M1 – Peptidomimetics and Signal Transduction Inhibition

M1–001
Tricking cancer cells to die

A. Levitzki
Unit of Cellular Signaling, Department of Biological Chemistry, The Alexander Silberman, Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel.
E-mail: levitzki@vms.huji.ac.il

Cancer cells develop strong anti-apoptotic signaling pathways and therefore escape many therapeutic regimens. Recognizing this feature of cancer cells, we have focused on two approaches: disarming the cancer cell from its anti-apoptotic weaponry [1, 2] and applying strategies aimed at enhancing pro-apoptotic signaling pathways selectively in the cancer cell [3]. The first goal has been achieved by developing highly selective Aktstatins [1, 2] that inhibit PKB 100 times better than PKA or PKC. These inhibitors are highly non-toxic, inhibit Akt/PKB induced phosphorylation in cells and in vivo and are highly effective as anti-tumor agents *in vivo*. The complementary strategy is to enhance pro-apoptotic

signaling pathways, selectively in cancer cells [3]. One of the key elements is to induce in the targeted cancer cells signaling pathways that induce strong by-stander effects, killing pretty fast not only the targeted cells but also the neighboring cancer cells that do not express the target, a common situation in the heterogeneous human tumor. We have achieved this goal for tumors over-expressing the EGF receptors, by targeting them with EGF guided non-viral vectors loaded with double stranded RNA. These dsRNA molecules are internalized by EGF receptor mediated endocytosis and kill only cells that over-express wild type EGFR, and neighboring tumor cells co-growing with the targeted cells. Using this targeted polyIC, we are able to cure mice bearing sizable intracranial human *Glioblastma Multiforme* (GBM), without harming normal brain tissue [4].

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M1–002
IkB kinases in innate immunity and cancer

M. Karin

Laboratory of gene regulation & signal transduction, Pharmacology, University of California, San Diego, La Jolla, California, CA, USA. E-mail: karinoffice@ucsd.edu

Mammals express five NF-κB proteins: NF-κB1, NF-κB2, RelA, RelB and c-Rel. These proteins can assemble into a variety of homo- and heterodimers that bind to κB sites on DNA and induce transcription of genes whose products play key roles in activation of innate and adaptive immune responses, inflammation and prevention of apoptosis. NF-κB1 and NF-κB2 require – proteolytic processing to produce the mature p50 and p52 NF-κB subunits that can associate with any of the other Rel proteins. Once formed, NF-κB dimers are stored in the cytoplasm through interaction with the IκB proteins, which need to be degraded via the 26S proteasome before NF-κB can enter into the nucleus and regulate transcription [1]. Ubiquitin-dependent degradation of IκBs requires their phosphorylation by the IκB kinase (IKK) complex, whose activity is rapidly stimulated in response to microbial and viral infections, proinflammatory cytokines and ionizing radiation. IKK is composed of two related catalytic subunits IKKα and IKKβ and a regulatory subunit IKKγ/NEMO, which is essential for activation of the complex [2]. We found that IKKα and IKKβ differ in their substrate specificities and as a result have distinct biological functions. Whereas IKKβ is a true IκB kinase, IKKα is a poor IκB kinase and instead is an efficient NF-κB2 kinase, whose activity is required for production of p52. As a result, IKKβ is required for general NF-κB functions, including activation of innate immune responses, inflammation and protection of cells from TNF-induced apoptosis, whereas IKKα is required for p52-specific functions, such as B cell maturation and formation of secondary lymphoid organs [3, 4, 5]. IKKα kinase activity is also required for inducing the proliferation of mammary epithelial cells in response to a TNF family member called RANK ligand. In this case, however, it is required for the canonical NF-κB activation pathway, which depends on IκB degradation. These findings reveal that IKKα and IKKβ may be differentially engaged by different members of the TNF receptor family. We used mice that lack IKKβ in defined cell types to study the physiological functions of the classical NF-κB activation pathway that depends on its activity. The results indicate that IKKβ plays a critical role in macrophage activation and inhibition of macrophage and neutrophil apoptosis in response to bacterial encounter [6]. IKKβ is also important for prevention of IL-1β secretion, although it is required for induction of IL-1β gene transcription. In addition to its role in the control of inflammation, IKKβ also plays an important role in carcinogenesis. We found that in a model of colitis-associated cancer the activation of IKKβ in intestinal epithelial cells suppresses the apoptosis of preneoplastic cells, whereas the activation of IKKβ in myeloid cells promotes the proliferation of transformed epithelial cells through a paracrine mechanism. Thus, IKKβ may provide a mechanistic link between inflammation and cancer. In addition, we have found that the IKK/NF-κB pathway is involved in inflammation-induced progression and metastatic growth. Inhibition of NF-κB activation in cancer cells converts inflammation-induced tumor growth to inflammation-induced tumor regression [7].

