally occurring protein known as cellular prion protein, $PrP^{C}$. The abnormal form of prion protein, $PrP^{Sc}$, accumulates in the brains of affected individuals.

Identification of peptides with high affinity for prion protein could be useful in prevention of prion diseases. It might also, lead to identification of other host molecules with high affinity to prion protein. The interaction of PrPC with exogenous peptides may lead to the inhibition of prion aggregation.

To search for consensus sequences that interact with $PrP^C$, we constructed and expressed human prion protein. The construct was cloned and expressed a significant amount of PrP protein. The recombinant protein was used to coat plates, which were then panned with a M13 phage display library of random heptamers expressed at the N terminal of the phage pIII protein. After several rounds of screening, groups of heptamer with consensus sequences were evident. These peptide sequences have been analyzed and investigated for their in vitro interaction with prion protein.

### New analogs of antiherpes virus drugs – synthesis and biological activity

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Acyclovir, 9-[(2-hydroxyethoxy)methyl] guanine (ACV) is an acyclic guanine nucleoside analogue that is widely used clinically as an antiherpetic agent. Its limited absorption (15–20%) in humans after oral administration prompted the search for prodrugs. A possible way to increase the bioavailability is by modifying the known antiviral drugs with various amino acids.

The aim of this study was to design and to synthesize of new thiazole containing amino acids (Val, Thz) ester prodrugs of acyclovir and to explore their activity on the HSV-1.

Synthesis of thiazole containing amino acid (Ala, Leu) ester prodrugs of acyclovir involved formation of N-Boc protected amino acid anhydrides, coupling of the N-Boc protected amino acid anhydride with acyclovir, and deprotecting the amino group of the amino acid ester of acyclovir.

The two examined derivatives and acyclovir as referent drug were applied in concentration 10, 5, 1 and 0.5 μg/ml. The Ala-thiazole-4-yl-acyclovir and Leu-thiazole-4-yl-acyclovir shown insignificant effects on the herpesvirus replication – 20 and 10% inhibition, respectively. Whereas the referent drug inhibited the viral replication completely in same dose (10 μg/ml).

These results suggest that Ala-thiazole-4-yl-acyclovir and Leu-thiazole-4-yl-acyclovir may be attractive in higher concentrations for antiviral chemotherapy obligatory with lower cytotoxicity effect in comparison with the effective nucleoside analogs.

Design of amino acid prodrugs seems to be an attractive strategy to enhance the solubility of the otherwise poorly aqueous soluble compounds and also to afford a targeted and possibly enhanced delivery of the activedrug.

An implicit proof of this assumption is the fact that L-valyl ester of acyclovir (valacyclovir) shows bioavailability of 60%.

## Metabotropic Glutamate

### In vivo characterisation of mGluR5 negative and positive modulators

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Recently, metabotropic glutamate receptors have gained a great deal of interest as therapeutic target. It is believed that in particular targeting modulatory site can lead to selective agents with high selectivity and good penetration to the CNS. In facet, several such substances have been introduced such as MTEP, MPEP, CDPPB or ADX47273. We performed a verification of the therapeutic potential of mGluR5 negative and positive modulators using various behavioural methods and compared to their brain concentrations assessed with microdialysis methods. mGluR5 negative allosteric modulator (NAM) MTEP produced analgesic effects in several pain models such as formalin, and Freund adjuvant model of inflammatory pain. Additionally MTEP attenuated L-DOPA induced dyskinesia and produced anxiolytic activity in several models of anxiety such as fear potentiated startle, context freezing. However, the brain concentrations at active doses were much higher than IC50 for mGluR5 receptor in vitro and efficacy was not better then respective reference agents. mGluR5 positive modulators (PAMs, e.g. CDPPB) are expected to have antipsychotic-like activity and improve learning. These expectations can be only partially fulfilled as no clear convincing picture arises from testing in various animals models of schizophrenia (amphetamine-induced hyperactivity or apomorphine-induced prepulse inhibition). In learning models positive effect was seen in object recognition task (spontaneous forgetting).

### Antidepressant-like effects of mGluR5 antagonists

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Glutamate system is involved in the pathophysiology and treatment of affective disorders. Both ionotropic and metabotropic glutamate receptors seem to participate in the mechanism of antidepressant action.

Antagonists of mGluR5 (group I mGlu receptors) exhibit antidepressant-like activity in preclinical tests. MPEP, MTEP, AIDA are active in forced swim/tail suspension tests or olfactory bulbectomy animal model of depression. Also, zinc, non-specific antagonist of mGluR5 and NMDA receptors, exhibits antidepressant activity in forced swim and tail suspension tests, and in animal models of depression (olfactory bulbectomy, chronic unpredictable stress, chronic mild stress). Chronic treatment with MPEP, MTEP or zinc induced adaptation in BDNF gene expression and serotonergic (5HT-2A and 5HT-1A) receptors, similar to that changes induced by most antidepressant treatments.

The preclinical data together with very limited clinical reports suggest that antagonism of mGlu5 receptors induce antidepressant effect.

### Activation of the mGlu7 receptor elicits antidepressant-like effects in mice

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Broad evidence indicates that modulation of the glutamatergic system could be an efficient way to achieve antidepressant activity. Metabotropic glutamate receptor (mGlu receptor) ligands seem to be promising agents to treat several CNS (central nervous system) disorders, including psychiatric ones.

**Objectives:** The aim of our study was to investigate potential antidepressant-like activity of the first, selective, and bio-available mGlu7 receptor agonist, AMN082 (N,N′-Dibenzyhydryl-ethane-1,2-diamine dihydrochloride), in wild-type (WT) and mGlu7 receptor knock-out (KO) mice.

**Methods:** The forced swim test (FST) and the tail suspension test (TST) in mice were used to assess antidepressant-like activity of AMN082.

**Results:** We found that AMN082, administered IP, induced a dose-dependent decrease in the immobility time of WT animals in the FST and TST suggesting antidepressant-like potency of an mGlu7 receptor agonist. Moreover, AMN082 did not change the behaviour of mGlu7 receptor KO mice compared to WT littermates in the TST, indicating an mGlu7 receptor-dependent mechanism of the antidepressant-like activity of AMN082. However at high doses, AMN082 significantly decreased spontaneous locomotor activity of both mGlu7 receptor KO mice and WT control animals, suggesting off-target activity of AMN082 resulting in hypo-locomotion.

**Conclusions:** These results strongly suggest that activation of the mGlu7 receptor elicits antidepressant-like effects.

### In vitro characterisation of mGluR5 negative and positive modulators

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Group I metabotropic glutamate receptors (mGluR1 and mGluR5) belong to the family of Gq-coupled GPCRs. Their activation leads via phospholipase C to the formation of inositol phosphates ($IP_3$) and mobilization of $Ca^{2+}$ from intracellular stores. It has been proposed that mGluR5 may play a role in various disease states of the central nervous system including Alzheimer's, Parkinson's, pain, anxiety, and schizophrenia, and therefore the development of modulators of mGluR5 may be of considerable therapeutic benefit. We have characterized the in vitro effects of various negative and (NAM) positive allosteric modulators (PAM) of mGluR5 – e.g. MTEP and MPEP (NAM), CDPPB and ADX47273 (PAM).

FLIPR is a screening platform that is widely used in the pharmaceutical industry to determine the agonist-stimulated $Ca^{2+}$-signal in cells stably or transiently expressing receptors such as mGluR5. Here we present data from cultured rat cortical astrocytes thus circumventing problems such as toxicity or constitutive activity of the receptor encountered with cell lines. We used a no-wash fluorescence calcium indicator kit that eliminates background fluorescence through a quenching technique. To validate the system, we compared the results with those produced by measuring the accumulation of $IP_3$ and by the displacement of [$^3$H]MPEP. Responses to selective and potent antagonists of the MPEP-family were highly comparable between the binding and the FLIPR experiments. However, some mGluR5 NAM and PAM scaffolds appear to interact with adjacent and partly overlapping sites, so that the tight correlation between binding and function was not always seen.

# Neurobiology

### Nanotechnology for neurosurgery

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Many neurosurgical procedures involve cellular/neuronal level nervous system disorders, e.g. primary brain tumors, epilepsy, and movement disorders such as Parkinson's disease. Although current neurosurgical techniques are "macro" – "cutting things out" (tumors, ruptured discs) or "implanting devices" (spinal hardware, deep brain stimulators) – nanotechniques will make possible the "sculpting" of aberrant nervous system function – neuron by neuron, if need be – rather than resecting or reversibly ablating the nervous system.

This presentation introduces nanotechniques for neuroprotection and neurosurgery:

1. Nanoimaging. Quantum dots allow both capillary/micron-level angiography and "nanobarcode" labeling of cells and subcellular entities (e.g. proteins, receptors). Other nanotechniques allow real-time tracking of the enzymatic actions of proteases and kinases.
2. Mechanical Nanoneuromodulation. Nanoscaffolds can greatly augment recovery from central nervous system lesions previously considered irreversible. Such nanoscaffolds, composed of amino acids which are later metabolized, provide a guide for regenerating axons and also limit scar formation.
3. Electrical and Chemical Nanoneuromodulation. Macro- and micro-level brain-machine interfaces are in clinical trials for closed loop neural feedback (neuroprostheses – e.g. spinal cord injured patients). Carbon nanotube arrays can monitor both electrical and chemical activity at the neuronal level, and can also stimulate both electrically and chemically with subcellular precision.
4. Nanoneurosurgery. Micron-level cutting and splicing of damaged axons has recently been described: a platform which combines a $< 10$ micron blade for axon cutting (or trimming), axon manipulation/alignment by dielectrophoresis, and axon splicing/repair by electrofusion.

These nanotechniques are applied to two neurosurgical issues: (1) neuromodulation, and (2) axonal regeneration following spinal cord injury.

Carbon nanofiber arrays can monitor – in real time – both electrical activity and chemical activity (e.g. neurotransmitter levels) at the cell body, intracellular, and/or axodendritic level. Electrical and chemical stimulation has similar precision, making feasible neuromodulation at the cellular or node of Ranvier level. The enhanced electrochemical properties of such arrays (improved capacitance and impedance) make feasible precise, multifocal neuromodulation without bulky power supplies/batteries. Furthermore, anti-inflammatory agents, neurotrophic factors, etc., can be released with similar spatial and temporal precision, opening possibilities for electrochemical neuromodulation heretofore impossible.

Axonal regeneration in the adult central nervous system can be augmented by appropriate electrical stimulation and chemical environment. A multimodality neural/axonal growth tube, incorporating a number of nanotechniques, is being developed to study and augment axon regeneration after spinal cord injury. This neural growth tube includes:

(1) intracellular nanosensors (e.g. quantum dots);
(2) nanoarrays to study the actions of proteases and kinases over time;
(3) carbon nanotube ring electrode arrays (for both recording and stimulating the axon) aligned in circular fashion on the inner surface of the neural growth tube;
(4) a nanoscaffold longitudinally aligned along the neural growth tube to foster axonal growth. Through electrical stimulation, the nanoscaffold can release nerve growth factors and other substances to promote axonal regeneration.

Multimodality nanotechniques for understanding nervous system function and repair offer great promise: the information gained by nanodevices such as carbon nanofiber arrays and the neural growth tube for spinal cord injury will far surpass the information gained by our admittedly crude "micro-" level approaches used until recently. Eventually much of neurosurgery will likely be performed by nano-sized devices on neurons and other cellular and sub-cellular level entities (e.g. tumor cells, aberrant neuron ensembles in epilepsy, and subtle neurochemical imbalances in mood disorders).

## Glutamate and glycine levels in the rat brain with weak and strong epileptic manifestations

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Our recently published data have shown significantly increased activity of GABA-ergic activities in the rat brains, 6 months after kainic acid (KA) injection. In present study relation between glutamate and glycine levels in the different brain regions of rats in the KA (10 mg/kg, subcutaneously)-induced spontaneous recurrent seizure model of epilepsy, 6 months after the initial KA-induced seizures, were investigated. Six months after KA injection there was found a significantly reduced glutamate levels in the amygdala/piriform cortex, in the septum and in the occipital cortex (68.6, 76.4 and 76.7% of control, respectively; $p < 0.05$) but notable increased in the caudate nucleus (171.8% of control; $p < 0.001$) of rats with spontaneous seizures. In KA rats with only wet dog shakes and rare focal seizures we found a moderately reduced glutamate in the septum but increased in the caudate nucleus (119% of control, $p < 0.05$). Interestingly, in KA rats with spontaneous recurrent seizure, six months after KA injection, increased glycine levels in the parietal (207% of control; $p < 0.001$) and frontal (197% of control; $p < 0.001$) cortices and in the caudate nucleus (148% of control; $p < 0.001$) were found. While, in KA rats with only wet dog shakes and rare focal seizures, six months after KA injection, a reduction of glycine levels in the occipital cortex (60.0% of control; $p < 0.01$) was observed. Obtained data would suggest that high levels of glycine in selected brain regions in KA rats with spontaneous recurrent seizures may significantly increase the response of the NMDA receptor activity. The over-stimulation of NMDA receptors is likely modulated by the increased inhibitory metabolic rate in the caudate nucleus of epileptic rats.

## Involvement of adrenergic system in analgesic effects of TYR-W-MIF-1 with or without stress

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Tyr-W-MIF-1 belongs to Tyr-MIF-1 family of peptides, which have been isolated from bovine hypothalamus and human brain cortex. Members of this family have opioid and anti-opioid like properties and are able to inhibit the expression of some forms of stress-induced analgesia (SIA).

The aim of our study was to examine whether adrenergic system is involved in analgesic effects of Tyr-W-MIF-1 after 1 h immobilization (IS), cold (CS) or hot (HS) stresses and in experimental model without stress. The $\alpha_2$-adrenergic agents such as clonidine (Clo), yohimbine (Yoh) and desipramine (Des) were used. All drugs were injected intraperitoneally in male Wistar rats. Nociception was measured by paw pressure test using an analgesimeter.

The obtained results showed that Tyr-W-MIF-1 (1 mg/kg) co-administered with Clo (2 mg/kg), Yoh (1 mg/kg) and Des (5 mg/kg) significantly decreased the pain threshold during stress or in model without stress.

In conclusion our data suggest that adrenergic neurotransmission is involved in the analgesic effects of Tyr-W-MIF-1 in normal conditions and during three models of stress – IS, CS and HS.

## Exposure to ultrafine particles enhances TLR-2 mediated responses in the brain of Apolipoprotein E knockout mice

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The etiology of most cases of neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and multiple sclerosis is generally unknown. While a genetic component has been identified for familial forms of these disorders, most cases are sporadic. Thus environmental factors are likely to play a role in initiation or progression of such disorders. In a previous study, we reported that exposure to either fine or ultrafine (nano) particles, which are present in urban air pollution, up-regulates neuroinflammation in the mouse brain. In the present study we hypothesized that this effect may be partially mediated by modulation of an evolutionarily well-conserved innate immune response.

Toll-like receptors (TLRs) are transmembrane receptors linked to signaling pathways which activate innate immune responses. To this date, thirteen different TLRs have been identified in mammalian species and they are shown to be specifically activated by unique structures present on pathogens known as pathogen-associated molecular patterns. The TLR-2 pathway is activated by considerably more ligands compared to other TLRs. In the brain and spinal cord, TLR-2 is mainly expressed in glial cells and this is increased during neuroinflammatory events.

In the present study, Apolipoprotein E knockout (ApoE−/−) mice were exposed to either fine or ultrafine particulates. Apolipoprotein E is a glycoprotein which functions in transport, uptake, and redistribution of cholesterol. In the CNS, it is produced by astrocytes and plays an important role in modifying brain inflammatory processes. ApoE(−/−) animals were exposed (5 h/day, 3 days/wk for 5 weeks) at a Site at Los Angeles, CA. Our results show that after exposure, there is a significant increase in the activation of NF-κB, CNS TLR-2 levels, and inflammatory cytokines.

Neuroinflammatory events have been shown to be a common underlying mechanism in the pathogenesis of several neurodegenerative impairments. Understanding how exposure to nanoparticles may contribute to disease progression will allow the proper identification and limi-

tation of such exposures. This will be important in reducing the incidence or severity of debilitating neurological disorders.

## Multiwalled carbon nanotubes: interconnecting solid-state electronics with biosystems

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Multiwalled carbon nanotubes (MWNTs) are essentially highly conductive metallic wires with extremely high aspect ratios. Vertically aligned MWNTs can be grown directly on prefabricated electronic circuits with nanoscale precision. Such materials are ideal metallic wires to interconnect solid-state electronics and biosystems. Developing biomaterial constructs that closely mimic the natural tissue microenvironment with its complex chemical and physical cues is essential for improving the function and reliability of implantable devices, especially those that require direct neural-electrical interfaces. We have developed the use of free-standing vertically aligned carbon nanotube arrays as multifunctional three dimensional (3-D) nano-engineered matrix that interpenetrates the neuronal network of PC12 cells. We found that PC12 neuron cells cultured on the nanotube arrays can form an extended neural network upon proper chemical and biochemical modification. The soft 3-D nanotube array architecture provides a new platform to fine-tune the topographical, mechanical, chemical, and electrical cues at sub-cellular scales. This biomaterial platform can be used for both fundamental studies of material-cell interactions and the development of multifunctional, chronically stable implantable devices. Furthermore, we have demonstrated the use of the free-standing nanotube arrays as nanoelectrode that interconnect with underlying circuits for the detection of nucleic acids to construct an ultrasensitive electrochemical sensor. The application of these devices and potential utility as a multifunctional platform for neurophysiology and biochemical studies will be discussed.

## Glutamate modulates G-protein coupled receptors natively expressed in neuronal and glial cells

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Glutamate is the main excitatory neurotransmitter in the Central Nervous System which is implicated in learning and memory processes. However, at high concentrations glutamate acts as a neurotoxin causing degeneration and neuronal death. Excitotoxicity has been implicated in the neurodegeneration detected in several disorders as Alzheimer, Parkinson and other dementias. Besides ionotropic receptors, action of glutamate is mediated by metabotropic receptors (mGluRs) which are coupled through G proteins to Phospholipase C activation (group I mGluR) or adenylate cyclase (AC) inhibition (groups II and III mGluR). Glutamate levels are modulated by adenosine which acts trough adenosine receptors (ARs) causing inhibition ($A_1$ and $A_3$) or stimulation ($A_{2A}$ and $A_{2B}$) of AC. The aim of the present work was to determine the excitotoxic effect of glutamate in neurons and in a line of glial cells (C6 glioma) and the implication of ARs and mGluRs in this process. Results show that glutamate causes excitotoxic damage only in neurons, being C6 glioma cells preserved. Loss of cell viability in neurons was glutamate concentration- and time-dependent, being maximal at 100 µM after 24 h of treatment. MGluRs were significant lost at 1 µM for 24 h or 100 µM for 2 h. However, adenosine $A_1$ and $A_{2A}$ receptors were significantly up-regulated at the later excitotoxic glutamate concentration. In C6 glioma cells, which have been used as a model of glial cells, glutamate treatment did not affect cell viability. In these cells, $A_1$ and $A_{2A}$ receptors were significantly increased and decreased, respectively, in a time-dependent manner. Nevertheless, mGluRs were regulated in a biphasic manner being decreased at 6 h, and increased after 24 and 48 h of glutamate treatment. Results suggest that although adenosine and metabotropic glutamate receptors are regulated by glutamate exposure being this regulation depending on the cell type and not associated to cell death.

## The excitotoxic hypothesis of schizophrenia and antipsychotic drugs as neuroprotective agents

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Excitotoxicity can play a role in the pathology of neurodegenerative disorders, possibly including schizophrenia. In schizoprenia, a possible hypofunction of NMDA receptors located on inhibitory GABAergic interneurons would result in a disinhibition of glutamatergic neurotransmission and excessive continuous stimulation of non-NMDA, AMPA and kainate receptors, and consequent neurodegeneration. 5-$HT_{1A}$ agonists are neuroprotective in animal models of stroke. The mechanism underlying 5-$HT_{1A}$ agonist-induced neuroprotection might involve inhibition of glutamate release. Kainic acid (KA) is an axon sparing neurotoxin and its injection into rodent striatum induces a neurodegeneration that relies upon glutamate release from cortical projections. We compared the effects of the antipsychotics, clozapine, ziprasidone and aripiprazole, that are partial agonists at 5-$HT_{1A}$ receptor, with those of haloperidol, which is devoid of 5-$HT_{1A}$ agonist properties, on kainic acid (KA)-induced striatal lesion volume, in C57Bl/6N mice. The involvement of 5-$HT_{1A}$ receptors was determined by antagonist studies with WAY100635, and data were compared with those obtained using the potent and high efficacy 5-$HT_{1A}$ receptor agonist, F13714. The atypical antipsychotics clozapine, ziprasidone and aripiprazole, but not haloperidol decreased KA lesion volume. F13714 decreased lesion volume at doses about 15–30 times lower than that of the antipsychotics. WAY100635 antagonized the effects of F13714 and those of aripiprazole and ziprasidone but not that of clozapine. WAY100635 alone did not affect lesion volume. These results show that 5-$HT_{1A}$ receptor activation protects against KA-induced striatal lesions and indicate that some atypical antipsychotic agents with 5-$HT_{1A}$ agonist properties may protect against excitotoxic injury, in vivo.

## Presence of *N*-methyl-D-aspartic acid (NMDA) in human brain and its involvement in neurotransmission

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NMDA (*N*-methyl-D-aspartic acid) is well known to be a potent agonist of L-glutamic acid at the ionotropic glutamate receptors. For this reason the glutamate receptors were termed L-glutamate receptors of NMDA type or NMDA receptors. The other glutamate receptors (kainate and quisqualate) are metabotropic receptors and are coupled to intracellular signal transduction through the G-protein. Our previous studies have demonstrated that NMDA is present as an endogenous compound in rat and tunicate nervous and endocrine tissues and in tissues of other vertebrates and invertebrates animals.

In this study we report the presence of NMDA in various regions of human brain and in synaptic vesicles. Human brain from normal subjects aged 60–80 were obtained from the Loyola University Brain Bank (Maywood, IL), from the Cooperative Human Tissue Network (Philadelphia, PA or Columbus, OH), or from the Dipartimento of Scienze Legali, University of Siena, Italy.

Tissues (0.5–1 g) were homogenized in 0.2 M perchloric acid or trichloroacetic acid in a ratio 1:20 and centrifuged at 20,000 *g* for 30 min. The supernatant was purified on a cation exchange resin (AG 50W-X8). Since in rat, it has been demonstrated that NMDA in vivo is synthesized by endogenous D-Asp, in this study we also have determined D-Asp according the described method. The purified sample was further purified with OPA treatment to eliminate all amino acids except NMDA, and then the NMDA was determined by an enzymatic-HPLC method. The synaptic vesicles were prepared as follows: tissues (1–2 g) were homogenized in 0.16 M NaCl in a ratio 1:20 and centrifuged at 10,000 *g* for 10 min. The supernatant was re-centrifuged at 48,000 *g* for 30 min. The pellet consisting of synaptic vesicles (verified by electron microscopy) was resuspended in 10 ml 0.16 M NaCl and recentrifuged as above. The new pellet was homogenized in 300 µl 0.05 M HCl. Then the total protein content was determined on a portion of this sample, while the remaining portion was centrifuged at 10,000 *g* for 10 min. The supernatant consisting of vesicular amino acids was used for the determination of NMDA.

The results obtained from this study demonstrated that NMDA is present in all brain regions examined and also in the synaptic vesicles, indicating a probable role in neurotransmission.

## D-Aspartic acid and *N*-methyl-D-aspartic acid in the nervous system of amphioxus and moth

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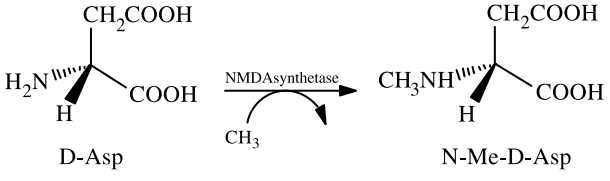
Synthetic *N*-methyl-D-aspartic acid (NMDA) is widely known for its ability to function as an agonist of the L-glutamate receptor of the NMDA-type. Recently, we demonstrated that endogenous NMDA is present in a mammal (rat) and a protocordate (*Ciona intestinalis*) and that NMDA has the ability to elicit release of the hormone GnRH in neuroendocrine tissues. In addition, it has been found that NMDA derives from D-aspartic acid (D-Asp) through *N*-methylation catalyzed by an NMDA synthase, according to the following reaction.




Thus D-Asp is expected to be found, as a biosynthetic precursor, in tissues that possess NMDA.

In the present study we determined the concentrations of D-Asp and NMDA in nervous tissues of a cephalochordate, *Branchiostoma lanceolatum*, and an insect, the sphinx moth *Manduca sexta*. The amphioxus is an interesting animal model, situated taxonomically at a key position between invertebrates and vertebrates. Like vertebrates, the amphioxus body is characterized by a dorsal, a notochord, segmental muscles, pharyngeal gill slits, and a post-anal tail. The insect *Manduca sexta* (Lepidoptera: Sphingidae) is a prominent representative of the large family of sphinx or hawk moths, which are known for their capacity for powerful, rapid, and sustained flight. This species has become a favored experimental model for studies of insect neurobiology, neuroendocrinology, postembryonic development, and herbivory.

The purpose of this study was to ascertain whether these representatives of two additional animal phyla possess D-Asp and NMDA as do mammals and tunicates and if so, to compare their levels of those D-amino acids with those previously reported for other animals and to examine the possible role(s) of these substances in these species.

We found that the nervous tissues of the amphioxus *B. lanceolatum* yielded D-Asp at a mean concentration of $280 \pm 30$ nmol/g tissue and NMDA at a mean concentration of $3.1 \pm 0.4$ nmol/g. Brain structures from *M. sexta* yielded D-Asp and NMDA at the following concentrations (expressed as nmol/g tissue): antennal lobes: D-Asp 60, NMDA 0.20; optic lobes: D-Asp 92, NMDA 0.30; "core" protocerebrum: D-Asp 68, NMDA 0.13; and whole brains, including core protocerebrum and optic and antennal lobes: D-Asp 75, NMDA 0.155. Notably, both the amphioxus and the moth exhibited a ratio of D-Asp to NMDA in the range 100–500, which is comparable to the ratio observed in mammalian and tunicate nervous systems. We hypothesize that NMDA could be an important effector that, like a hormone, has biological activity at very low levels.

## TPH2 gene in suicide and major psychosis

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The tryptophan hydroxylase isoform-2 gene (*TPH2*) is located on chromosome 12 and is expressed primarily in brain tissue. Although the tryptophan hydroxylase isoform-1 gene (*TPH1*) has been reported to have a genetic association with bipolar disorder and schizophrenia, the Tph1 isoform is expressed at much lower levels than Tph2 (150-fold less in the mouse brain). We hypothesized that bipolar disorder and schizophrenia are associated with abnormal levels of *TPH2* mRNA in the brain. *TPH2* and β-actin mRNA levels in postmortem brain were quantified using real-time PCR. mRNA samples provided by the Stanley Foundation Array Collection were derived from the dorsolateral prefrontal cortex (Brodmann Area 46) of 35 bipolar, 35 schizophrenic, and 35 control subjects. There were significant differences in the mRNA levels among bipolar, schizophrenic, and normal subjects ($F(2,102) = 3.58$; $p = 0.031$). A greater amount of TPH2 mRNA was found in the bipolar group in comparison with control subjects (Tukey's test: $p = 0.024$). Further investigations of *TPH2* are needed to clarify the potential role of this gene in the pathophysiology of major psychosis and suicidality.

## Effects of D-kyotorphin on immobilisation stress-induced analgesia in rats

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D-Kyotorphin (D-Kyo), the most potent isomer of the endorphin-like dipeptide kyotorphin (Kyo), induced Met-enkephalin release at rates of approximately 4 times basal release. It is known that endogenous opioid peptides, released during stress, produce analgesia referred to as stress-induced analgesia (SIA) and relevant to the perception and the response to pain. Several factors have been reported to induce analgesia due to stress. One of these is immobilization stress (IS) which evokes extremely variable endocrine, physiological, and behavioral responses by activating motor, autonomic, and HPA systems. Complexities are involved in stress-induced analgesia (SIA) which is due to the activation of both opiate and non-opiate mechanisms.

The purpose of the present study was to examine the effects of D-Kyo on 1 h immobilization stress-induced analgesia (ISIA) in rats. A non-competitive antagonist of opiate receptors as naloxone (Nal) was used to determine whether analgesic activity is mediated by the opioid system. The experiments were carried out on male Wistar rats. Nociceptive

effects were measured by the paw pressure and hot plate tests. All drugs were administered intraperitoneally (i.p.).

In summary our findings indicate that D-Kyo (5 mg/kg) inhibited 1 h ISIA. Nal (1 mg/kg) injected immediately after 1 h IS and 20 min before the peptide decreased significantly the pain threshold compared to ISIA. This suggests an involvement of the opioid system.

## Protein phosphatase inhibition by okadaic acid impairs astrocytes-mediated neuroprotection against oxidative stress

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Okadaic acid (OKA) is a phycotoxin commonly contaminating shellfish seafood, that potently inhibits protein phosphatase 2A (PP2A). OKA induces apoptotic degeneration of cultured neurons, and we investigated whether it could also affect glial cells. Exposure of cultured glial cells from the rat cerebellum to increasing concentrations of OKA resulted in glial degeneration and death, with 80% of cell death at 50 nM OKA, after 8 h. The time course of glial degeneration was characterized by progressive shrinking of the cell body and significant morphological changes at earlier time than in neurons. OKA glial toxicity was associated with DNA laddering in agarose gel electrophoresis, chromatin condensation and fragmentation and activation of caspase-3. All these molecular events are compatible with the activation of an apoptotic process and occurred in cultured neurons as well. However, differently from neurons, OKA toxicity in glial cells was potentiated by treatment with protein synthesis inhibitors such as cycloheximide or actinomycin D. Glial cells treatment with toxic concentrations of OKA promoted oxygen free radical formation, as detected by confocal laser microscopy, and significantly reduced both catalase and glutathion peroxidase activity, as well as reduced glutathion cellular levels.

The presence of antioxidants such as α-tocoferol, in the growth media during exposure to OKA, partially reduced both oxidative stress and toxicity. The effect of OKA treatment on the phosphorylation levels of several proteins in neurons and glial cells will be discussed.

## Protecting the brain against seizure-induced neuropathology: excitatory mechanisms mediated by the amygdala

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Human and animal studies clearly demonstrate that prolonged seizure activity induces neuronal cell death, and ultimately brain damage. Seizures can be initiated by a number of brain insults, including traumatic brain injury, status epilepticus, and exposure to neurotoxic compounds such as organophosphate agents (OPs). The severity of the brain injury closely correlates with the intensity of the seizures, independently of the nature of the insult that initiated the seizure activity. The amygdala, a temporal lobe structure known to play a central role in the generation and spread of seizures in the brain, is particularly sensitive to seizure-induced brain damage. However, the mechanisms underlying the high vulnerability of the amygdala are not well understood. Several lines of evidence implicate GluR5-containing kainate receptors (GluR5KR), a type of ionotropic glutamate receptor, in the amygdala's vulnerability to seizures and epileptogenesis. Here we will present evidence that GluR5KRs play a major role in the modulation of neuronal excitability in the amygdala, and actively participate in the induction and expression of seizure activity in vivo. In addition, we will provide evidence that GluR5KR antagonists are very efficacious in protecting the brain against seizures and seizure-induced neuropathology. These results support the concept that GluR5KRs represent a novel target for antiepileptic/neuroprotectant drug development.

## Alterations in plasma kynurenate and glutamate in ketamine-treated rats

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Several lines of evidence suggested that hypofunction of *N*-methyl-D-aspartate (NMDA) receptor might be related with schizophrenia. In the present study, alterations of kynurenate (KYNA), an endogenous antagonist of glycine binding site for NMDA receptor, and glutamate (Glu), endogenous agonist of NMDA receptor were investigated in the ketamine-treated rats, which were reported to be an animal model of schizophrenia.

An NMDA receptor antagonist, ketamine, was administered consecutive 5 days (once a day) to male Sprague-Dawley rats (30 mg/kg), and at 4–5 weeks after cessation of the administration, approximately 200 µl of blood was drawn from jugular vein of each rat.

Plasma KYNA and Glu in ketamine-treated rat were determined by our HPLC methods previously developed. As a consequence, plasma KYNA and Glu concentrations in Ket-treated rats were significantly increased as compared to those in saline-treated rats ($p < 0.05$). A precursor of KYNA, kynurenine (KYN) was also investigated, but the plasma KYN concentration was not significantly changed in the Ket-treated rats. These results suggested that increased KYNA and Glu might be related with ketamine-induced abnormal behaviors. In this conference, recent our data on the KYNA, KYN, and the other amino acids will be also presented.

## Few examples of ways to interpret data from expression profiling studies

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Typically, results from expression profiling experiments provide lists of gene transcripts or gene products (proteins) that are changed in disease or upon some treatment. Those changes have to be understood as biological messages from the specimens. The messages, however, are encrypted, and it is up to the scientist to find out about their meanings. It is certainly not very helpful to sort the lists into transcripts or proteins that belong to functional groups like receptors or transcription factors, since that does not reveal anything about perturbed molecular interactions.

Two examples are presented how to obtain a better understanding of the biological "language" of lists from expression profiling studies. The first is a meta-analysis of lists retrieved from studies on chronic inflammatory diseases of the brain. The second is derived from Affymetrix chip data obtained from cultured rat microglia treated with the chemokine fractalkine. Both examples show, that it is possible to develop interactive molecular networks from those lists. These networks can be used to perform investigations on mutual molecular influences by computer-assisted simulations in both spatial and temporal dimensions.

## Biomarkers of glaucoma

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Glaucomatous damage is a neurodegenerative eye disease and one of the leading causes of blindness with 67 million patients worldwide. Major currently challenging questions include early diagnosis, risk evaluation, and follow-up.

The role of biomarkers in medicine is to specify molecular alterations/reactions/pathways attributable to concrete pathologic condition. Molecules can change qualitatively (gene mutation/s) or quantitatively (altered gene expression). Although, gene mutations are well documented in glaucoma, the known mutations are involved in a negligible minority of glaucoma patients. Therefore, quantitative approaches comparing gene expression levels of glaucoma patients with controls are currently under our consideration in terms of potential diagnostic application.

Disease specific expression difference observed in circulating leukocytes of glaucoma patients gave us useful information on molecular pathomechanisms of glaucoma. Following key molecular pathways have been shown to be affected by glaucoma pathology: stress response, apoptosis, DNA-repair, cell adhesion, tissue remodelling, transcription regulation, multi-drug resistance, energy metabolism, etc.

Our ultimate goal is the application of such information for diagnostic and potentially even screening purposes for the identification of groups of risk. A non-invasive molecular diagnostic approach based on disease specific gene expression patterns in leukocytes has been recently suggested for glaucoma [Golubnitschaja and Flammer, International Patent No. IB02/00648].

This diagnostic test foresees a precise expression profiling of selected genes in fresh blood samples. A clinical application of the test is currently under consideration, and novel nanotechnological approaches, which should provide a possibly easy and cheap routine application of the test, are under development by the authors.

## Permissive and protective genes in alcoholism: Yin and Yang. Protection against alcoholism in a genetic model of alcohol dependence

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A number of studies have shown that the susceptibility to develop alcoholism is 50–60% genetic, due to multiple genes. Given a permissive environment these genes allow the full spectrum of alcohol dependence to develop. However, two genes can prevent alcoholism development; one of which provides total protection.

Studies in two rat lines bred as either alcohol drinkers or abstainers show that enzymes that metabolize ethanol and oxidize its metabolite acetaldehyde greatly influence the development of alcohol dependence. We describe the amino acid and gender differences responsible for these changes. Overall, any modification that leads to an arterial acetaldehyde burst generates an aversion to ethanol. We show that modifications of gene expression by gene therapy greatly blunt alcohol consumption in drinker rats previously rendered ethanol dependent by chronic administration of ethanol. In addition to the therapeutic implications, these studies shed light on the mechanism by which individuals who carry fast isoforms of alcohol dehydrogenase are protected against alcoholism.

The tools should form part of the inventory available to the neuroscientist to reduce harmful alcohol consumption.

## Nitric oxide and oxygen radical attack on GDP-dissociation inhibitor 2 (GDI-2) in spinal cord injury of the rat

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Apart from quantitative changes of protein levels, qualitative alterations in terms of post-translational modifications (PTMs) have been reported in spinal cord injury (SCI). The reported PTMs associated with SCI were induced by active oxygen species or nitric oxide. In SCI protein oxidation is determined by detection of protein carbonyl content by western blotting and immunohistochemistry. As to nitric oxide-induced protein modification (protein nitration), formation of 3-nitrotyrosine (3-NT) has been reported in SCI of the rat by using immunohistochemistry and HPLC of a protein hydrolysate.

Presently, no site-specific identification of oxidative modification and protein nitration were published to the best of our knowledge and this formed a rationale to carry out present study. Protein profiling in SCI of the rat revealed that, at 8 h following the traumatic lesion, levels of a signalling protein, GDP-dissociation inhibitor-2 protein (GDI-2) (synonym: Rab GDP dissociation inhibitor beta), were increased about 3-fold, and we therefore decided to use this potentially important signalling structure to study the presence of oxidation-and nitration-induced PTMs in SCI.

## Nanoparticles as drug delivery systems for the brain

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The blood–brain barrier (BBB) represents an insurmountable obstacle for the delivery of a large number of drugs to the central nervous system (CNS). One of the possibilities to overcome this barrier is drug delivery to the brain using nanoparticles. Drugs that have been transported into the brain and led to a pharmacological effect after intravenous injection using this carrier include the hexapeptide dalargin, the dipeptide kyotorphin, loperamide, tubocurarine, doxorubicin, and the NMDA receptor antagonists MRZ 2/576 and MRZ 2/596. In order to achieve a significant transport across the blood brain barrier the coating of the nanoparticles with certain surfactants or the adsorption or the covalent linkage of special apolipoproteins – A-I (apo A-I), B-100 (apo B-100), or E (apo E) – is required.