M1–003
Structure-based lead optimisation of kinase inhibitors: facts or fantasy?

G. Müller

Axxima Pharmaceuticals AG, Munich, Germany. E-mail: gerhard.mueller@axxima.com

Lead finding and optimization attempts towards selective kinase inhibitors frequently rely on 3D structure information derived for kinase-inhibitor complexes. This talk highlights structural aspects of protein kinases determining the selectivity of low-molecular weight inhibitors, emphasising aspects of conformational changes of the target proteins upon ligand binding. In this context, the KinaTorTM technology developed by Axxima Pharmaceuticals will be introduced as a tool to experimentally determine the selectivity profile of kinase inhibitors following a chemo-proteomics approach.

M1–004
Receptor tyrosine kinase inhibition in cancer therapy: from monospecific to multi-targeted drugs

A. Ullrich[1]

[1]*Molecular Biology, Max Planck Institute for Biochemistry, Martinsried, Germany,* [2]*Singapore Oncogenome Laboratory, Centre of Molecular Medicine, Institute of Molecular and Cell Biology, Singapore, Singapore. E-mail: ullrich@biochem.mpg.de*

Cancer represents a disease prototype that is connected to defects in the cellular signaling 1network that controls proliferation, motility, survival and recognition by the immune system. The spectrum of genetic alterations identified in cancer cells includes mutations in various genes leading to structural and functional dysfunctions in signal transmission as well as over- or under expression of positive or negative signal generating proteins. For the past years we have investigated various aspects of signaling systems in tumor cells in order to identify critical switch points in the pathophysiological process that results in malignancy. These efforts aim at the selective blockade of abnormal, disease-promoting signaling mechanisms rather than the eradication of all growing cells in the body as in the case of currently used chemotherapeutic drugs. This strategic approach began with the cloning of the EGF receptor cDNA and the related receptor HER-2/neu. The work that began in 1983 yielded the first specific oncogene-based FDA-approved (1998)

therapeutic "Herceptin" for the treatment of metastatic breast cancer. Analogous "target-driven drug development" efforts have led to the identification of the receptor tyrosine kinase Flk-1/VEGFR2 as a critical signaling element in tumor angiogenesis which served as basis for the development of anti-angiogenic small molecule drugs SU5416, SU6668 and SU11248 which block the function of this receptor. The drug discovery process that led to SU11248 represents a prototypical example for the adaptation of cancer therapeutics from highly specific to multi-targeted drugs. SU11248 is in phase III clinical trials for kidney carcinoma and GIST. New insights that were gained over the past twenty years of targeted cancer therapy development will be discussed.

M1–005
Structure-based discovery of non-peptidic small molecule inhibitors of Caspase-3

J. Sakai[1,2], A. Yoshimori[3,4], R. Takasawa[2] and S.-I. Tanuma[1,2,3,4]

[1]Department of Biochemistry, Tokyo University of Science, Chiba, Japan, [2]Genome and Drug Research Center, Tokyo University of Science, Chiba, Japan, [3]Research Institute for Biological Sciences, Tokyo University of Science, Chiba, Japan, [4]Institute for Theoretical Medicine, Inc., Tokyo, Japan. E-mail: j3201042@ed.noda.tus.ac.jp

Caspases are cysteine aspartyl proteases that play critical roles during the execution of apoptosis. The caspase cascade in apoptosis maintains and amplifies the original apoptotic stimulus, and their disregulation is involved as a key factor in the development of a variety of diseases, including Alzheimers's disease, Parkinson's disease and cancer. To data, many peptide inhibitors have been reported. However, in generally, peptide inhibitors are of limited utility in a clinical setting. Through computational structure-based screening of an in house virtual library, followed by in vitro testing of selected candidate compounds, we identified CS4566 as a small-molecular weight non-peptidic inhibitor that inhibits the caspase-3 activity. CS4566 inhibits caspases-3 activity with IC_{50} value of 15.1 μM. The predicted binding interaction of CS4566 to S1 and S2 subsites of caspase-3 was very similar to that of caspase-3 selective inhibitor Ac-DNLD-CHO which was designed by our computational method. Furthermore, in Jurkat cells, CS4566 inhibited internucleosomal DNA fragmentation in a dose-dependent manner. At 200 μM, the nucleosomal DNA fragmentation was almost completely inhibited. Taken together, our results showed that CS4566 is a new class of small-molecule inhibitor for caspases-3 and represents a promising lead compound for designing an non-peptidic agent for caspase-mediated diseases, such as neurodegenerative disorders and viral infection diseases.

M1–006
Inhibitors of a mycobacterial protein kinase target and their conversion into novel drug candidates for *Mycobacterium tuberculosis* infected patients

L. Õrfi[1,4,5], A. Koul[2], D. Hafenbradl[2], B. Klebl[2], E. Hoppe[2], A. Missio[2], G. Müller[2], A. Ullrich[3], J. Pató[4], F. Wáczek[4,5], P. Markó[4], P. Bánhegyi[4,5], Z. Greff[4] and G. Kéri[6,4,5]

[1]Department of Pharmaceutical Chemistry, Semmelweis University, Budapest, Hungary, [2]Axxima Pharmaceuticals AG, Munich, Germany, [3]Department of Biochemistry, Max Planck Institute, Munich, Germany, [4]Vichem Chemie Ltd., Budapest, Hungary, [5]Cooperative Research Center, Semmelweis University, Budapest, Hungary, [6]Peptide Biochemistry Research Group, Hungarian Academy of Sciences, Budapest, Hungary. E-mail: lorfi@vichem.hu

Surprisingly, the genome of Mycobacterium tuberculosis contains 11 genes encoding for functional serine/threonine protein kinases. By genomic and genetic validation, the protein kinase G (PknG) was isolated as a critical virulence factor, being responsible for the survival of the mycobacteria in macrophages. Pathogenic mycobacteria manage to survive in a specialized organelle structure within macrophages, the phagosomes. Mycobacterium tuberculosis secretes PknG into the phagosome, from where it also moves into the cytoplasm of the macrophage. Secreted PknG prevents the fusion of endosomes with the phagosomes and thus ensures that the mycobacteria can survive within the macrophages. Macrophages represent an important reservoir of mycobacteria in infected individuals. Therefore PknG represents a novel target, which might have an important therapeutic impact in the fight against tuberculosis. Active PknG has been expressed and purified from *E. coli*. A substrate was identified for this novel kinase and a biochemical assay has been established. High throughput screening of Nested Chemical LibraryTM and commercial compound libraries in biochemical assay resulted in numerous hits from different compound families. AX20017, a tetrahydro-benzothiophene derivative was selected as potential hit for chemical optimization and as a starting point for a drug discovery program. AX20017 inhibits PknG in the submicromolar range. Apart from the goal of improving potency, medicinal chemistry had to fix a few liabilities of the hit compound, like the poor metabolic stability, off-target and cytochrom P450 activity. Novelty search on the compound family showed a heavily patented field. Our aims were to come up with patentable new variants of the selected hit, which would also have optimal ADMET properties. More than six hundred derivatives were synthesized in an iterative development process in a two years project while PknG inhibition, kinase selectivity, metabolic stability, solubility and membrane permeability were monitored and results were fed back to synthetic plans. PknG inhibition of the new and protected compounds is now in the range of single digit nM IC50 values. Water solubility and permeability are acceptable and the selected leads showed a high level of selectivity against a kinase panel, consisting of >40 human protein kinases. The compounds are non-toxic and PK/PD studies are underway. Here, we present an innovative and successful integrated drug development strategy against a deadly disease killing millions of people worldwide every year.