Intravenous injection of polysorbate 80-coated nanoparticles loaded with doxorubicin achieved very high brain levels while all the controls, including uncoated nanoparticles and doxorubicin solutions mixed with polysorbate, did not reach the analytical detection limit. Moreover, experiments with the extremely aggressive glioblastoma 101/8 transplanted intracranially showed a long term survival for 6 months of up to 40% of the rats after intravenous injection of the polysorbate 80-coated nanoparticle preparation. The surviving animals showed a total remission by histological investigation. Untreated controls died within 10–20 days, the animals in the doxorubicin control and uncoated doxorubicin nanoparticle groups died between 10 and 50 days.

The mechanism of the drug transport across the blood–brain barrier with the nanoparticles appears to be endocytotic uptake by the brain capillary endothelial cells followed either by release of the drugs in these cells and diffusion into the brain or by transcytosis. After injection of the

nanoparticles, apo A-I, apo B-100, or apo E adsorb on the particle surface and then may promote the interaction with the LDL receptor (apo E and apo B-100) or the scavenger receptor class B type I (SR-BI) (apo A-I) followed by endocytotic uptake. The nanoparticles thus would mimic the uptake of naturally occurring lipoprotein particles. This hypothesis was supported by the achievement of a antinociceptive effect with dalargin-loaded poly(butyl cyanoacrylate) nanoparticles with adsorbed apo E or with loperamide-loaded albumin nanoparticles with covalently bound apo A-100, apo B-100, or apo E.

## Genes and neuropsychiatric disorders

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Despite strong evidence from family and twin studies that psychiatric disorders are heritable, there has been limited progress in identifying the actual genes involved. Genetic heterogeneity, where clinically similar cases are affected for different genetic reasons, may be a major reason. The focus of this discussion will be on anxiety disorders, which as a group are the most common form of mental illness in the United States. Current understanding of underlying mechanisms of anxiety disorders rests on the fact that drugs with ''calming'' qualities are used for treatment, and include alcohol, barbiturates, opiates, and benzodiazepines. In addition, selective serotonin reuptake inhibitor antidepressants (SSRIs, such as paroxetine and citalopram) have been used in treating anxiety because they are not addictive. In animal models, SSRI treatment causes changes in multiple target genes, including the gene encoding brain-derived neurotrophic factor (BDNF). Results from our laboratory and others have supported a role for gene variation at the serotonin transporter gene (*SLC6A4*) and the BDNF gene (*BDNF*) in risk of different anxiety disorders. A better understanding of the genetic diathesis of anxiety will likely lead to improved treatment strategies for this disorder and other comorbidities such as major depression and the addictions.

## Characterization of blood stress proteome in breast cancer

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Breast cancer (BC) is worldwide one of the most frequent cancer diseases with fatal outcome. Following key features designate BC:

– etiology is unclear,
– high incidence of BC in the population (8–12 per 100 women worldwide),
– high probability of metastases already in early stages of the disease,

if any, early diagnosis of BC is extremely difficult yet.

Proteomics technologies allow us to address the complexity of tumorigenesis in BC at a level of global protein profiling. Profiling of BC specific proteins revealed extensive alterations in stress-regulated proteins followed by destabilization of the genome – the tumor promoting characteristic.

The goal of our project was to investigate expression patterns of proteins which have been shown to be a member of ''Minimal Stress Proteome'', i.e. essential proteins an activity of which is obligatory for each organism, in order to survive and to keep its genetic integrity under diverse stress conditions. This comparative analysis was performed in circulating leukocytes isolated from fresh blood samples of breast cancer patients versus controls.

Protein expression patterns were investigated by two-dimensional polyacrylamide gel electrophoresis followed by protein spot identification using tandem mass spectrometry analysis. Specific expression levels of selected proteins were quantified by Western-blotting. Comparative DNA damage levels were evaluated using ''Comet Assay''-analysis.

Extensive alterations were demonstrated in expression patterns of heat-shock and detoxification proteins, key-molecules for DNA-integrity/repair/synthesis/transcription. Investigated proteins were products of both chromosomal and mitochondrial DNA.

**Conclusion:** Blood Stress Proteome might provide novel targets for early diagnosis and therapy in BC.

## Parkinson's disease: proteome and effect of neuroprotective drugs

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The current concept regarding Parkinson's disease (PD) and other neurodegenerative disorders, considers them diseases of multiple etiological nature where several mechanisms are implicated in a cascade/s of events involving many biochemical and signaling pathways. An unresolved question, however, is to determine which of these factors constitute the primary event, the sequence in which they act and where is the point of convergence or the final pathway by which the dopaminergic neurons of the substantia nigra pars compacta (SNpc) dye. It is at this point where high throughput transcriptomics and proteomics tools come to the help of neuroscientists to assist in dissecting signaling pathways and find networks that may shed light on the pathobiology of PD. Our group was the first to conduct a large scale gene expression profiling study of post-mortem SNpc of parkinsonian brains and compared them to matched controls. This study revealed a significant dysregulation of genes from biological processes linked to previously established neurodegenerative mechanisms both in sporadic and hereditary PD, such as protein handling and aggregation, mitochondrial dysfunction, heat shock proteins and oxidative stress, vesicle trafficking and synaptic transmission, cell cycle, dopamine metabolism and cell adhesion/cytoskeleton maintenance. Recently, proteomic studies have confirmed to a great extent these results, suggesting these pathways may play significant roles in the initiation of PD. The signatures unveiled by these approaches could provide crucial information on diagnosis and development of surrogate markers for PD, reliable candidate as predictive early biomarkers and to future development of CNS ''magic bullets'' targeted drugs.

## Noradrenergic mechanisms in neurodegenerative disorders: evidence from in vivo animal studies

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A deficiency in the noradrenergic system of the brain that originates from cells in the locus coeruleus, is theorized to play a critical role in the

progression of a family of neurodegenerative disorders that includes Parkinson's and Alzheimer's disease. Consideration is given to evidence that several neurodegenerative diseases and syndromes share common elements, including profound cell loss in the locus coeruleus, and may in fact be different manifestations of a common pathophysiological process. Findings in animal models of Parkinson's disease indicate that the modification of locus coeruleus-noradrenergic activity alters electrophysiological, neurochemical and behavioral indices of neurotransmission in the nigrostriatal dopaminergic system, and influences the response of this system to experimental lesions. In models related to Alzheimer's disease, noradrenergic mechanisms appear to play important roles in modulating the activity of the basalocortical cholinergic system and its response to injury, in modifying cognitive functions including memory and attention, and in altering anatomical, neurochemical and behavioral phenotypes in transgenic mouse models of cerebral beta-amyloidosis. Mechanisms by which noradrenaline may protect or promote recovery from neural damage are reviewed, including effects on neuroplasticity, neurotrophic factors, neurogenesis, inflammation, cellular energy metabolism and excitotoxicity, and oxidative stress. Based on evidence for facilitatory effects on transmitter release, motor function, memory, neuroprotection and recovery of function after brain injury, a rationale for the potential of noradrenergic-based approaches, specifically alpha2-adrenoceptor antagonists, in the treatment of central neurodegenerative diseases will be presented.

## The sharp-2 transcription factor: a new candidate for protecting the brain against ischemia

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Brain ischemia results in dysfunction and overt clinical neurological signs. Glutamate, the neurotransmitter required for normal physiological excitation, is also involved in the pathophysiology of ischemia. Although this neuropathological process can be mediated by any of the glutamate receptors, the *N*-methyl-D-aspartate (NMDA) glutamate receptor subtype plays a major role. In addition, NMDA receptors mediate adaptive responses important for synaptic plasticity during development and in the adult. However, the molecular mechanisms by which NMDA receptors mediate these opposing effects are poorly understood. Brain-derived neurotrophic factor (BDNF) is a member of the family of neurotrophins that has been shown to reduce infarct size. Promoter IV of the *bdnf* gene is the major promoter that mediates activity-dependent BDNF transcription. However, BDNF exon IV mRNA levels remain low in ischemia despite increases in neuronal activity. Here we show that Sharp-2, a member of the basic helix-loop-helix family of transcription factors, represses promoter IV-dependent BDNF gene transcription. Low level NMDA receptor activation, which establishes a neuroprotective state in cultured neurons, increased binding of CREB and NF-κB to the promoter while decreasing Sharp-2 occupancy, consistent with idea that overcoming Sharp-2-mediated repression of promoter IV is necessary for transcriptional activation. In a Sharp-2 knock-out mouse model, basal and activity-dependent exon 4-specific *bdnf* mRNA levels are enhanced significantly. Taken together these findings suggest that Sharp-2 may be a new target for drug development to protect the brain against ischemia.

## What we can learn from a genome scale model of unicellular metabolism?

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Systems biology combines experimental and computational approaches to understand the structure and dynamics of biology systems. Dynamic models have-mainly due to the lack of appropriate experimental data-been restricted to relatively small intracellular networks of interacting components, mostly associated with a particular biological function. Genome scale stoichiometry-based models of metabolism have however been successfully used to analyze properties of metabolic networks including growth phenotype prediction of organisms. We show that such models, given clearly defined growth conditions, can also be used to analyze in detail specific aspects of metabolism and to reconstruct the genome scale fluxome. *Halobacterium salinarum* is an extreme halophile that has complex nutritional requirements based on amino acids. After manually reconstructing of the organism's metabolic network, we went beyond the normal static flux balance analysis and report genome scale dynamic growth simulation, which show remarkable agreement with observed physiological behaviour. The predictions can serve as approximations of the in vivo fluxome while genome scale measurements of fluxes are still infeasible.

## Effect of poly(ADP-ribose)polymerase-1 (PARP-1) inhibition on metabolic insults occurring at birth: promises and limitations

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Interruption of oxygen availability and re-oxygenation at birth implies a severe metabolic insult, affecting the development of the central nervous system (CNS), increasing its vulnerability to challenges occurring at adult stages. It has been reported that perinatal asphyxia (PA) produces regionally specific neuronal decrease and neurite atrophy in basal ganglia, and hippocampus. In hippocampus, a concomitant increase of neurogenesis and neurite hypertrophy has also been observed. The potential neuroprotection of nicotinamide, a non-selective inhibitor of poly(ADP-ribose) polymerase (PARP-1), has been investigated), based on the idea that PA can trigger the overactivation of PARP-1, leading to $NAD^+$ exhaustion and energy crisis, and to a caspase-independent apoptosis. In agreement, it has been found that nicotinamide, administered 1–24 h after the insult (0.8 mmol/kg, i.p., 24, 48 and 72 h after birth) can prevent some of the effects of PA, mainly in neostriatum, preventing a PA-induced decrease of the number of nNOS cells, and neurite atrophy. The effect of PA on neurite development has further been studied by lentivirus-mediated gene transfection of green fluorescence protein (GEP), monitored in vitro until the cultures are further treated for histochemistry.

## Effects of α-ketoglutarate on neutrophil (PMN) free amino and α-keto acid homeostasis or immunocompetence

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The objective of this study was to determine the dose as well as duration of exposure-dependent effects of α-ketoglutarate (KG) on polymorphonuclear neutrophil (PMN) free amino and α-keto acid profiles and, in a parallel study, on PMN immune functions. PMN were incubated with different KG concentrations (5, 10, 20, 50 and 100 μM) for 120 min which corresponded to 1/2-, 1-, 2-, 5- and 10-fold the clinically achieved plasma concentrations as well as with 100 μM KG for 10, 60 or 120 min to examine if there is a critical duration of exposure necessary to produce any significant effects.

KG supplementation to whole blood significantly increased intragranulocyte KG as well as pyruvate levels but also the content of important amino acids (glutamine, glutamate, aspartate, asparagine, ornithine, arginine, alanine, serine, glycine) in a dose and duration of exposure-dependent manner, a fact that indicated an increased uptake of KG by PMN and further utilization into derivatives. Enzymes required for this (e.g. tricarboxylic acid cycle, glutaminase, aspartate aminotransferase, alanine aminotransferase, etc.) are certainly present in PMN cells as others have shown. For PMN, the demand-controlled intracellular conversion of KG to the intragranulocytic carbon and nitrogen stores glutamine and glutamate is of special metabolic relevance because the glutamate arising from this reactions, especially, plays a key metabolic role since it can be very rapidly transformed into other essential cell substrates as a so-called ''intracellular turntable'' for amino nitrogen metabolism. Moreover, KG is particularly important for the metabolization by the enzymes of the TCA cycle (i.e. KG can be converted to pyruvate, the starting point for the „de novo synthesis" of glucose from KG) effects.

But the metabolization of KG by the TCA cycle does not just supply intracellular carbon and nitrogen precursors for the above-mentioned metabolic pathways, since it also provides NADPH. In PMN cells, NADPH plays a special and essential role because of its further metabolism by the membranous NADPH oxidase. The activation of this enzyme complex provides superoxide anions and in so doing forms an essential component of the granulocytic immune defense. An increase in KG metabolism due to an inflammatory event does not just lead to essential metabolic precursors, but much more significantly it increases the participation of the immediately available intragranulocytic redox systems. For this reason it is not surprising that the increase in important intragranulocytic amino and α-keto acids upon application of KG is also associated with an increase in extragranulocytically produced superoxide anion and hydrogen peroxide as well as a clear increase in extracellular myeloperoxidase activity.

## Effects of pyruvate on neutrophil (PMN) free amino and α-keto acid homeostasis or immunocompetence

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The objective of this study was to determine the dose as well as duration of exposure-dependent effects of pyruvate (PYR) on polymorphonuclear neutrophil (PMN) free amino and α-keto acid profiles and, in a parallel study, on PMN immune functions. PMN were incubated with different PYR concentrations (0.05, 0.1, 0.2, 0.5 and 1 mM) for 120 min which corresponded to 1/2-, 1-, 2-, 5- and 10-fold the clinically achieved plasma concentrations as well as with 1 mM PYR for 10, 60 or 120 min to examine if there is a critical duration of exposure necessary to produce any significant effects.

Exogenous PYR significantly increased PMN intracellular PYR content in a dose as well as duration of exposure-dependent manner, indicating an increased uptake of PYR by PMN from whole blood. When intracellular PYR increases, the concentrations of important amino acids (alanine, glutamate, aspartate, glutamine, asparagine, ornithine, arginine, serine, glycine) and α-ketoglutarate were significantly elevated, which favour the hypothesis that increases in PMN free PYR concentrations are followed by PYR conversion into amino and α-keto acid derivatives.

Moreover, the findings of high intragranulocytic PYR levels [$\approx 6 \times 10^{-17}$ mol per PMN cell ($\approx 150$ μmol/l)] may also represent a metabolic correlate for the underlying (patho)physiological functions and requirements of neutrophils as well as the enzymatic constellation required for a direct or indirect PYR metabolisation or synthesis is certainly present in neutrophils. Alanine aminotransferase for example transfers the amino group of glutamate to PYR resulting in the production of α-ketoglutarate (and alanine) which can enter the tricarboxylic acid cycle or be converted into oxalacetate (and glutamate) by aspartate aminotransferase. Additionally, PYR is also regarded as a precursor for the de novo synthesis of glucose from α-ketoglutarate (i.e. via fructose-1,6-bisphosphatase) or oxalacetate (i.e. via PYR-carboxylase) as well as for intragranulocytic serine and glycine synthesis. Especially worthy of mention here is the condensation to the tripeptide glutathione (γ-Glu-Cys-Gly) which protect PMN cells against attack by reactive oxygen species such as the highly membrane permeable $H_2O_2$. Moreover, once metabolized by the TCA cycle (i.e. via acetyl-CoA), PYR not only becomes indirectly available as a substrate for the enzymes of other cell cycles (i.e. urea cycle) but also increases NADPH.

Immunofunctionally, NADPH has an essential role for neutrophils, because of its further metabolisation by membranous NADPH oxidase. The activation of this enzyme complex supplies superoxide anions ($OH^-$) and in this way forms an essential component of granulocytic immune defence. An increase in PYR metabolisation during an inflammatory event therefore leads not only to essential metabolic precursors, but also improve PMN immunocompetence by increases in the relative activity of immediately available intragranulocytic redox systems (i.e. $OH^-$, $H_2O_2$) or enzymes (i.e. myeloperoxidase).

## Nanoparticles induced neurotoxicity and neurorepair in hyperthermia. Influence of a new antioxidant compound H-290/51

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Previous reports from our laboratory show that rats received mild doses of engineered nanoparticles from metals, e.g., Ag, Cu or Al (50–60 nm, 50 mg/kg, i.p. for 1 week) exhibit mild cognitive dysfunction, blood–brain barrier (BBB) disruption and neurotoxicity. However, when these nanoparticles treated rats were exposed to additional heat stress at 38 °C for 4 h, their cognitive impairment, BBB dysfunction and brain damage was exacerbated, compared to untreated heat-stressed animals. These observations suggest that hyperthermia could enhance brain pathology and cognitive dysfunction in nanoparticle treated animals.

There are reasons to believe that nanoparticles enhance oxidative stress in the CNS. Thus, it is likely that increased lipid peroxidation and/over-production of hydroxyl radicals in nanoparticles treated hyperthermic rats is responsible for enhanced neurotoxicity. Keeping these views in consideration, we pretreated rats (after 1 week administration of nanoparticles) with a new antioxidant compound H-290/51 (an inhibitor of lipid peroxidation, 50 mg/kg, p.o.) before subjecting them to hyperthermia. One group of nanoparticles treated rat received H-290/51 and kept at room temperature for comparison.

Our results show that pretreatment with H-290/51 significantly attenuated heat stress induced cognitive dysfunction, BBB impairment and neurotoxicity in nanoparticle treated rats. However, no significant diminution of nanoparticles induced BBB breakdown, cognitive impairment or neurotoxicity was observed in H-290/51 treated rats kept at room temperature.

These observations suggest that nanoparticles aggravate oxidative stress following hyperthermia that could be primary contributing factor in exacerbating neurotoxicity and cognitive dysfunction. Thus, use of antioxidants may attenuate nanoparticle-induced neurotoxicity. Taken together, our observations are the first to suggest that nanoparticles induced neurotoxicity is somehow mediated through oxidative stress-related mechanism.

## Peculiar vulnerability of rat nigral dopamine neurons to the combination of antioxidant defense decline and excitotoxicity triggered by glutamate transporter dysfunction. New insights into the pathophysiology of Parkinson's disease

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Glutamate transporters clear extracellular glutamate and provide substrates intracellularly for glutathione synthesis. Because nigral glutathione depletion is the earliest biochemical event in Parkinson's disease (PD) pathogenesis and nigral dopaminergic (DA) neurons express the neuronal glutamate transporter EAAC1/EAAT3, we here investigated the consequences of glutamate transporter dysfunction on DA neuron viability both in vitro and in vivo. In rat embryonic mesencephalic cultures, L-trans-pyrrolidine-2,4-dicarboxylate (PDC), a substrate inhibitor of glutamate transport, triggers early and sustained increase in extracellular glutamate levels and delayed glutathione depletion, ROS production, and death of both astrocytes and neurons. DA neurons mostly express EAAC1 and are more vulnerable to PDC treatment than non-DA neurons. PDC-induced death of DA neurons shows the following selective features: it combines oxidative and NMDA receptor-mediated excitotoxic processes, is prevented by cysteine, a substrate of EAAC1 and the limiting precursor for glutathione synthesis, and is independent of astrocyte demise. Moreover, PDC renders these neurons vulnerable to non-toxic NMDA treatment. Altogether, these data suggest that PDC is directly toxic for DA neurons by decreasing the availability of glutathione precursors and lowering the threshold for glutamate toxicity through NMDA receptors. Finally, we show in adult rat that acute intranigral injection of PDC induces regionally massive and dose-dependent loss of nigral DA neurons, and α-synuclein aggregates formation. These data suggest for the first time that glutamate transporter function is primarily directed toward antioxidant defenses in DA neurons and that their dysfunction is a candidate mechanism for the selective death of DA neurons such as occurring in PD.

## Selenium content and glutathione peroxidase activity in epileptic patients

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Cognitive impairment is often associated to epileptic syndromes, and at times neurodegeneration of important cognitive regions, such as the hippocampus, can be demonstrated. The neurodegenerative process may imply the formation of free radicals in amounts exceeding the physiological mechanisms devoted to their elimination. One of them, glutathione peroxidase (GPX), requires selenium for its activity. We investigated whether GPX specific activity in the serum of epileptic patients may change according to both, their type of epilepsy and the presence of neurodegeneration. Epileptic patients were divided in 2 reference groups namely Focal Epilepsy and Generalized Epilepsy. Patients with Focal Epilepsy were further subdivided in two groups of patients, with and without Mesial Sclerosis, and patients in the Generalized Epilepsy group were subdivided in three groups, namely Juvenile Myoclonic Epilepsy, Progressive Myoclonic Epilepsy and Lennox-Gastaut syndrome. Selenium associated with GPX, protein P and albumin was measured in the serum by inductively coupled plasma mass spectrometry after affinity chromatography. Serum GPX activity was also determined. A statistically significant 135% increase of GPX specific activity was found in the Focal Epilepsy group, for patients without Mesial Sclerosis as compared with both, control subjects and patients with Mesial Sclerosis. A similar GPX activity increase was observed for the patients of the Lennox-Gastaut group. No significant differences were observed for the levels of either Se-protein P or Se-albumin. No differences in GPX specific activity, Se-protein P or Se-albumin were observed in relation with the pharmacological treatment. Further studies will be important to clarify the clinical significance of our results.

## The anticonvulsant drug Levetiracetam does not reduce the survival of cultured neurons

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Levetiracetam is a recent antiepileptic drugs (AED), with minimal cognitive side effects, whose mechanism of action is still largely unknown. We have used cultured cerebellar neurons to investigate whether levetiracetam may affect glutamatergic neurotransmission. Upon depolarizing stimuli such as veratridine (5 μM), cerebellar neurons undergo first an early phase of swelling of the cell body between 10 and 30 min, followed by a complete degeneration after 24 h. Both phases can be prevented by the presence of several AEDs such as carbamazepine. The early phase of neurotoxicity can be prevented by NMDA receptor antagonists. Pretreatment of neurons with increasing concentrations of levetiracetam did not ameliorate either early or late phases of toxicity. Furthermore, levetiracetam failed to reduce neurodegeneration 24 h after the direct exposure to either, glutamate, a toxic stimulus involving NMDA receptors, or domoate, an agonist of both kainate and AMPA receptors. Furthermore, intracellular concentrations of $Ca^{2+}$ following L-type VSCC stimulation with the ago-

nist BAY-K 8644 were not reduced by levetiracetam. Because neuronal development and survival in culture are very sensitive to the modulation of both L-type VSCC and glutamatergic neurotransmision, we then studied whether prolonged exposure to levetiracetam could affect those two parameters. Differently from carbamazepine, levetiracetam did not alter neuronal survival and macroscopical development, such as the length of neurite network and neuronal movements in culture. Thus, we measured the phosphorylation of two protein kinases that are known to be involved in trophic effects: ERK and p38. The results will be presented and discussed in comparison with the results obtained using other AEDs.

## Nanoparticles and the brain: cause for concern?

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The propensity of NP to translocate across cells, along neuronal pathways and distribute to lymph and blood circulation makes them uniquely suitable for therapeutic and diagnostic uses. Thus, one aspect of nanomedicine is the targeting of specific organs by using NP. However, this very mechanism exposes target organs to potential adverse effects (oxidative stress) due to subcellular distribution in sensitive target organs such as the central nervous system (CNS). In addition, most recent data indicate a potential of nanoparticles to impact protein folding and the possibility of contributing to neurodegenerative disorders. Translocation pathways in the respiratory tract for inhaled insoluble nano-sized particles (<100 nm) along sensory neurons will be discussed, with special emphasis on the upper respiratory tract and the potential of delivery of nanomaterials to the CNS. The neuronal pathway is contrasted with CNS delivery of blood–borne substances, and examples of the importance of particle size and solubility will be presented. Implications of species differences in respiratory tract anatomy, breathing pattern and brain anatomy for extrapolation to humans of NP effects observed in rodents need to be considered. Although there are anecdotal data indicating a causal relationship between long-term ultrafine particle exposures in ambient air (e.g., traffic related), at the workplace (e.g., metal fumes), and neurotoxic effects in humans, more studies, experimental and toxicological, are needed to test the hypothesis that inhaled nano-sized particles cause neurodegenerative effects.

## Association of *GRIK4* with outcome of antidepressant treatment in the STAR*D cohort

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Association tests were carried out in order to test for possible correlation between citalopram treatment response and 768 markers genotyped in 1816 eligible patients from the STAR*D cohort, divided into discovery and replication samples to control for multiple testing. In addition to the previously identified marker in the serotonin receptor gene *HTR2A*, our analysis revealed a new marker (*rs1954787*) in *GRIK4*, which met the prior *p*-value thresholds in both the discovery and replication samples. *GRIK4* codes for the kainic-acid type ionotropic glutamate receptor KA1. The effect size of the *GRIK4* marker association alone was modest, but homozygote carriers of the treatment-response-associated marker alleles of both *GRIK4* and *HTR2A* were 23% less likely to experience non-response to treatment as participants carrying none of these alleles. Results presented herein demonstrate that genetic variation in a kainic-acid type glutamate receptor is significantly associated with response to the antidepressant citalopram. This finding suggests an important role for the glutamate system in modulating response to SSRIs such as citalopram.

## Synthetic polymers that mimic the RGD ligands as stationary phases for their selective separation

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Molecularly Imprinted polymers are the state-of-the art in production of man-made 'smart' materials that selectively recognize a target molecule, the template. They are produced by the 'frozen' of monomers around the template, which are initially pre-organized into a solution, by polymerization. After template removal the remaining into polymer cavity are both in shape and chemical functionality complementary to the template, so selectively could recognize it. The monomers methacrylic acid (MAA, functional monomer) and ethylenoglycol dimethacrylate (EDMA, cross-linker) are used extensively in such process, considering as the most appropriate. In present study were used as template the peptide the Arg-Gly-Asp with two common functional monomers, MAA and acrylamide, and as cross-linker the EDMA. As it is already well known the RGD peptide is the major integrin binding site present in a variety of integrin ligands. The molar ratio of template-functional monomer-cross-linker was kept constant for both monomers and their effect in recognition properties of produced polymers was reported. The RGD rebinding results are indicating that in case of MAA the produced polymers have smaller dissociation constant $K_D$ values in a Langmuir monosite model isotherm meaning better recognition, but the values of the percentage of theoretical maximum binding sites (% $B_{max}$) are lower than the case of acrylamide. These results suggest that the acrylamide, despite the lower somewhat $K_D$, are the monomer of choice for the production of such polymers for RGD recognition leading to higher imprinting efficiency.

## The role of different ions in molecular recognition process of C-terminal cholecystokinin pentapeptide in polymeric receptors

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The production of molecularly imprinted polymers (MIPs) for the recognition of C-terminal cholecystokinin pentapeptide (CCK-5P) in

the presence or absence of metal ions is reported. Metal ions play an important role in recognition process allowing the monomers to assemble around the template in protic solvents by ligand formation. The MIPs were prepared under the same molar ratio of template to monomers (acrylamide, N,N′-methylene bisacrylamide) in the presence or absence of nitriloacetic acid nickel complex (Ni-NTA). Subsequently the produced Ni-NTA polymers were washed with EDTA (pH 8) to remove the $Ni^{2+}$ and incubated in aqueous solution of $MgSO_4$, $FeSO_4$, $ZnSO_4$, $CoSO_4$ or $CuSO_4$ respectively at initial concentration 20 mM in order to examine the contribution of these ions to recognition process. From the rebinding data, a Langmuir adsorption isotherm was obtained for each metal ion used and the subsequent Scatchard plot analysis showed that the above ions exhibited different dissociation constant values, $K_D$. It was found that polymers containing the metal ion complex with the order Fe-NTA, Ni-NTA and Cu-NTA gave lower dissociation constant values and thus exhibit the stronger guest binding activity. The percentage of theoretical maximum binding sites $B_{max}$ is almost constant for these ions, meaning that the ion-template coordination is responsible for their binding strength and not for the number of active sites.

## Excitatory amino acid antagonists and rheumatoid arthritis

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**Objectives:** Rheumatoid arthritis (RA) is a systemic, chronic, autoimmune, inflammatory disease causing progressive joint destruction, deformity and disability. It affects about 1% of the population and is most common among those aged 40–70 years. The etiology and pathogenesis of the RA is largely unknown. Recently, evidence is accumulating that the synovial fibroblasts are important in all aspects of the etiopathogenesis of RA and that these cells are dominant players in rheumatoid synovium. It has been found that they have aggressive, proliferative and cartilage-attacking properties, and are directly responsible for cartilage destruction. Recently, it has been demonstrated that glutamate antagonists inhibit proliferation of rabbit fibroblasts and suppress cancer growth in vitro and in vivo.

Therefore, the aim of this study was to assess the level of glutamate antagonist, kynurenic acid (KYNA), in synovial fluid of patients with active arthropathies and to determine effect of glutamate antagonists on the viability of human rheumatoid arthritis fibroblast-like synoviocytes and rabbit synoviocytes in vitro.

**Methods:** Samples of synovial fluid were collected from the knee joints of patients with RA, spondyloarthritis (SpA) and osteoarthritis (OA). KYNA level was analyzed by HPLC. For primary cultures synovial tissue was obtained from the knee of patients which fulfilled the American College of Rheumatology 1987 criteria for diagnosis of RA at the time of synovectomy during total joint replacement as a standard clinical procedure. Rabbit synoviocytes cell line HIG-82 was obtained from American Type Culture Collection, Menassas, VA, USA. Cell viability was assessed by means of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.

**Results:** As compared to OA patients the significantly lower level of KYNA was found in synovial fluid of RA and SpA patients. KYNA level in serum did not differ in all three studied groups. Glutamate antagonists, KYNA and riluzole reduced viability of human rheumatoid arthritis fibroblast-like synoviocytes in primary cell culture and rabbit synoviocytes HIG-82. Similarly, viability of these cells was reduced by antirheumatic drugs, diclofenac and sulfasalazine.

**Conclusion:** Our data suggest that glutamatergic mechanisms may contribute to peripheral inflammatory processes and glutamatergic antagonists may be beneficial in the treatment of RA.

## Tryptophan metabolites in canine epilepsy

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L-Kynurenine is a major intermediate in the oxidative metabolism of L-tryptophan leading to niacin formation. Peripheral as well as central tissues are excessively involved in kynurenine degradation and alteration of kynurenine metabolites level could be found under different pathological conditions. Kynurenines are characterized by significant different activities. While kynurenic acid is known as an antagonist at the excitatory amino acids receptors and at $7\alpha$ nicotine cholinergic receptors triggering in part neuroprotective and anticonvulsive activities, 3-hydroxykynurenine is a cytotoxic agent and free radical generator, 3-hydroxyanthranilic acid is carcinogen, quinolinic acid is a potent neurotoxin and the function of anthranilic acid is still unclear. The aim of this study was the measurement of tryptophan metabolites e.g. tryptophan, L-kynurenine, anthranilic acid, kynurenic acid, 3-hydroxykynurenine and 3-hydroxyantharnilic acid in the serum of epileptic dogs using high performance liquid chromatography (HPLC) system coupled with fluorescence or UV detector. The detection limit for kynurenic acid was in low fmol range and for tryptophan, L-kynurenine, anthranilic acid, 3-hydroxykynurenine and 3-hydroxyanthranilic acid was in low nmol range. In the serum of corresponding controls we found $103.98 \pm 45.83$ pmol/µl of tryptophan and $6.46 \pm 1.89$ pmol/µl of L-kynurenine. In the serum of dogs with idiopathic epilepsy tryptophan was moderately reduced, while in the serum of dogs with secondary epilepsy L-kynurenine was moderately increased comparing to corresponding controls. Obtained preliminary data would suggest different kynurenine metabolisms in the periphery (serum) depending on the type and intensity of epileptic manifestation.

## D-Aspartate induces secretory activity in rat Harderian gland through ERK pathway

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Harderian gland (HG) is an orbital gland found in the majority of land vertebrates possessing a nictitating membrane. Although its main role is to lubricate the eye, other functions have been suggested,

including roles in thermoregulation or photoprotection, as part of the retinal-pineal axis, and as source of either pheromones or growth factors. High concentrations of D-aspartate (D-Asp) are found in the HGs of various vertebrate species. Recent studies suggest a role of D-Asp in the glandular secretion in some species of amphibians and reptilians. The present study was designed to investigate the effects of D-Asp i.p. injection (2 μmol/g b.w.) on rat HG. The results revealed that HG takes up and accumulates D-Asp. At 2 h after D-Asp injection a strong positivity to Sudan Black (reaction for lipid detection) was observed in glandular acini. The increased lipid secretion was accompanied by an increased expression of mitochondrial uncoupling protein-3 (UCP3), a member of the family of mitochondrial protein carriers involved in the export of fatty acid anions and/or fatty acid peroxides, to counteract their damaging effect when they accumulate in the mitochondrial matrix. The activation of extracellular regulated kinase (ERK), a MAP kinase that occupies a focal point in signal transduction, observed already at 30 min after D-Asp administration, suggests that this amino acid could activate the lipid secretion through ERK pathway. The kinase pathway could be triggered by linkage of the D-Asp at the NMDA receptor. The presence of the mRNA of the NMDA receptor subunits (NR1, NR2A, NR2B, NR2D) in the rat HG supports this hypothesis.

## Influence of nanoparticles on blood–brain and spinal cord barriers in relation to neurotoxicity and neurorepair in CNS injuries

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Nanoparticles are small sized (1–100 nm) particles having novel properties that can be highly desirable for applications within the medical and environmental fields. Most of these nanoparticles are formed from transition metals, silver, copper, aluminum, silicon, carbon and metal oxides. Due to their size, these nanoparticles can either easily cross the blood brain barrier (BBB) and/or produce damage to the barrier integrity by altering endothelial cell membrane permeability. In spite of our increased understanding on the BBB function, influence of nano particles on blood–brain and spinal cord barriers is still unknown. It is quite likely that nanoparticles when reach to the CNS compartments may induce profound cellular and molecular stress leading to disruption of the BBB either directly or through a cascade of secondary cellular and molecular events.

On the other hand, drug delivery to the spinal cord or brain following injury is still a serious problem because of the presence of blood–brain and blood-spinal cord barriers. Recently, drugs attached to nanowires are considered as a suitable method for enhanced delivery within the central nervous system (CNS). However, most of these investigations are carried out in vitro models. Thus, these novel aspects of therapeutic enhancement of drugs or compounds tagged with nanoparticles require additional investigation using in vivo models.

Interestingly, few reports suggest that nanoparticles from metals will cause membrane damage when they enter into the brain fluid environment. However, it is still unclear whether inclusion of nanoparticles in the body fluid environment will influence biological response of the organisms to stress caused by external environments, such as extreme heat or cold. Thus, the role of nanoparticles on the function of central nervous system as ''friend'' or ''foe'' requires detailed investigations using cellular and molecular approaches. Our laboratory is engaged to find out the role of nanoparticles derived from various metals on the structure and function of the blood–brain and blood-spinal cord barrier in relation to neurotoxicity and/or or neurorepair. Our investigation is focused on the following 3 aspects:

*(a) Nanoparticles influence Blood–brain barrier function*

Intravenous (30 mg/kg), intraperitoneal (50 mg/kg) or intracerebral (20 μg in 10 μl) administration of either Silver, Copper or Aluminum nanoparticles (size 50 to 60 nm) influenced the BBB function to Evans blue albumin in rats and mice in a highly selective and specific manner. The leakage of Evans blue dye was observed largely in the ventral surface of the brain and in the proximal frontal cortex. The dorsal surfaces of cerebellum and the thoracic spinal cord show mild to moderate Evans blue staining. The ventral surface of the spinal cord exhibited mild largely in the thoracic and lumbar segments of the spinal cord. Intraperitoneal administration of nanoparticles were least influenced the visual disruption of the BBB to the Evans blue dye. The effect of aluminum nanoparticles on the BBB function was much less intense compared to the silver and copper nanoparticles. These observations suggest that nanoparticles are able to alter BBB function in vivo.

*(b) Nanoparticles enhances neuroprotection in CNS injury*

We used 3 different compounds named AP-173, AP-713 and AP-364 (Acure Pharma, Sweden) having potential neuroprotective efficacy in CNS injury were identified and tagged with $TiO_2$-based nanowires using standard procedure. Normal compounds were used for comparison. Spinal cord injury (SCI) was produced by making a longitudinal incision into the right dorsal horn of the T10-11 segments under equithesin anaesthesia. In separate group of rats AP-173, AP-713 and AP-364 alone or tagged with nanowires were applied topically within 5 to 10 min after SCI and the rats were allowed to survive 5 h. In these animals, behavioral outcome, blood-spinal cord barrier (BSCB) permeability, edema formation and cell injury were examined at 5 h. A focal SCI resulted in severe motor paralysis, widespread disruption of the BSCB to Evans blue albumin (EBA), $^{[131]}$Iodine or lanthanum tracers and exhibited profound edema formation. Cell or tissue destruction was present around the lesion site extending up to T8 and T12 segments. Topical application of normal compounds AP-173, AP-713 or AP-364 in high quantity (10 μg in 20 μl) markedly attenuated behavioral dysfunction that are prominent around 2–3 h after SCI. BSCB disruption, edema formation and nerve cell, glial cell and axonal injuries are less pronounced in drug treated injured animals. These beneficial effects are most marked in animals that received SCI2 treatment compared to AP-173 or AP-364. Interestingly, when these compounds were administered tagged with nanowires, their beneficial effects on functional recovery and spinal cord pathology were further enhanced. Thus, AP-713 tagged with nanowires was able to attenuate functional disturbances up to 5 h after trauma. Spinal cord cell and tissue destruction was minimal in nanowire-tagged AP-713 treated group compared to AP-713 treatment alone. In nanowire-tagged group also the most larked beneficial effects were seen in AP-713 treatment compared to other compounds. Topical administration of nanowires alone did not influence spinal cord pathology or motor function after SCI. Taken together, our results probably for the first time indicate that the drug-delivery and their therapeutic efficacy is enhanced when the compounds are administered with nanowires.

*(c) Nanoparticles alter heat stress induced brain pathology*

Research carried out in our laboratory suggests that subjection of rats to whole body hyperthermia (WBH) pretreated with nanoparticles derived from Cu, Ag and Al (≈50 to 60 nm) resulted in worsen outcome in terms of brain pathology compared to naive rats. Thus, the nanoparticle treated rats exhibited enhanced cognitive dysfunction, blood–brain brain barrier (BBB) disruption, edema formation and brain pathology. These novel observations suggest that nanoparticles react differently within the body during heat stress and lead to enhance neurotoxicity.

*(d) Nanoparticles and drugs of abuse*

New results in our laboratory show that rats treated with nanoparticles are more susceptible to psychostimulants induced neurotoxicity and behavioural dysfunction. Thus, development of morphine tolerance and dependence in nanoparticles treated animals was seen much earlier compared to normal groups. In these nanoparticle treated rats, spontaneous withdrawal symptoms were also most prominent on day 1 compared to normal rats. Breakdown of the BBB and BSCB were most marked on the day 1 in nanoparticles treated rats subjected to morphine withdrawal compared to normal animals in which BBB dysfunction was highest on the day 2 and 3 following morphine withdrawal. These observations probably for the first time show that nanoparticles influence the psychostimulants dependence and withdrawal.

*General conclusion:* Our results suggest that nanoparticles are able to influence the brain function at the cellular mad molecular levels. Using right kinds of strategies the power of nanoparticles can be used to induce neuroprotection and enhanced drug delivery to achieve neuroprotection. On the other hand, nanoparticles depending on their physico-chemical properties may induce neurotoxicity as well, probably via disrupting the blood–brain barrier function.

## Imaging vascular and neural retinal pathology using functionalized quantum dots

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Degenerative retinal disorders such as Age Related Macular Degeneration (AMD) are the leading cause of blindness in the western world. AMD in particular is characterized by the progressive disorganization of the retinal pigment epithelium-neural retinal interface, leading to the degeneration of photoreceptor neurons. Although the pathology of AMD is not completely understood, it includes the formation of pathological blood vessels from the underlying choroid (choroidal neovascularization), the disruption of Bruch's membrane, cellular disorganization, reactive gliosis, and apoptotic cell death. Although a genetic basis for AMD has recently been identified, neuroprotective strategies remain a principle clinical approach. However, an incomplete understanding of the molecular and cellular events and processes responsible for this highly multidimensional disease makes it difficult to propose and systematically investigate physiologically grounded treatment approaches. In an attempt to address this, we have optimized antibody functionalized quantum dot imaging protocols specifically for central nervous system neurons and glial cells which allow high resolution imaging of cellular details and anatomy. Semiconductor quantum dot nanocrystals are fluorescently stable materials that have wide absorption spectra, so that they can be excited with a wide range of wavelengths, especially towards the ultraviolet, but have very narrow emission spectra. This allows multiplexing quantum dots of different colors. Although our own work has shown that one must be careful with the conjugation methods used to couple antibodies to quantum dots in order to avoid false labeling, in general these properties make them extremely robust tools for specific labeling of biological preparations. Different conjugation strategies for quantum dots specifically tested with neurons and glia will be discussed, including how to derive an estimate of the number of bound functional antibodies (i.e. antibodies that are sterically available for target protein binding), and its application within the context of neurocellular fluorescence in vitro and in situ imaging of dissociated central nervous system cells and the retina, respectively. The goal is to develop novel nanotechnology research tools that will allow the investigation of the healthy and diseased nervous system in novel ways.

## Strain-dependent effects of SGS742 in the mouse

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SGS742 has been reported to increase spatial memory in rodents. However, effects of SGS742 have not been systematically assessed in different strains so far and indeed strains show different cognitive abilities per se. The aim of the study was therefore to examine the effect of SGS742 in three different inbred (C57BL/6J, DBA/2, BALB/c) strains and three outbred strains (CD1, CF1, OF1). Mice were administered intra-peritoneal (3-aminopropyl)n-butylphosphinic acid (SGS742; GABA (B)-receptor antagonist) and tested in the Morris water maze (MWM). Open field, elevated plus maze, neurological observational battery and rota rod were carried out to support interpretation of data. SGS742 enhanced performance in the MWM in an inbred strain, C57BL/6J and in the outbred strain OF1 in terms of learning and memory formation at the consolidation level. A series of side effects as e.g. reduced motor coordination and proprioception were noticed. However, those may not have been influencing results observed in the cognition task. In conclusion, SGS742 enhanced cognitive performance in two mouse strains and we conclude that testing compounds for effects on spatial memory should be carried out in several strains or even different species in order to claim cognitive enhancement.

## Functional neuroprotection following ischemia using an inhibitor of NMDA receptor –PSD95 interaction

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Although it has been known for over 25 years that glutamate excitotoxicity in ischemic brain injury is mediated largely by NMDA receptors, clinical trials of NMDA receptor antagonists in stroke have proven unsuccessful. This is largely due to unacceptably severe side effects resulting from extracellular blockade of essential NMDA-mediated function. An alternative approach is to block the interaction between NMDA receptor subunits and intracellular scaffolding proteins responsible for excitotoxic protein-protein interactions such as activation of nitric oxide synthase. We have been investigating such a strategy in a rat model of transient ischemia (temporary occlusion of the middle cerebral artery) (tMCAO). Interactions between the NMDAR subunit NR2B and PSD95 were inhibited by systemic administration of a peptide (NA1) consisting of the C-terminus of NR2B rendered cell permeant by fusion with the protein transduction domain of the HIV-1-Tat protein. We first confirmed that escalating doses of NA1 did not result in significant impairment of relevant behaviours in the desired dose range. We subsequently administered NA1 (or control peptide) either 1 or 3 h post tMCAO (or sham) and evaluated functional neuroprotection over 2 months using an extensive battery of tests designed to measure physical, sensory, motor and cognitive function. Following testing rats were euthanized and infarct volumes were determined. We report significant long-term functional and anatomical neuroprotection

in the absence of observable side effects, and conclude that interference with NMDA-PSD95 interactions is a viable approach for new drug development in stroke.

## Occurrence of D-aspartate oxidase in the neuronal post-synaptic membrane

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D-Aspartate oxidase (D-AspO; EC 1.4.3.1) is the enzyme that catalyzes the oxidization of D-aspartic acid (D-Asp) to oxaloacetate. Previously, our results have suggested that D-Asp could be a novel neurotransmitter in mammals and mollusks. This hypothesis is supported by the following results: 1) D-Asp is present in synaptosomes and in synaptic vesicles of rat and *Aplysia limacina*; 2) If purified synaptosomes are incubated with $K^+$ ions or $Ca^{++}$ ionomycin, endogenous D-Asp is released in the medium; 3) If cultured neuron are incubated with D-Asp, cAMP (the second messenger) increases significantly.

In addition to the above properties, another criterion has to be fulfilled in order to assert that D-Asp is a neurotransmitter. A mechanism must be present for a rapid clearing of the D-Asp from the synaptic cleft after its binding to an hypothetic receptor: either an efficient uptake system, or alternatively, an enzymatic system for the destruction of the molecule.

In this study, we indeed show that a D-AspO is present in the post-synaptic membrane which could represent the enzymatic system for the destruction of D-Asp. This result constitutes an important support for the role of D-Asp as neurotransmitter.

The post-synaptic membranes, from rat brain and from ganglia of *Aplysia limacina*, were prepared as follows: 1 g of tissue was homogenized in 10 volumes of 0.32 M sucrose in 50 mM Tris–HCl, pH 7.5, using an Elvehjem class potter with clearance of 0.2 mm. The homogenate was centrifuged at 3,000 *g* for 20 min to remove nuclei and cellular debris, and the supernatant was re-centrifuged at 40,000 *g* for 15 min. The precipitate consisting of crude synaptosomes was resuspended in a minimum volume of 0.32 M sucrose in 50 mM Tris–HCl, pH 7.5, and layered onto a sucrose gradient consisting of 15 ml of 1.2 M sucrose (bottom) and 15 ml of 0.87 M sucrose (top), then centrifuged at 50,000 *g* for 30 min. The synaptosomes, visible as an opaque colored band floating 1 cm under the top of 1.2 M sucrose gradient, were collected and resuspended in about 10 ml 0.32 M sucrose and re-centrifuged at 50,000 *g* for 15 min. The pellet consisting of purified synaptosomes was homogenized with 5 ml of 0.05 M Tris–HCl pH 7.5 with a potter homogenizer and dialyzed extensively against a solution containing 2 M NaCl in 0.1 M Tris–HCl pH 8.2 to remove the amino acids still binding to the membranes. Finally this sample was used for the determination of D-AspO.

The determination of D-AspO was carried out as follows: 200 µl of post-synaptic membranes (1 mg of protein) were mixed with 50 µl of 1 M Tris–HCl, pH 8.2 and 50 µl of 1 M D-Asp (substrate) and incubated at 37 °C for 60 min. The reaction was stopped by the addition of 50 µl of 4 M TCA. After centrifugation, 250 µl of the supernatant were mixed with 50 µl of 5 mM 2.4-dinitrophenylhydrazine in 5 M HCl. After 10 min, 250 µl of 4 M NaOH were added, mixed and read at 445 nm against a blank (same sample without D-Asp). The enzymatic activity was expressed as O.D. One unity was the enzyme quantity that developed a color corresponding to 1.0 O.D. in the assay conditions. The specific activity was expressed as unity/mg.

Our results support the hypothesis. However, in order to declare that D-Asp is really a novel transmitter, it need still to demonstrate the presence of the receptors for D-Asp in the post-synaptic membranes and ultimate by electrophysiological demonstration.

## "Elements" of mental disorders: neural networks, cells, proteins, genes? – The systems biology perspective!

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*Biological psychiatry* is searching for the "generators" of symptoms and/or the diseases. This approach is using the top-down procedure to genetics and molecular biology. This analytical program appears to be fruitful as a "master unit" that "orchestrates" the clinical phenomena is expected to be found. For instance, in schizophrenia several genes are found that are determining the molecular processes in signal transmission in the dopamine synapses in the various brain areas.

But this finding alone does not "explain" hallucinations or formal thought disorders: *philosophy of science* and *systems philosophy* shows that the explanation of a global complex phenomenon by single units has its difficulties: a broken vase shows that not the parts alone but their relations to each other determine the whole. Therefore not the structure of the elementary units alone but the *mode of their interaction* is important for understanding brain dysfunctions. Mathematically speaking, *systems philosophy* (or: *systems methodology*) conceives a system as a network, i.e. a set of nodes with parallel distributed, diverging and converging forward and backward directed edges (graph theory). For this reason the interaction networks of intracellular molecular networks have to be reconstructed by computer-based modeling.

In the talk, present attempts to model dopamine signaling processes with regard to schizophrenia will be presented.

## Selective recognition and separation of amino acids by molecularly imprinted polymers

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Molecularly imprinted polymers (MIPs) are smart tailored-made materials, used for the sensitive and selective recognition of small molecules, and/or biologically important substances. The target molecule, acting as a molecular template, is copolymerized with an excess of a functional monomer and a cross-linker. The template is entrapped in the formed polymer and following its removal, complementary cavities, in structure and in properties to the template, are formed in the polymer. Consequently, the polymer is able to rebind the template molecule with high affinity and specificity.

The chemical and physical stability of MIPs in both aqueous and organic solvents, their resistance at extreme pH and temperature conditions and their reusability revealed their promising applications in the field of biocatalysis, diagnostics, immunoassays, bioseparations, biomimetic sensors, affinity chromatography while imprinting molecules of biological origin, such as amino acids, oligopeptides and even proteins have gained great attention.

In this study a number of molecularly imprinted polymers (MIPs) have been produced via non-covalent imprinting for L-methionine

and L-lysine and screened for their rebinding characteristics. The MIPs were produced with alterations in the molar ratio of template:monomer:cross-linker and the rebinding affinity for both amino acids was evaluated by guest batch-wise rebinding experiments. The percentage of net rebinding and the imprinting factors were used for the measurement of imprinting efficacy. Scanning electron microscopy images of MIPs and BET specific surface area measurements were obtained in an attempt to correlate the adsorption characteristics with polymer morphology. In the case of L-methionine, the MIPs that were produced presented better characteristics indicated that these polymers may be used in the solid phase extraction of L-methionine, or for diagnostics assays. Further experiments need to be performed for the molecularly imprinting of L-lysine to facilitate better rebinding characteristics of the polymers.

## Application of disease proteomics to evaluation of therapy efficiency in malignant gliomas

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Malignant gliomas are the most common primary brain tumours in adults. Due to their invasive character, surgery is unable to remove all tumour tissue, and ancillary therapies such as three-dimensional conformal radiotherapy, stereotactic radiosurgery, and concurrent chemo-radiotherapy are routinely used postoperatively. Despite this multidisciplinary approach the prognosis of gliomas remains poor with a median survival in the range of 1 year.

Little is known about the molecular and cellular mechanisms of the resistance of gliomas towards current therapies.

The goal of our project was to investigate expression patterns of proteins with pro-invasive activity in surviving human glioblastoma cells under clinically relevant treatment conditions. Malignant glioma cells underwent either radiation or temozolomide treatments alone, or combined chemo/radio treatment.

Protein expression patterns were investigated by two-dimensional polyacrylamide gel electrophoresis followed by protein spot identification using tandem mass spectrometry analysis. Specific expression levels were quantified by Western-blotting. Extracellular gelatinase activities were determined by zymogramms. DNA damage was comparatively evaluated using ''Comet Assay''-analysis.

Survival curves indicated no effective suppression of glioma cells under all treatment conditions tested. Morphological changes demonstrated sub-lethal effects of both temozolomide and combined treatments.

Markers for brain tumour progression and angiogenesis such as MMP-2, MMP-9, vimentine, lysozyme c and others were highly up-regulated under clinically relevant treatment conditions.

**Conclusion:** Activation of proteins supporting pro-invasive tumour activity indicates an increasing malignancy grade of surviving glioma cells under the treatment conditions tested. This correlates well with clinical observations of recurrences by treated glioblastomas.

## Profiling of endogenous metabolites in spinal fluid – before and after a trauma. Role of nanomedicine

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There are three main objectives when profiling endogenous metabolites;

- Diagnosis, i.e. classification
- Identification of biomarkers
- Dynamic studies i.e. the study of a treatment in order to identify effects from adding a compound, foodstuff or other to a living species a human, animal, tree, plants, fish . . .

The profiling of endogenous metabolites in biofluids is done by different analytical methods such as LC-NMR, LC-MS, LC-MS/MS and GC-MS. These techniques generate huge amounts of data, i.e. a large number of metabolite peaks for each sample. In biology, chemometric methodology has still been largely overlooked in favour of traditional statistics. It is not until recently that the overwhelming size and complexity of the 'omics' technologies has driven biology towards the adoption of chemometric methods.

Chemometrics provides strategies and tools to make good use of such measured data, enabling practitioners to make sense of measurements and to quantitatively model and produce visual representations of information. The combination of metabolic profiling and chemometrics is also called metabonomics.

In this presentation, a general outline of a strategy for investigating changes/difference in metabolite patterns is described. The chemometrical-based approach is presented step by step and exemplified by analysing the changes in the endogenous metabolite profile in the spinal fluid after a trauma. We will also highlight the use of nano-particles in the treatment of spinal cord injury.

## Role of growth hormone and amino acids on the brain protein synthesis in rats given proteins of different quantity and quality contained wheat gluten

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The purpose of the present study was to determine whether the regulation of brain protein synthesis was mediated through changes in the plasma concentrations of insulin and growth hormone (GH), and whether the concentrations of amino acids in the brain and plasma regulate the brain protein synthesis when the quantity and quality of dietary protein is manipulated. Two experiments were done on three groups of aged rats given diets containing 20% casein, 5% casein or 0% casein (Experiment 1), and 20% casein, 20% gluten, or 20% gelatin (Experiment 2) for 1 d (only one 5-h period) after all rats were fed the 20% casein diet for 10 d (only 5-h feeding per day). The aggregation of brain ribosomes, the concentration in plasma GH, and the branched chain amino acids in the plasma and cerebral cortex declined with a decrease of quantity and quality of dietary protein. The concentration of plasma insulin did not differ among groups. The

results suggest that the ingestion of a higher quantity and quality of dietary protein increases the concentrations of GH and several amino acids in aged rats, and that the concentrations of GH and amino acids are at least partly related to the mechanism by which the dietary protein affects brain protein synthesis in aged rats.

### Protein aggregation and neurodegeneration: focus on oligomers of beta amyloid peptides

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This study took advantage of a specific protocol designed to obtain separately different aggregated species of Amyloid beta peptide (Abeta). Using this protocol we investigated the intracellular pathways activated by $Abeta_{1-42}$ oligomers and fibrils. Oligomers were found to be the most potent neurotoxic species, being active at nanomolar concentration range. Confocal microscopy studies showed that oligomers but not fibrils internalized into the cells. of the peptide suggesting that fibrils exert its effects extracellulary, while the effects of oligomers were, at least in part, mediated by the internalization. Both fibrils and oligomers activate ROS generation but with different kinetics. Fibrils activated ROS already 3 h after the addition of the peptide. At this time fibrils were localized outside cells and eventually bound to cell surface membrane. At variance, oligomers induced ROS generation was temporally associated with the internalization of the peptide.

Oligomers and fibrils, although with a different kinetics, increased p53 expression and transactivation of p21-luc promoter. Finally, oligomers but not fibrils activated the extrinsic apoptotic pathway with the induction of TRAIL cascade involving caspase8. The specific involvement of the intrinsic and extrinsic apoptotic pathways after oligomer or fibrils was supported by data that demonstrated that the treatment with a specific inhibitor of caspase 9 prevented both oligomers and fibrils-induced cells death, while the inhibition of caspase 8 reduced cell death only in cells exposed to oligomers but not in cells treated with fibrils.

### Vasospastic individuals demonstrate significant similarity to glaucoma patients as revealed by gene expression profiling in circulating leukocytes

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**Purpose:** There is a growing body of evidence that vasospatic individuals could be predisposed to glaucoma. The goal of this study was to look for possible similarities in gene expression profiles of circulating leukocytes between vasospastic individuals and glaucoma patients.

**Methods:** Normal-tension (NTG) and high-tension (HTG) glaucoma patients as well as individuals with vascular dysregulation (VD) and healthy controls – both latter groups with no history of glaucoma disease – were recruited for the gene expression analysis. ''Atlas Human Cardiovascular Array'' technique with altogether 588 human genes known to play an important role in cardio-vascular processes was used for the expression profiling in the selected groups on the level of gene transcription. Individual gene expression rates were concomitantly quantified using ''Real-Time''-PCR.

**Results:** Compared to the control group the expression of 146, 68, and 60 genes were found to be altered in NTG, HTG, and VD groups respectively. Thirty-four genes demonstrated similar expressional alterations in NTG, HTG, and VD groups versus controls, and only 21 genes demonstrated similar expressional alterations in NTG and HTG having no overlap with VD group. Among 146 genes differentially expressed specifically in NTG group we monitored 48 and 53 genes which were similarly expressed in either VD or HTG groups, respectively. Among 68 genes differentially expressed specifically in HTG group we found 43 genes to be similarly expressed in VD group only.

**Conclusion:** There are pronounced similarities in gene expression profiles among NTG, HTG and VD groups which strongly support a potential predisposition of vasospastic individuals to glaucoma.

## Plant Amino Acids

### Functions of amino acids and antioxidant proteins in heavy metal-induced oxidative stress in plants

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The contamination of soils and water with metals has created a major environmental problem, leading to considerable losses in plant productivity and hazardous health effects. Exposure to toxic metals can intensify the production of reactive oxygen species (ROS), which are continuously produced in both unstressed and stressed plants cells. Some of the ROS species are highly toxic and must be detoxified by cellular stress responses, if the plant is to survive and grow. The aim of this talk is to assess the mode of action and role of antioxidants (enzymatic and non-enzymatic) in protecting plants from stress caused by the presence of heavy metals in the environment. Heavy metals can be extremely toxic to cells but normally occurring in low concentrations in the environment. However, human activities have considerably altered such a scenario and many events of environmental contamination have been widely reported in recent years. Research on bioremediation techniques are being carried out extensively. The metal cadmium (Cd) is particularly toxic and has been found in agricultural land due to antropogenic activities. Cd can generate the production of reactive oxygen species (ROS), which must be dismutated by a group of antioxidative enzymes. We have been studying the biochemical and physiological aspects related to the antioxidative responses by plants (tomato, coffee, soybean, tabacco, rice, radish, sugarcane and crotalaria) to heavy metal (Cd, Ni, Al and Se). The analyses carried have shown that glutathione reductase (GR) normally respond more effectively to heavy metal stress. Other enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST) and other peroxidases vary considerably in their responses, which appear to be dependent of plant tissue, metal concentration and developmental stage. Analysis of amino acids has also shown significant alterations in the pool of soluble amino acids, for instance an increase in proline in tomato tissues, in response to Cd-induced stress. An ongoing project in our laboratory has now been initiated using proteomic and molecular approaches to gain more insights into the antioxidative stress responses to these metals.

### Improvement of amino acid content in rice (*O. sativa*)

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Rice is the most important staple crop worldwide. Among other problems, amino acid content of the rice grain is unfavourable with respect to the essential amino acids lysine, methionine and tryptophane. Various approaches have been followed to improve rice grain quality in this respect, among them conventional breeding strategies, e.g. IR64 high protein rice, and also strategies exploiting molecular knowledge and employing transgenic approaches. We will provide information on the improvement of rice grain, especially with respect to the sulphur containing amino acids, i.e. methionine and cysteine.

### Lysine metabolism in rice seeds: expression profile of *DHPS* and *LOR-SDH* genes

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The major plant sources of protein are cereal and legume seeds. Rice is the staple food of half of the world population. The content of essential amino acids such as lysine, threonine, methionine and isoleucine is limited in cereals leading to the low nutritional quality of storage proteins in mature seeds. The essential amino acid lysine is synthesized in higher plants in the aspartic acid biosynthetic pathway, which also leads to the formation of threonine, methionine and isoleucine. Recent genetic, molecular, and biochemical evidence suggests that lysine synthesis and catabolism are regulated by complex mechanisms. The first enzyme unique to lysine biosynthesis is the dihydrodipicolinate synthase and there is substantial evidence that this enzyme exerts a major control over the synthesis this essential amino acid. However, lysine 2-oxoglutarate reductase and saccharopine dehydrogenase, which regulate the catabolism of lysine have only been studied in more detail recent years. In this work we performed Real Time PCR to analyzed different rice cultivars to determine the profiling of the expression of *DHPS*, *LOR/SDH* genes in developing rice seeds. Total soluble amino acids and soluble lysine content were determined by the ninhidryn method and HPLC, respectively. The IAC-165 cultivar presented the lower *LOR/SDH* expression while Japanese cultivar 153-J Col Fuzui the higher expression level. In contrast, *DHPS gene* expression was higher in the IAC-165 cultivar while 256-J Shizuoka genotype was the lowest. IAC-165 cultivar presented higher levels of soluble lysine content. Together, the results suggest that both, lysine biosynthesis and catabolism, are important for the accumulation of lysine in rice seeds (Financial support by FAPESP, Brazil).

### Cadmium-induced oxidative stress in tomato plants: antioxidant enzyme responses

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Cd can induce excessive production of reactive oxygen species (ROS) which can cause serious damage to cellular metabolism, leading to growth inhibition. A class of antioxidant enzymes acts in the scavenging of ROS and plants exposed to heavy metals may exhibit alteration in enzyme activity. In this study we observed alterations in CAT, GR and peroxidases activities in tomato cv Micro-Tom plants. SOD isoenzymes activities were also shown to be altered. The results suggest that in tomato cv Micro-Tom the main defence system to Cd stress is variable depending on the heavy metal exposure period and concentration. The study of such responses may allow the determination of tolerance levels and specificity of the response to distinct pollutants in the environment. The understanding of such responses may also be useful in breeding programs or biotechnological alternatives to produce and/or select tolerant plants with potential to be used in phytoremediation in order to reduce the amount of heavy metals in contaminated areas.

### Physiological effects of cadmium on hormone tomato mutants

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Cadmium (Cd) uptake is harmful to most plants since it can cause oxidative stress, reduction in chlorophyll synthesis and inhibition of shoot and root growth. The reactive oxigen species (ROS) production process may severely affect the functional and structural integrity of biological membranes, and malondialdehyde (MDA) is one of several products formed via the decomposition of certain primary and secondary lipid peroxidation products. Moreover, in many plants under various forms of stress, the concentration of proline increases relatively within the amino acid pool, playing an important role to protect plants against free radical-induced damage. In this study we observed alterations in MDA, chlorophyll and proline synthesis in tomato mutant's *diageotropica* (*dgt*) and *Never ripe* (*Nr*) submitted to Cd exposure. Our results have indicated a toxic effect of Cd, variable during the heavy metal exposure period and concentration, as well as the mutant studied. Induction of lipid peroxidation and an increase of proline to Cd exposure were observed in response to Cd when compared to untreated plants. According to the information described above, the increased contamination and subsequent accumulation of Cd in the soil may have serious consequences, since the concentration of heavy metals may reach high levels in the soil and become a limiting factor for normal plant growth and productivity of field crops and thus affect human health.

### Higher lysine or threonine levels affect the methionine level in higher plants

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Lysine, threonine and methionine are three essential amino acids whose levels limit the nutritional quality of cereals and legume plants. These amino acids synthesized through the aspartate family biosynthesis pathway, in which lysine was produced through a different branch of threonine and methionine. To elucidate the relationship between these biosynthetic branches and to study the factors that regulate the methionine synthesis, we crossed between transgenic tobacco plants overexpressing Arabidopsis cystathionine $\gamma$-synthase (AtCGS), the first unique enzyme of methionine biosynthesis, which exhibits higher levels of methionine and two different lines. The first line overexpressed feedback-insensitive bacterial enzyme dihydrodipicolinate synthase (bDHPS) that contains a significantly higher lysine level, and the second line overexpressed the feedback-insensitive bacterial enzyme aspartate kinase (bAK). The results of the analysis of the progenies of plants expressing bDHPS/

AtCGS together with the analysis of feeding plants demonstrated that lysine reduced the expression level of *S*-adenosylmethionine (SAM) synthase. As a result, the level of SAM reduced, which led to a higher expression level of AtCGS and an increase in methionine level. Testing the second set of crosses (AtCGS/bAK), we next found that plants co-expressing both foreign genes have significantly higher methionine and threonine levels compared to levels found in wild-type plants. However, the methionine level does not increase beyond that found in plants expressing the AtCGS alone. This finding can be explained by the feedback inhibition regulation mediated by SAM on the expression level of AtCGS. To test this assumption, plants expressing bAK were crossed with plants expressing the AtCGS versions in which the domains responsible for the feedback regulation have been deleted. Indeed, significantly higher methionine levels accumulated in the newly produced plants. The results of this study suggest new ways of producing transgenic crop plants containing increased methionine and lysine levels, as well as methionine and threonine levels, and consequently having improved nutritional quality.

## Occurrence of amino acids in soil and their uptake in Barley

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Occurrence of amino acids in soil solution was studied in a field experiment and the results served as guidance for uptake experiments. Barley (Hordeum vulgare) was grown in hydroponic culture. The simultaneous uptake of the five amino acids, L-Serine, L-Glutamic acid, Glycine, L-Arginine and L-Alanine was recorded as disappearance of amino acids from incubation solutions. All tested amino acids were clearly taken up and a concentration dependency was observed for the uptake rate. The dependency of uptake rate on solution concentration of amino acids could be described by Michaelis-Menten kinetics. Control experiments using labelled amino acid showed a strong correlation between depletion of amino acid in the incubation solution and presence of label in plants, thus verifying that solution depletion could be used to measure root uptake of amino acids. Our results support the hypothesis that amino acids can be of importance in the nitrogen budget of plants.

## Do catabolic enzymes play major regulatory roles in plant amino acid metabolism?

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Plant amino acid metabolism is a highly regulated metabolic network, which plays a central role in plant growth and development. Previous studies have shown that Lys metabolism in plants is principally regulated by two major components: (i) the biosynthetic enzyme dihydrodipicolinate synthase (DHPS) whose activity is subject to allosteric feedback inhibition by Lys and (ii) the *LKR/SDH* gene encoding a bifunctional polypeptide possessing the first two enzymatic reactions of lysine catabolism, namely lysine-ketoglutarate reductase (LKR) and saccharopine dehydrogenase (SDH). Yet, the regulatory interaction between DHDPS and LKR/SDH as well as their relative importance is still unknown. Using a bioinformatics approach, we are addressing the interactive regulatory nature of DHDPS and LKR/SDH in the regulation of Lys metabolism and also the interactive regulatory nature of the biosynthetic/allosteric and catabolic enzymes in metabolic pathways of other amino acids in plants. The results of this bioinformatics approach will be presented.

## Antioxidant enzymes activity pattern in developing tomato plants

**C. C. Monteiro, P. L. Gratão, L. E. P. Peres, P. J. Lea,** and **R. A. Azevedo**

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Tomato mutants have been used as an important model for genetical studies. For instance, some mutations have been identified such as those involved in auxin, cytocinine and ethylene synthesis and sensitivity. Although the *lutescent* and *green flesh* tomato mutants present alterations at senescence, this aspect do not seem to be involved with sensibility to cytocinine and ethylene. In this study our objective was to evaluate the antioxidant system (SOD, CAT and GR) in the *lutescent* and *green flesh* tomato mutants, trying to establish an interaction between the senescence process and enzyme activity. The results showed alterations in SOD, CAT and GR, suggesting that the defence system varies considerably depending on mutants and their tissues (root, leaves and fruits). However, it was observed low activity of CAT and GR during the development. Others enzymes are been evaluated to understand the possible interaction mechanism between senescence and antioxidant systems.

## The Lysine Histidine transporter 1 mediates plant uptake of amino acids

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Plant nitrogen uptake is a key process in the global nitrogen cycle and is usually considered a ''bottleneck'' for biomass production in land ecosystems. Organic nitrogen, predominantly in the form of amino acids, represents a significant proportion of the total N pool in many soils and recent studies have shown that plants may access amino acids in field settings. Although plants have the ability to take up soil amino acids, the transporters responsible have until recently not been identified. We have isolated one candidate transporter involved in the uptake of soil amino acids. D-amino acids such as D-Ala and D-Ser are toxic to plants, providing an excellent opportunity for screening mutants that have defective root amino acid absorption mechanisms. Mutated *Arabidopsis thaliana* seeds were selected on D-Ala, one EMS plant and a T-DNA knockout line survived, these were later found to be mutated in the same gene, LHT1 (Lysine and Histidine transporter 1). Both mutant lines display reduced uptake levels of amino acids in a short-term depletion experiment and a long term labelling experiment. Mutant plants have reduced biomass when grown on agar with L-Gln. When grown on agar for 21 days (LD), with nitrate as the sole nitrogen source, the biomass of mutant lines does not differ from wild type, suggesting that endogenous amino acid transport is not affected under the first three weeks. We are currently doing molecular work on the LHT1 transporter, to investigate the function of the transporter in more detail. We believe that the discovery of this transporter is an important step for

the understanding of whether or not plants benefit from the uptake of amino acids.

## Uptake and growth utilization of D- and L-amino acids by plants

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Amino acids can be used as nitrogen sources by plants but the actual mechanisms enabling this process are largely unknown. We studied the capacity of the model plant *Arabidopsis thaliana* to absorb and utilize different amino acids for growth. Several L-amino acids were shown to promote growth of plants cultivated in axenic culture but the growth promoting effect was not correlated to the capacity for root uptake.

A mutation in the gene encoding the Lysine Histidine Transporter1 affected the capacity of plants to absorb several amino acids both in short- and long-term studies. The effect of the mutation on plant amino acid acquisition was found to be more severe on low, than on high external amino acid concentrations.

None of the tested D-amino acids promoted growth and several compounds, in particular D-Ser and D-Ala were shown to inhibit plant growth. Transgenic plants expressing genes encoding D-amino acid metabolising enzymes were, however, able to utilize the toxic D-amino acids for growth, suggesting that poorly developed metabolism of such compounds may be the major bottleneck for plant utilization of D-amino acids. We conclude that plant uptake of amino acids depends on specific amino acid transporters such as the Lysine Hisitidine Transporter 1. We also conclude that the ability to use amino acids for growth depends on the capacity to metabolize the absorbed compound as well as the resulting products of this metabolism.

## High-lysine maize endosperm mutants: proteomic analysis of glutelin storage proteins

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Maize is widely cultivated in the world and is an important source of dietary protein. The seed storage proteins albumin, globulins, zeins and glutelins determine the nutritional quality of the grain. Zeins correspond to 50–60% of the total proteins and lack lysine and tryptophan in their amino acid sequences, while the non-zein fractions have adequate amounts of these essential amino acids. These attributes cause this cereal to be of poor nutritional quality. To better understand seed storage protein composition, a study was conducted to compare, through bidimensional polyacrylamide gel electrophoresis (2D-PAGE), the amount of glutelins in the endosperm of the opaque and floury mutants *o1*, *o2*, *fl1* and *fl2*, respectively, which contain higher lysine contents than the wild type line Oh43+. Glutelins were extracted and quantified. Glutelin contents in the mutants were 28–88% greater than in Oh43+. Profiles were obtained from 50 µg of proteins focused on a non-linear pH3-10 18-cm strip and separated by gradient PAGE and silver staining. Proteins ranged from 6–100 kDa with p*I* values of 3.3 to 9.0. Image analyses showed differences in the abundance (%Volume greater than 1.5×) and the presence or absence of proteins when compared to Oh43+. *o1* and *fl2* had 10 differentially expressed proteins, while *o2* had 16 and *fl1* had 15. Moreover, 7 proteins were found only in *o1*, 8 in *o2*, 6 in *fl1* and 10 in *fl2*. These differences indicate a possible correlation with the increased lysine levels in these mutants, which is consistent with breeding and biotechnological programs to increase the levels of lysine in maize.

## Roles of gene families of *O*-acetylserine thiol-lyase and serine acetyltransferase in *Arabidopsis*: just redundancy or hidden secret?

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*O*-Acetylserine thiol-lyase (cysteine synthase: CSase) and serine acetyltransferase (SATase) are committed in the biosynthesis of cysteine in plant. CSase forms cysteine from hydrogen sulfide and *O*-acetylserine produced by the action of SATase. In the genome of *Arabidopsis thaliana*, there are nine CSase-like genes (*Bsas*) and five SATase-like genes (*Serat*).

Judging from gene expression levels and *K*m values, Bsas1:1, 2:1 and 2:2 are likely the major CSase isoforms and Bsas3:1 is β-cyanoalanine synthase (CASase), which is responsible for the formation of β-cyanoalanine from cysteine and cyanide, rather than CSase. T-DNA knockout mutants of *Bsas* genes were isolated and analyzed for their gene expressions, enzyme activities and metabolite profiles. In all mutants, expressions of knocked-out *Bsas* genes were repressed, but expression levels of other *Bsas* besides the knocked-out gene were not significantly changed. In *bsas1:1*, CSase activity was decreased to 54% of wild-type and cysteine content was decreased. In *bsas3:1*, CASase activity was decreased to 36% of wild-type. We have also conducted targeted- and non-targeted-metabolite profiling analyses. These results suggested that Bsas1:1 and Bsas3:1 play the most important role for cysteine biosynthesis and β-cyanoalanine synthesis, respectively.

To investigate the function of each *Serat* gene, we have isolated quadra-knockout mutants, in which only each single *Serat* gene remains, by crossing the each T-DNA mutant. Phenotypic and metabolic analyses of these mutants are undertaken to investigate the actual function of each *Serat* gene.

## The role of cellular compartmentation for sulfur amino acid biosynthesis

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The heteromeric cysteine synthase complex (CSC) consists of 10 subunits and constitutes the entry of reduced sulfur into metabolism. Dominant-negative mutation of the rate-limiting subunit (serine acetyltransferase) and constitutive overexpression in the cytosol of tobacco plants showed the crucial function of the CSC for regulation of cysteine biosynthesis. In comparison to overexpression of the active subunit in tobacco, contents of OAS, cysteine, glutathione, methionine and total sulfur were even more enhanced in transgenic tobacco expressing the inactivated subunit. Up to 25-fold increased cysteine levels were observed. Transgenic serine acetyltransferase was demonstrated to unequivocally interact with the endogenous tobacco second subunit (O-acetylserine thiol lyase) of the CSC in planta. Thus, the de-regulation of CSC was based on competition of inactive transgenic CSC subunit with endogenous tobacco subunit for binding in the CSC and probably involved contributions of plastidic and mitochondrial cysteine synthesis.