M1–007P
Structural studies with the importin alpha complexed with nuclear localization sequence peptidomimetics

M. R. M. Fontes[1], A. A. S. Takeda[1], T. Teh[2], R. F. Standaert[3] and B. Kobe[2]

[1]Department Physics and Biophysics, Sao Paulo State University, Botucatu, SP Brazil, [2]Dept. Biochemistry and Molecular Biology, University of Queensland, Brisbane, QLD Australia, [3]Dept. Chemistry, University of Illinois at Chicago, Chicago, IL, USA. E-mail: fontes@ibb.unesp.br

Importin alpha (ImpA) is the nuclear import receptor that recognizes cargo proteins with classical monopartite and bipartite nuclear localization sequences (NLS) and facilitates their transport into the cellular nucleus. The NLSs are characterized by one or two clusters of basic amino acids. Crystal structures of native mammalian ImpA, their complexes with monopartite NLS peptide from SV40 and with the bipartite NLS peptides from nucleoplasmin, RB protein, and N1/N2 protein have been solved by us [1–3]. The various ImpA isoforms exhibit specific cargo preferences in vivo and in vitro. The more general trend is that many cargoes are recognized by multiple isoforms but have a preference for one. Isoform-selective NLS mimetics would provide an

excellent tool for studying the physiological consequences of the substrate specificities and lead to the development of new ligands that can distinguish between isoforms. The potential applications include drugs (anti-inflammatory, anti-cancer, and anti-fungal), gene therapy, drug delivery, and diagnostics. Here, we present the co-crystallization experiments and preliminary crystallographic studies of six NLS peptidomimetics and non-autoinhibited impA complexes. The crystal structures of all six complexes were solved in the resolution range 2.0–2.5 Å. Electronic density calculations reveal the presence of a clear electron density in the major NLS binding site of all complexes, corresponding to the peptidomimetic molecules. The structures provide the information on how the ligands interact with the protein, and how they might be improved most effectively.

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M1–008P
A cell-penetrating peptide combined with the $G\alpha_s$ C-terminal sequence as inhibitor of A_{2A} adenosine receptor signaling in PC12 cells

F. Porchia[1], L. Giusti[1], A. M. D'Ursi[2], S. Albrizio[3], C. Gargini[1], C. Esposito[2], G. Caliendo[2], E. Novellino[3], P. Rovero[4] and **M. R. Mazzoni**[1]

[1]Department of Psychiatry, Neurobiology, Pharmacology and Biotechnology, Uni. Pisa, Pisa, Italy, [2]Department of Pharmaceutical Sciences, Uni. Salerno, Fisciano (Sa), Italy, [3]Department of Medicinal Chemistry and Toxicology, Uni. Naples 'Federico II', Naples, Italy, [4]Department of Pharmaceutical Sciences, Uni. Florence, Sesto Fiorentino (Fi), Italy. E-mail: mariarm@farm.unipi.it