# Polyamines – Transglutaminases

## Toxicity of enzymatic oxidation products of spermine to human cancer cells: sensitization by lysosomotropic compounds

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The in situ formation of cytotoxic metabolites by an enzyme-catalyzed reaction is a recent approach in cancer chemotherapy. Cytotoxicity induced on human cancer cells by bovine serum amine oxidase (BSAO) and spermine is attributed to $H_2O_2$ and aldehydes produced by the reaction. We observed that cytotoxicity induced by spermine metabolites was greater in multidrug resistant cell (MDR) than the corresponding wild type cells lines (WT). This represents an aspect of particular importance with respect to potential therapeutic applications of the method, since conventional cancer therapy suffers from the development of drug resistance. An increasing of the incubation temperature from 37 °C to 42 °C enhanced cytotoxicity in both WT and MDR cells exposed to $H_2O_2$ and aldehydes, the enzymatic oxidation products. Hydrogen peroxide was mainly responsible for the loss of cell viability. With about 20%, the aldehydes formed from spermine contribute also to cytotoxicity. The combination of BSAO/spermine is not only able to prevent tumour cell growth, but also prevents mass tumour growth, particularly well if the enzyme has been conjugated with a biocompatible hydrogel-polymer. In fact, the growth of a mouse melanoma (B16-F0) was reduced by 70% after injection of the immobilized enzyme, in comparison with 32% inhibition after injection of native enzyme. Combination with MDL 72527 ($N^1$,$N^4$-bis(2,3-butadienyl)-1,4-butanediamine dihydrochloride), a lysosomotropic compound, improves the cytocidal effect of polyamines oxidation products, mainly in MDR cells than their wild-type counterparts, as was evident from the decrease of cell survival. This phenomenon due to an increased mitochondrial activity in MDR cells, makes this new approach attractive in combating cancer and in treating MDR cancer patients.

## Catabolism of protein-bound polyamine derivatives as potential target for cancer therapy

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Protein modifications with polyamines are constituted by a covalent amide linkage to the γ-carboxyl groups of protein glutamic acid residue. Conjugation of the amines in this manner is catalyzed by transglutaminases (TGases, E.C. 2.3.2.13), $Ca^{++}$-dependent enzymes which promote exchange of primary amines for ammonia at the carboxamide groups of certain glutaminyl residues.

In epidermal cells undergoing terminal differentiation, γ-glutamyl-bound polyamines were found to be components of keratinocyte protein. This finding suggested that the levels of protein-polyamine crosslinks may be strongly affected by antiproliferative or differentiative stimuli. In tumor cells, TGase activity is generally low and the level of unbound polyamines is higher compared to normal cells, due to the hyperproliferative activity. Therefore, the possibility to increase both TGase activity and consequently the amount of intracellular protein-polyamine conjugates is intriguing, in the light of the control of both proliferative and metastatic activity of cancer cells.

Several studies have been developed about polyamine oxidase (PAO) inhibition and tumor cell proliferation. Searching for a mechanism responsible for the antiproliferative effect of MDL 72527 (A selective PAO inhibitor), we investigated the possibility that PAO may act on the protein-bound spermidine. SDS-PAGE performed on the soluble proteins of B16-F10 cells incubated with guinea pig liver TGase and $^3$[H]-spermidine, showed that the amount of protein-bound radioactive polyamine was significantly reduced after treatment with PAO. This finding suggests that the selective oxidation by PAO may be one of the cellular mechanisms regulating protein cross-links levels, affecting the rate of proliferation of normal and neoplastic cells.

## Proapoptotic effects of dexamethasone on polyamine metabolism in rat thymus

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Glucocorticoids (GCs) are a class of steroid hormones that exert a wide range of anti-inflammatory, immunosuppressive, and antineoplastic activities, including the ability to induce apoptosis in T and B lymphocytes. Despite many experimental and clinical results the molecular mechanism of GC-induced apoptosis is still incompletely understood.

The aim of our investigation was to elucidate the effect of dexamethasone, synthetic glucocorticoid, on polyamine metabolism in rat thymus. We have done the experiments with male albino Wister rats, weighing 180–230 g, were used. The animals were divided into two groups: control and experimental groups. Experimental group received dexamethasone, intraperitonealy during 3 days, in a daily dose of 4 mg/animal. The last dose of hormone was applied on the 3$^{rd}$ day, one hour before sacrificing. Control group received 0.9% NaCl instead of hormone. The animals were killed by decapitation. The thymus was removed quickly and rinsed with ice cold saline. The polyamines were extracted from thymus by using butanol. After extraction the amount of polyamines was investigated by electrophoresis. For estimation of PAO activity, 10% water homogenate of thymus we have prepared.

Our results suggest that the supplementation of experimental animals during three days with dexamethasone significantly decreases spermine and spermidine levels in rat thymus tissue – Sp Control $= 362.56 \pm 25.33$ nmol/g wet w.; Sp Exp. Group $= 313.01 \pm 21.16$ nmol/g ww; Spd Control $= 673.81 \pm 30.95$ nmol/g ww; Spd Exp. Group $= 410.21 \pm 17.26$ nmol/g ww. PAO activity significantly decreases under the influence of hormone in comparison with the control group-PAO Control $= 0.449 \pm 0.121$ U/mg Prot.; PAO Exp. Group $= 0.312 \pm 0.096$ U/mg Prot. Our results suggest that the decreasing of polyamine amounts in rat thymus is not the change of polyamine oxidase activity.

PS During experimental period the natural thymus size diminishes for 1/3 compared with the thymus size at the beginning of hormone application.

## Tissue transglutaminase and NF-κB cross-talk in THP-1 differentiating macrophages

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Monocytic leukemia THP-1 cells can be differentiated into macrophages by TPA. THP-1 cultures are an useful model to study the

mechanisms of inflammatory and immune responses. It is well known that the transcription factor NF-κB, involved in cell growth, differentiation, and apoptosis inhibition, also takes part in the expression of numerous cytokines and adhesion molecules regulating immune response. Recently, tissue type transglutaminase (TG2) up-regulation has been reported in several inflammatory conditions promoting NF-κB activation.

In this study, we investigated the expression of TG2 and other TG isoenzymes during TPA-induced maturation of THP-1 monocytes into macrophages. Further, we evaluated whether a NF-κB/TG2 cross-talk may be involved in THP-1 differentiation.

TG activity increased by 25-fold in TPA-differentiated macrophages in comparison to monocyte cultures and was inhibited by GTP-γ-S. This effect was concomitant with a dramatic changes in TG2 expression throughout differentiation, while both TG3 and TG1 were present at low levels.

The reduction of NF-κB activation by SN50 (30 μg/ml), in the early phase of TPA-induced differentiation, showed a 45% decrease in TG2 expression. Notably, a 60% reduction of TG2 activity by TG inhibitor 1,3-dimethyl-2[(oxopropyl)thio]imidazolium) (R283, 250 μM), was associated with a significant reduction of NF-κB nuclear translocation.

These data suggest that TPA-induced maturation of THP-1 monocytes to macrophages relies on increase in TG2 expression, which is associated with NF-κB activation and reciprocal regulation between NF-κB and TG2.

## Functional characterization of AtoS-histidine protein kinase in *E. coli*

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AtoS is the membrane signal-transducing histidine kinase (HK) that together with the response regulator AtoC, plays a pivotal role in the transcriptional regulation of *atoDAEB* operon in *Escherichia coli*. The carboxyl-terminal cytoplasmic region of an HK, called transmitter domain, consists of an ATP-binding region and a so-called H-box that includes the conserved histidine residue, which represents the site for self-phosphorylation. Upon acetoacetate induction, the AtoS kinase autophosphorylates and subsequently transfers the phosphoryl group to AtoC, thus leading to its activation.

To localize AtoS autophosphorylation site, chemical stability tests and site directed mutagenesis were performed. These experiments identify the histidine-398 which is located within the conserved H-box as the site of phosphorylation. In order to elucidate the mechanism of AtoS autophosphorylation, cross-linking experiments with recombinant his-tagged protein AtoS and its cytosolic portion (lacking the two N-terminal transmembrane domains) were performed. Ligand-induced dimerization would be expected to cause kinase activation by shifting the equilibrium between inactive histidine kinase monomers and active dimers. Cross-linking experiments in vivo, in the presence or absence of acetoacetate, also demonstrated the importance of signal-binding for the dimerization or oligomerization of AtoS procedure. In addition, two inactive forms of AtoS, lacking the histidine autophosphorylation site and the ability of ATP binding in the G-box respectively, were found to be necessary for the autophosphorylation reaction in vitro. These results indicate that the autophosphorylation reaction of AtoS occurs within the dimer by a trans-intersubunit mechanism and dimerization may be a prerequisite for trans-phosphorylation and activation of AtoS.

## Quercetin affects B16-F10 melanoma cell metastatic potential through the increase of intracellular transglutaminase activity

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Quercetin, a plant flavonol, possesses antioxidant and antitumour properties. The mechanism(s) responsible for these effects have not fully elucidated, although it has been shown that in different cancer cells several enzyme systems, such as cytochrome P450 isoenzymes, protein tyrosine kinase, RNA and DNA polymerases and epoxide hydrolase can be affected. So far, no data are available on the effect of quercetin on transglutaminase (TGase) activity, which is known to be related to the induction of cell differentation and consequently to the inhibition of cell growth.

The aim of this work was to investigate the effect of quercetin on TGase activity in highly metastatic murine melanoma cells B16-F10.

B16-F10 cells were incubated with 10 μM quercetin for 24, 48 and 72 h. We evaluated different parameters related to cell proliferation and differentiation: cell counting, polyamine levels and TGase activity. Cell proliferation was reduced by 30% after 48 hrs and by 28% after 72 h of treatment, as well as the level of intracellular polyamines while, TGase activity was enhanced (172% increase after 48 h and 35% after 72 h). Proteins were extracted from treated and untreated cells and 2-D PAGE was performed to detect changes in protein pattern. Gels have been scanned and analyzed by PDQuest (Biorad). Average gels from at least three independent experiments were compared to each other and spots differing at least four fold in intensity were considered and picked for further analysis. Comparison of protein patterns revealed the following changes in treated cells compared to the control: 11 proteins of de novo synthesis and 35 up-regulated, including TGase enzyme.

The data obtained suggest that quercetin modulates its anticancer property by enhancing synthesis and activity of TGase.

## Directed mutagenesis on the gene of coagulation factor IX and their effect on the proteolytic activity

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In this study site-directed mutagenesis was performed on coagulation factor IX (fIX) gene and their effect on the protein's proteolytic activity was studied. Blood coagulation factor IX (fIX) is a vitamin K-dependent plasma serine protease that plays a major role in coagulation mechanism and haemostasis. It is synthesized in hepatocytes as a precursor protein of 461 amino acids. After several post-translational modifications the mature protein is secreted in the circulation as inactive zymogen. The zymogen is then activated during the coagulation cascade into an active serine protease.

A truncated form of fIX gene (rf9) inserted in a pET22b vector was used in our experiments, so as to be able to overexpress the protein in prokaryotic cells. Two mutations were induced on this gene. Both of them are described in databases as polymorphisms. The first one (A21975G) is on the protein's activation peptide and causes the substitution of $Thr^{194}$ by an Ala residue. The prevalence of this G containing allele in general population is 23%. However in our previous study we found that in a certain group of thrombophilic patients the prevalence of the G containing allele was 50%. The second mutation (A32881C) is also described as polymorphism and causes the substitution of $Thr^{461}$ by a Gly residue and involves the 2% of the population.

Both mutated and native forms of the truncated coagulation factor IX (rf9) were overexpressed in *E. coli* BL21 cells after induction with IPTG. The truncated factor rf9 lacks its first N-terminal domains; therefore it doesn't require posttranslational modifications in order to gain activity. Proteolytic activity of rf9 was studied using an amidolytic assay. Rf9 was first activated using Russell's viper venom FX activating protein (RVV-X). The specific tripeptide MS-D-Phe-Gly-Arg-pNA was used as a substrate. Proteins were purified from the cytoplasm using a Q-Sepharose ff column. They were activated and their proteolytic activity was studied. A difference in the activity of the two mutants was observed compared to the native protein, thus a correlation of the two mutations and factor's activity was attempt.

## From a protein inhibitor of polyamine biosynthesis to a transcriptional regulatory factor

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Antizyme, long known to be a protein non-competitive inhibitor, induced by polyamines, the end product of the enzymic reaction of ornithine decarboxylase. Recently, antizyme proved to be the *atoC* gene product, encoding the response regulator of the bacterial two-component system AtoS-AtoC. The gene located just upstream of *atoC* encodes the sensor kinase, named AtoS, that modulates AtoC activity. In vitro phosphorylation of purified $His_{10}$-tagged AtoC by AtoS a membrane protein in *E. coli*, is affected by acetoacetate the inducer of expression of *atoDAEB* operon involving in the short-chain fatty acid metabolism. Antizyme is thus referred to as AtoC, functioning both as a post-translational and transcriptional regulator. The AtoS-AtoC signal transduction system in *E. coli* has a positive regulatory role on poly-(R)-3-hydroxybutyrate biosynthesis. A number of polyamine analogues were synthesized and tested for their effects on the transcription of the above mentioned genes, as well as for their ability to support growth of a polyamine-dependent *E. coli* strain. Deletion of the *atoS-atoC* locus results in diminished accumulation of cPHB compared to $atoSC^+$ cells. Spermidine can induce the accumulation of cPHB in *E. coli* similarly to acetoacetate, possessing a functional AtoS-AtoC TCS, but not in cells lacking either or both components of this signal transduction system or in cells expressing AtoC with mutations in the phosphorylation site. Moreover, the AtoC protein exhibited ATPase activity enhanced by AtoS-catalysed phoshorylation as well as by specific oligonucleotide sequence containing the whole region for the cooperative binding to two adjacent sites of AtoC on the enhancer of *atoDAEB* operon. Based on the known three-dimensional structures of two-component regulatory systems a structural model of AtoC in complex with a proposed homodimeric domain of AtoS containing a putative phosphohistidine was constructed.

## Antizyme inhibitor 2 (ODC-like): another player in polyamine metabolism

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Ornithine decarboxylase (ODC), a key enzyme in the biosynthesis of polyamines, is regulated at post-translational level by antizymes (AZs), a family of proteins composed by three members in mammals (AZ-1, AZ-2 and AZ-3), which are induced at the translational level by polyamines through a mechanism of +1 ribosomal frameshifting. AZs interact with ODC, decreasing the decarboxylating activity of the enzyme and promoting the degradation of ODC by the proteasome in a ubiquitin-independent process. AZ-1 and AZ-2 also have the ability to inhibit the transporter for the uptake of extracelular polyamines. Another target of antizymes is the antizyme inhibitory protein (AZIN). AZIN shares a significant degree of sequence homology to ODC, is enzymatically inactive and blocks the ability of antizyme to promote ODC degradation. A few years ago, a novel human gene highly homologous to ODC, termed ODC-like or ODC paralogue (ODCp) was cloned, but the studies aimed to determine its functions rendered contradictory results. Our studies with mouse ODCp using transient transfected HEK 293T cells have demonstrated that ODC-like has no intrinsic ornithine or arginine decarboxylating activity, but mimics the action of AZIN, rescuing ODC from the effects of antizymes. Moreover, a direct interaction between ODC-like and the three antizymes was detected by immunoprecipitation experiments, and the expression of ODC-like activated polyamine uptake by COS cells and counteracted the inhibitory effect of the three antizymes on polyamine transport. These results clearly indicate that ODC-like functions as a novel antizyme inhibitory protein, and accordingly it should be named AZIN2. The gene is expressed in brain and testes, and its expression pattern suggests that the protein may participate in processes of terminal differentiation of neural cells and spermatogenesis, respectively. In conclusion, our studies reveal the existence of another player in the complex regulation of polyamine metabolism and uptake, whose properties and physiological function warrant further investigation.

## Peculiar effect of polyamines biosynthesis inhibitors on experimental mammary tumors growth

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**Aim:** to study peculiarities of ornithine decarboxylase inhibitors effect on mammary tumors growth.

**Results:** Effect of intraperitoneal injections of α-difluoromethylornithine (α-DFMO, 500 mg/kg, for rats also 1000 mg/kg per one injection) and polyhexamethylenguanidine (PMG, 10 or 15 mg/kg per one injection) on growth of Ca-755 mouse mammary carcinoma and Walker W-256 rat mammary carcinosarcoma was studied. It was found that under conditions of tumor strain adaptation to modified way of transplantation, tumor-grafted animals may divide into 2 subgroups in one of which quick growth of control tumors and retardative effect of PMG and α-DFMO (a standard effect) was observed and in another, in contrary, slow growth or even no growth of control tumors and its acceleration or even initiation under PMG and α-DFMO (a paradoxal effect). Time interval between tumors appearance in the first and second subgroups was 6 days (equal to average longitude of estrus cycle for mice and rats) for Ca-755 and 3 days (a half of this longitude) for W-256. Study of polyamines content, polyamine oxidase (PAO) activity and immunohistochemical expression of ornithine decarboxylase (ODC) shows that modulations of polyamines (mainly spermine) interconversion (1) and interrelations between more than one

physiological reactivity levels of ODC expression control system (2) are involved to a mechanism of a paradoxal effect. It is necessary to point the (1) factor is the main for Ca-755 and (2) factor – for W-256.

## Molecular analysis of the regulation of the *atoDAEB* operon by AtoC/Antizyme

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Bacteria adapt rapidly to ever-changing environments in order to survive by using networks of two-component signal transduction systems consisting of a sensor histidine kinase and a response regulator, which is usually a transcription factor. Signal sensing by the sensor kinase triggers its autophosphorylation, on a histidine residue, and then the subsequent phosphate transfer to the response regulator, which is thus activated.

The catabolism of short-chain fatty acids requires enzymes encoded by the *atoDAEB* operon, i.e. the AtoE membrane transporter, the heterodimeric (AtoD-AtoA) acetyl-CoA: acetoacetate-CoA transferase and the AtoB thiolase II. This operon is highly inducible by acetoacetate, whose catabolism requires the *atoDAEB* operon-encoded enzymes, and the induction requires the transcription factor AtoC. AtoC has a dual function since it acts as both an antizyme (Az), i.e. post-translational inhibitor of polyamine biosynthetic enzymes and transcriptional regulator of the *atoDAEB* operon.

We have shown that AtoC/Az constitutes a TCS with the sensor kinase AtoS, which phosphorylates AtoC upon acetoacetate induction. By performing in vitro experiments we have shown that AtoS-mediates the phosphorylation of AtoC and by using in vivo approaches we demonstrated that the induction of *atoDAEB* expression requires both functional AtoC and AtoS. By fusing a reporter gene, i.e. *lacZ*, to *atoDAEB* promoter sequence and performing promoter deletion analysis, we identify the DNA region that mediates the induction of the *atoDAEB* promoter in the presence of acetoacetate. In vitro protein-DNA, binding experiments proved that AtoC binds to the same sequences that are involved in acetoacetate-mediated induction. That AtoC binds these sequences was also confirmed by chromatin immunoprecipitation experiments that showed acetoacetate-inducible AtoC/Az binding to the same DNA region in vivo. The acetoacetate effect seems to be indirect, as its presence in DNA-protein binding reactions in vitro did not enhance AtoC binding to its target DNA. In contrast, the polycationic polyamines were found to enhance the in vitro DNA-binding of AtoC/Az albeit modestly. DNAse I protection footprinting analysis revealed that AtoC/Az binds two 20 bp stretches, constituting an inverted palindrome, that are located at −146 to −107 relative to the transcription initiation site. Analysis of promoter mutants obtained by in vitro chemical mutagenesis of the *atoDAEB* promoter verified both the importance of AtoC/Az binding for the inducibility of the promoter by acetoacetate and the $\sigma^{54}$-dependence of the *atoDAEB* expression. Integration host factor was also identified as a critical component of the AtoC/Az-mediated induction of *atoDAEB*.

## Differential effects of thyroid hormones, estradiol and histamine on the cellular stress response in yeast

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Upon exposure to adverse microenvironmental conditions, cells adapt and protect themselves via the inducible cellular stress response (CSR). Hormones and inflammatory mediators may modulate the CSR; in part by altering the expression of heat shock proteins (HSPS). This study aimed at investigating the possible role of thyroxine ($T_4$), triiodothyronine ($T_3$), 17β-estradiol and histamine (HI) on the heat shock (HS) response in *Saccharomyces cerevisiae*, an established experimental model for studying both the universal CSR and the function of several biologically important components. The HS response was evaluated by determining microscopically the viability of post-logarithmic phase grown yeast cells, after exposure to HS (53 °C, 30 min). The expression of hsps was determined by western blotting. The effects of $T_3$, $T_4$, 17β-estradiol or HI were investigated following administration of the agents either through to the post-logarithmic phase of growth (24 h, long-term) or for 2 h prior to HS (short-term). Upon long-term exposure, the thyroid hormones $T_3$ and $T_4$ conferred dose-dependent thermotolerance to the yeast culture that seemed to be independent of hsp60, hsp70 and hsp104 induction. On the contrary, 17β-Estradiol and HI showed no effect on yeast viability after HS. Upon short-term exposure, HI elicited a biphasic effect on yeast viability and an inverted dose-related elevation of hsp60, hsp70 and hsp104, in contrast to the thyroid hormones and 17β-estradiol, which showed no effect on yeast viability after HS. In conclusion, these preliminary data provide evidence for the differential action of hormones and inflammatory mediators on the induction of the CSR in eukaryotic cells.

## Polyamine catabolism in testes of rats treated by dexamethasone

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**Background and aims:** Dexamethasone is one of synthetic steroids with a more powerful glucocorticoid activity than cortisol. It is known that dexamethasone affects testosterone production as well the concentration of LH receptors in testes of both experimental animals and men. However, the exact mechanism responsible for inhibitory effect of glucocorticoids on testicular steroidogenesis and spermatogenesis, is not known. On the other side, polyamines (spermine, spermidine and putrescine), quantitatively the most abundant in reproductive organs, have a significant effect on synthesis of testosterone, DNA and RNA as well as on the mobility and survival of spermatozoa. The aim of the experiment was to examine the effect of dexamethasone on the metabolism of polyamines in rat's testes.

**Methods:** Dexamethasone (1 mg/100 g b.w.) was administred intraperitoneally once a day during 7 days. The control group of rats received the same amount of saline. The activity of polyamine oxidase (PAO) and diamine oxidase (DAO), enzymes responsible for polyamine catabolism and their interconversion, was estimated by measuring the formed aminoaldehydes, and enzyme activity was expressed as U/mg proteins. Separation and detection of polyamines from rat's testes were performed by paper electrophoresis and subsequent colorimetric determination with ninhydrine.

**Results:** The obtained results point out the statistically significant decrease of PAO ($3.07 \pm 0.06$) as well as the significant decrease in spermidine level ($48.42 \pm 7.5$ nmol/g tissue) in comparison to corresponding control values of PAO ($3.27 \pm 0.01$; $p < 0.05$) and spermidine ($64.45 \pm 3.18$; $p < 0.01$). The activity of DAO and the level of spermine and putrescine were not significantly changed.

## Influence of fungicides treatment on free polyamines content in *Vitis vinifera* grapes

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Polyamines, are ubiquitous in nature and are important regulators of growth and differentiation. At cellular pH they carry a net positive

charge, bind to negatively charged macromolecules like DNA, and are essential for cell division. Abiotic stress conditions such as low pH, high $SO_2$, high salinity, osmotic shock, nutrient stress, low temperature all result in an increase in cellular polyamine levels.

A recent work examining the concentration of polyamines in plant has been aimed at using polyamine concentrations as indicators of stress.

It is now well established that analogues of the naturally occurring polyamines also possess the ability to perturb polyamine metabolism and, for example, a number of putrescine and spermidine analogues have been shown to control a variety of plant fungal diseases and to alter biosynthetic enzyme activities and intracellular polyamine concentrations in fungal cells.

Indeed these analogues have already been shown to control powdery mildew on barley under experimental conditions, although such effects were not always associated with altered polyamine biosynthesis.

Downy mildew induced by *Plasmopara viticola* is a major illness that affects wild and cultivated grapes (*Vitis species*).

This fungus occurs throughout all-important wine growing regions in temperate climates. In order to achieve an effective control of this fungus it is necessary to use fungicides at the right stage of growing. Among the chemicals currently used to control this pest there are copper compounds and a new fungicide belonging to the benzamide family, zoxamide, [(*RS*)-3,5-dichloro-*N*-(3-chloro-1-ethyl-1-methyl-2-oxopropyl)-4-methylbenzamide] that shows a strong preventive activity combined with excellent rainfast and residual properties. This fungicide stops fungal growth by inhibiting cell division, preventing the late blight fungus from reproducing.

In this work the fungicides above mentioned have been compared, in a southern Italian vineyard, by using them several times, to test the concentration of polyamines in grapes and evaluate their ability to alter polyamine concentrations.

Grape samples were picked up one, two and four week after the treatments. Each sample was homogenized with 0.2 M perchloric acid (PCA), kept 30 min on ice and centrifuged at $28{,}000 \times g$ for 10 min. The supernatants were derivatized using the benzoylation method described by Flores and Galston slightly modified.

The data obtained showed that Spermidine is the most represented polyamine in grapes, mainly in the black variety.

During fruit ripening both total and individual polyamine concentrations evidenced a constant decrease in all the samples analysed. Moreover also a week after the last treatment, the results were comparable with the control samples.

In conclusion, the slight differences respect to the control, showed by the grape samples after the fungicide treatments, pointed out that the chemicals applied in the present study do not modify the standard behaviour of polyamines during grapes ripening.

## The influence of N$^{\omega}$-nitro-L-arginine methyl ester on arginine and polyamine metabolism in rat's brain tissue during exposition to microwave radiation

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**Introduction:** Exposition to microwave radiation (MW), from mobile phones, satellite communications, radio relays, radars and microwave devices in medicine induce disturbances in different organ systems. It has been shown that MW from mobile phones induce increasing of oxidative stress and apoptosis of neurons with impairment of blood brain barrier, disturbances of memory and space orientation. Citrulline and nitric oxide – NO are products of L-arginine by NO sintase-NOS. L-ornithine and polyamines are products of L-arginine by arginase. N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME) competitive inhibits NOS and exerts neuroprotective effects.

The aim of this investigation is the determination of arginase, PAO and DAO activity, concentration of citrulline, as well as the effects of L-NAME on arginine and polyamine metabolism in brain tissue of rats exposed to MW.

**Material and methods:** Four groups of Wistar rats were investigated during 60 days: I-control – sham exposed, II (L-NAME) – rats treated with L-NAME (5 mg/kg b.w. i.p.), III (MW) – rats exposed to MW (4 h/day), IV (MW + L-NAME). The source of MW was mobile test telephone.

**Results:** Decreasing activity of arginase ($0.19 \pm 0.04$ vs. $0.25 \pm 0.05$ mmol/mg prot; $p < 0.01$) and increasing of citrulline concentration ($10.34 \pm 0.49$ vs. $7.83 \pm 0.41$ mmol/mg prot; $p < 0.001$) were registered in brain of MW exposed rats compared to control. In L-NAME group there were decreasing of citrulline level ($p < 0.05$), and increasing of arginase activity ($p < 0.05$), compared to control. In brain of exposed rats activity of PAO was significantly increased while activity of DAO was significantly increased vs. control ($1.12 \pm 0.10$ vs. $0.79 \pm 0.09$ U/mg prot; $p < 0.001$ and $0.51 \pm 0.06$ vs. $0.65 \pm 0.06$ U/mg prot; $p < 0.05$, prospectively). In MW + L-NAME group we registered increasing of DAO activity ($0.61 \pm 0.04$ vs. $0.51 \pm 0.06$ U/mg prot; $p < 0.05$), in brain tissue compared with MW group.

**Conclusion:** From the results obtained we can conclude that L-NAME exerts neuroprotective effects by preventing polyamine and arginine metabolism disturbances in rats' brain under exposition of MW.

## Brain polyamine and nitric oxide interactions during Fas-induced apoptosis

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Apoptosis is a natural cell phenomenon by which damaged cells are removed in order not to prevail in organism. One of the factors influencing apoptosis is nitric oxide, diffusible, and highly reactive molecule acting as apoptosis promoter in some cells, while in the others it can inhibit apoptosis. It is synthesized from L-arginine, also the precursor of polyamines, modulators of numerous processes in organism. The aim of the work was to examine brain NO synthesis and polyamine metabolism interactions during anti-Fas antibodies-induced apoptosis and treatment by IFN and IGF.

Mice of Balb/c species, divided into 6 experimental groups, were used: control group treated intraperitoneally by 0.85% NaCl, II – anti-Fas group treated by mice anti-Fas antibodies ($2 \times 40$ µg per mouse or 0.8 µg/kg body mass), III – treated by IFN, IV – anti-Fas + IFN, V – treated by IGF and VI – anti-Fas + IGF. The animals were sacrificed 48 h after. Arginase, diamino oxidase and polyamine oxidase activities, nitrate + nitrite concentration, as well as oxidative stress and antioxidative defense parameters were determined in brain homogenates.

Diminished NO production and diminished arginase activity confirm that NOS and arginase interaction is not based on simple competition for the same substrate in Fas-induced apotposis. The decreased activity of polyamine oxidase and the consequent impossibility of higher polyamines conversion into putrescine together with documented increased SSAT activity in apoptosis prove that acetylated polyamines release into extracellular space, which is the most probable cause of putrescine intracellular pool decrease, proved in apoptosis. The obtained results point out the importance of brain NO and polyamine metabolism interactions during anti-Fas antibody-induced apoptosis.

## The role of polyamines in etiopathogenesis of schizophrenia

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The work is devoted to the study of polyamine's role in etiopathogenesis of schizophrenia. Polyaminemia and polyaminuria have been shown in patients with schizophrenia. Correlation between the dynamics of clinical presentations and polyamine levels in blood serum during treatment was examined before treatment beginning, after the active psychotic semiotics recession, and in discharge from the hospital. During treatment the reduction in clinical rates intensity (BPRS score) was observed equally with the decreasing of polyamine levels in patient blood serum. After the active psychotic symptoms recession in patients with shift-like schizophrenia it was appeared positive correlation between the degree of reduction of such symptoms as disorganization of thinking, depressed mood, delusions and jealousy, and decreasing of putrescine concentration in blood. High extant of the correlation was detected for spermidine too. The degree of diminishing of many clinical symptoms also correlates positively with declining of polyamine levels. The highest coefficients of multiple correlation were detected between the decreasing of polyamine quantity and such symptoms as disorganization of thinking ($p<0.001$), anxiety ($p<0.001$), depressed mood ($p<0.001$), delusions and jealousy ($p<0.001$), and decreasing of total amount of steps by BPRS score ($p<0.001$). During the active psychotic semiotics recession in patients with paranoid schizophrenia the reliable range of correlation between declining of polyamine levels and smoothing of such clinical symptoms as: somatic anxiety ($p<0.001$), autism ($p<0.001$), dullness of affect ($p<0.001$), was observed equally with high correlation between declining of polyamine levels and decreasing of symptom intensity (for anxiety, $p<0.001$; for delusions and jealousy, $p<0.001$). At discharge from the hospital patients with shift-like schizophrenia showed the closest positive correlation between declining of polyamine levels and reducing of such clinical symptoms as emotional isolating ($p<0.001$), disorganization of thinking ($p<0.001$), exertion ($p<0.001$), hallucinatory behavior ($p<0.001$), motional lethargy ($p<0.001$), unusual thoughts ($p<0.001$). For the patients with paranoid schizophrenia the most close degrees of multiple correlation were detected between declining of polyamine levels and decreasing of such clinical symptoms (by the BPRS score) as guilt feeling ($p<0.001$), delusions and jealousy ($p<0.001$), disorganization of thinking ($p<0.001$), autism ($p<0.001$), unusual thoughts ($p<0.001$). At the same time for these patients, like for patients with shift-like schizophrenia, the important contribution into multiple correlations was brought by spermine concentration. At discharge from the hospital for patients with paranoid schizophrenia the most close correlation was observed between spermidine concentrations and such clinical symptoms as somatic anxiety ($p<0.001$), anxiety ($p<0.001$), emotional isolating ($p<0.001$), motional lethargy ($p<0.001$), autism ($p<0.001$), dullness of affect ($p<0.001$). For patients with shift-like schizophrenia the closest correlation was detected between spermidine levels and clinical symptoms, such as emotional isolating ($p<0.001$), disorganization of thinking ($p<0.001$), autism ($p<0.001$), dullness of affect ($p<0.001$). Thus, the statistical treatment by correlation analysis of clinical and biochemical data exposed positive correlation between serum polyamine levels and symptoms of patients with schizophrenia.

It was established that levels of polyamines in blood serum and in urine in patients with schizophrenia were dependent on type of course, leading syndrome and duration of disease. Endotoxicosis in schizophrenia is due to significantly more high concentration of polyamines in average molecular mass fraction of blood serum in patients with schizophrenia then in normal persons. Correlation between concentration of polyamines in blood serum and in urine in patients with schizophrenia and the phase of the course of disease (exacerbation, remission) has been shown. There is correlation between polyamine levels in blood serum in patients with schizophrenia and such clinical manifestations of disease (BPRS) as ''anxiety'', ''the disorganization of thinking'', ''hostility'' as well as sum of points on brief psychiatric estimative scale BPP. In the process of treatment the degree of decrease in polyamine levels has been correlating with reduction such clinical disorders as ''the disorganization of thinking'', ''depression'', ''suspiciousness'', ''somatic concern'', ''motor slowness down'' as well as with decrease of sum of points on brief psychiatric estimative scale BPRS.

Direct action of neuroleptic drugs (structural analogs of polyamines) activated decomposition, depressed synthesis and normalized polyamine levels in model non-cellular test-systems, obtained from tissues with increased cellular proliferation. It is considered as a possible mechanism of therapuetical action of these psychotropic medicines. It is proposed the polyamine participation in etiopathogenesis of schizophrenia.

## Prediction of antiproliferative and carcinogenic properties of benzimidazole and azafluorene derivatives as synthetic structural analogs of polyamines

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The purpose of this research is to study the biological properties of polyamines' (PA) structural analogs from benzimidazole and azafluorene derivatives.

The objective is to research into the effect of benzimidazole and azafluorene derivatives (polyamines' structural analogs) on polyamines' main metabolic indicators (diaminoxidase and polyaminoxidase activity, PA levels and ornitinedecarboxylase activity) in tissue with an enhanced mitotic activity.

Basis for the research objects and methods. Preliminary results and data available in books on the subject suggest that changes in the mitotic activity of tissues correlate in a certain way with the PA level. Thus, during an intensive cell division (regeneration, tumor and embryo growth) an enhanced polyamines level is observed. This is why PA metabolism can be a suitable experimental target in studying the proliferative activity of tissues and the biological properties of substances that influence that activity.

Two different mechanisms of increasing the polyamines levels in tissues with ''normally'' and ''pathologically'' enhanced cell proliferation have been described earlier. Thus, in the hepatoma tissues and as a result of hepatocancerogenesis caused by diethylnitrosamine, the increase in the PA levels is due to a sharp reduction or a total loss of diaminoxidase and polyaminoxidase activity in a greater degree than as a result of an enhanced ornitinedecarboxylase activity. A higher polyamines level in a regenerating liver depends on a sharp increase in the synthesis rate of these substances since the rate of their disintegration by way of an oxidative desamination remains constant. Hence, substances inhibiting an oxidative desamination of polyamines are likely to manifest carcinogenic properties. And vice versa, chemical compounds activating the process of oxidative disintegration of putrescine and PAs may have an antitumoural potential.

The biochemical properties of *12 derivatives of benzimidazole (1,2) and azafluorene (3-12)* were studied: 1) 7-aminopyrido[1,2-α]benzimidazole, 2) 7-nitropyrido [1,2-α] benzimidazole, 3) 1-amino-4-azafluoren, 4) 1-amino-4-azafluorenol, 5) 9-dicyanomethylene-4-azafluoren, 6) 9-

[α-(β-hydroxyethyl)aminomethylene]-4-azafluoren, 7) 9-[α-pyridilaminomethylene]-4-azafluoren, 8) 1-amino-9-phenylamino-4-azafluoren, 9) 1-amino-4-azafluorenon-9, 10) 1,4-diazoacetonaphthylene[1.2-f]-fluorantene, 11) 2-methoxycarbonyl-(β-benzoilethyl)aniline, 12) 5-(2-methoxycarbonyl)-phenyl-α-furfural.

The effect of substances on PA disintegration, levels and synthesis has been quantitatively assessed on a model cell-free testing system of tissue with a retained system of controlling the rate of the cell proliferation: the rat regenerating liver. The carcinogenic and carcinostatic properties of chemically modified polyamines analogs have been assessed by character and level of their effect on the rate of polyamines disintegration and synthesis during incubation. The total quantity of the substance for the whole research cycle is 2 μM. This small quantity is a favourable distinction of this method as compared to other approaches to this problem. The total testing time does not exceed several hours. These advantages allow for use of this testing system in primary collection of low-toxic substances with a potential antiproliferative activity and in forecasting carcinogenic properties of chemical compounds.

Any excess over the control figures has been expressed as a percentage. In total it has enabled assessment of the effect of each substance on the diaminoxidase, polyaminoxidase and ornitinedecarboxylase activity in general.

Standardization of the used method of testing chemical compounds would allow for monitoring carcinogenic and carcinostatic properties of the existing drugs and forecasting the quality of new medications.

**In-house results:** All tested substances, except (1), notably increase spermidine disintegration. Substances (3), (10) and (12) accelerate the rate of putrescine oxidative desamination, substances (4), (7), (9) and (10) enhance spermidine oxidative degradation. All studied 12 original chemical compounds decrease ornitinedecarboxylase activity in regenerating liver. Substances (3), (8), (9) and (10) appeared to be the most effective inhibitors of putrescine and PA synthesis rate. The same compounds (3, 8, 9, 10) decreased PAs levels in the most active way.

**Conclusions:** 1) Among 12 substances of original synthesis 7-aminopyrido[1,2-α]benzimidazole and 7-nitropyrido [1,2-α] benzimidazole decrease in 1,5–1,7 times aminoxidase activity in regenerative liver so they are potentially carcinogenic agents.

2) 1-Amino-9-phenylamino-4-azafluorene and 1,4-diazoacetonaphthyleno[1,2-f]fluorantene were the most powerful polyamine disintegration activators. They decreased the level of polyamines 50–70% less. Therefore, these substances could suppress carcinogenic proliferation.

3) 1-Amino-4-azafluorene, 1-amino-9-phenylamino-4-azafluorene and 1,4-diazoacetonaphthyleno[1,2-f]fluorantene, lowering ornitinedecarboxylase activity 30–50% less, may be potential antiproliferative agents.