Cell-penetrating peptides (CPPs) are amphipathic or cationic oligopeptides able to transport covalently attached cargo across cell membranes. The peptide penetratin identified as a segment of the antennapedia homeodomain protein that allows its penetration across biological membranes has been used as carrier of peptides and oligopeptides. We and others have shown that peptide aptamers corresponding to the C-terminal fragment of Gα subunits mimic and thus perturb interactions with heptahelical G protein-coupled receptors "*in vitro*" systems. The combination of aptamer and CPP technology can generate pharmacological reagent effective in cell culture models and "*in vitro*". Therefore, we designed, synthesized and tested a 37 residue fusion peptide containing the 16 residue of penetratin (carrier) on the N-terminal side and the 21 residues of Gαs C-terminal sequence on the C-terminal side (cargo). This membrane-permeable Gαs peptide, which acquired a defined structure with 2 α-helix segments in a SDS micellar solution was able to inhibit adenosine receptor mediated cAMP production in PC12 cells while the carrier peptide by itself had no effect. The carrier and fusion peptide did not affect basal accumulation of cAMP. The inhibitory effect of the fusion peptide was concentration dependent (EC50, 10.80 ± 0.34 μM; n = 4), significantly reducing the maximal efficacy of the adenosine receptor agonist, NECA. PC12 cells showed a marked plasma membrane fluorescence when treated for 30 min with the fluorescein labeled fusion peptide. This and similar fusion peptides may represent a new class of pharmacological agents with potential research and therapeutic applications.

M1–009P
Identification of chalcone derivatives that stimulate glucose uptake in 3T3-L1 adipocytes

S. Oikawa, R. Kamei, M. Kadokura and Y. Kitagawa

Daiichi Suntory Biomedical Research Co., Osaka, Japan. E-mail: Shinzo_Oikawa@dsup.co.jp

A chalcone derivative, 3-nitro-2′-benzyloxychalcone was identified by a cell-based glucose uptake screening assay. The compound stimulated glucose uptake and potentiated insulin-stimulated glucose uptake in a concentration-dependent manner in 3T3-L1 adipocytes. When cells were treated with various concentrations of insulin in the presence of the compound, marked enhancement of insulin-stimulated glucose uptake was observed at each concentration, suggesting that the compound might function as an insulin sensitizer. Preliminary study on the structure-activity relationships revealed that two aromatic benzene rings tolerated several substituents, but substitution by acidic or highly polar groups abolished the activity. The hydrophobicity of the substituents appeared to play a part in determining the extent of activity or lack thereof. Among several chalcone derivatives, 4-chloro-2′-benzyloxychalcone showed the highest level of activity. The chalcone derivative-stimulated glucose uptake was almost completely inhibited by wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase. These results suggest that the action of chalcone derivatives is mediated via a pathway involving phosphatidylinositol 3-kinase.

M1–010P
Structure-based design of a potent and selective peptide inhibitor of caspase-3

A. Yoshimori[1,2], R. Takasawa[3], J. Sakai[3,4], T. Kobayashi[3,4], S. Sunaga[3,4] and S.-I. Tanuma[1,2,3,4]

[1]Research Institute for Biological Sciences, Tokyo University of Science, Chiba, Japan, [2]Institute for Theoretical Medicine, Inc, Tokyo, Japan, [3]Genome and Drug Research Center, Tokyo University of Science, Chiba, Japan, [4]Department of Biochemistry, Tokyo University of Science, Chiba, Japan. E-mail: yosimori@rs.noda.tus.ac.jp

The structure-based design of potent and selective inhibitors of members of the caspases is an important strategy for chemical knockdown to define the critical role of each caspase in apoptosis and inflammation. Recently, we have developed computational system named Amino acid Positional Fitness (APF) Method for designing potent peptide inhibitors [BMC Pharmacol. 4, 7]. The APF Method allows the rapid prediction of binding affinity between all peptides being tested and a target protein. In this study, we have modified the APF method to design potent and selective peptide inhibitors. To date, no tetrapeptide inhibitor potent and selective for caspase-3 has yet to be identified. A well-known caspase-3 inhibitor, Ac-DEVD-CHO, inhibits other caspases with similar Ki values. Therefore, the selective inhibitor could become an important tool for investigations of the biological function of caspase-3 in apoptosis signaling pathway. By using the APF method, Ac-DNLD-CHO was designed as the first-rank candidate for the potent and selective inhibitor of caspases-3. As expected, Ac-DNLD-CHO had similar potent inhibitory activity (Ki = 0.680 nM) to a well-known inhibitor Ac-DEVD-CHO (Ki = 0.288 nM). It is noteworthy that Ac-DNLD-CHO exhibits an approximate by 80–1000 fold selectivity for caspase-3 over caspases. Ac-DNLD-CHO could also be useful in determining whether caspase-3 acts in cells that respond to various apoptotic stimuli such as drugs and viruses. Furthermore, Ac-DNLD-CHO may be an attractive lead compound to generate novel effective non-peptidic pharmaceuticals for caspase-mediated diseases, such as neurodegenerative disorders and viral infection diseases.

M2 – Bioconjugates of Peptides and Proteins

M2–001
Advances in infinite binding of proteins to targets

C. F. Meares, T. M. Corneillie, P. A. Whetstone and N. G. Butlin
Chemistry Department, University of California, Davis, CA, USA. E-mail: cfmeares@ucdavis.edu

Engineering the permanent formation of a receptor-ligand complex has a number of potential applications in chemistry and biology, including targeted medical imaging and therapy. These systems can be prepared by a combination of protein engineering and synthetic chemistry, for example using the site-directed incorporation of nucleophiles at the periphery of an antibody's binding site, paired with the chemical design of a weakly electrophilic ligand, to produce a receptor-ligand pair that associates efficiently and permanently. An exemplary system involving metal-DOTA complexes shows that this approach can lead to the straightforward production of infinite binding ligand-protein pairs beginning from weakly binding starting materials. In contrast to combinatorial strategies for strong binding, which seek binding sites with the best complementarity to a single structure, infinite binding of a set of structurally related ligands –- such as a set of probe molecules – can be easily achieved. A greater challenge is engineering a tumor-binding single-chain antibody (scFv) to permanently attach to its protein target. We will describe progress toward this goal.

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M2–002
Endothelial cell directed drug-targeting strategies for therapeutic intervention of inflammatory diseases and cancer

G. Molema
Endothelial Cell and Vascular Drug Targeting, Pathology and Laboratory Medicine, Medical Biology section, Groningen, Groningen, The Netherlands. E-mail: g.molema@med.umcg.nl

Vascular endothelial cells actively participate in leukocyte recruitment and neovascularization, both hallmarks of chronic inflammatory diseases such as rheumatoid arthritis, and of tumor growth. This feature together with their easy accessibility for systemically applied drugs makes endothelial cells an important target for therapeutic intervention. Vascular drug targeting aims at selectively delivering pharmacologically active entities (drugs, genes, siRNAs) into the activated endothelial cells at the diseased sites. Drug targeting constructs consist of carrier molecules (protein, liposomes, viruses) complexed or chemically conjugated with pharmacological agents. Specificity for the activated endothelium is created by using carriers with an intrinsic binding domain or by conjugating homing ligands such as peptides, antibodies, antibody fragments, or sugar molecules to the carrier molecules. For pharmacological effectiveness, essential characteristics of the drug targeting constructs include drug efficacy, drug loading of the constructs, internalization capacity of the target cells, and cellular handling which determines the fate of the drug in the cell. Examples of vascular drug targeting systems will be presented and their effects will be discussed in relation to these characteristics. Furthermore, the main challenges in the development of these therapeutic entities for future clinical application will be addressed.