## A role for polyamine signalling in chemoprevention

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Cancer is a major health concern worldwide and is a significant cause of mortality. In the UK, for example, there are approximately 20,000 deaths each year from colorectal cancer and the projected 5-year survival is between 45 and 50%. Similarly in the USA, it is estimated there will be over 90,000 new cases of bladder cancer with approximately 25,000 deaths. Therefore although treatments such as surgery followed by chemotherapy/radiotherapy are available for both types of cancer better therapy is required. An alternative strategy to chemotherapy is chemoprevention, the use of chemical agents, drugs or food supplements to actively prevent the disease development.

We have investigated a number of potentially active chemopreventative agents (natural and synthetic) in a range of human cancers and shown that they can all kill cells in culture. However the mechanism of action of these agents is not clear. The aim of this study was to determine whether there is a common signal that leads to cell death using both natural and synthetic chemopreventative compounds.

Non steroidal anti-inflammatory drugs (NSAIDs), ellagic acid, curcumin and quercetin were all tested and compared to know cytotoxic agents. All agents tested showed cytotoxicity in the systems used. Decreases in polyamine content and inhibition of polyamine biosynthesis were observed as were changes in catabolism and polyamine export. Adding back exogenous polyamines reversed the cytotoxicity suggesting that the polyamine pathway is an important intermediate in the signalling to cell death caused by these putative chemopreventative agents.

## Gene expression of alanine racemase in kuruma prawn *Marsupenaeus japonicus*

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Some free D-amino acids are found in animal kingdom. Marine invertebrates such as crustacean and bivalve mollusk species contain a large amount of free D-alanine in their tissues as a major osmolyte for isosmotic regulation. In the muscle and hepatopancreas of the kuruma prawn *Marsupenaeus japonicus* D-alanine concentration is almost equivalent to that of L-form, and D-alanine has been clarified to be biosynthesized from L-alanine by alanine racemase [EC 5.1.1.1], which catalyzes the interconversion between alanine enantiomers. However, the metabolism and physiological functions, and the accumulation mechanism of D-alanine are still indistinct.

In *M. japonicus*, free D-alanine was found in heart, gill, intestine, nervous tissues, and testins besides muscle and hepatopancreas. Moreover, gene expression of alanine racemase was also detected in all of these tissues. Under salinity stress of *M. japonicus* from 50 to 100% seawater, D- and L-alanine increased largely in muscle heart, gill, intestine, and testis. Moreover, D-alanine was also detected in hemolymph. In hepatopancreas, however, D-alanine content was not changed under this stress conditions, but the contents of D- and L-alanine elevated significantly during acclimation from 100 to 150% seawater. On the other hand, no remarkable change was seen in the other tissues under this high-salinity stress conditions. The gene expression of alanine racemase as well as the enzyme activity also elevated in hepatopancreas according to the seawater acclimation from 100 to 150%. These data indicate that the synthesis of D-alanine is controlled through the gene expression level of alanine racemase.

## Polyamines – markers of tumor growth and targets for anticancer therapy

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The aim of the work was study of the peculiarities of polyamines metabolism (PA) during tumor growth; to investigate the inhibitors of PA metabolism as an anticancer agents, and to evaluate the interrelationship between PA metabolism in tumors and transcription mediated by NF-κB.

It was shown that the activation of synthesis and regular increase of individual fraction of PA is a characteristic features for experimental tumors and human malignant neoplasms with different histological genesis. It was detected that PA-test may be used as acceptable criteria of the therapy efficacy as well as prognostic factor of clinical outcome in patients with ovarian cancer, breast cancer and malignant lymphoma.

It was shown that characteristic modulations of PA metabolism in tumor cells take place during forming of resistance to cisplatine and

remain in cisplatine-resistant tumors. These are: increase of the ODC activity and putrescine content, and part of spermine in total PA amount, decrease of DAO and γ-glutamiltranspeptidase activity.

Experiments in vivo have allowed to shown that promising targets for anticancer therapy are the intermediates of PA metabolism. It was found that the polyhexamethylenguanidine and green tea biocomposites have significantly inhibited the growth of experimental tumors, prolonged the mean survival time of tumor-bearing animals, and may be recommented for the further creation as a means for target therapy of oncological patients.

The significant evidences were obtained that PA participation in the regulation of activity of NF-κB transcription factor is one of the molecular functions of PA by tumor growth. It was found that antitumor action of inhibitors of PA metabolism is linked with of NF-κB signaling pathway.

### Effect of polyamines biosynthesis inhibitors on growth of drug-resistant experimental tumors and lifetime of tumor-bearing animals

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**Aims:** To study polyamines (PA) metabolism indices in experimental tumors during development of resistance to Cisplatine (CsP) and Doxorubicine (Dox) and effect of polyamines biosynthesis inhibitors on growth of drug-resistant experimental tumors and lifetime of tumor-bearing animals.

**Results:** During development of resistance to CsP, certain characteristic modulations take place in PA metabolism in Ehrlich ascites carcinoma cells, L1210 Leukemia cells and NK/ly lymphoma cells. These modulations are the followings: significant increase of putrescine concentration, magnification of spermine part in total PA amount, decrease of diamine oxidase (DAO) and γ-glutamiltranspeptidase. These modulations remain in developed CsP-resistant tumors. CsP injections to animals with CsP-resistant tumors, in contrary with CsP-sensitive ones, leads to still more magnification of PA content in tumor cells.

In CsP- and Dox-resistant substrains of Guerin's carcinoma, essential magnification of putrescine content and ODC activity, decrease of DAO and γ-glutamiltranspeptidase activities and acetylspermidine take place. In plasmalemma fraction of these cells, significant increase of both acid-soluble and acid-insoluble PA was found. This may affect membrane fluidity and then transmembrane transport of antitumor drugs as well as PA themselves.

Inhibitors of PA biosynthesis, α-DFMO and polyhexamethylenguanidine (PMG), retarded growth of CsP-resistant tumors in vivo and significantly magnify average lifetime of tumor-bearing animals. Combined application of PMG and CsP led to additive effect.

These results show that the application of PA biosynthesis inhibitors is prospective for combined therapy of CsP-resistant tumors.

### Histamine levels in the conjunctiva, costosternal cartilage and brain of normal and cromolyn-treated rats

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Synthesized from histidine, histamine (HI) is primarily stored as a preformed inflammatory mediator released upon activation of multifunctional, yet heterogeneous mast cells (MCs) identified in virtually every organ. Additional HI sources include central histaminergic neurons and inflamed cartilaginous tissue. Besides their function in hypersensitivity responses, mounting evidence implicates MCs in inflammatory disorders like rheumatoid arthritis and atherosclerosis. Aiming eventually at evaluating HI anatomical distribution, cellular origin and contribution in these diseases and in therapeutic interventions, this study sought to determine HI levels in various tissues of normal and cromolyn (CRM)-treated rats. Adult male Wistar rats were divided into 4 groups ($n = 4$–6). Group A was injected sc with 30 mg/kg CRM qdx10d. Group B received 0.9% saline accordingly. In group C, a 10 µl drop of 40 mg/ml CRM was instilled into the lower conjunctival fornix of one eye, the contralateral eye receiving saline. Normal group D remained untreated. Following sacrifice, HI was quantified fluorophotometrically in the conjunctiva, cartilaginous parts of the $9^{th}$–$10^{th}$ costosternal junction and in various brain regions. The respective conjunctival and cartilaginous HI levels of $4.67 \pm 0.2$ and $1.34 \pm 0.3$ ng/mg tissue were not significantly altered by either saline or CRM administration. In the brain, HI levels ranged from $0.504 \pm 0.04$ in the hypothalamus to $0.05 \pm 0.01$ ng/mg tissue in the cerebellum. Hypothalamic HI content was not modified by CRM, but it was significantly reduced in group B animals. In conclusion, normal HI levels were unaffected by MC stabilization, while the reduced hypothalamic HI content could be attributed to the stress induced by experimental handling.

## Proteins – Analysis

### Structural and functional analysis of hypothetical proteins in mouse hippocampus from two-dimensional gel electrophoresis

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Protein profiling in five individual mouse strains showed strain-specific expression of three hypothetical proteins (HPs). As functional and structural assignment of HPs were based on predictions and low identity to known structures, HPs were identified by MALDI-TOF/TOF, and their proposed tentative function was determined by enzyme assays. Three identified HPs were extracted from gels and renatured, and pyridoxal phosphate phosphatase, inorganic pyrophosphate phosphatase, and antioxidant activities were revealed, findings in agreement with functional predictions.

### Application of FT-IR spectroscopy in the assessment of changes in the secondary structure of food proteins taking legumes as a model

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Structural analysis of plant oligomeric proteins, such as legume proteins, and of conformational changes that occur during processing is

hampered by their low solubility. Consequently, structural aspects that account for stability of several proteins of nutritional interest upon heat-processing and/or gastro-intestinal digestion have not been thoroughly investigated so far. Fourier transform infrared (FT-IR) spectroscopy, a vibration spectroscopic technique, is a powerful tool to get information on the secondary structure of proteins by studying the different contributions to the amide I band, which arises from the stretching vibrations of C=O in the peptide bonds. In the present study, the secondary structure of proteins in common bean (*Phaseolus vulgaris* L.), the most important economic species of the genus *Phaseolus* grown worldwide, was investigated by FT-IR spectroscopy. To obtain reliable information on the structural changes induced by different thermal treatments, FT-IR measurements were performed on powdered samples embedded in KBr pellets, which is convenient in the case of proteins with limited solubility, such as heat-treated legume proteins. The amide I band of suitable model proteins, such as 7S globulin purified from common bean seed and commercially available concanavalin A, was first analyzed by using both Fourier Self-Deconvolution and curve fitting analysis methods. Then, the secondary structure of proteins in common bean was investigated by studying the amide I infrared absorption band in whole seed flour, before and after thermal treatments (dry-heating and autoclaving). 7S protein was found to be characterized by a high amount of β-sheet structure (28%). The amount of β-sheet structure in concanavalin A (36%) was higher than in 7S globulin. The same analysis procedure showed that the content of β-sheet structure in whole common bean was 32%. The dry-heating caused a reduction of β-sheets to 13%, the appearance of random coil structures and the formation of aggregated species. The autoclaving treatment increased content of random coil conformation and produced very high amounts of beta aggregates (60%). These results indicate that multimeric heat-induced complexes of legume proteins have a high stability because of the high content in β-sheet structures, which may adversely affect protein utilization.

## Age determination of two mammoths from Hungarian and Transylvanian regions based on amino acid racemization in tusk and bone

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We have determined the age of woolly mammoth (Mammuthus primigenius) remains found near Pécs in Hungary, and near Csíkszereda in Transylvania by a method based on amino acid racemization (AAR). The mammoth skeletal remnants were found in fine-grained yellow, clayey loess of late glacial aeolian origin. Calibration curves – D- and L-amino acid ratios of bones of known age determined by radiocarbon method as a function of age – earlier used for age determination of human bones, was employed in case of the mammoth tusk (Csíkszereda), and mammoth tusk as well as mammoth cranial-bone (Pécs). As the racemization of the amino acids is considerably affected by the pH and heavy metal concentration of the environment, therefore we examined the composition and micro element concentration of the soil. It was established that the pH of the loess (7.14 Csíkszereda; 7.77 Pécs) did not affect substantially the racemization of the amino acids, therefore the AAR method is suitable for determination of age of these samples. D-allo-Ile contents of the samples were measured by INGOS AAA 400 amino acid analyzer, using postcolumn derivatization with ninhydrin, while D- and L-Asp, -Glu and -Ala contents were measured by MERCK Hitachi LaChrom high performance liquid chromatograph using precolumn derivatization with OPA/TATG. Crude protein contents of mammoth tusk from Csíkszereda were measured to be 11.7%, whereas those of mammoth tusk from Pécs were measured to be only 1.13%, and those of cranial-bone from Pécs to be 8.17%. From these results the conclusion can be drawn that mammoth tusk from Csíkszereda has been preserved better. In the course of our examinations it was established that the sample practically did not contain D-allo-Ile, thus this amino acid cannot be used for the age estimation. In the D-amino acids it was established that for Glu and Ala the D/L ratios were below 0.1, therefore based on our earlier results we could not use these data in age determination. The amount of D-Asp was appropriate, nearly optimal in all three samples. Based on the D/L-Asp ratio of the mammoth tusk from Csíkszereda measured to be 0.229, using our calibration curve we could estimate the age of the tusk to be $9460 \pm 470$ years. D/L-Asp ratio of the mammoth tusk from Pécs was measured to be 0.247, while that of the cranial-bone to be 0.241, thus based on the tusk the age could be estimated to be $10200 \pm 500$ years, based on the cranial-bone to be $9960 \pm 500$ years. The difference between the tusk and the bone is 240 years, which is within the error limit of the method.

## Redesign and altered ion-binding of a natural calcium regulated biosensor, photoprotein aequorin

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Aequorin is a calcium regulated photoprotein which was originally isolated from the jellyfish Aequorea victoria. The holoprotein consists of the apoaequorin polypeptide chain and non-covalently bound substrate coelenterazine. Trace amounts of calcium ions trigger a conformational change in the protein which results in the intramolecular oxidation of coelenterazine, and concominant production of $CO_2$ and blue light.

For the event of calcium binding the protein employs the ubiquitous motifs called EF-hands. The EF-hands are helix-loop-helix structures responsible for $Ca^{2+}$-binding. They are found in a broad range of functionally diverse calcium binding proteins, known as the EF-hand protein superfamily.

Due to its high sensitivity to $Ca^{2+}$ and its non-invasive nature, aequorin has been used as a $Ca^{2+}$ indicator in biological systems for approximately 30 years. Aequorin's light emitting reaction can also be triggered by a range of other divalent and trivalent cations, leading however to significantly lower and in some cases barely detectable light yields. Based on this promiscuity towards other ions, this project has tested the hypothesis that aequorin's preference for certain cations can be manipulated through mutations engineered in its three ion binding loops (EF-hands). Subsequent protein variants with altered ion-binding specificity would be useful as biosensors for cations in biomedical research and in environmental monitoring.

We developed a high-throughput screening assay for the expression of apoaequorin and reconstitution of the active protein complex in 96-well plates. Wild type and 48 mutant variants of the protein were tested for activity against the following seven cations: calcium, zinc, copper, lead, cobalt, cadmium and lanthanum. Several mutants exhibited significantly altered specificity towards the new ions. One selected mutant was purified, characterised and compared to wild type protein. The effect of the primary screen mutations on the ion selectivity and activity of the protein will be discussed. The altered specificity of the selected mutant will be assessed towards the efficiency of the light emitting reaction and its kinetics.

## NMR structural analysis of the HIV-1 GP120 V3 – CCR5 co-receptor N-terminal peptide interaction

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Standard multidimensional and multinuclear NMR spectroscopy was applied to probe the structural and physicochemical determinants of three representative peptides from V3 domain of the HIV-1 and a 22-residue peptide, representing the amino terminal of the chemokine receptor CCR5, in their free or interacting state. Titration of CCR5 peptide with V3-peptides was performed in NMR tube, at 286 K. 1D $^{1}$H NMR spectra and $^{1}$H-$^{15}$N HSQC were recorded after each addition of V3 peptides. Data analysis of HSQC and NOESY spectra verifies the interaction of the V3-CCR5 peptide constructs and according to it three sites of interaction can be noticed at the N terminal of CCR5 with different, most likely, binding affinity for the different V3 sequences. The participation of the three interacting sites seems to be enhanced with the increase of the positively charged amino acids at the 7/9-residue V3 N-terminal peptide fragment, which especially in V3 LAI peptide is rich in basic residues on either side the highly conserved GPGR motif, for which a β turn conformation is observed. SF2 and LAI V3 peptides adopt a β-hairpin like conformation, while MN V3 peptide preserves the loop that is being formed in the GPGR region but the C terminal and the N terminal are located in opposite directions. The analysis of the V3 peptides calculated structures reveals important similarities and differences which provide valuable information about the extent and the amino acids involved in their interaction with CCR5 co-receptor.

## Separation and detection of yeasts by electromigration techniques

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The yeasts became one of the most important etiological agents of nosocomial infections. Especially the nosocomial blood stream infections are characterized by high mortality rate, early detection and exact identification can help to effective and succesful therapy. Microbial infection is preceded by adherence and biofilm formation. Biofilm formation represents the most common form of yeasts.

In this study, ways and means of the separation and possibly identification of the selected strains of yeasts are outlined. The capillary isoelectric focusing and capillary electrophoresis with UV/Vis and fluorometric detection were successfully used for the on-line rapid separation and focusing of the yeasts, native and/or dynamically modified by the non-ionogenic tenside. The possibilities to distinguish *C. albicans* from *C. dubliniensis* and biofilm-positive *C. parapsilosis* from biofilm- negative by capillary and/or gel isoelectric focusing are introduced here. The pH gradients were traced by the low-molecular-weight PI markers. These methods need further modification to detect or eventually identify and specify clinically important yeasts for their successful use in the diagnostic microbiology.

## Utilization of epitope extraction technique for study of clinically significant peptides

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Today, epitope extraction, based on combination of protein proteolysis with peptide finger-print mass spectrometric analysis, is one of the powerful techniques used for protein characterization. Peptides can be identified by the ability of the antibody to protect one region of the protein more than others from proteolysis. Peptides represent clinically significant group of substances applied in large measure in the field of medical diagnostic and therapy. Identification of short linear peptide sequences in accordance with main allergogenic epitopes of the whole allergens provides the future of safe epitope-based peptide vaccines for a wide range of allergic sensitizations.

Allergogenic epitopes are usually located at the surface of allergens and they are recognized by specific IgE molecules. New developed enzyme (IMERs) and immunoaffinity (IMARs) magnetic microreactors integrated into μ-chip were used for epitope mapping of model food allergen ovalbumin. Magnetic enzyme microreactors with immobilized native and/or TPCK-trypsin, pepsin, α-chymotrypsin and proteinase K were utilized for proteolysis of ovalbumin and subsequently the magnetic immunoaffinity microreactors with immobilized rabbit polyclonal and/or mouse monoclonal anti-ovalbumin antibodies were utilized for capturing of allergogenic epitopes from the mixture of peptide fragments. The peptide fractions eluted from immunosorbent were analyzed by MALDI-TOF-MS without further purification and peptide sequence (KIKVYLPR, M/z 1016.6615) was confirmed by tandem mass spectrometric sequencing on QTOF Ultima API (Waters, UK). The fragmentation pattern of the peptide was compared to theoretical pattern of the studied sequence using PepSeq script in MassLynx 4.0 (Waters, UK) and very good match was found.

The utilization of chip-based epitope extraction technique has a plenty of advantages and provides the proper tool for rapid and simple isolation of epitopes with immunogenic potential.

## Non-inhibitory properties of α1-proteinase inhibitor: binding with lipophilic ligands

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Human $\alpha_1$-proteinase inhibitor ($a_1$-PI), also known as $\alpha_1$-antitrypsin, is the most abundant plasma serine protease inhibitor (serpin). It is best recognized for inhibiting neutrophil elastase in the lungs. This study addresses a challenging question of whether, in addition to its inhibitory activity, $\alpha_1$-PI may also possess non-inhibitory functions, such as binding of non-peptide exogenous ligands, as reported for non-inhibitory serpins. Among putative ligands of bio-medical interest examined in this study, *all-trans* retinoic acid (RA) appears to be the most prominent candidate for $\alpha_1$-PI. RA exhibits multiple biological activities. Moreover, its recently proposed role in the regeneration of the lung tissues destroyed by elastase, may complement and enhance the efficiency of currently used $a_1$-PI therapy. The results of our study strongly suggest that $a_1$-PI does bind RA in vitro to non-covalent complexes of up to 2 moles of RA per one mole of the protein. To the best of our knowledge, this is the first report that

provides experimental evidence of the binding between RA and $\alpha_1$-PI. The insights gained from our findings will be discussed in context of the interactions of $a_1$-PI with (1) small non-peptide ligands that may have a potential to prevent the undesirable aggregation of this protein, and (2) small molecules of pharmaceutical interest that can be utilized towards the development of bi-functional drugs for combination therapy.

## Arabino-Galactan Proteins from *Pistacia lentiscus* var. *chia*: isolation, characterization and biological function

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Arabino-Galactan Proteins (AGPs) were isolated from Chios mastic gum (CMG) by using a buffer containing 0.1 M NaCl, 20 mM Tris-HCl, pH 7.5. Protein analytical methods combined with specific procedures for carbohydrate characterization, indicated the presence of protein backbone highly glycosylated. In particular, staining of the electrophoretical separated molecules by Yariv reagent, revealed the existence of arabinose and galactose and such a modification is characteristic for AGPs.

After extensive dialysis of the isolated extracts against water and atomic absorption, experiments evidenced the existence of zinc ions that are probably covalently bound to the AGPs. By using anion-exchange chromatography, capillary electrophoresis, colorimetric methods and GC-MS was found that the extracts were separated in three major populations (A, B, and C) which were consistent with their respective negative charge content, namely uronic acid. The characterization of neutral sugars that was investigated with GC-MS showed the existence of arabinose and galactose in different amounts for each group.

Experiments concerning the inhibition of growth of Helicobacter pylori in the presence of AGPs, as it is shown for other CMG constituents, showed that the extracts of at least 1.4 g CMG affected the viability of the bacterium. Whether the AGPs provoke abnormal morphologies of *H. pylori*, as it is reported for the total CMG or for O-glycans that possess terminal $\alpha$1,4-linked N-acetylglucosamine and are expressed in the human gastric mucosa, is not evidenced and it has to be further elucidated.

## HP-NAP protein activates neutrophils by its C-terminal region even without dodecamer formation which is prerequisite for DNA protection: novel approaches against *H. pylori* inflammation

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*Helicobacter pylori* Neutrophil Activating Protein (HP-NAP) protects DNA from free radicals as dodecamer by its ferroxidase activity without however binding to it.

By replacing His25, His37, Asp52 and Lys134, that are located within the ferroxidase site, with Ala, a total loss of ferroxidase activity, dodecamer formation and DNA protection in environments rich in free radicals is observed.

Molecular Dynamics Simulations revealed that dimer formation is highly unlikely following mutation of the above amino acids, since the ferrous ion is not attracted equally strongly by both subunits. These findings indicate that iron plays an important role in the conformation of HP-NAP by initiating the formation of stable dimers that are indispensable for the ensuing dodecamer structure.

Very surprisingly, neutrophil activation is stimulated by structural elements that are localized within the C-terminal region of both HP-NAPmut and dodecamer HP-NAPwild type. In particular, it was found that the dodecamer conformation is not necessary for activation and that helices H3 (Leu69-Leu75), H4 (Lys89-Leu114) or the linking coils (His63-Thr68 and Thr76-Ser88) are critical in stimulating neutrophils activation.

## Discovery and validation of protein and peptide biomarkers with novel mass spectrometry based workflows

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There is a critical lack of validated early biomarkers for most conditions and diseases. Early diagnosis does enable treatment of less severe disease states, the use of less invasive techniques and could potentially reduce the costs of healthcare systems.

Biomarker discovery and verification/validation are two distinct workflows. During the discovery phase, a relatively small number of samples with a high number of potential biomarker candidates are screened. The high-throughput provided by the iTRAQ$^{TM}$ reagent strategy allows for simultaneous analysis of such samples. Once biomarker candidates have been identified with initial statistical significance, these have to be validated. This validation workflow involves analyzing a large number of samples with a relatively small number of candidates to establish the biological significance of the biomarker candidates. Rather than switching to immunological techniques for this validation step, we suggest a mass spectrometry based approach. This orthogonal strategy is a novel targeted, high throughput quantitative multiplexed multiple reaction monitoring (MRM) approach. The approach relies on assay development using a combination of MRMs to target specific peptides identified in discovery, followed by MS/MS to confirm that the quantitative MRM signal results from the target peptide.

Several published examples of discovery and validation of protein and peptide biomarkers using these mass spectrometric workflows will be presented.

## Do proteases accept 1,2-amino ketones as nucleophiles in enzymatic synthesis?

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The advantageous use of proteases as biocatalysts for specific formation of peptide bonds is currently well established. Protease-catalysed coupling reactions with protected amino acid or peptide derivatives may be carried out under mild conditions like room temperature and neutral pH value.

The thiol-endopeptidases papain, bromelain and ficin are natural products of *Carica papaya*, *Ananas comosus* and *Ficus carica*, respectively. All show a primary specificity for aromatic or bulky aliphatic moieties in the $P_2$ position. Hydrophobic amino acid derivatives are the best nucleophiles to bind to the $S_1'$ position of the proteases, especially papain.

The enzymatic synthesis of *Z*-L-aminoacyl-antipyrine amides from *Z*-protected amino acid esters and 4-aminoantipyrine (AAP) has been already accomplished by utilising papain in aqueous-organic and biphasic media as well as in suspension. Product yields of 80 and 68% for *Z*-Gly-AAP and *Z*-Ala-AAP, respectively, could be obtained.

Our further experiments demonstrate that the general catalytic potential of thiol-endopeptidases with regard to *N*-components is broader than known so far. Thus, these proteases are able to catalyse the reaction of *N*-protected amino acid esters and *N*-protected amino acids with the heterocyclic 1,2-amino ketone 4-aminoantipyrine. There are so far no examples in literature for application of AAP in enzymatic synthesis with bromelain and ficin.

## Spectroscopic and structural approach to search smallest active analogue of an antimicrobial peptide from frog

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Recently, antimicrobial peptides have become a potential source of new antibiotics to combat the increasing emergence of drug-resistant bacteria. Several antimicrobial peptides such as magainin, a 23-residue antimicrobial peptide, have been successful in pharmaceutical and commercial development. Six kinds of peptides, named gaegurins (GGNs), 24–37 residue peptide, have been isolated from the Asian frog *Rana rugosa*. Gaegurin 5 is a 24-residue, membrane-active antimicrobial peptide. We recently reported the antimicrobial activities of two novel undecapeptides derived from an inactive N-terminal fragment (residues 1–11) of gaegurin 5. The anticancer activities of the two antimicrobial undecapeptide analogues were additionally identified. The relationships between their structural properties and biological activities were assessed by characterizing the fundamental structural determinant for the basic membrane interaction. The circular dichroism and nuclear magnetic resonance results revealed that in a membrane-mimetic environment, the active peptides adopt a more stabilized helical conformation than that of the inactive fragment, and this conformation conferred an overall amphipathicity to the active peptides. Therefore, the most decisive factor responsible for the activity and selectivity could be the intramolecular amphipathic cooperativity, rather than the amphipathicity itself. Especially, the tryptophan residue of the active peptides seems to play a crucial role at the critical amphipathic interface that promotes and balances the amphipathic cooperativity, by stabilizing both the hydrophilic and hydrophobic interactions with the membrane.

## Different acidic hydrolysis methods of proteins in order to determine the enantiomers of tryptophan

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We have previously elaborated a method for the resolution and determination of tryptophan enantiomers subsequent to a precolumn derivatization with o-phthaldialdehyde/1-thio-β-D-glucose tetra-acetate (OPA/TATG) using high performance liquid chromatography. For the determination of tryptophan bonded in proteins we need an acidic hydrolysis method giving an appropriately high recovery rate, as during the commonly employed hydrochloric acid hydrolysis Trp decomposes quantitatively, whereas alkalic hydrolyses result in considerable racemization. In our earlier investigations we achieved good results in determination of total Trp contents using mercaptoethane sulfonic acid (MES-OH) as an acidic hydrolysing reagent, therefore first this reagent was examined. In case of amino acid enantiomers, however, not reacted excess of MES-OH prevents the separation, in its presence OPA/TATG derivatives of the amino acids do not form, presumably because the MES-OH forms a derivative with OPA and the amino acid instead of TATG. We tried several methods for the removal of MES-OH and transformation of its sulfhydryl group, respectively. By converting of MES-OH into copper mercaptide and performic acidic oxidation of the sulfhydryl group, respectively, the expected results could not be achieved. Excess of MES-OH was converted into carboxymethyl derivative with iodoacetic acid, so a small portion of tryptophan could transform into the OPA/TATG derivative. Subsequently, *p*-toluenesulfonic acid (PTS) hydrolysis method, giving the second highest recovery rate for tryptophan among the acidic hydrolysis methods, was examined. In the presence of PTS the derivatization took place appropriately, however, using only PTS as hydrolysing reagent, recovery rate for the tryptophan was low, around 45–50%. Racemization occuring during the hydrolysis is not considerable. If also tryptamine is added in order to protect the indole ring of tryptophan during the hydrolysis, the derivatization does not take place, presumably due to the amino group of tryptamine. Trp amounts obtained after hydrolyses carried out without protective agent are sufficient for the determination of D/L Trp ratio, however. Assumed that decomposition of Trp is of the same degree for both D- and L-enantiomers, the amount of the individual Trp enatiomers can be calculated based on total Trp contents and the enantiomer ratio. We used this method for determination of D- and L-Trp contents of pork samples roasted in different manners and for different durations.

## On the possible involvement of mouse rpS5 phosphorylation in ribosomal biogenesis and cellular differentiation

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Ribosomal proteins are integral components of the ribosome subunits involved in protein biosynthesis. Also, evidence exists to indicate that several ribosomal proteins regulate various other cellular functions, suggesting that these proteins may act as multifunctional entities. We have found that induction of differentiation of MEL cells with chemical inducers is associated with down-regulation of rpS5 gene, a gene encoding the mouse S5 ribosomal protein. To further explore the rpS5 role in cell differentiation, MEL cells were transfected with the mouse full-length rpS5 cDNA and a subline that constitutively express this cDNA was established. Transfected MEL cells induced by several inducers (DMSO, HMBA, UDP-4) exhibited a delay in the onset of differentiation without any significant change in their cell proliferation as compared with mock-transfected and parental MEL cells. By trying to analyze rpS5 function into this process, we found that rpS5 possesses a domain within the first 37aa at its $NH_2$-terminal region that functions in vitro as a substrate for phosphorylation by casein kinase II (CK II) probably acting as PEST region. This is in agreement with observations showing that rpS5 belongs to the early-assembled proteins during ribosomal biogenesis and its nucleolar association may be due to specific functional activities e.g. reactivation or modification of specific functional regions. By following these observations, we transfected HeLa cells with the eukaryotic expression pEGFP-C1 vector that bears either the wild-type (GFP-S5) or a truncated form of rpS5 lacking the $NH_2$-terminal region (GFP-TrS5) all tagged with the GFP protein (a gift from Dr. S. Huang) and then analyzed the cells by confocal microscopy in order to explore

protein localization intracellularly. Our results show that GFP-S5 is mostly accumulated into the nucleoli, while its truncated form GFP-TrS5 protein that lacks the 37aa portion is found to be diffused into the nucleoplasm. This early observation suggests that the phosphorylation of rpS5 would be probably a critical modification for its function on ribosomal biogenesis and localization into the nucleolar compartment. Whether these changes in rpS5 protein localization into the nucleolar compartment are involved in the delay of onset of MEL cell differentiation is an interesting proposal that needs further investigation.

## Advances in the o-phthalaldehyde derivatization of amino acids and amines for their high performance liquid chromatographic analysis

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Recently, the special behavior of the *o*-phthalaldehyde (OPA) derivatives of amino acids (AAs) and amines (As), applying various SH-additive containing reagents, has been investigated and documented.

Due to basic studies, optimum conditions could be achieved with the OPA/ET/FMOC reagent: maximum stability of the OPA labeled primary amino groups and excellent response of the double labeled, primary and secondary amino group containing spermidine and spermine.

The application of the procedure to biological samples proved to be a challenging task.

This paper shows the quantitation of 32 compounds, including the classical amino acids, γ-amino butyric acid, proline, OH-proline, aliphatic mono- and diamines, histamine, agmatine and tyramine, in a single run, present in biological tissues of medical interest, applying a simple sample preparation method:

(i) Without any preliminary neutralization, in the presence of perchloric acid used for deproteinization,
(ii) – furnishing,
– side reaction free derivatizations,
– stable and high response values,
– fast, selective chromatographic elution protocol, and,
– quantitative recovery from the matrix.

Optimum analytical conditions will be characterized by the reproducibility of at least three separate derivatizations of various amounts of all 37 OPA-ET-(FMOC) derivatives, subsequently to the sample preparation method, characterized by the RSD percentages of determinations.

## The physiologic effect of human thioesterase II (hTE) inhibition in the CD4 cell surface down-modulation by HIV-1 Nef

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The down-modulation of CD4 receptor expression is one of the most important events during the HIV-1 infection. Among the three viral proteins involved in this process Nef, Env and Vpu, the first one is the most relevant. Results obtained by our group and others showed a clear relationship between the virus mediated receptor down-modulation, the increasing of infectivity and viral replication of HIV-1, suggesting a pathogenic role in this phenomenon including disease progression. More recently, we provided proof-of-concept that specific inhibition of Nef mediated CD4 down-modulation could be an efficient therapeutic strategy. Based on these results and aiming to identify new targets and therapeutic strategies, we investigated the physiological role of the human tioesterase II (hTE) in the down-modulation of CD4 receptor mediated by Nef. The hTE was described as one of the main cellular partners of Nef in this process, but the CD4 down – modulation mechanism involving hTE remains unclear. Protein palmitoylation or, more specifically, *S*-acylation enzyme activity is a reversible post-translational lipid modification. Palmitoyl-protein thioesterase is a lysosomal long-chain fatty acyl hydrolase acting by removing fatty acyl groups from modified cysteine residues in proteins. Recently showed that hTE greatly enhances its enzymatic activity by association with Nef. The implication of the specific interaction with Nef function in HIV-infected T cells is not so clear. Using the interference RNA (RNAi) mechanism, as a molecular tool to block the expression of cellular and viral proteins expression involved in this phenomenon, we observed that hTE plays a relevant role on Nef-CD4 modulation, differently as assigned by previous works. We showed here the hTE participation in the regulation of CD4 cell surface levels possibly by a depalmitoylation process of CD4 molecules, resulting in the independent Nef internalization. In 293T cells co-transfection experiments with CD4 and Nef we can see approximately 66% of CD4 cell surface down – modulation, and when we used a RNAi against the previously verified down – modulation process was reduced in 30%, showing that blocking hTE expression can reduce the CD4 down – modulation by Nef.

However, when the cell is co-transfected only with CD4 and interference RNA against the, the CD4 level increases by 63% approximately showing that hTE have a great and important function in the CD4 surface levels renewal, but Nef probably has other pathways of CD4 down modulation as the hTE reduction by iRNA is not enough for all depletion by the Nef effect in the CD4 levels variation.

We hypothesize that hTE has an important function in the palmitoilation of CD4. The CD4 molecule amount and its turn over is sensible for inhibition by hTE expression. These data are supported by experiments blocking the expression of hTE which do not alter the pattern of CXCR4 expression (This receptor is not palmitoyled) therefore we can not see the increasing or decreasing of CXCR4 expression levels.

The hTE enzymatic action on Nef-NL43 transfected cells showed a probable synergism action between Nef and hTE. Probably, this synergism is involved in the palmitoylation and depalmitoylation of CD4 membrane receptor. The confirmation of these preliminary findings as well as, a more thoroughly understanding of the general biological consequences arising from the interaction between hTE and Nef, requires additional studies.

## DNA and protein isolation from the 290 million year-old amphibian *Discosauriscus austriacus* and applications of biotechnology in palaeontology

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During the last years, DNA and protein isolation from fossils has created a previously undiscovered source of interest in the fields of molecular biology, genetics and palaeontology. Fossil DNA sequencing seems to be problematic, while protein isolation tends to provide more promising results. Here we report the DNA and protein preservation in the prehistoric animal *Discosauriscus austriacus*, from the Lower (Early) Permian Period of Czech Republic. *Discosauriscus* was a small sized (∼30 cm) amphibian, a well adapted land-dweller with wide jaws and the general appearance of a crocodile. It belonged to the family Discosauriscidae in the order of Seymouriamorpha, within the clade Reptiliomorpha (Anthracosauria). Sey-

mouriamorpha were primitive amphibians that lived in the Permian period (290-245 million years ago). Little is known about these creatures, like the fact that they were predators, as can be judged from the presence of sharp, conical teeth. Juveniles are considered to have adopted an aquatic lifestyle, whereas adults were terrestrial. Their phylogenetic position stays also unresolved, with many palaeontologists to believe that they lay somewhere near the stem clade of the reptiles.

In our experiments we used bone pieces from the animal. The fossil was exceptionally preserved, having big amounts of original bone (not stone copy). For control we used a much younger neolithic sample from a mammal (a wild pig – species *Sus scrofa*). The procedure contained mixing of RNA Blue with chloroform, followed by precipitation of DNA with pure ethanol in RNA Blue reagents. Centrifugation, precipitation of the supernatant, drying and dissolution of the protein pellet, were the next steps. Gel electrophoresis and staining with "Coomassie" Blue R-250 were used to visualize the polypeptide bands appeared in the sample. For the DNA analysis we applied PCR reaction for amplifying short sequences (62 bp) from mitochondrial genome (primers were chosen according to conservative genome parts from modern amphibians and reptiles).

The bone analysis of *Discosauriscus austriacus* showed a small amount of DNA (few ng), most probably a crushed nucleotide. No short sequenced DNA was detected. In protein analysis we found two main bands between 110–160 kD, but it is possible to exist other proteins too. Taking into consideration recently published data, reporting protein sequences from the 600,000 year-old mastodon *Mammut americanum* and from the 68 million year-old dinosaur *Tyrannosaurus rex* and on the base of molecular weight, we assume that the preserved protein is a kind of collagen, but further analysis is needed, in order to clarify its type and sequence.

The *Discosauriscus* protein presented here is claimed to be the oldest preserved protein that has been found until now. Comparing protein and DNA sequences, provides supporting evidence for the taxonomic status and relationships between recent and extinct animals. Thus, its phylogenetic significance points the beginning of a new era for the science of Palaeontology, combining techniques and knowledge obtained from Biotechnology.

## Critical steps in preparation of affinity immunosorbents

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New diagnostic methods reliant on biochemical biomarkers of disease hold the potential to provide effective measures of biological activity and markers that account for clinical benefit of the therapy. Detection of biomarkers is often based upon the use of specific antibodies immobilized on some type of support. The significance level of bioaffinity sorbents is in their versatile and potential utilization.

This principle of bioaffinity chromatography was used in many applications, i.e. isolation and purification of biomolecules, detection and identification of biomarkers, selective decontamination of mixtures and use of immobilized enzymes for modification of target biomolecules.

We focus on neurodegenerative Alzheimer disease and its possible markers, especially the immunospecific supports dedicated to capturing and pre-concentration of target Aβ peptides and Tau proteins. The goal in the construction of an efficient immunosorbent is to immobilize the antibody to the solid-phase support without adversely affecting the antibody's function to capture antigen. The choice of support plays an important role, generally low non-specific sorption and chemical and physical stability are preferred. Rapid and simple manipulation and possible application into microfluidic device are facilitated by using magnetic supports.

The aim is in particular high steric accessibility of the active sites, high operational and storage stability of binding activity, and low non-specific sorption of protein to minimize the contamination of the target antigen. Therefore, suitable immobilization method of the specific anti-Aβ and anti-Tau antibodies with use of newly developed magnetic and non-magnetic carriers with controlled non-specific sorption will be described. In addition, new sights in quantification of isolated markers will be implemented.

## Oxidative folding, assembly and function of the small TIM mitochondrial chaperone

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Nearly all cellular processes are carried out by nanoscale assemblies of proteins that make up most of the dry mass of a cell. In addition, the architecture and function of all cells depends on the correct spatio-temporal targeting of proteins to a site of biological function different to where they are made. The magnitude of these targeting events is reflected by the fact that up to 50% of the human proteome consists of membrane proteins or proteins that have to be translocated across an intracellular membrane. It is hence not surprising that more than 50% of the current Pharma Industry targets are membrane proteins. Our focus is on the characterization of the nanostructure and function of mitochondria, the organelles that control several key cellular processes in normal physiology (ATP production, metal ion homeostasis) but also medical conditions (neurological disorders, cancer) and apoptosis. As nearly all mitochondrial proteins (on average 10–15% of the cell proteome) are imported from the cytosol, these organelles have elaborate and versatile protein machineries (called translocases) to cope with these import processes. These are dynamic assemblies that are modular (consisting of more than 30 protein subunits) that are very precisely coordinated. Our recent work impinges on three main areas: (i) how membrane proteins are specifically targeted to the mitochondrial inner membrane, (ii) the discovery of the novel mitochondrial TIM9-10 chaperone system that facilitates membrane protein assembly, (iii) the molecular basis of a novel oxidative folding pathway that operates in mitochondria. The latter findings strongly argue for an expanding view of de-novo disulfide bond formation in all eukaryotic cells. The specific mitochondrial oxidoreductase system that we discovered consists of the oxidase protein Mia40 and the flavin-linked sulfhydryl oxidase Erv1. We have demonstrated a cascade of electron transfer in this process from the small Tims via Mia40 to Erv1 and finally onto Cytochrome c. This pathway illustrates an important link between the protin import and folding process and the respiratory chain in mitochondria. Our approach ranges from in vivo studies (intact cells, about 6000 proteins), through *in organello* studies (isolated mitochondria and subcellular structures, about 900 proteins) down to in vitro studies (isolated single proteins and reconstituted molecular machines). Potential applications of this research in nanobiotechnology and nanomedicine will be discussed.

## Methods and updated instrumentation for proteomics

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Genome analysis yields information of the static state of the inherited information. In order to understand the dynamic cell situations, e.g. during cell differentiation and regulation or under disease conditions, the gene products, the proteins must be directly studied. Proteome studies allow analysis of the entire protein content of a cell or tissue at individual

stages. Proteomics enhances our knowledge in cell differentiation, molecule trafficking and signalling, in translation, regulation and evolution. Proteomics work is being applied to study antibiotics resistant strains or human tissues of various brain-, lung-, and heart-diseases. It may cumulate in the identification of antigens for the design of new vaccines.

These advances in Proteomics have been possible through high resolution two-dimensional gel electrophoresis (2DE-)systems in combination with highly sensitive mass spectrometric approaches. The new 2DE-techniques can resolve up to 10,000 protein spots of entire cells or tissues. High protein resolution is well suited for the identification of protein modifications linked to protein functions. The new technologies provide studies in biochemistry, cell biology, molecular medicine and in drug design.

### Electrophoretic separation of amyloid beta peptides

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The main protein component of the amyloid deposits found in the brains of Alzheimer's disease patients is a peptide termed amyloid-β (Aβ or βA4). The amyloid-β peptides (molecular weight about 4.5 kDa) are approximately 42-amino acid residue peptides. Peptides deposition and aggregation are thought to be important pathological events in the formation of Alzheimer's disease plaque.

The concentrations of Aβ peptides and tau protein(s) in cerebrospinal fluid and/or blood belong to the candidate biomarkers supporting diagnose of Alzheimer's disease and they have been proven to fulfill the criteria for good diagnostic tests intra vitam.

Conventional SDS-PAGE system is not suited for separating of amyloid-β peptides Aβ(1-38), Aβ(1-40) and Aβ(1-42). Alternative electrophoretic techniques have been compared for their usefulness in separation analysis of peptides corresponding to amino acids (1-38), (1-40) and (1-42) of the amyloid-β sequence. The first system is the fundamental Tris-Tricine electrophoresis, while the second one employs acetic-urea-polyacrylamide gel electrophoresis denaturing system. We have also applied the urea version of the bicine/bis-tris/Tris/sulfate SDS-PAGE to separate Aβ peptides. Here the clear resolution was achieved by addition of 8M urea to the separation gel and use aforementioned multiphasic buffer.

All electrophoreses procedures were performed using the Bio-RAD Mini PROTEAN 3 electrophoresis gel system and gels were stained using Coomassie Brilliant blue R-250 or by silver staining.

The separation by means of acetic-urea-PAGE was not sufficient enough. We proved that electrophoretic separation of these peptides is practicable by the bicine/Tris/SDS-PAGE system with the separation gels containing 8M urea. Under these conditions the longer peptide migrated faster than the one ending at amino acid 38 or 40. All peptides are resolved as cleraly separated sharp bands, even on minigels which we used. A good separation of these peptides could also be achieved with 8 M urea added on to the Tris-Tricine system, but the bands are slightly misshapen and clouded.

## Sports Exercise and Health

### Effect of branched chain amino acids on protein signalling in resting and exercising muscle

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**Background:** The molecular mechanisms responsible for the anabolic effects of resistance exercise and nutrition probably involve activation of the Akt-mTOR-p70S6 kinase ($p70^{S6k}$) pathway, which is believed to play a major role in regulating protein synthesis. In a previous study, a single bout of resistance exercise did not affect mTOR or $p70^{S6k}$ phosphorylation, only in combination with intake of branched-chain amino acids (BCAA) was an increase found 1 and 2 h after exercise. However, it was not possible to separate the effects of exercise and BCAA on $p70^{S6k}$. Hence, the aim of the present study was to try and separate the effect of BCAA from the exercise-induced effect. To study this, subjects performed resistance exercise with one leg and the effect of BCAA intake on phosphorylation of Akt, mTOR and $p70^{S6k}$ was measured in both the exercising and resting leg.

**Methods:** Four healthy young subjects, one male and three females, performed resistance exercise with one leg. The exercise protocol consisted of 4 sets of 10 repetitions at 80% of 1RM followed by 4 sets of 15 repetitions at 65% of 1 RM with the dominant leg. Subjects were supplemented with a BCAA solution (45% leucine, 30% valine, and 25% isoleucine) or flavoured water (placebo) using a randomised cross-over design. Muscle biopsies from the vastus lateralis were taken before, immediately after and 1 h after exercise. Muscle biopsy specimens were freeze-dried, homogenized and analysed for Akt ($Ser^{473}$), mTOR ($Ser^{2448}$) and $p70^{S6}$ kinase ($Ser^{424}$/$Thr^{421}$ and $Thr^{389}$) phosphorylation by Western blot technique. Proteins were visualised and quantified by enhanced chemiluminescence detection.

**Results:** Exercise caused a pronounced increase in $Ser^{424}/Thr^{421}$ phosphorylation of $p70^{S6k}$ in both trials, an increase that persisted for 1 h into the recovery period and was 3-fold higher in the BCAA trial. A small effect after exercise and a 3.7-fold increase 1 h after exercise was found in the resting leg in the BCAA trial. $Thr^{389}$ phosphorylation of $p70^{S6k}$ was unaltered immediately after resistance exercise in both trials, but was increased 5.4-fold in the exercising leg 1 h after exercise in the BCAA trial. The same pattern, although to a lesser extent, was seen in the resting leg; 1 h into recovery the $Thr^{389}$ phosphorylation of $p70^{S6k}$ was 2.7-fold increased when BCAA were ingested. A 2 to 3-fold increase in phosphorylation of mTOR on $Ser^{2448}$ was found 1 h after exercise in the BCAA trial; the increase was similar in the resting and exercising leg. A smaller increase was found 1 h after exercise in the placebo trial, 66 and 74% in the resting and exercising leg, respectively. Phosphorylation of Akt on $Ser^{473}$ was not influenced by exercise or BCAA supplementation at any time point.

**Conclusion:** Resistance exercise in combination with BCAA causes a more pronounced effect on $p70^{S6k}$ phosphorylation in human muscle than BCAA alone. In contrast, the effect of BCAA on mTOR phosphorylation was similar in the exercising and resting leg, suggesting that $p70^{S6k}$ is activated not only by mTOR but also via an alternative pathway. Furthermore, the effects of exercise and BCAA seem to be mediated via an Akt-independent pathway.

### The leucine metabolite HMB: mechanistic considerations and clinical outcomes in exercise performance

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β-Hydroxy-β-methylbutyrate (HMB) is a metabolite of leucine, which has a history of sports nutrition use, mainly for enhanced muscle mass

and strength gains. It is also utilized as a component of a medical nutritional product in the US, with claims around cancer- and AIDS-induced lean mass loss, as well as wound healing claims. HMB has also been shown to partially normalize protein synthesis and protein degradation in the skeletal muscle of cachectic mice. The mechanism of action has been elucidated in this model, and involves well-known signalling pathways involving NFκB, PKC and the ubiquitin-proteosome system, thus regulating protein degradation, as well as mTOR, the 70 kDa ribosomal S6 kinase ($p70^{S6k}$) and initiation factor 4E binding protein (4E-BP1) impacting protein synthesis. Thus, at least in this model of tumor-induced weight loss, HMB treatment was able to at least partially reverse the signalling events that result in rapid lean mass loss. Fortification with arginine and glutamine additionally impacted protein synthesis rates.

Intense physical training also places a significant stress on muscle tissues, and can result in production of a catabolic state with some similarities to the cachexia in cancer. Clinical studies have been conducted testing HMB supplementation in intense resistance training, with mixed results. A recent clinical study looked at a nutritional product containing HMB, glutamine, arginine and taurine to assess it is impact on training gains (strength and lean mass) as well as biochemical markers of muscle stress when subjects engaged in a rigorous resistance training program. Subjects on the product achieved significantly greater performance and lean body mass gains and fat mass losses. Additionally, cortisol and plasma CK levels were reduced, and testosterone levels increased in the supplemented group compared to the control product (isocaloric and isonitrogenous to the test product), indicating a greater anabolic response to exercise, and reduced exercise-induced muscle damage.

Protein homeostasis in skeletal muscle is affected by a number of stressors, and both protein synthesis and degradation rates can be impacted. Orally administered HMB attenuates protein degradation in muscle by preventing induction of the ubiquitin-proteasome proteolytic pathway as well as at least partially preventing protein synthesis shutdown, which involves mTOR signaling, similar to leucine. We will discuss the implications of these observations to the design of future trials in exercise performance, as well as the implications for other uses of this interesting compound.

## Muscular buffer capacity as a determinant of performance

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Intense muscle contractions result in large ionic changes and an increased non-mitochondrial ATP turnover, contributing to the accumulation of hydrogen ions ($H^+$). The removal of $H^+$ during intense skeletal muscle contractions occurs via a number of different transport systems and via intracellular buffering. Intracellular buffer capacity ($\beta m_{in\text{-}vitro}$) is typically estimated by titrating a muscle homogenate with a fixed acid and measures the contribution to physicochemical buffering by proteins, dipeptides (e.g., carnosine) and phosphates within the muscle. $H^+$ release is also facilitated by a number of transporters such as the monocarboxylate transporters (MCTs) and sodium bicarbonate co-transporter (NBC). A greater buffer capacity may help to delay fatigue and hence improve performance during repeated, high-intensity exercise. We have reported significant correlations between repeated-sprint ability (RSA) and muscle buffer capacity ($r = -0.68$). Furthermore, we have shown that despite being matched for total work, and producing similar increases in $\dot{V}O_{2max}$ (10–12%), interval training, when compared to continuous training, produces significantly greater improvement in $\beta m_{in\text{-}vitro}$ and RSA (4.5%). An increased anaerobic energy contribution to the latter sprints may be one mechanism by which increases in $\beta m_{in\text{-}vitro}$ contribute to improved RSA. While these results suggest that $\beta m_{in\text{-}vitro}$ may be an important attribute for RSA, we have recently observed significant improvements in RSA despite a significant decrease in $\beta m_{in\text{-}vitro}$ following very high-intensity training. Thus, further research is required to investigate the relationship between RSA and both intracellular and extracellular buffer capacity. It has been suggested that the local formation of $H^+$ within the skeletal muscle may be an important stimulus for adaptations of the muscle pH regulating systems. There is some support for this hypothesis as $\beta m_{in\text{-}vitro}$ has been reported to increase in response to high-intensity, but not moderate-intensity training. However, while a minimum accumulation of $H^+$ may required to improve $\beta m_{in\text{-}vitro}$, it appears that the degree of $H^+$ accumulation is not the main factor stimulating further improvement in $\beta m_{in\text{-}vitro}$ and the synthesis of transporter proteins. While initially counter-intuitive, too large an accumulation of $H^+$ during training may, in fact, have a detrimental affect on the pH regulatory systems within the muscle. Recent findings from our laboratory have shown that, despite being matched for training intensity and total work performed, interval training with short rest periods (1 min; end-exercise pH ≈ 6.81) resulted in a consistent decrease in $\beta m_{in\text{-}vitro}$, while interval training with longer rest periods (3 min; end-exercise pH ≈ 6.90) resulted in a consistent increase in $\beta m_{in\text{-}vitro}$. We hypothesised that this may have been due to the previously reported affects of acidosis on protein synthesis and/or the cumulative effect of repeated, transient decreases in $\beta m_{in\text{-}vitro}$ following intense exercise. These acute decreases in $\beta m_{in\text{-}vitro}$ appear to largely be attributable to decreases in protein buffering.

## Effect of exercise training on protein signalling

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Regular exercise training is known to cause adaptations within skeletal muscle that depends on the type of exercise. For example, resistance exercise increases the synthesis of contractile proteins leading to an increase in muscle mass and strength, whereas endurance exercise increases the synthesis of mitochondrial proteins leading to an increase in fat oxidation and endurance. The molecular mechanisms behind these adaptations are not known, but are likely to be mediated through phosphorylation and activation of enzymes that regulate translation initiation. A number of studies in experimental animals have linked phosphorylation of protein kinase B (PKB or Akt), the mammalian target of rapamycin (mTOR) and the subsequent activation of p70S6 kinase ($p70^{S6k}$) to increased protein synthesis and muscle growth. However, such a relationship has not yet been established in human muscle. Various results have been reported concerning the effect of resistance exercise on the Akt-mTOR-$p70^{S6k}$ pathway, which probably can be explained by different types of contractions, work load and duration, the subjects' training status and nutritional supply. One single exercise session induces an increase in mTOR and $p70^{S6k}$ phosphorylation in relatively untrained subjects but not in resistance-trained subjects, except when exercise is performed in combination with nutritional intake. Furthermore, maximal eccentric (lengthening) but not submaximal eccentric exercise or maximal concentric (shortening) exercise is found to activate $p70^{S6k}$, supported by a parallel increase in phosphorylation of the ribosomal protein S6, suggesting a combined effect of muscle tension and contraction type. Most studies report that when resistance exercise is performed in a fasting state, Akt phosphorylation remains unchanged in the early recovery period, suggesting that mTOR and $p70^{S6k}$ can be activated via alternative pathways. In contrast, sustained endurance exercise increases Akt and mTOR phosphorylation but has no effect on $Thr^{389}$ phosphorylation of $p70^{S6k}$, only a partial phosphorylation of $p70^{S6k}$ is found but no activation of the kinase. Instead there is a decrease in the phosphorylation of the elongation factor eEF2, suggesting an activation of translation elongation and possibly protein synthesis in the recovery period after sustained endurance exercise. The lack of $p70^{S6k}$ activation suggests that translation initiation is activated via alternative pathways, possibly via activation of eukaryotic initiating factor 2B.

## Some amino acids associated with physical and mental fatigue

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Physical and mental fatigue, particularly in relation to immunodepression and the incidence of illness have been of interest to my group for many years. The amino acids of particular interest have been glutamine (Gln), the branched chain amino acids (BCAA), tryptophan (Trp), glutamate (Glu) and alanine (Ala). Our studies have investigated these in endurance athletes, in patients after major surgery or stroke or with chronic fatigue syndrome, and in short-term sleep deprivation. In addition to aspects of immune function, mood profiles were assessed. Trp was investigated in relation to fatigue using functional magnetic resonance imaging of the brain. After prolonged, exhaustive exercise, individuals involved in our studies had a notably high incidence of upper respiratory tract infections (URTI).

Clinical studies indicate that Gln feeding contributes to the alleviation of infections and improved gastrointestinal function. A reduction in URTI in endurance athletes has been attributed to supplementary feeding with Gln or the Gln precursors, BCAA. However, the situation is not clear with regard to the precise effect on the immune system of Gln, which is decreased after prolonged, exhaustive exercise. Glutaminase has recently been observed to be present on the secondary granules of human neutrophils, indicating that these cells utilize Gln. The transient immunodepression observed after prolonged, exhaustive exercise may be sufficiently short term for the body to recover rapidly and effectively with adequate nutrition. Nevertheless, repeated bouts of exhausting exercise undertaken in close succession, may lead to more chronic immunodepression for which rapid Gln repletion might prove beneficial.

In addition to being precursors of Gln, the BCAA have been discussed as having an important role in the central fatigue hypothesis, as has tryptophan. The plasma glutamine/glutamate ratio has been suggested as a marker for unexplained underperformance syndrome (also known as overtraining). Alanine may be a marker of fatigue.

## Kinetics of creatine ingested as a component of a food bar

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The purpose of the present study was to test if the consumption of creatine incorporated in food bars modifies creatine plasma kinetics, erythrocyte retention and loss in urine and in feces when compared with its ingestion in the form of an aqueous solution (AS). Seventeen healthy young men ingested 2 g creatine either in the form of AS, or incorporated in a protein-(PP) or in a beta-glucan-(BG) rich food bar. Kinetics of plasma creatine was measured during 8 h and urinary excretion during 24 h. Then, the subjects received three times a day the same treatment for one week at the end of which creatine contents were determined in erythrocytes and in feces ($n = 4$ for feces). All subjects were experienced with the three treatments interspaced by a wash-out of four weeks. Absorption of creatine was slowed down by 8-fold in the presence of BG ($P < 0.001$) and by 4-fold with PP ($P < 0.001$) whereas the velocity rate constant of elimination and the area under the curve were not modified. Urinary loss of creatine in the first 24 h following ingestion was $15 \pm 1.9\%$ in AS and $14 \pm 2.2\%$ in PP conditions (NS) whereas it was only $8 \pm 1.2\%$ with BG ($P = 0.004$). Increase in creatine concentration in erythrocyte was similar whatever the form of creatine ingested. Creatine seems to be totally absorbed since no creatine or creatinine was detectable in feces. No side effect was reported. Ingestion of creatine combined with BG facilitates its retention by slowing down its absorption rate to a sufficient extent to reduce urinary excretion.

## Short-term changes in gene expression by creatine and resistance exercise in human skeletal muscle

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Having previously shown that creatine supplementation with or without subsequent resistance exercise has no short term effects on muscle protein synthesis. we were interested in testing the hypothesis that there might be longer term effects which are preceded by alterations in gene expression. The present study was designed to assess short-term changes in gene expression and activation of signalling pathways induced by a single bout of exercise after creatine supplementation. Nine subjects received either creatine or a placebo ($3 \times 7$ g) for 5d in a double-blind fashion before undergoing 4 muscle biopsies: at rest, immediately after exercise, and 24 and 72 h later. All biopsies were taken in the fasted state. The exercise consisted in 10 sets of 10 repetitions of one leg-extension at 80% of 1-RM. Previous creatine supplementation decreased the phosphorylation state of PKB on Thr308 at rest by 60% ($P < 0.05$) and the phosphorylation state of 4E-BP1 by 30% 24 h post-exercise ($P < 0.05$). Creatine increased collagen 1($\alpha$1), GLUT-4 and MHCI (myosin heavy chain I) mRNA at rest by 250% ($P < 0.05$), 45% ($P < 0.05$) and 80% ($P < 0.05$), respectively, and MHCIIA (myosin heavy chain IIA) mRNA immediately after exercise by 70% ($P < 0.05$). Immediately after exercise, the mRNA transcription of MAFbx (muscle atrophy F-box), MHCIIA, PGC-1$\alpha$ (peroxisome proliferator-activated receptor gamma coactivator-1$\alpha$) and IL-6 (interleukin-6) were up-regulated (+60% to +350%, $P < 0.05$) and the phosphorylation state of p38 was increased both in the sarcoplasm (12 fold, $P < 0.05$) and in the nucleus (25 fold, $P < 0.05$). At the same time, the phosphorylation state of PKB (protein kinase B) on Thr308 and 4E-BP1 (eukaryotic initiation factor 4E-binding protein) on Thr37/46 was decreased by 50 and 75%, respectively ($P < 0.05$). Twenty-four hours post-exercise, MAFbx, myostatin and GLUT-4 mRNA expression decreased below pre-exercise values (−35%−−50%, $P < 0.05$); calpain 1 increased by 70% 72 h post-exercise ($P < 0.05$) and at no other time. In conclusion, 5d of creatine supplementation affects the expression of certain genes to a limited extent. The creatine-induced changes are unlikely to enhance those related to exercise alone. There seem to be little or no interaction between an increase in availability of creatine and other putative molecular changes leading to hypertrophy such as gene expression or anabolic signalling pathways.

## Taurine and muscle activity

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Taurine, 2-amino ethanesulfonic acid, is a two carbon chain with an amino terminal function on one end and a sulfonic function on the other end. At physiological pH, most of the taurine molecules in solution are in a highly charged zwitterionic form which prevents their passage through the membrane lipid bi-layers. Taurine is the most abundant amino acid in many mammalian tissues with a typical concentration around 20 mM in

human skeletal muscle. In plasma and in interstitial fluids, taurine concentrations reach only the çmolar range. This makes necessary the existence of an active transport system to build up concentration gradients between cellular and extracellular spaces. A taurine transporter clone (*taut*) localized on chromosome 3 has been isolated from human tissues. It is a member of the $Na^+/Cl^-$-dependent transporters, the stoichiometry of which is 2:1:1 for $Na^+/Cl^-$/taurine, respectively.

Although skeletal muscles store about 70% of the whole body taurine, the function of taurine during muscle activity remains uncertain. There is considerable evidence that contracting muscles release taurine leading to a higher plasma taurine concentration at the completion of endurance events such a marathon or a 100-km run. This phenomenon is probably a consequence of the well known osmoregulatory function of taurine, but the effects on muscle performance are speculative. During contractions of isolated muscle in vitro, the larger the taurine release is, the larger the loss of tension. On the other hand, changes in muscle taurine content of mice supplemented with taurine or with guanidino-ethanesulfonic acid (GES) do not affect maximal tension of EDL and soleus muscle suggesting that taurine is not directly responsible for the force decline during prolonged exercise. Nevertheless, due to the multiple actions of taurine on ion channels and transport system, depletion potentially impairs excitation-contraction coupling and $Cl^-$ conductance leading to muscle fatigue.

In rat, pre-administration of taurine allows to maintain the taurine concentration in skeletal muscle during exercise and improves endurance performance possibly via inhibition of oxidative stress. A lower DNA damage induced by exhaustive exercise in white blood cells has been reported in humans supplemented with taurine (6 grams/day for 7 days). Moreover, taurine supplementation was associated with an improved exercise performance. But, to date, there is no evidence that oral taurine supplementation increases muscle taurine content in humans.

## The effects of diet and training on muscle carnosine

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pH Homeostasis in muscle during intense exercise (athletic, or in nature, for the purposes of hunting or escape) or with prolonged exposure to hypoxic conditions (diving) is maintained by export of $H^+$ from muscle and by physico-chemical buffering. Intracellular buffering provides the first defence against local increases in $H^+$ with increased carboxylate anion formation. However, few compounds in muscle have pKa's in the critical range, pH 6 to 7, and at the same time are present in sufficient amounts to enable them to contribute significantly to physico-chemical buffering. Of these the most important in human muscle are carnosine (pKa 6.83), ATP, and (during exercise) inorganic phosphate (pKa 6.88) and organic phosphates such as glu-6-P, fru-6-P, glyc-3-P (pKa's 6.1–6.8), with lesser contributions from $HCO_3$ present in muscle at the start of exercise and protein bound histidine. In species adapted for high speed running or prolonged diving, carnosine and methyl derivatives of this may occur in exceptionally high concentrations exceeding even the level of carbohydrate.

Carnosine is a dipeptide of β-alanine (β-Ala) and histidine occurring in high concentrations in muscle (M-Carn).

a) **Normal content:** Vegetarian humans have approximately half the M-Carn content of meat eaters. Vegetarians: UK lacto-ovo-vegetarian/ovo-vegetarian ($n=6$; $28.7 \pm 10.0$ yrs; F; 5 active/1 sedentary) $12.9 \pm 2.8$ mmol · kg$^{-1}$ dm. Meat eaters: UK pescetarians ($n=2$; $19.5 \pm 2.1$ yrs; 1 F/1 M; active) $17.3 \pm 2.5$ mmol · kg$^{-1}$ dm; Korean cyclists ($n=12$; $19.9 \pm 1.2$ yrs; M; endurance trained) $22.3 \pm 4.6$ mmol · kg$^{-1}$ dm; UK Sports Science students ($n=39$; $26.5 \pm 5.4$ yrs; M; active) $23.3 \pm 5.4$ mmol · kg$^{-1}$ dm; Vietnamese PE students ($n=12$; $22.0 \pm 2.4$ yrs; M; active) $26.5 \pm 4.1$ mmol · kg$^{-1}$ dm; Australian Sports Science students ($n=5$; $19.4 \pm 2.6$ yrs; F; active) $33.6 \pm 13.2$ mmol · kg$^{-1}$ dm.

b) **Supplementation:** Synthesis of M-Carn occurs in situ and is limited by the availability of β-Ala. Supplementation with β-Ala (4 g · d$^{-1}$ in wk 1 increasing to 6.4 g · d$^{-1}$ by wk 4 and maintained at this for 6 more weeks) increased M-Carn ∼60 and ∼80% at 4 and 10 wks. Increase occurred in both type I and II fibres and by the same amount. Supplementation with Carn itself had the same effect as an isomolar dose of β-Ala and showed no further advantage. In different studies β-Ala supplementation has been shown to increase cycle exercise capacity as well as the work rate corresponding to the ventilatory threshold in a graded exercise test, and, isometric (knee-extension) endurance at 45% MVC. The increase in isometric endurance was consistent with the estimated increase in buffering and rate of $H^+$ formation. These findings are the first to demonstrate an enhancement of exercise performance by an increase in intracellular buffering, in any form of whole body exercise. Most recently it has been possible to demonstrate non-invasively an increase in M-Carn with β-Ala supplementation by proton-MRS.

c) **Training:** In 4 studies using different modalities of training (knee-extension/flexion, cycling, mixed endurance/weight training, mixed whole body resistance training) maintained up to 12 wks, M-Carn was unaffected by training alone and only increased when β-Ala supplementation was included. The absence of any training effect agrees with an earlier 16 wk study but is in contrast with other work.

It is concluded that in humans, M-Carn synthesis is limited by the natural synthesis of β-Ala and the small amount available through the normal diet. Only when the dietary level of this is increased is the level of M-Carn increased.

## Effect of 4 weeks β-alanine supplementation upon isometric and isokinetic exercise performance in humans

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The histidine containing dipeptide carnosine ([carn], β-alanyl-L-histidine) is ubiquitous in muscle of all vertebrate species. Due to a pKa of 6.83 a major role of carnosine in muscle will be to buffer protons and thus moderate changes in muscle pH associated with intense exercise. Carnosine is synthesised in muscle from β-alanine (β-ala) and histidine of which β-ala is limiting. The aim of this investigation was to determine a method of elevating muscle [carn] with β-alanine supplementation and to establish if such increases can improve the performance of high intensity isometric and maximal intensity isokinetic exercise.

**Methods:** Subjects in all studies were physically active, untrained males (Mean ± SD, age: $25.7 \pm 6.3$ y, height: $1.80 \pm 0.07$ m, mass $79.0 \pm 9.0$ kg). Study 1: Biopsies of the *M. vastus lateralis* were freeze dried and analysed via HPLC pre and post supplementation for [carn]. Supplements were taken 8ñd$^{-1}$ for 4 weeks. Total doses were: 4 wk β-ala (β4) 145.6 g ($n=8$), iso-molar 4 wk carn (C4) 343.0 g (iso-molar for β-ala 143.3 g) ($n=5$). Study 2: 12 subjects performed 30 maximal isokinetic contractions of the quadriceps/hamstring muscle groups between 0 and 90° at 180° s$^{-1}$ pre and post 4 weeks supplementation with either β4 ($n=6$) or matching placebo ($n=6$). Study 3: 13 subjects performed a sustained isometric muscle contraction of the knee extensors at 45% maximal voluntary isometric contraction (MViC) pre and post supplementation with β4 ($n=7$) or matching placebo ($n=6$).

**Results:** Pre supplementation muscle [carn] was 22.6 mmol kg$^{-1}$ dm, both treatment groups showed similar increases ($p<0.05$) in muscle [carn], β4 = 66.5%, C4 = 65.8%, compared to the control group 9.9% ($n=6$). Maximal isokinetic exercise performance was not affected by β4 supplementation, however endurance hold time at 45% MViC was

prolonged by 13.2% ($p < 0.05$), with increases in buffering capacity predicted from performance and supplementation data in reasonable agreement (10.7 vs. 7.0 mEq $H^+ \cdot kg^{-1}$ dm, respectively).

**Conclusion:** This is the first investigation to show intracellular pH to limit exercise performance in whole body exercise in humans. The results of this investigation provide a working hypothesis that elevating muscle [carn] can delay fatigue by attenuating the decline in muscle pH associated with fatigue in high intensity isometric exercise.

## Ageing, protein–protein cross links, protein–carbonyl group interactions and carnosine

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The dipeptide carnosine (β-alanyl-L-histidine) was discovered over 100 years ago, but its function remains enigmatic. There have been a number of observations indicating that carnosine can suppress senescence in cultured cells and in some animals. The most common molecular symptom of ageing is the accumulation of altered proteins formed mostly by post-synthetic oxidation and glycation. This lecture will discuss the observed effects of carnosine on the formation of altered proteins, particularly cross-linked species which can inhibit proteolysis of aberrant polypeptides. Carnosine's reaction with methylglyoxal will also be highlighted because this highly reactive bicarbonyl may play important roles in age-related pathology, ageing in general and diabetic complications. The lecture will also contain a number of additional but highly speculative mechanisms by which carnosine may mediate other homeostatic activities which collectively could help suppress onset of the senescent phenotype.

## β-Alanine supplementation and performance

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β-Alanine is a non-proteinogenic amino acid that is synthesized naturally in the liver in small quantities and can be consumed from dietary meat intake. Together with histidine it can be synthesized into carnosine within skeletal muscle. Carnosine has been shown to enhance muscle buffering capacity during high intensity exercise. Considering that histidine is a non-essential amino acid and is found in plentiful amounts with carnosine synthetase within the body, β-alanine concentration is likely the rate limiting step in carnosine synthesis. When β-alanine is ingested it has been shown to be effective in increasing carnosine concentrations within skeletal muscle, and subsequently may provide a significant ergogenic benefit for athletes participating in high-intensity exercise by reducing the rate of fatigue in muscle. Studies examining the efficacy of β-alanine supplementation on high-intensity exercise performance have reported significant improvements in physical work capacity, lactate threshold and enhanced time to exhaustion in untrained or physically active males and females consuming between 3.2 and 6.4 $g \cdot d^{-1}$ of β-alanine during 4–10 weeks of supplementation. Studies examining the efficacy of this supplementation in competitive athletes have shown that β-alanine ingestion can lead to a greater exercise stimulus, specifically reflected in a higher volume of exercise during resistance exercise training sessions. In addition, greater gains in lean tissue mass and fat loss have been reported in competitive athletes supplementing with β-alanine. Discussion will focus on review of these studies, including the potential influence of body mass, duration of supplement regimen and training status of individual. Additional discussion will examine the effect of β-alanine supplementation on endocrine response to an acute training session, and the compounded effect that occurs when β-alanine supplementation is combined with creatine.

## Effect of carbohydrate/protein supplements on exercise performance and recovery

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The addition of protein to a carbohydrate supplement has been found to improve physical performance and recovery beyond that produced by carbohydrate supplementation alone. Work time to exhaustion during moderate intensity exercise is improved, and based on plasma enzyme levels, muscle damage significantly reduced. Consumption of a carbohydrate/protein supplement during exercise has also been found to enhance subsequent exercise performance, and taken post exercise found to speed many aspects of exercise recovery. Recovery from prolonged strenuous exercise requires that the body be rehydrated, depleted fuel stores be replenished, damaged tissue be repaired, and training adaptations be initiated. An important and initial concern for exercise recovery is rehydration. For effective rehydration not only is sufficient fluid ingestion required, but the body must retain the fluid ingested. Recent research suggests that performance drinks that contain small amounts of protein are better retained than traditional performance drinks containing only carbohydrate. Muscle glycogen is an essential fuel for intense exercise and also must be replenished. Glycogen synthesis is a relatively slow process, and therefore the restoration of muscle glycogen requires special considerations when there is limited time between training sessions or competition. To maximize the rate of muscle glycogen synthesis, it is important to consume a sufficient amount of carbohydrate immediately post exercise. The addition of protein to the carbohydrate supplement has been found to increase the supplement's effectiveness for promoting glycogen storage. The ingestion of protein will also stimulate protein synthesis and reduce protein degradation, thus having the added benefit of promoting muscle tissue repair and training adaptation. Furthermore, research suggests that carbohydrate/protein supplementation will support the immune system during prolonged stressful exercise. Evidence will be presented demonstrating the effectiveness of a carbohydrate/protein supplement consumed during exercise on exercise performance and post exercise to speed exercise recovery. Moreover, the effects of carbohydrate/protein supplementation on hydration, glycogen storage, and muscle protein synthesis will be discussed.

## Distribution of the histidine containing dipeptides: anserine and carnosine, within Turkey muscle

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Anserine (Ans) and carnosine (Carn) are naturally occurring histidine containing dipeptides (HcD) that occur in high concentrations within muscle where they contributes to $H^+$ buffering. In humans Carn (β-AlaHis dipeptide) can be increased 60% or more by supplementation with β-alanine (β-Ala), which is rate limiting to Carn synthesis in human muscle. β-Ala is obtained from meat ingestion or from synthesis of uracil in the liver. Current data on the HcD content of meats primarily refers to a limited number of cuts within a species. Poultry data predominately refers to the Inner breast fillet.

**Purpose:** To determine the distribution of HcD in different commercial cuts of turkey muscle meats.

**Method:** Samples were obtained from 5 Hens (93 days, $6.15 \pm 0.48$ kg) and 5 Stags (144 days, $17.35 \pm 0.92$ kg) at standard kill time points. Samples were homogenized with water and analysed via HPLC.

**Results:** HcD contents of turkey muscle: Outer Breast $61.96 \pm 8.36$ mmol $\cdot$ kg$^{-1}$ dm; Inner Breast Fillet $61.38 \pm 4.36$ mmol $\cdot$ kg$^{-1}$ dm; Wing Oyster $38.04 \pm 5.84$ mmol $\cdot$ kg$^{-1}$ dm; Prime Wing $31.72 \pm 3.46$ mmol $\cdot$ kg$^{-1}$ dm; Mid Wing $26.46 \pm 1.61$ mmol $\cdot$ kg$^{-1}$ dm; Thigh $21.66 \pm 2.54$ mmol $\cdot$ kg$^{-1}$ dm; Drumstick $17.30 \pm 5.66$ mmol $\cdot$ kg$^{-1}$ dm; Gizzard $0.89 \pm 0.54$ mmol $\cdot$ kg$^{-1}$ dm.

**Conclusion:** The breast muscles contain significantly higher levels of HcD than the other major muscle cuts of a turkey, with the gizzard containing significantly lower levels than any other cut.

## Influence of exercise on kynurenic acid levels in the serum

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Tryptophan is metabolized over the kynurenine pathway to NAD, which is an essential factor for energy metabolism. Ito already reported that exercise in humans activates kynurenine pathway and Ito demonstrated in a rat study that treadmill exercise activates the kynurenine pathway of tryptophan. Within the kynurenine pathway of tryptophan degradation several neuroactive substances are synthesized that play a significant physiological role. Kynurenic acid, a product of L-kynurenine transamination acts as an antagonist at the glutamate EAA receptors as well as at the 7 alpha nicotine cholinergic receptors. Increased kynurenic acid levels in the serum has been demonstrated under various pathological conditions including epileptic jerks but also in conditions with increased wet dog shakes and enhanced motor activities. In the present study we investigated the levels of kynurenic acid in serum of rats exposed to the treadmill exercise. Our data provide the evidence that exercise leads to alterations of kynurenic acid levels in the serum depending on the length of exercise. A transient decrease of kynurenic acid was observed at the 1$^{st}$ and the 14$^{th}$ day of experiment, while at the 21$^{st}$ day of experiment the values were comparable to controls. Revealed data indicate the involvement of kynurenic acid metabolism during exercise.