M2–003
Peptides for cellular delivery. Targeting the MDM2 oncogene using Peptide Nucleic Acid (PNA)

P. E. Nielsen
Department of Medical Biochemistry and Genetics, University of Copenhagen, Copenhagen, Denmark. E-mail: pen@imbg.ku.dk

Antisense strategies, including siRNA, for targeted control of gene function are regaining interest both for basic research as well as for drug discovery and development. However, bioavailability (and cellular delivery) is a major hurdle for which robust and effective solutions are still lacking.The MDM2 oncoprotein is abnormally up-regulated in several human tumors by gene amplification, increased transcript levels and/or enhanced translation. MDM2 protein is a negative-feed back regulator of p53 and thus probably a key player in the control of cell proliferation and apoptosis. Furthermore, decreasing the cellular level of MDM2 will increase p53 activation by DNA damage and thereby synergistically increase the therapeutic effects of DNA damaging chemotherapeutics. Therefore, MDM2 should be a relevant target for cancer therapy. Using peptide nucleic acids (PNA), a pseudopeptide DNA mimick, and a novel lipofection mediated delivery of PNA-acridine conjugates, we have identified PNA oligomers targeted to the 5' proximal end of the MDM2 mRNA that are toxic to JAR cells (which over-express MDM2 as a growth requirement), that show inhibition of MDM2 synthesis, show significant up-regulation of p53 activity, and that increase the cell toxicity of the anticancer drug camptothecin. We have also explored the use of cationic peptides, such as oligoarginine or the Tat peptide, as cellular delivery agents, and we have discovered novel modifications that significantly improve the cellular uptake as well as the cellular antisense effects of the PNAs. Various aspects of these results including *in vitro* and *in vivo* bioavailability of PNA-peptide conjugates will be discussed.

M2–004
Chemically modified catalase for prevention of ROS-mediated injury and tumor metastasis

M. Nishikawa[1], Y. Takakura[1] and M. Hashida[2]
[1]Department of Biopharmaceutics and Drug Metabolism, Kyoto University, Kyoto, Japan, [2]Department of Drug Delivery Research, Kyoto University, Kyoto, Japan.
E-mail: makiya@pharm.kyoto-u.ac.jp

Reactive oxygen species (ROS) including superoxide anion and hydrogen peroxide are powerful oxidants that, at high concentrations, are toxic to cells and cause tissue damage. Therefore, antioxidant enzymes, such as superoxide dismutase (SOD) and catalase, are promising compounds for preventing ROS-mediated tissue injury. However, the delivery of these enzymes to sites where ROS are generated is a prerequisite for preventing it. We have demonstrated that the tissue distribution of SOD and catalase can be controlled by chemical modification: alteration of electric charge, glycosylation and conjugation of polyethylene glycol (PEG). We found that targeted delivery of the enzymes to liver nonparenchymal cells was effective for prevention of hepatic ischemia/reperfusion injury in mice. The combination of mannosylated SOD and succinylated catalase was the most effective in inhibiting the hepatic injury. On the other hand, a sublethal concentration of ROS, especially hydrogen peroxide, may accelerate tumor metastasis by increasing the expression of matrix metalloproteinases, angiogenic factors and growth factors. Then, inhibition of the metastasis by targeted delivery of catalase was

examined. A hepatic metastasis of colon26 cells in mice was inhibited by galactosylated catalase targeting to hepatocytes. We also found that PEG-catalase effectively inhibited the metastasis of colon26 cells to the lung. Using melanoma cells permanently labeled with luciferase gene, we clearly demonstrated that PEG-catalase prevent the multiple processes of metastasis including the adhesion and proliferation of tumor cells. These results indicate that chemically modified catalase having diverse tissue distribution characteristics prevents ROS-mediated tissue injury as well as tumor metastasis.