## Effect of prophylactic supplementation of N-acetyl-cysteine and epigallocatechin gallate on markers of oxidative stresss, inflammation and apoptosis after eccentric contraction-induced injury in untrained males

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The purpose of this study was the examine the effects of 14 days of prophylactic supplementation of N-acetyl-cysteine (NAC) and epigallocatechin gallate (EGCG) on changes in peak torque production, muscle soreness, muscle damage, oxidative stress, inflammation and apoptosis after a single damaging bout of eccentric muscle contractions. Thirty healthy, recreationally active males ($20.0 \pm 1.8$ years, $160 \pm 7.1$ cm, $167.4 \pm 37.3$ kg) were randomized in a double-blind fashion to ingest daily either 1800 mg of NAC, 1200 mg of EGCG, or 1500 mg of glucomannan placebo (PLC) in a prophylactic fashion for 14 days. After supplementation, subjects completed one eccentric exercise bout (100 repetitions at $30o \cdot sec^{-1}$) of the dominant knee extensors. Participants completed strength and soreness assessments while providing fasting blood samples and vastus lateralis muscle biopsies (no 72 h biopsy) before and after supplementation, as well as 6, 24, 48 and 72 h post-exercise to assess changes in muscle damage, oxidative stress, inflammation, and apoptosis. Separate $3 \times 6$ repeated measures ANOVA with a probability level of 0.05 was used for the statistical analysis with LSD post-hoc comparisons for all significant interactions. Significant reductions in peak torque as well as increases in soreness and creatine kinase at 6 h post-ex were found in all groups. No changes ($p > 0.05$) were observed for the indicators of oxidative stress, serum superoxide dismutase, serum 8-isoprostane and muscle glutathione reductase, as well as the indicators of stress and inflammation, serum cortisol and tumor necrosis factor-alpha. For the apoptosis markers, significant increases ($p < 0.05$) in muscle levels of bax, bcl-2, caspase-3 enzyme activity and decreases in total DNA content were observed in all groups with no significant differences among groups, whereas no changes ($p > 0.05$) were reported for cytochrome C. At all time points, PLC demonstrated greater caspase-3 enzyme activity and lower levels of total DNA content in comparison to either supplementation group. An eccentric bout of muscle contractions appears to significantly increases muscle damage and apoptosis with no changes in oxidative stress and inflammation. More research is needed to determine the impact supplementation with EGCG and NAC may have on changes related to apoptosis and eccentric exercise.

## Effect of creatine supplementation on cognitive performance in young and elderly

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The energy for cognitive performance depends on the hydrolysis of adenosinetriphosphate (ATP) to adenosinediphosphate and inorganic phosphate. The re-synthesis of ATP by creatine kinase is dependent upon phosphorylcreatine and the process results in the degradation of phosphorylcreatine, which requires the presence of creatine for replenishment. Deficiency in creatine is a major limitation in this process. However, magnetic resonance spectroscopy studies have shown that creatine monohydrate supplementation results in significant increases in creatine concentrations in the human brain. Furthermore, the positive effects of creatine supplementation have been shown to be due to the fact that the presence of creatine improves mitochondrial membrane stimulation, intra-cellular handling, antioxidant mechanisms and glutamate re-uptake in synaptic vesicles, with a resultant neuroprotective effect on several chemicals in the brain particularly dopamine and glutamate. Dopamine concentrations are increased while those of glutamine are lowered. Dopamine is a neurotransmitter that plays a major role in working memory tasks; while glutamate is an excitatory neurotransmitter, which at high levels can disrupt cognitive performance due to excitoxicity of neurons. Positive effects of creatine supplementation on cognitive functioning have been shown on vegans and vegetarians, who are likely to be low in creatine. Similarly positive effects have been demonstrated on sleep deprived individuals, although only with complex task which activate the prefrontal cortex. Research with the elderly has shown improved visual and verbal short-term memory and long-term memory. Of even greater

interest is the promising work with individuals with Parkinson's and Huntington's diseases.

## Converting science into medals using amino acids and proteins to support performance in elite athletes

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The difference between winning and losing in elite sport is often measured in thousandths of a second. The concept of Performance Nutrition focuses on the nutritional factors that influence the performance of the athlete, starting with the actual performance demands itself and then working backwards to provide an approach that is out-comes based and not process driven. As such the role of the Performance Nutritionist is to work in partnership with academics and leading experts to develop strategies that are evidence-based to support elite sport performance. There is a growing understanding and evidence base that amino acids and other nitrogenous compounds may influence performance of elite athletes. There is immense pressure on athletes to perform on the World stage, but often athletes do not receive the specialist support needed. This may lead to athletes using banned substances. There are two main reasons why an athlete may use drugs either for an actual ergogenic effect and/or to support recovery and 'health'. The selective and strategic use of proteins and amino acids may form an approach that not only supports performance but may provide an alternative to drugs.

**Practical rigor:** Working as an elite Practitioner it is critical to have an innovation and research focus, however the traditional controlled biomedical designs do not easily fit into the fluid world of elite sport. Reliable performance measures are difficult to use, we often rely on actual performances such as World championships. A challenge to the Practitioner is to use and develop applied research techniques that can be scrutinized and challenged. Action Research provides the practitioner with an opportunity to critique and explore solutions to practical problems and expand the practitioner evidence base. This paper focus's on the systematic approach taken to developing a strategy of transferring the science into practice, with a focus on the use of protein and amino acids in elite sport.

**Innovative projects:** Beta Alanine feeding has been shown to increase intra muscular carnosine. A diet and supplement strategy has been developed and employed with elite endurance cyclists. It was recently used as part of the support for riders competing in the recent World Championships held in Palma (Majorca). Performance measures included the most successful World Championships of any British Sport to date.

Weight management in elite sport is under represented in the literature, this has led to developing some unconventional but effective weight management strategies, using low energy high protein diets appears to help minimize muscle loss in weight loss in elite athletes e.g. a 4 kg loss of fat in an elite athlete in 2 weeks (estimate negative energy balance of 2000 kcal per day) with no loss in muscle mass then wining a major Championships 3 days later.

The 'brittle' athlete is a term that may describe the athlete who struggles to cope with the normal high training loads required by an elite athlete. It has been suggested that these athletes may have a compromised anti oxidant capacity. Whey protein can be used as a cysteine donor to support glutathione synthesis. This has formed part of a multi system support for athletes. Outcomes include a reduction in days lost from training and improved performances.

**Conclusion:** To help maximize the performance impact the practitioner and academic need to work in partnership to develop innovation research that is impacting on elite sport competition. The elite practitioner has the unique opportunity to inform the scientist and help shape pertinent questions that will impact on the performance of elite athlete.

## Fatty acid metabolism in humans during exercise

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Fatty acids are important fuel at rest and during exercise of low or moderate intensity. Their concentrations in human skeletal muscle are in the micromolar range, but large amounts can be released through the process of lipolysis from triacylglycerols stored in the lipid droplets of muscle fibers and adipocytes. Lipolysis in muscle and adipose tissue is stimulated during exercise through the activation of a triacylglycerol hydrolase known as hormone-sensitive lipase, or HSL, by reversible phosphorylation. In adipose tissue, HSL phosphorylation results from stimulation of the cyclic AMP cascade by increased catecholamine and decreased insulin secretion; in muscle, HSL phosphorylation results from stimulation of the cyclic AMP cascade by increased epinephrine secretion and from activation of the calcium-dependent protein kinase C. An additional factor responsible for exercise-induced lipolysis is the translocation of HSL to the lipid droplets of adipocytes and muscle fibers. Lipolysis in muscle is stimulated within 1 min of moderate- or high-intensity exercise, while the lipolytic rate in adipose tissue peaks within 5 min of endurance or resistance exercise. Plasma triacylglycerols (packaged in lipoproteins) do not normally contribute considerable amounts of fatty acids during exercise.

Fatty acids released from adipocytes to the circulation enter muscle fibers by way of facilitated transport thanks to an inward concentration gradient. There, both ''imported'' and ''domestic'' fatty acids enter the mitochondria with the help of L-carnitine (an amino acid) to undergo β oxidation. Acetyl coenzyme A, the product of β oxidation, is then oxidised to $CO_2$ in the citric acid cycle. The full oxidation of fatty acids releases large amounts of energy (about 106 ATP from palmitate, the prototype of fatty acids), albeit at low rates (0.2–0.3 mmol ATP per kg muscle per sec, as compared to 0.5 from carbohydrate oxidation, 1.5 from anaerobic carbohydrate breakdown, and 2.6 from creatine phosphate breakdown). Fatty acid oxidation is maximal at 45–65% $VO_2$max, in both absolute (as mass per unit of time) and relative terms (as percentage contribution to total energy expenditure). The reason for the drop in the rate of fatty acid oxidation at higher exercise intensities is not known with certainty, but a possible explanation is the diversion of carnitine to acetyl carnitine formation because of overproduction of acetyl coenzyme A. This may slow down fatty acid entry to the mitochondria but may also speed up carbohydrate oxidation by lowering the [acetyl CoA]/[CoA] ratio, thus lifting the inhibition of pyruvate dehydrogenase.

Apart from exercise intensity, exercise duration modulates fatty acid utilisation: the longer one exercises at a constant moderate intensity, the more fatty acids relative to carbohydrates one burns. Nutrition, another factor, affects fatty acid utilisation in a rather predictable way: a carbohydrate-rich diet in the days preceding prolonged moderate-intensity exercise lowers the share of fatty acids in energy expenditure. Likewise, carbohydrates ingested during such exercise suppress fatty acid oxidation. By contrast, a fat-rich diet for several days or weeks increases the uptake of plasma fatty acids by the exercising muscles and the use of plasma triacylglycerols during prolonged moderate-intensity exercise. Both effects result in increased contribution of fatty acids to energy expenditure. Training also affects the use of fatty acids during exercise. In particular, endurance training and, according to recent reports, high-intensity interval training improve fatty acid oxidation during prolonged exercise at a given absolute moderate intensity. A high fatty acid turnover in skeletal muscle has been linked to muscle insulin sensitivity, an important contributor to whole-body insulin sensitivity.

## Creatine supplementation and kidney function: the odds and ends

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Nine years ago, two London nephrologists (Pritchard and Kalra) introduced the first case of kidney damage induced after creatine (Cr) supplementation on a 25-y-young man with a focal segmental glomerulonephrosis. A year later, another nephrologist (Koshy) reported an interstitial nephritis in a young healthy patient having absorbed Cr for 4 weeks. Based on these anecdotal reports, several national agencies (France, USA) claimed that Cr supplementation should be taken with high precaution due to the lack of definite evidences related to detrimental effects, kidney function included. The ''French Agency of Food and Health Security (AFSSA)'' even discouraged publicity of exogenous Cr due to hypothetical carcinogenic and mutagenic compounds issued from excess Cr metabolism.

For the last 10 years, we accumulated evidences in healthy young human subjects on some kidney functions after Cr supplementaion from 5 days up to 5 years. We did measure the glomerular filtration rate-GFR (creatinine Crn clearance), glomerular membrane permeability (plasma albumin excretion rate and clearance) and tubular reabsorption (urea clearance) before and after Cr supplementation. Renal clearances of Crn, urea and albumin did not reveal any statistical differences between the control data and the values collected after Cr supplementation. These results on the stability of the GFR were also confirmed by 8 other scientific teams (a total of 160 subjects). Moreover, albumin urinary excretion rates also remained stable within the physiological range of healthy subjects ($<20\,\mu g/min$). As ''microalbuminuria'' is a well-known predictor of kidney impairment, it may be argued that, in healthy subjects, the glomerular membrane permeability is not affected by the different loads of Cr supplementation.

A few animal studies (mice, rats) were published on the progression of renal disease under Cr supplementation. Conflicting results were obtained but recent investigations by Kreider, Taes and Tarnopolsky did not confirm any adverse effects on renal function of healthy animals.

Based on the excellent and extended review by Wyss and Kaddura-Daouk, the AFSSA claimed unequivocally that excess consumption of Cr might induce derived carcinogenic and mutagenic compounds (methylamine, formaldehyde, heterocyclic amines). We did measure the urine excretion rate of methylamine, formate and formaldehyde in healthy subjects submitted to Cr supplementation ($21\,g/day$–2 weeks). Despite the fact that the urine excretion rate of formaldehyde and methylamine increased respectively to 4.5- and 9.2-fold after Cr supplementation, the GFR and albumin excretion remained stable. Moreover, Derave (2006) showed that exogenous Cr ingestion did not increase the urinary excretion of the carcinogen N-nitrososarcosine.

To conclude, kidneys are keeping their functionality in healthy subjects supplemented with Cr, even during several months. However, regular checkups should be the elementary tactic to follow the potential dysfunction.

## Peripheral adaptations to creatine supplementation: what are the mechanisms?

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The effects of creatine supplementation on exercise performance have been heavily studied, and creatine is widely used by athletes. Additionally, the effects of creatine supplementation in patient populations (e.g. Sarcopenic elderly, Amyotrophic Lateral Sclerosis, Parkinson's disease, Muscular Dystrophy, congestive heat failure, etc.) and in models of muscle atrophy/disuse have been studied. The improvement in sports performance, and the increase in strength and muscle mass following creatine supplementation have been well documented, however, the mechanisms responsible for these muscular adaptations have been less studied. Several potential mechanisms underlying muscular adaptations to creatine supplementation have been identified. The potential mechanisms include: metabolic adaptations (e.g. increased muscle glycogen, increased muscle phosphocreatine, faster phosphocreatine resynthesis), changes in protein turnover, hormonal alterations (e.g. increased growth hormone, increased testosterone, decreased cortisol), stabilization of lipid membranes (leading to less muscle damage), molecular modifications (e.g. increased expression of myosin heavy chain mRNA, increased expression of myogenin and MRF-4 mRNA, and increased expression of IGF-I and IGF-II mRNA), or as a general training aid (e.g. increased training volume, increased training intensity, reduced recovery time between sets and/or exercises). The available evidence suggests that creatine exerts ergogenic effects through metabolic adaptations and molecular modifications, which in turn may increase training volume/intensity. Creatine supplementation alters skeletal muscle directly, via enhanced energy metabolism (increased muscle glycogen and phosphocreatine, faster phosphocreatine resynthesis), increased expression of endocrine and growth factor mRNA, and indirectly by allowing training volume/intensity to be increased. It is likely that these mechanisms work synergistically rather than independently.

## Protein turnover in the human musculoskeletal system: effects of nutrition, physical activity/inactivity and age

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We have learned a great deal about the control of the size of the lean body mass over the last quarter century, much of it distinct from what might be expected in animals. Over the short term modulation of the translational stage of protein synthesis appears to be a more important regulator of protein mass than protein breakdown, with the latter appearing to have a trimming role in physiological situations. The major modulator of protein synthesis appears to be the availability of amino acids and that of protein breakdown is insulin, with little cross over. The links between the ''accelerator'' and ''brake'' pedals appears to be a system of signalling molecules connected to PKB, with the P70/mTOR axis responding to amino acids (particularly leucine) and FOXO-Atrogin systems being inhibited by insulin with little other cross talk. There are good dose response relationships between muscle protein synthesis and availability of amino acids and between insulin availability and inhibition of proteolysis. However it is impossible to build muscle bulk simply by increasing supply of amino acids because of the kinetics of the catabolic enzymes of essential amino acids, their inducibility by excess dietary protein and the anaphylaxis of the anabolic system to continued amino acid availability.

Acute exercise halts protein synthesis during contractile activity and stimulates proteolysis; by mechanisms which are not known in detail but probably involve Ca release. After exercise both arms of protein turnover are stimulated in the fasted state with supply of amino acids boosting synthesis and supply of glucose inhibiting breakdown by causing insulin release. It appears that exercise in the lengthening mode has only a small extra effect in stimulating MPS compared to concentric exercise. However there are marked differences in the effects of exercise in the lengthening and shortening modes on gene expression with greater up regulation of genes associated with muscle building in the lengthening mode. Exercise at above 60% of 1 RM appears to saturate the anabolic increase and it is surprising that in terms of mixed muscle mass

dynamic exercise seems to be as effective as resistance exercise in causing the effect.

Muscle and tendon collagen synthesis appear to be unresponsive to nutrition but bone collagen synthesis seems to be amino acid responsive, but unaffected by lipid or glucose/insulin. However muscle and tendon collagen turnover are very responsive to physical activity being acutely stimulated by exercise and down regulated by disuse.

Age appears to decrease the capacity and sensitivity of muscle to anabolic stimuli, decreasing not the basal state but the ability to increase synthesis and decrease breakdown in response to nutrients.

## Slowing down the aging process through nutrition and exercise

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There is evidence that show that some people age slower than others. The ''successful agers'', as they have been called, present greater mental functioning and physical health in later years compared with the ''normal'' people.

Many factors are involved in the aging process, impacting on the cardiovascular, respiratory, nervous, musculoskeletal and immune systems. The speed of deterioration in the function of these systems differs significantly among people. However, there are some common characteristics that can be found. First, people live longer now, i.e. age slower compared to a few decades back. Second, everyone will, eventually, age and die!

With these two in mind, we will review the literature to find the wonder anti-aging ''pill''! We will focus specifically on the role of nutrition and physical activity. There are too many ''anti-aging'' candidate factors, some more promising than others. Vitamins and minerals, vegetables, wine, n-3 PUFA, Q-10, ginger, antioxidants, carnosine, hypo-caloric diet, aerobic and resistance exercise, and even crosswords and Nintendo!

What is very exciting from the latest research is that several of the above factors have shown promising results in animal models. Whether these apply to humans, is a question waiting to be answered. It is easier for example to impose caloric restriction to a group of mice living in a cage than to do so in people living a normal life.

## Glutamine, glutamate, alanine as possible markers of fatigue in treadmill exercise

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**Objective:** To investigate further the plasma concentration ratio of glutamine to glutamate, p[Gln/Glu], as a potential marker of fatigue in exercise but using separate enzymatic assays for each amino acid. Plasma tryptophan p[trp] and alanine p[Ala] concentrations, together with the rate of perceived exertion (RPE, a marker of fatigue) were also measured.

**Background:** Previously a low p[Gln/Glu] <3.58 has been linked to an overstrained (fatigued) state and a high p[Gln/Glu] >5.88 to a well-trained (non-fatigued) state. However, in the original study, a single colorimetric (glutaminase) assay was used to establish, first p[Gln] and then from this, p[Glu], preventing independent variation.

**Methods:** Eleven male recreational runners consented to 40 min treadmill running at 5% below lactate threshold. Blood was sampled pre, post, 30 min post and 1 h post exercise. Separate enzymatic, colorimetric assays were used to determine p[Gln] and p[Glu]. High pressure liquid chromatography (HPLC) was used to measure p[Ala] and p[Trp]. Rate of perceived exertion (RPE) was measured during exercise and immediately after using the Borg scale.

**Results:** There was a marked, significant decrease in p[Gln/Glu] at 30 min post exercise (50%; $p < 0.01$) but this did not correlate with RPE, which increased during exercise. There was an overall increase in p[Ala] (18%, $p < 0.05$) post-exercise which correlated with RPE. Five athletes with high RPE (fatigue) had significantly higher p[Ala] ($p < 0.05$) compared with those who had low RPE ($n = 6$). The p[Gln/Ala] ratio was significantly decreased at 30 min post exercise ($p < 0.05$) but did not correlate with RPE. There was no change in p[Trp].

**Conclusion:** p[Gln/Glu], and possibly p[Gln/Ala], may be a marker of fatigue in exercise. However, in the present study, as assessed by RPE, there was more support for increased p[Ala] as a marker of post-exercise fatigue.

## A central role for carnitine in the in vivo regulation of fuel metabolism in skeletal muscle

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More than 95% of the body's total carnitine store resides within skeletal muscle. Here it plays essential roles in: (i) maintaining the mitochondrial acetyl-CoA/CoASH ratio, particularly under conditions when the rate of pyruvate dehydrogenase complex flux exceeds the rate of acetyl group utilisation by the tricarboxcylic acid cycle (e.g. intense exercise), and (ii) in the translocation of long-chain fatty-acids into the mitochondrial matrix for subsequent β-oxidation.

This presentation will highlight research published over the past decade demonstrating the importance of carnitine as a key regulator of muscle fuel metabolism in vivo. In particular, it will focus on the evidence that PDC flux (and thereby acetyl group availability) limits mitochondrial ATP production during the rest to exercise transition period (the acetyl group deficit), which can be overcome by pharmacologically activating muscle PDC and acetylating the free carnitine pool at rest prior to exercise. Given that muscle free carnitine availability may also be limiting to fat oxidation, particularly during intense sub-maximal exercise, this presentation will also focus on recent evidence demonstrating that acutely increasing muscle total carnitine content, using hyperinsulinaemia-mediated stimulation of muscle carnitine transport, can reduce muscle glycolysis, blunt PDC activation (under conditions of fixed glucose delivery), increase glycogen storage, and apparently increase in muscle fat oxidation.

The demonstration that muscle carnitine availability can be readily manipulated in humans, and significantly impacts on physiological function under a variety of conditions, will result in renewed business, clinical and scientific interest in this compound.

## Applications of creatine: aging, exercise and muscle disease

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Human aging is associated with a loss of strength and muscle mass (sarcopenia) that can impair function. We, and others have found a potentiation of the resistance exercise induced training improvements in fat free mass and strength with simultaneous creatine monohydrate

supplementation. Creatine supplementation in older adults does not appear to be of significant benefit in the absence of an exercise stimulus. In younger adults, creatine supplementation during a period of resistance exercise training has been shown to potentiate the increases in fat free mass and strength. Possible mechanisms include an increase in the total number of contractions over a period of time (increased training volume) and/or direct effect on skeletal muscle. We have found that acute creatine supplementation can increase steady state mRNA content for a variety of genes involved in cell volume regulation and cell cycle, indicating a direct effect on skeletal muscle. In vitro studies, and more recently, a human training study have found that creatine monohydrate supplementation can enhance satellite cell proliferation and this is likely a further mechanism whereby resistance training induced gains may be further enhanced by creatine supplementation. An extensive number of animal studies have found potential clinical therapeutic benefit from creatine monohydrate supplementation in models of Huntington's disease, Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, head trauma and anoxia (stroke). We, and others have found low muscle creatine content in patients with a variety of muscle diseases including mitochondrial myopathy, dystrophy, and inflammatory myopathy. Several studies have found beneficial effects from creatine monohydrate supplementation in children and adults with muscular dystrophy and a recent Cochrane review concluded that creatine was of therapeutic benefit in muscular dystrophy. More specifically, we have looked at Duchenne muscular dystrophy and found an increase in dominant hand strength, fat-free mass with a trend towards an attenuation of the global reduction in muscle strength seen in this condition. Interestingly, we also confirmed an earlier report of a reduction in *N*-telopeptide excretion (a marker of bone breakdown). Cell culture work has suggested that creatine may be of benefit from a bone mass perspective and recent animal studies in our laboratory have shown improvements in some aspects of bone metabolism. Several studies have found that creatine monohydrate can attenuate some of the side effects of corticosteroid use. Given the wide spread use of corticosteroids in the treatment of inflammatory myopathies, creatine monohydrate may be beneficial in such conditions. A recent publication has demonstrated the therapeutic efficacy of creatine monohydrate supplementation in adults with dermatomyositis and polymyositis who are simultaneously taking predinsone. Overall, it appears that creatine monohydrate supplementation may be of adjunctive benefit in primary myopathies and can enhance resistance training mediated gains in strength and fat free mass particularly in older adults.

## Influence of sex differences in endurance exercise on amino acid metabolism and the implications for dietary protein requirements

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Women oxidize more lipid and less carbohydrate during endurance exercise as compared with men. Consequently, it is not surprising that women have lower amino acid oxidation as compared with men. The mRNA transcriptome pattern in skeletal muscle is consistent with an increase in fat oxidation for women, yet there is no consistent pattern in the mRNA for genes involved in carbohydrate or amino acid oxidation. Together, this data suggests that the main sex difference is in fat oxidation, with carbohydrate and ultimately amino acid oxidation following, by metabolic demand. Animal and human studies have shown that 17-β-estradiol is a major factor mediating these sex differences in endurance exercise metabolism. Again, fat metabolism appears to be primarily regulated, with carbohydrate and protein following by metabolic demand. Amino acid oxidation is a minor contributor to total substrate oxidation during endurance exercise with estimates of 2–5% being most commonly reported. Endurance exercise training in sedentary individuals decreases amino acid oxidation at the same relative and absolute exercise intensities. This is due to a lesser activation of branch chain 2 oxo-acid dehydrogenase (BCOAD). This training adaptation is likely due to higher basal glycogen concentration and attenuated carbohydrate utilization during endurance exercise, resulting in higher BCOAD phosphorylation (less active). For this reason, it is not surprising that amino acid and protein requirements are not elevated with recreational and modestly trained athletes. In highly trained athletes; however, the total mitochondrial volume is high and both training volumes as well as intensities are correspondingly elevated. In well-trained athletes, dietary protein intakes of $0.8\,g/kg/d$ are slightly suboptimal and in top sport elite male athletes the maximal dietary protein requirement is at most $1.65\,g/kg/d$, based upon nitrogen balance studies and habitual dietary intakes. Metabolic data in women would suggest that protein requirements would be lower than in men. In moderate to well trained women athletes, dietary protein requirements are minimally elevated above those of a sedentary individual and estimates for top sport elite female athletes would be in the order of 25% lower than top sport male athletes ($\sim 1.2\,g/kg/d$). Fortunately, most athletes consume adequate energy, carbohydrate and protein to easily meet these needs; however, for the elite athlete on an energy restricting diet, nutritional counseling may be required to obtain an adequate protein intake to meet the demands of oxidation and muscle damage and repair during intensive training.

## Does supplementation with proteogenic amino acids and proteins influence post-exercise protein turnover? (e.g. synthesis, protein loss, cell damage)

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The importance of post-exercise nutrition has long been recognized. Nutrition following exercise is important, not only for athletes and other healthy exercising individuals, but perhaps even more so for populations for which muscle hypertrophy is critical for health and function. The elderly, those recovering from illness and bed rest, astronauts and others benefit from information that optimizes nutritional and exercise practices.

Protein and amino acid supplementation has become very popular as evidenced by a multibillion dollar market. It is clear that protein nutrition is important for the response of muscle to resistance exercise; however there are many factors that determine the nature of the response.

The metabolic basis for muscle hypertrophy is the balance between protein synthesis and breakdown. Amino acids are taken up by the muscle and utilized for synthesis of muscle proteins. Muscle hypertrophy results only from positive net muscle protein balance over a given time period, i.e. muscle protein synthesis must exceed muscle protein breakdown. Thus, over a given time of training, periods of increased muscle protein synthesis and/or decreased breakdown are necessary for muscle protein accretion due to resistance exercise training. At any given time, net muscle protein balance can be either positive or negative. The balance is determined by the combination of nutrition and exercise. Muscle protein accretion occurs only when the sum of the positive periods exceeds the sum of the negative periods.

Nutrition has been shown to influence the response of muscle protein balance following exercise. Resistance exercise will increase net balance, but ingestion of an amino acid source is necessary be-

fore positive balance results following exercise. Amino acid ingestion prior to or following exercise stimulates muscle protein synthesis resulting in net muscle protein balance and thus accretion of muscle proteins. The combination of resistance exercise and ingestion of an amino acid source results in greater net muscle protein balance than at rest.

Following exercise, essential amino acids (EAA) stimulate protein synthesis resulting in positive net muscle protein balance, even without the nonessential amino acids. Whey proteins and casein also have been shown to stimulate net muscle protein balance. Any differences in protein accretion between proteins following exercise seem to be due to their EAA content. Recent evidence suggests that leucine may be the key component for stimulation of muscle protein synthesis, however the evidence is still not entirely clear.

The timing of amino acid ingestion in relation to exercise influences the response of net muscle protein balance. A mixture of EAA and carbohydrate ingested prior to exercise results in greater amino acid uptake than when the mixture is ingested immediately or one hour following exercise. However, whey protein ingestion before resistance exercise engendered a response similar to that when ingested following exercise. Thus the response of muscle protein balance seems to be due to a combination of the source of amino acids and the timing. There is no difference in amino acid uptake when EAA are co-ingested with carbohydrates vs. one h following carbohydrates. Milk and beef ingestion have been shown to stimulate net muscle protein balance to an extent similar to individual proteins and free amino acids.

Thus, it seems that ingestion of a source of EAA, including whole proteins and proteins in foods, stimulates uptake of amino acids and results in the accretion of protein in muscle following exercise.

### Effect of intake of different dietary protein sources on plasma amino acid profiles

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Although blood amino acid concentrations, particularly leucine, affect body protein metabolism, little is known of the pattern of amino acid responses to consumption of protein-rich foods. We employed a controlled single shot crossover design to characterise the plasma amino acid responses to the intake of 20 g of protein from various dietary sources. Fifteen untrained subjects consumed 20 g protein serves of skim milk, soy milk, grilled beef steak, boiled eggs, and a liquid meal supplement (PowerBar Protein Plus$^{TM}$) after an overnight fast. Plasma amino acids concentrations were measured immediately before ingestion of food and for 180 min afterwards using a gas chromatography-flame ionisation detection technique. For leucine, the lag time into plasma, time to maximum concentration and duration of absorption was shorter with liquids (skim milk, soy milk, liquid meal) than solid foods ($P < 0.01$). The maximum plasma concentration of leucine was greater with skim milk than all other treatments, while maximum leucine concentrations were lower with soy than steak and liquid meal ($P < 0.05$). The area under the plasma leucine concentration-time curve (AUC) of soy milk was less than steak, egg and liquid meal, while the AUC of skim milk was lower than liquid meal. These results may be useful to plan the type and timing of intake of protein-rich foods to maximise the protein synthetic response to various stimuli such as resistance training.

# Synthesis Amino Acids – Medicinal Chemistry

### Synthesis of linear and cyclic analogs of 558–565 loop of the A2 subunit of factor VIIIa blood coagulation

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The platelet activation and aggregation on the extracellular matrices is an important procedure in the initiation and growth of thrombus, leading either to prevent bleedings at the lesion of the blood vessel or to cause thrombotic disorders by an undesirable production of thrombin and its transportation to the general blood circulation. Under pathological conditions where the capacity of the natural thrombin inhibitor is depleted the thrombin generation and its action has to be controlled. Factor VIII (FVIII) is an essential protein for the blood coagulation cascade. Its lack causes bleeding symptoms whereas high level concentration is conected with venous and arterial thromboembolism. Factor VIII is comprised of a heavy (A1-A2-B) and a light (A3-C1-C2) peptide chain and includes three types of domains (A1-A2-B-A3-C1-C2). FVIII is activated by proteolytic cleavages caused by thrombin or by factor Xa. Thrombin production is depended on Factor IXa (FIXa), which plays a crucial role in curtailing of thrombin generation and accordingly on the additional activation of platelets. Peptides, which are expected to inhibit selectively the maximization of thrombin production, are based on the regions in which the FVIII interacts with the factor IX. Both FVIII and FIX are essential for normal coagulation and deficiency of either is associated with the bleeding diathesis.

Based upon the acceptance that the 558-565 loop of A2 subunit domain of FVIIIa interacts with factor IXa, we have synthesized a number of linear and cyclic analogs of 558–565 loop A2 and tested their biological behaviour. The sequence 558–565 of A2 subunit is the following:

$Ser^{558}$-$Val^{559}$-$Asp^{560}$-$Gln^{561}$-$Arg^{562}$-$Gly^{563}$-$Asn^{564}$-$Gln^{565}$

It is known that cyclic peptides have many advantages. They are important tools in biological tests because they exhibit metabolic stability, higher rigidity and generally they enhance the ligand-receptor binding compare to linear analogs. All the syntheses of the linear precursors were carried out stepwise in SPPS via an ester linkage, using the Fmoc/$Bu^t$ methodology on the solid support of 2-chlorotrityl chloride resin and DIC/HOBt as coupling reagent. All the peptides were purified (HPLC) and identified (ESI-MS). They are under investigation for their biological activity.

$H_2N$-Ser-Val-Asp-Gln-Arg-Gly-$Asn^{564}$-Gln-COOH
$H_2N$-Ser-Val-Asp-Gln-Arg-Gly-$Asp(OBzl)^{564}$-Gln-COOH
$H_2N$-Ser-Val-Asp-Gln-Arg-$Asp(OBzl)^{564}$-Gln-$NH_2$
$cyclo_{558\text{-}565}$[Ser-Val-Asp-Gln-Arg-Gly-$Asp(OBzl)^{564}$-Gln]
$H_2N$-Ser-Val-Asp-Gln-Arg-Gly-$Asp(OMe)^{564}$-Gln-COOH
$H_2N$-Ser-Val-Asp-Gln-Arg-Gly-$Asp(OMe)^{564}$-Gln-$NH_2$
$cyclo_{558-565}$[Ser-Val-Asp-Gln-Arg-Gly-$Asp(OMe)^{564}$-Gln]
$H_2N$-Ser-Val-Asp-Gln-Arg-Gly-$Asp^{564}$-Gln-COOH
$H_2N$-Ser-Val-Asp-Gln-Arg-Gly-$Asp^{564}$-Gln-$NH_2$
$cyclo_{558\text{-}565}$[Ser-Val-Asp-Gln-Arg-Gly-$Asp^{564}$-Gln]

## Nociceptive effects of newly synthesized arginine mimetics

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Non-proteinogenic, unnatural α-amino acids have been widely used as components of peptides to enhance biological activity, proteolytic stability, and bioavailability. It is well known that unnatural amino acids with guanidine functionality exhibit diverse pharmacological effects when introduced in biologically active systems. Our previous efforts were focused on the preparation and the characterization of unnatural amino acids, particularly those containing a basic functionality (oxyamino and sulfoguanidino) in the side chain. We have synthesized numerous unnatural amino acids, structural analogs arginine, which demonstrated certain biological effects.

Now, we have set as an objective to look further in the analgesic effects and the structure effect relationship between derivatives, and this paper reports the preparation of new derivatives of unnatural amino acids nor-canaline (NCan), nor-canavanine (NCav) and NsArg, and their antinociceptive effects using the paw pressure (PP) and HP tests.

## A convenient one-pot synthesis of benzyl- and p-toluensulfonyl esters of N-protected hydroxyl containing amino acids

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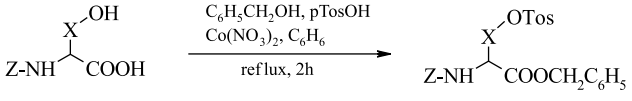
In the present work we present the direct synthesis of benzyl- and *p*-toluensulfonyl esters of N-protected hydroxyl containing amino acids: serine, tyrosine, homoserin and threonine.

Tosylation of alkohols is often used transformation in organic chemistry. The tosyl group can be displaced by wide variety of nucleophiles under conditions of $S_N2$-reactions and they react easier than alkylhalogenides.

We proposed a useful method of simultaneous preparation of bensyl and tosyl esters of N-protected amino acid, which is combination of well-known synthetical procedures:




1(a-d)

| X | $CH_2$ | $CH_2C_6H_4$ | $CH_2CH_2$ | $CH(CH_3)$ |
|---|---|---|---|---|
| comp. | a | b | c | d |

## Hormonal risks from non ionized telecommunication system

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Mobile phone telecommunication systems are becoming increasingly integrated into our life. Millions of individuals are exposed to radiofrequency emission on a daily basis and concerns are being raised as to potential risks to human health from the energy content in such emission. The main aim of the present study is to give an overview on the range of radiofrequency relevant to telecommunication in the Arab world and its impact on human health. This study elucidates the effect of the emitted radiofrequency on cognitive function, memory loss, neurotransmitters and hormonal profile in teenagers as well as in adults. In view of our results, recommendation will be stated as to precautionary principle with the object to minimize possible health risks which might affect a large number of person.

The purpose of this treatise is to give an overview of presently available data on radiofrequency (RF) relevant to telecommunication (800–900) MHZ (Global System for Mobile Telecommunication) and 1.6 GHZ, particularly neighbours of cell towers and its impact on human health, focusing on cognitive function, memory loss, neurotransmitters and hormonal profile in teenagers (12–18) years old) as well as in adults up to 50 years old. The study will be extended to develop precautionary principles for restricting hazards involved with exposure to radiofrequency transmission.

There are growing concerns about the possible hazards of electromagnetic waves emitted by mobile phones on human health. One of the biggest concerns is their possible association with increased risk of cancer and their possible effects on cellular DNA. Electromagnetic waves can inflict their results through both thermal and non-thermal effects. There are many animal studies that show that electromagnetic waves have a wide range of damaging effects on the male reproductive system and sperm parameters.

## Polyfluoromethyl-containing amino acids – new structures and synthetic strategies

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The introduction of polyfluoromethyl groups into amino acid molecules is an effective and useful method for goal modification of their important properties like basicity, acidity and lipophilicity. Numerous examples of polyfluoromethyl-containing amino acids are potent enzyme inhibitors: β-fluoroalanine and α-difluoromethylornithine are irreversible inhibitors of alanine racemase and ornithine decarboxylase, correspondingly.

In the present study, two new approaches to the synthesis of new various polyfluoromethyl-containing amino acids from readily available fluorinated carbonyl compounds, mainly – β-alkoxyvinyl polyfluoromethyl ketones 1, were attempted. The first approach bases on a sequence of transformations of acyclic compounds, which is resulted in new GABAs 2 and homoprolines 3 with polyfluoromethyl and hydroxy groups at geminal position. The second one is the reduction of polyfluoromethyl-containing pyrones 4 or pyridones 4 and 5 into corresponding δ-(hydroxyamino)-α-amino acids 6 and δ-amino acids 7.

The new fluorinated amino acids were obtained both as racemic and as enantiomerically pure forms. The synthetic pathway details, pros and cons of our approaches will be discussed.

## Monocyte-induced carbonylation of laminin-1 and collagen IV in the presence of glucose and insulin

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Monocytes are a key source of producing reactive oxygen species (ROS) such as superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$). Monocyte-derived ROS have multiple actions, including the oxidation of proteins on their immediate environment. Monocyte-extracellular matrix

(ECM) interactions have been implicated in atherosclerosis pathophysiology. In the present study we evaluated monocyte-induced carbonylation of laminin-1 and collagen IV, key structural and functional components of the basement membranes, in the presence of glucose and insulin. Moreover, superoxide anion and hydrogen peroxide production by monocytes in the presence of glucose and insulin were estimated.

Laminin-1 and collagen IV carbonylation was tested by a sensitive ELISA assay using dinitrophenol (DNPH) and an anti-DNP antibody. Superoxide anions were detected intracellularly, with the use of nitroblue tetrazolium (NBT). Hydrogen peroxide levels were also detected intracellularly, with the use of the fluorescent indicator 2.7-dichlorodihydrofluorescein diacetate (DCFH-DA). Monocytes were incubated with glucose, insulin and several inhibitors for 30 minutes at 37 °C. The inhibitors used, were Cariporide, that inhibits the $Na^+/H^+$ exchanger, NHE-1, Gf and Go, inhibitors of different protein kinase C (PKC) isoforms, DPI, an inhibitor of NADPH oxidase, and Wortmannin, that inhibits the phosphoinositide 3-kinase (PI3K). The activator of PKC, PMA was also used. The level of statistical significance of the differences was estimated using the Student t-test.

The level of laminin and collagen carbonylation was higher when monocytes were incubated in the presence of glucose and insulin than in controls. On the contrary, the presence of the inhibitors of NHE-1, PKC, NADPH oxidase and PI3K, together with either glucose or insulin counteracted the increase in protein carbonylation. Furthermore, the presence of PMA showed higher laminin-1 and collagen IV carbonylation in relation to controls. It was also observed that in the presence of glucose and insulin, monocytes produced increased levels of ROS in relation to controls. In the case that superoxide anion levels were measured, the increased levels induced by glucose and insulin were inhibited after treatment with the inhibitors of NHE-1, PKC, NADPH oxidase and PI3K. On the contrary, in the case that hydrogen peroxide levels were estimated, the increased levels induced by glucose and insulin were inhibited only after treatment with the inhibitors of NADPH oxidase and PI3K.

Our results indicate that both glucose and insulin affect monocyte-induced carbonylation of laminin-1 and collagen IV, while they also affect ROS production by monocytes. In the case of carbonylation, it is possible that the above substances act through activation of NHE-1, PKC, NADPH oxidase and PI3K, while the same signalling molecules seem to play a significant role in ROS production by monocytes.

## Synthesis of RGD carboxamide analogues and incorporation of resveratrol to the side chain of aspartic acid. Evaluation of the biological activity

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Peptides containing Arg-Gly-Asp (RGD) sequence are considered to be potent inhibitors of platelet aggregation. That is so because the RGD sequence is recognized from the integrin GPIIb/IIIa, a glycoprotein receptor found on the surface of the platelets, which is primarily responsible for platelet aggregation. Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene), on the other hand, is a naturally occurring phytoalexin found in grapes, which demonstrates a broad range of biological properties, one of which is antiplatelet activity.

The fact that both resveratrol and RGD containing peptides have shown antiplatelet activity prompted us to combine them in the same molecule and investigate the effect of the coupling products on platelet aggregation. Initially, the peptide amides were synthesized by the solid phase technique, using the Rink amide MBHA resin, as a stationary phase, by the method of carbodiimides (DIC/HOBt). The N-terminal amino groups of the peptides were either acetylated or left unprotected, while all the other protecting groups used during the synthetic procedure were removed. The second stage involved the incorporation of resveratrol to the side chain of aspartic acid, through the formation of an ester bond between aspartic acid's side chain carboxylic group and one of resveratrol's phenolic hydroxyl-groups. Coupling reaction took place in solution using *N*,*N*-dicyclohexylcarbodiimide (DCC) as coupling reagent and 4-dimethylaminopyridine (DMAP) as a catalyst. The reaction products were purified by reversed phase HPLC and identified by ESI-MS. Finally, the biological activity of the coupling products, concerning the inhibition of human platelet aggregation, was determined in vitro against several peptide agonists.

## Synthesis of acetylenic analogues of glutamate

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Glutamate ((*S*)-Glu) is the major excitatory amino acid in the central nervous system. It acts by stimulating ionotropic and metabotropic glutamate receptors (iGluR and mGluR, respectively). Glutamate has been shown to be involved in many neuropathologies such as anxiety, pain, ischemia, Parkinson's disease, epilepsy and schizophrenia.

mGlu receptors are G-protein-coupled receptors and eight subtypes (mGluR1–8) have been identified and classified into three groups (I–III) based upon sequence homology, transduction mechanism and pharmacological profile.

Because of their modulating properties, mGlu receptors are recognized as promising therapeutic targets and many ligands (agonists and antagonists) have been prepared to better understand the pharmacology of mGlu receptors in order to selectively activate the different groups and subtypes of receptors.

An α-amino acid moiety can be found in all mGlu receptors competitive ligands and most of the side chains hold an acidic function. Examination of the glutamate binding site in the mGlu receptors and pharmacological data of some ligands shows that sterically constrained structures with an optimal distance between functional groups could lead to potent and selective new ligands.

It is known that introducing an unsaturation in a biologically active structure could modify the conformation of the molecule and thus the biological activity. In this respect, results in the synthesis of acetylenic analogues of glutamate will be described.

## In vitro assessment of the antineoplastic and antiangiogenic potential of a series of L-canavanine analogues

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Three novel L-aminoacid mimetics (nitrocanavanine, nitroarginine and methylnitrocanavanine) where synthesized and characterized. Their antiproliferative and cytotoxic potential was examined in a panel of human tumor cell lines as well as against VEGF-stimulated human umbilical vein endothelial cells (HUVECs). Throughout the experiments the prototype aminoacid L-canavanine (CAV) and the cytotoxic/antiangiogenic agents thalidomide (THAL) and hydroxyartemisinine (HART) where used as positive controls. The preliminary screening for antiproliferative and cytotoxic effects of tested compounds against a spectrum of six human cell lines revealed that the newly synthesized agents display only marginal effects. In contrast all of the tested com-

pounds inhibited the proliferation of VEGF-stimulated HUVECs, whereby nitrocanavanine and nitroarginine did not affect the viability of HUVECs, while methylnitrocanavanine caused some cytotoxicity at concentrations exceeding 200 μM. The lack of cytotoxicity was corroborated by an DNA-fragmentation assay which indicated that nitrocanavanine, nitroarginine and methylnitrocanavanine did not induce apoptosis in HUVECs. The abnormal proliferative activity of vascular endothelium is a crucial event in tumor-induced angiogenesis and hence the established inhibitory activity of the novel aminoacid mimetics and CAV, together with their low cytotoxicity give us reason to consider these compounds as prospective leads for development of antiangiogenic agents.

## Synthesis of squaric acid-containing amino acid analogs

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The carboxyl group in a-amino acids is an important functional group as a proton-donating or an peptide bond-forming group. It has been recognized that sulfonic acid, phosphonic acid, boronic acid, and tetrazole can serve as an important surrogate for the carboxyl group of a-amino acids. These analogs have attracted significant attention in view of their metabolic stability, inhibitory effects to proteases, and haptens to generate catalytic antibodies. In addition to these carboxylic acid surrogate, we have been interested in 2-hydroxy-3,4-dioxocyclobut-1-enyl (Sq) group known as a planar squaric acid which belongs to a class of oxocarbons and exhibits unique physicochemical properties, for example, strong acidity, aromaticity, strained ring, electron deficiency, and metal chelating ability. The Sq group has received considerable attention as a carboxylic acid mimic in medicinal chemistry, novel chromophore in material science, new chelator in inorganic chemistry, and useful building block in organic synthesis. We considered that the replacement of the carboxyl group involved in biologically important amino acids with a Sq group would provide a new and useful entry as a novel amino acid surrogate. In this paper, we wish to describe the methods and syntheses of Sq group-containing amino acids, named a-amino squaric acids, where the Sq group is incorporated into the amino acid structure by a carbon-carbon bond or carbon-sulfur bond.

## Oligomeric products of N-acetyltyrosine bromination

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Myeloperoxidase (MPO), a neutrophil enzyme, utilizes hydrogen peroxide and ubiquitous in the medium halide anions to form hypochlorite ($HOCl/OCl^-$) and hypobromite ($HOBr/OBr^-$). These reactive oxidants and halogenating agents react, in turn, with amino groups of peptides and proteins to form mono- and di- chloro- or bromamines. Although the chlorinating MPO activity is the main one, it has been demonstrated by Gaut et al., that the bromination pathway also operates when $Br^-$ concentration is $10^3$–$10^4$ times lower that physiological $Cl^-$ concentration – therefore it is believed to be physiologically relevant. It was also shown that in MPO-$H_2O_2$-$Cl^-/Br^-$ system hypochlorite is produced as a first and then it reacts with $Br^-$ to form hypobromite. The both halogenating agents are able to brominate/chlorinate/oxidize various compounds containing phenol residue. It is widely known that at the inflammation site, proteins containing Tyr moiety form 3-(bromo)chlorotyrosine, 3.5-di(bromo)chlorotyrosine, 3,4-dihydroxytyrosine (DOPA) and dityrosine (DT). Moreover, 3-bromo- and 3-bromotyrosine could serve as a marker of sepsis or inflammation.

We examined the MPO and hypobromite mediated bromination of N-acetyltyrosine. The formation of 3-bromotyrosine and other oligomeric products was monitored in aerobic conditions in μM-mM concentration range, 2–7 pH range and at the presence of proteins and peptides. Sample were analyzed with the reversed-phase HPLC and fractions examined with electrospray mass spectroscopy (ESI). HPLC profiles revealed that that bromination in examined systems leads to the formation of N-acetyl-3-bromotyrosine and a new oligomeric product for the 50–500 μM reactants concentration range. The formation of oligomeric product predominated in pH range 3–5 (70–95%) whereas at the pH 7 the monomeric N-acetyl-3-bromotyrosine was the main product. At a presence of taurine, Ala–Ala, N-acetyl-Ala–Ala and albumin HPLC profiles showed similar pattern although the total yield of brominated products was lower. The same procedures applied for lower reactants concentrations (10–25 μM) resulted in the higher 3-bromotyrosine yield. The structure of oligomer was examined with ESI and nuclear resonance methods: $^1$H-NMR, $^{13}$C-NMR, COZY $^1$H–$^1$H, $^1$H-$^{13}$C. NMR spectra showed that oligomer is built of four bromotyrosine units linked with isodityrosine or dityrosine bonds. $^1$H-NMR spectra revealed a presence of 1,2,3,5-tetrasubstituted benzene moiety. ESI spectra showed the presence of the molecular ion of $m/z = 1200$ and isotope pattern corresponding to four bromine atoms. Fragment ions of $m/z = 780$, $m/z = 420$, 384, 358 and 321, 287, 277 in the ESI mass spectrum implicated the presence of the fragments containing respectively four, two and one monobromobenzene moieties.

It is likely that oligomer (cyclic tetramer) is formed via N-acetyl-3-bromotyroxyl radicals although the mechanism of radical formation is still unclear. Our results suggest that bromination of N-acetyl-tyrosine and concomitant tetramer formation could be mediated with N-bromamines moieties of the peptide and protein lysine residues. Tetramer is cytotoxic and attenuates the proliferation of human prostatic cancer (PC-3) cells in the 5-70 μM concentration range.

## Synthesis of linear analogue peptides of 1811–1818 loop of the A3 subunit of the light chain A3-C1–C2 of FVIIIa blood coagulation

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Factor VIII is a protein cofactor for the serine protease Factor IXa, which catalyzes the conversion of Factor X–Xa and is decreased or defective in individuals with hemophilia A. Factor VIII is synthesized as a multidomainal single-chained molecule (A1-A2-B-A3-C1-C2), with a molecular mass of 300 kDa. Factor VIII circulates as a partial proteolyzed protein containing a heavy chain (A1-A2-B domains) and a light chain (A3-C1-C2 domains), which are held together by a metal ion dependent linkage.

In the intrinsic pathway of blood coagulation, FVIII functions as a nonenzymatic cofactor in the Factor X (FX)-activating complex.

Recent studies have identified the FVIII light chain region Glu1811-Lys1818 as being involved in FIXa binding and in the assembly of the FX-activating FIXazFVIIIa complex.

Here we report on the synthesis of linear analogue peptides of the 1811–1818 loop of the A3 subunit of the light chain A3-C1-C2 of FVIIIa, in order to examine their anticoagulant activity.

The peptides were synthesized by the solid phase technique, using the 2-Chlorotrityl chloride resin, as a stationary phase, by the method of carbodiimides (DIC/HOBt), while for the peptide amides synthesis the Rink amide MBHA resin was used. The N-terminal amino groups of the peptides were either acetylated or left unprotected, while all the other protecting groups used during the synthetic procedure were removed. The reaction products were purified by reversed phase HPLC

and their effect on human platelet aggregation in vitro experiments was also investigated.

## Synthesis of cyclic RGD peptides incorporating thiosalicylic acid and their antiplatelet activity in vitro

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Integrins are trans-membrane glycoprotein (GP) receptors that mediate cell–cell or cell-extracellular matrix interactions. The GPIIb/IIIa, which serves as a receptor for plasma protein fibrinogen, is a member of superfamily of cell adhesion proteins (integrins). During last decades much research has been directed to the field of platelet membrane receptor GPIIb/IIIa, which mediates platelets aggregation process. The GPIIb/IIIa undergoes significant conformational changes on platelet activation usually by thrombin, ADP or collagen and results in high affinity for plasma protein fibrinogen, which binds to receptor and assembles adjacent platelets into aggregation through the RGD (Arg-Gly-Asp) sequence occurring in fibrinogen. The interest of researchers is essentially focused on the design, synthesis and development of compounds, which prevent a thrombosis through the inhibition of platelet aggregation. A fundamental physiological role of a platelet, as a blood element, is the interaction with fibrinogen, which leads to the platelet aggregation and thereby initiates the formation of hemostatic clot, preventing bleeding at the site of a vascular injury. Various RGD containing peptides are able to block the native ligand from binding to its receptor.

Our team has already demonstrated that small linear and cyclic peptides, containing the sequence RGD, show extremely strong antiplatelet activity. Continuing this research project we have synthesized a new series of cyclic RGD peptides with a variety of ring sizes incorporating thiosalicylic acid, by conventional solution techniques and/or by solid phase. The synthesized RGD cyclopeptides were tested for inhibitory activity on human platelet aggregation in vitro, by adding common aggregation reagents (collagen, ADP, ristocetin) to citrated platelet rich plasma.

The cyclic peptides have many advantages. They are important tools in biological tests because they exhibit metabolic stability, higher rigidity and generally they enhance the ligand-receptor binding compare to linear analogs. All the syntheses of the linear precursors were carried out stepwise in SPPS via an ester linkage, using the Fmoc/$Bu^t$ methodology on the solid support of 2-chlorotrityl chloride resin and DIC/HOBt as coupling reagent. All the peptides were purified (HPLC) and identified (ESI-MS). They are under investigation for their biological activity.

## o-Quinone methides of bis-naphthalene and perylene as alkylating agents of selected peptides

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Quinone methides (QMs) have been proposed as intermediates in a large number of chemical and biological processes. The reactivity of QMs is mainly due to their electrophilic nature, which can also be directly correlated to their toxicological properties. QMs have been proposed as intermediates in the biosynthesis of lignin and enzyme inhibition. Among hydrolase inhibitors, QMs have been recently used as covalent α-lactamase, phosphatase, and ribonuclease A7 inactivators. It has been suggested that quinone methides play a key role in the chemistry of several classes of antibiotic drugs and antitumor compounds such as mitomycin C and anthracyclines. With simple modifications suggested o-Quinone Methides (o-QM) could cross-link two biologically useful molecules, such as nucleic bases or peptides and proteins. If the alkylation process is achieved under mild (ideally biological) reaction conditions, it could be used in a number of biomolecular applications. In fact these reactive intermediates have been used as DNA alkylating agents and cross-linkers.

Here we present the synthesis of a new series of synthetic o-QMs (bis-napthalene and perylene type) conjugated with RGD analogue peptides. The biological activity of these compounds is under investigation.

## New cinnamoyl amides of nonproteinogennic fluorinated amino acids and their biological activity

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N-hydroxycinnamic acid amides (HCAAs) constitute a diverse class of phenolic amides present in several plant species. The role of N-phenylpropenoyl amino acid amides in the plants is still not clear. Many studies have suggested that these compounds might play an important role in the chemical defense of plants against fungal and bacterial pathogens.

Our research program is focused on obtaining of new bioactive HCAAs. In this study, cinnamoyl and hydroxyl cinnamoyl amides of mono fluorinated α-amino acid have been synthesized. The incorporation of these unusual amino acids in cinnamoylamides is of increased interest because of their potential pharmacological utility in drugs.

The structure of the obtained compounds has been proved by UV, $^1$H-NMR and MS.

A study of antiviral activity of the synthesized compounds is in progress.

## Halogenation of substituted cinnamic acid amides

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It is well known that cinnamic acid derivatives show antiviral, antibacterial, vasoactive, antiflammatory, antioxidant and other properties. To study the effect of removal of double bond in the side chain of such compounds on antimicrobial and radical scavenging activities, several *trans*-cinnamoyl amino acid amides were brominated and chlorinated. The dihalogeno cinnamoyl amides were generally obtained in good yields and purified by recrystallization from 96% $EtOH/H_2O$.

A study of radical scavenging activity against $DPPH^*$ test and antimicrobial activity of the synthesized compounds is in progress.

## Synthesis and antioxidative activity of cinnamic acid amides of thiazole containing amino acids

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Cinnamic acid derivatives as esters, amides and glycosides are known to have antibacterial, antiviral, antiinflammatory, antiproliferative, immunostimulatory, etc. properties. Some of them (amide and ester analogues of caffeic acid with natural amino acid esters) exibit stronger antioxidative activity. These activities are associated as well with their function as antioxidants and the enzyme inhibitors as with their binding activity with specific receptors.

In the last two decades, inprecedented biologically active natural products containing directly linked azoles, have been isolated from natural sources. Many of these compounds are candidates for drug development. In particular thiazole, oxazole and imidazole amino acids that may play a key role in biological activities of unusual peptides are also important intermediates for natural product synthesis and peptidomimetics.

Here we report the synthesis of novel cinnamic acid amides with thiazole containing valil-ethyl ester as potential antioxidants.

The amides were synthesized from sinapic, coumaric, ferulic acids and the corresponding valyl-ethyl ester hydrochloride form using N-ethyl-N-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 4-N,N-(dimethylamino)-pyridine (DMAP).

Their efficiency as radical scavengers was evaluated by 2,20diphenyl-1-picrylhydrazyl DPPH (1,1-diphenyl-2-pycrylhydrazyl) test.

## Aminoacylation of unprotected saccharides

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Despite the attractive properties of sugar esters, their industrial uses remain limited, as they are difficult to synthesize chemically. In the synthetic design of a newly sugar esters it is very important to control the degree of substitution and the regioselectivity. Even with similar physicochemical properties, regioisomers might differ in their toxicity, smell, taste or biodegradability.

In this study, we described a simple method for aminoacylation of unprotected sugars with mildly activated aminoacids esters. As a model reaction, we investigated esterification of sucrose – "royal carbohydrate" with cyanomethyl ester of benzyloxycarbonyl-phenylalanine.

We find the experimental condition to realized *one pot synthesis* in organic solvent, of esters of unprotected carbohydrate compounds using chloroacetonitrile as a coupling reagent.

## Synthesis of new α,α-disubstituted cyclic amino phosphonates whit potential antitumor activity

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Aminophosphonic acids are very interesting because of their diverse biological activity, including anticancer, antiviral, antibacterial, antifungal and insignificant toxicity in mammalian cells. These compounds are closely related to the amino acids, thus acting as competitive inhibitors and they can serve as false substrates during the course of amino acid metabolism.

The objectives of the present study is the synthesis of new *N*-(phoshonomethyl)glycine derivatives prepared from cycloalkaneaminocarboxylic acids with 5-, 6-, 7-, 8- and 12-membered rings as well as investigation of their antitumor activity. The new compounds were synthesized by three-component *Kabachnik-Fields* reaction. The corresponding aminocycloalkanecarboxylic acid was treated with paraformaldehyde in the presence of triethylamine in methanol. When the reaction mixture became homogeneous, dimethyl hydrogen phosphonate was added. All the compounds were obtained in high yield and their structure was proved by $^{1}H$, $^{13}C$ and $^{31}P$ NMR spectroscopy. The cytotoxicity of the compounds was evaluated in a panel of human tumor cell lines, the clinically utilized drug cisplatin being used as a positive control. The cytotoxicity determination was extended against the human embryonal kidney cell line 293T chosen as a model of normal, non-malignant cellular population. The 8- and 12-membered cyclic analogues proved superior to the remaining compounds, and were found to trigger apoptotic cell death in DOHH-2 cells. The latter compound caused 50% inhibition of the viability of hemobastose-derived cell lines at concentrations ranging from 20–67 μM.

## Determination of chirality of novel amino acid, α-phenyl-α-(2-pyridyl)glycine in peptides

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Nonproteinogenic α,α-disubsutituted glycines are of particular importance in developing small, acyclic, conformationally restricted peptides. Recently, we have synthesized peptides containing novel α,α-disubstituted glycines having 2-pyridyl group(s), such as α,α-di(2-pyridyl)glycine (2Dpy), α-phenyl-α-(2-pyridyl)glycine (2Ppg) and α-methyl-α-(2-pyridyl)glycine (2Pmg). NMR studies of the tripeptides containing 2Ppg revealed that 2Ppg has propensity to induce a β-turn structure by an intramolecular hydrogen bond between 2Ppg-NH and a pyridine nitrogen.

Herein, we describe the determination of chirality of 2Ppg residue in peptides. We have prepared 2Ppg-containing tripeptide (Z-L-Val-L/D-2Ppg-Aib-OMe) and tetrapeptide (Z-L-Val-L/D-2Ppg-Aib-L-Leu-OMe) using the Ugi reaction in good yields. Diastereomers of both peptides could be preparatively separated by silica-gel column chromatography. The crystal structure of one diastereomer of the tetrapeptide was clarified by X-ray analysis. Thereby, the configuration of a 2Ppg residue in the diastereomer was determined to be R, and the β-turn structure was revealed. CD spectra of a pair of the diastereomers, which differ only in the configuration of the 2Ppg residue, look like those of enantiomers each other in dichloroethane. This means that the configuration of 2Ppg residue may mainly contribute to CD spectra of peptides and the (R)-2Ppg residue indicates the positive Cotton effects at 227 nm and the negative one at 272 nm.

We have also tried to prepare and separate diastereomeric mixtures of tripeptide esters, Z-AA-D/L-2Ppg-AA-OR* (AA = Gly or Aib), in which various optically active alcohol (R*-OH) were used. Diastereomers of Z-Aib-D/L-2Ppg-Aib-OR*, where R*-OH was (S)-methyl manderate or (R)-1-phenylethanol, could be chromatographically separated. The configurations of 2Ppg residue in these diastereomers were determined from CD spectra.

### Effect of glucose on atherosclerosis-related functions of human monocytes

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Peripheral blood monocytes can bind to the vascular epithelium and to extracellular matrix molecules, like laminin and migrate to the subendothelium space. In subedothelium space monocyte CD36 scavenger receptor recognise and bind oxidized low density lipoproteins (oxLDL). OxLDL are then phagocytosed and accumulate in the lysosomes converting monocytes into foam cells. Foam cell formation is the first step towards atherosclerosis development. The monocyte exposure to oxLDL and oxLDL binding to CD36 scavenger receptors, activates Peroxisome Proliferator-Activated Receptors γ (PPAR-γ) transcription. Hormones related to diabetes, like insulin or glucose, activate atherosclerosis-related monocyte functions.

In the present study was investigated the effect of high glucose concentrations, insulin and PPARγ activators (rosiglitazone) in atherosclerosis-related functions of human monocytes. The atherosclerosis-related functions studied were monocyte adhesion to laminin, collagen type IV or endothelial cells, monocyte migration through the same substrates, CD36 scavenger receptor expression and oxLDL phagocytosis. In our experiments we used cariporide (the inhibitor of $Na^+/H^+$ exchanger – NHE1), Go (the inhibitor of PKCα and PKCβ isoforms), Gf (the inhibitor of all PKC isoforms) and wortmanin (inhibitor of PI3K). In the experiments for CD36 expression and oxLDL uptake we also used puromycin (protein synthesis inhibitor) and cytochalasin D (inhibitor of actin polymerization).

The results of our experiments confirmed that high glucose concentrations, insulin and rosiglitazone induced all the studied atherosclerosis-related functions of the monocytes. In all these functions the addition of cariporide counteracted the activity of glucose, insulin and rosiglitazone. The data of the present study also suggests that PPARγ activation in monocytes induces atherosclerosis, and that NHE 1 exchanger plays an important role in the beginning of atherosclerosis. The addition of Go, Gf and wortmanin reversed the effect of glucose, insulin or rosiglitazone, indicating the involvement of PKC isoforms and PI3K in the signal transduction leading to atherosclerosis-related functions of monocytes. Besides, puromycin and cytochalasin D treatment indicated that the increase in the number of CD36 receptors can be attributed to CD36 mRNA transcription and underscored the essential role of actin in the oxLDL uptake.

### Synthesis of new GNRH-I analogues with modifications in position 6 and biological evaluation on pituitary and breast cancer cells

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GnRH analogues have been extensively used in oncology to induce reversible chemical castration. In addition to the classic hypophysiotropic action of GnRH, it has been shown that many malignant cells, such as breast cancer cells, locally produce GnRH-I and express the GnRH-I receptor/s. We synthesized seven new GnRH-I analogues with modifications in position 6 and studied their pituitary binding affinity (in αT3-1 cell membranes) and effect on breast cancer (MCF-7 and MDA-MB-231) cell proliferation. $Gly^6$ was substituted by D-Lys alone or covalently linked to Gly, Ala, Sar, 2-Amino-4-pyrrolidinothieno[2,3-*d*]pyrimidine-6-carboxylic acid (ATPC), a-Me-Val, N-Me-Val. All GnRH analogues lacked the carboxy-terminal $Gly^{10}$-amide of GnRH and an ethylamide residue was added to $Pro^9$, a modification common in many potent GnRH agonists, such as leuprolide ([D-$Leu^6$, *des*-$Gly^{10}$]-GnRH-NHEt. Results show differential impact of these modifications on the binding affinity to the GnRH receptor in mouse pituitary cells and on the inhibition of human breast cancer cell proliferation. Substitutions in position 6 always resulted in high binding affinities. In particular, [D-$Lys^6$(Gly), *des*$Gly^{10}$]-GnRH-NHEt and [D-$Lys^6$(Sar), *des*$Gly^{10}$]-GnRH-NHEt have higher pituitary binding affinity than leuprolide, but only the latter had significant antiproliferative effect on both MCF-7 and MDA-MB-231 cells.

# Transport

### The actin-binding protein α-adducin interacts with a pool of EAAT3 glutamate transporters in C6 glioma cells

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The ubiquitous EAAT3 transporter belongs to the family of the $Na^+$-dependent glutamate carriers expressed mainly in the CNS. Although several studies have demonstrated that EAAT3 dysregulation is associated with neurologic disease, the mechanisms underlying EAAT3 regulation are not yet fully understood. We have recently reported that, in C6 glioma cells, both basal activity and membrane targeting of EAAT3, but not its up-regulation by phorbol esters, require the integrity of actin cytoskeleton. Here we show that, in the same cell model, EAAT3 partially co-localizes with actin filaments as well as with the actin binding protein α-adducin. While EAAT3/actin co-localization is mostly detected on cell processes of cells with bipolar morphology, co-localization between EAAT3 and adducin is more evident in the perinuclear zone, where the transporter is distributed in vesicle-like structures. The interaction between EAAT3 and α-adducin is also confirmed by co-immunoprecipitation experiments. However, neither co-localization nor co-immunoprecipitation patterns are modified by cell treatment with phorbol esters. Moreover, co-immunoprecipitation experiments indicate that the interaction between the two proteins is also resistant to Latrunculin A, a toxin which causes the disorganization of F-actin. In conclusion, these data point to a PKC- and F-actin-insensitive interaction between α-adducin and a pool of intracellular transporters, suggesting that the cytoskeletal protein may be involved in EAAT3 trafficking.

## Transport of angiotensin converting enzyme inhibitors by $H^+$/peptide transporters – revisited

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The $H^+$/peptide cotransporters PEPT1 and PEPT2 transport di- and tripeptides as well as peptidomimetics such as certain β-lactam antibiotics across the apical membrane of intestinal and renal epithelial cells. It has been reported several times that PEPT1 and PEPT2 also accept many angiotensin converting enzyme (ACE) inhibitors as substrates. Today, the view that the oral bioavailability of ACE inhibitors such as captopril and enalapril is due to intestinal PEPT1 activity is discussed uncritically in most reviews on peptide transport. Because we did not find an appreciable interaction of captopril with hPEPT1 we re-evaluated this concept in competition assays using [$^{14}$C]Gly-Sar as a reference substrate for hPEPT1 expressed in Caco-2 cells and rPEPT2 expressed in SKPT cells. In a second approach, the interaction of ACE inhibitors with hPEPT1 and hPEPT2 was investigated after heterologous expression of the transporters in the membrane of human cells. Fourteen ACE inhibitors including new compounds were tested with regard to their interaction with peptide transporters. Medium affinity was observed for spirapril, trandolapril, ramipril, benazepril, quinapril, moexipril and cilazapril. High affinity inhibitors were fosinopril and zofenopril. For perindopril, enalapril, quinaprilat, lisinopril and captopril interaction was very weak or not measurable. When PEPT1 and PEPT2 were expressed in *Xenopus laevis* oocytes and analyzed by the two-electrode voltage clamp technique, ACE-inhibitor (10 mM at PEPT1, 2 mM at PEPT2) induced inward currents were in most cases less than one fifth compared to those elicited by Ala–Ala–Ala and Gly-Gln. For half of the compounds no currents were observed. In our opinion, the concept of ACE inhibitor transport by peptide transporters has to be put into perspective more critically.

## Recombinant expression of the amino acid transporter PAT1 and the peptide transporters PEPT1 and PEPT2

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Most membrane carriers for amino acids and peptides transport certain drugs because of their sterical resemblance to the natural substrates. Much of the current development is being driven by the pharmaceutical industry which looks on epithelial transporters as vehicles for drug delivery in general and for purposes of increasing the oral bioavailability of drugs such as ß-lactam antibiotics and proline- and GABA-related drugs. Progress is limited by the lack of structural data for all members of the solute carrier (SLC) family, and this applies also for the amino acid transporter PAT1 and peptide transporters PEPT1 and PEPT2. At the moment there are only 124 structures of integral membrane proteins in the protein data bank available, in contrast to several thousands of non-membrane proteins. This is mainly due to difficulties in getting sufficient amounts of purified protein.

Here we present the first results of our studies regarding the recombinant expression of the carrier proteins hPEPT1, hPEPT2 and hPAT1. We cloned the transporter cDNAs into different *E. coli* expression vectors and tested the expression in different expression strains at varying growing conditions. The next step was a large scale fermentation where PEPT1 could be expressed as insoluble *inclusion bodies*. After solubilisation of the precipitated protein and an enrichment step via IMAC, the identity of the carrier was confirmed after tryptic cleavage by mass spectroscopy. The recombinant expression of hPEPT2 and hPAT1 in a large scale was so far not successful. Hence, we tested the cell free expression of both proteins using the EasyXpress Protein Synthesis Mini Kit from Qiagen. With this *E. coli* lysate-based system we could show expression for each membrane protein. The expression of hPAT1 was also tested at large scale using the EasyXpress Protein Synthesis Maxi Kit (Qiagen). The insoluble precipitate was solubilized and purified via IMAC and hPAT1 protein could be detected as a prominent band on a Coomassie stained SDS-gel.

## Mutational analysis of histidine residues in the $H^+$/amino acid cotransporter PAT1

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The human $H^+$/amino acid cotransporter 1, hPAT1, is mainly expressed in intestine, brain and lung. The substrate specificity of the system and the transport mechanism have been investigated in detail in recent years. The system received very much attention recently because of its ability to transport therapeutically relevant amino acid derivatives such as cis-4-hydroxy-L-proline, L-azetidine-2-carboxylic acid, 3-amino-1-propanesulfonic acid, nipecotic acid and D-cycloserine. For several other $H^+$/solute cotransporters essential histidine residues have been identified to function as $H^+$ acceptors/donors. We investigated the influence of an inwardly directed proton gradient on the kinetic parameters of L-[$^3$H]proline uptake in Caco-2 cells. At an outside pH of 7.5 the transport affinity of L-proline and several drugs decreased dramatically, the $K_t$ of L-proline uptake increased from 1.8 to 22 mM. $H^+$ did not affect the maximal velocity of transport. Similarly, diethylpyrocarbonate (DEPC), known to chemically block histidine residues decreased only affinity of L-proline transport but not the maximal velocity of transport. Both extracellular protons and DEPC treatment strongly increased the inhibition constants ($K_i$) of other substrates at hPAT1. Three histidine residues are conserved among all PAT proteins (PAT1-4) and different species, H55 and H93 located in the first and second transmembrane domain and H135 located in the second cytoplasmatic loop. We individually mutated histidine residues and transiently transfected HRPE cells with N-terminal HA-tagged hPAT1-mutants to compare their catalytic function. The expression was examined by cell surface biotinylation and immunoblotting. These site-directed mutagenesis analyses indicate that His55 seems to be the most critical histidine residue required for the transport activity of PAT1.

## Phosphate specific transporter system (PSTS) in *Thermus thermophilus* involves a DING phosphatase

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Phosphate transport in bacteria occurs via a phosphate specific transporter system (PSTS) that belongs to the ABC family of transporters, a multisubunit system, containing an alkaline phosphatase. DING proteins were characterized due to the N-terminal amino acid sequence DINGG-GATL, which is highly conserved in animal and plant isolates, but more variable in microbes. Most prokaryotic homologues of the DING proteins often have some structural homology to phosphatases or periplasmic phos-

phate-binding proteins. In *E. coli*, the product of the inducible gene *DinG*, possesses ATP hydrolyzing helicase enzymic activity. An alkaline phosphorolytic enzyme of the PSTS system was purified to homogeneity from the thermophilic bacterium *Thermus thermophilus*. N-terminal sequence analysis of this protein revealed the same high degree of similarity to DING proteins especially to the human synovial stimulatory protein P205, the steroidogenesis-inducing protein and to the phosphate ABC transporter, periplasmic phosphate-binding protein, putative [*P. fluorescens* Pf-5]. The enzyme had a molecular mass of 40 kDa on SDS/PAGE, exhibiting optimal phosphatase activity at pH 12.3 and 70 °C. The enzyme possessed characteristics of a DING protein, such as ATPase, ds endonuclease and 3′phosphodiesterase (3′-exonuclease) activities and binding to linear dsDNA, displaying helicase activity on supercoiled DNA.

In this work the purification and biochemical characterization of a *T. thermophilus* DING protein was achieved. The biochemical properties, N-terminal sequence similarities of this protein implied that the enzyme belongs to the PSTS family and might be involved in the DNA repair mechanism of this microorganism.

## ADMA-efflux from endothelial cells

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Elevated plasma concentrations of the endogenous nitric oxide synthase (NOS) inhibitor asymmetrical dimethylarginine (ADMA) represent an established risk factor for cardiovascular disease. However, the ADMA plasma levels found in various clinical settings, such as renal failure, coronary heart disease, hypertension, diabetes, and pre-eclampsia, are not high enough to explain an efficient competitive inhibition of endothelial NOS (eNOS). Because ADMA derives from protein degradation, one hypothesis is that ADMA may accumulate intracellularly at the site of its generation. Protein degradation provides also arginine for NO synthesis. It thus seems crucial that ADMA is efficiently removed from this important eNOS substrate source. We thus ask the question which transporter(s) is responsible for ADMA efflux from endothelial cells. Transport studies with Xenopus laevis oocytes identified the system y + L amino acid transporter-1 (y + LAT1) as an efficient ADMA efflux path that mediates the exchange of neutral amino acids and $Na^+$ against intracellular ADMA. The expression of this transporter was reduced in peripheral blood mononuclear cells of a patient with vasospastic angina. The patient had severe coronary and peripheral endothelial dysfunction accompanied by eNOS-derived superoxide production (=uncoupling). Treatment with 6 g Arg orally for a three months period normalized endothelial dysfunction and reversed eNOS uncoupling. Acute challenge with Arg or Orn (9 g each) markedly increased the plasma levels of ADMA, compatible with an intracellular accumulation of ADMA in the patient that was overcome by high concentrations of cationic amino acids facilitating ADMA export through cationic amino acid transporter (CAT). In agreement with this hypothesis, ADMA efflux from peripheral blood mononuclear cells of the patient was accelerated by cationic, but not neutral amino acids. Our data suggest that y + LAT is the major export route for ADMA under physiological conditions. An impairment of this transporter may cause intracellular (endothelial) ADMA accumulation leading to eNOS uncoupling and systemic endothelial dysfunction, and may thus represent a novel mechanism underlying vasospastic angina.