M2–005
Mechanisms of protein cellular delivery with transportans

M. Pooga[1,2], K. Padari[2], P. Säälik[1], K. Koppel[1], M. Hansen[3], R. Raid[2] and Ü. Langel[3]

[1]*Estonian Biocentre, Tartu, Estonia,* [2]*Department of Zoology and Hydrobiology, University of Tartu, Tartu, Estonia,* [3]*Department of Neurochemistry and Neurotoxicology, Stockholm University, Stockholm, Sweden. E-mail: mpooga@ebc.ee*

Introduction of oligonucleotide and peptide or protein-based drugs has been seriously hampered by the poor cellular uptake. Cell penetrating peptides (CPP) facilitate translocation of hydrophilic compounds across the plasma membrane and are often used for delivery of bioactive macromolecules into cells. We studied the uptake of streptavidin complexed with transportan or TP10 by HeLa and Bowes melanoma cells in order to better characterize the mechanism of protein transduction. Transportan–protein complexes associated preferentially at cholesterol-rich subdomains of plasma membrane and with filopodia or microvilli as judged by electron and fluorescence microscopy. The peptide–protein complexes localized on the cell surface or in the close proximity, suggesting two different modes of interaction – direct contact with the plasma membrane or binding to exoplasmic structures, probably proteoglycans. Depletion of the plasma membrane of cholesterol markedly decreased interaction of transportan–protein complexes with cells. Transportan–protein complexes were observed to translocate into cells mainly in vesicular structures of different size and morphology. Induction of vesicular structures and internalization of the complexes were strongly inhibited at low temperature, suggesting the prevalence of endocytotic pathways in the uptake process. However, not all transportan–protein complexes were confined to the vesicular membrane-surrounded structures of cells but localized also in cytoplasm. Localization in the cytoplasm beneath the plasma membrane was more typical for TP10-protein than for transportan-containing complexes. Internalization of peptides transportan and TP10 themselves resulted in rather similar distribution pattern in the cells. Majority of the nanogold-labeled peptide was confined to vesicular structures with different size and electron density.

M2–006
Intracellular targeting of calpastatin derived peptides

Z. Bánóczi[1], Á. Tantos[2], P. Tompa[2], Z. Kristóf[3], G. Csík[4], P. Friedrich[2] and F. Hudecz[1]

[1]*Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös L. University, Budapest, Hungary,* [2]*Biological Research Center, Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary,* [3]*Department of Plant Anatomy, Institute of biology, Eötvös L. University, Budapest, Hungary,* [4]*Research Group for Biophysics, Institute of Biophysics and Radiation Biology, Hungarian Academy of Science, Semmelweis University, Budapest, Hungary. E-mail: banoczi@ludens.elte.hu*

Calpastatin is the endogenous inhibitor of calpain, the inhibitory domains of calpastatin contain three highly conserved regions, A, B, and C. The region B inhibits calpain on its own, whereas A and C regions do not have this activity [1]. Tompa et al. described that peptides related A and C regions activate m- and m-calpains [2]. Based on these results the corresponding peptides were conjugated to penetrating for intracellular delivery and the conjugates were tested on COS7 cells. The conjugates contained amide, thioether or disulfide bond. Two sets of conjugates were also prepared with 4-(7-methoxycoumaryl)acetic acid (Mca) or with 4-(7-hydroxycoumaryl)acetic acid (Hca). To measure the calpain activity inside the cell a fluoresence substrate was synthesised, too. This was built up using 4-(4-dimethylaminophenylazo)benzoic acid (DABCYL) at the N-terminal, and 5-[(2-aminoethylamino)]naphtalene-1 sulfonic acid (EDANS) at the C-terminal of TPLKSPPPSPRC(R8-NH2) which contained octaarginine, as cell penetrating unit and TPLKSPPPSPR as ''supersubstrate'' of calpain [3]. We found that the calpastatin conjugates maintained their calpain activating effect *in vitro*. The intracellular activating effect of conjugates will be reported.

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M2–007P
The use of the phenylacetyl group for protecting amino groups of peptides

T. Barth[1], J. Velek[1], J. Barthová[2], J. Ježek[1], L. Hauzerová[1] and T. Vaníik[1]

[1]*Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic,* [2]*Faculty of Sciences, Department of Biochemistry, Charles University, Prague, Czech Republic. E-mail: barthtom@yahoo.com*

Penicillin amidohydrolase (EC 3.5.1.11) is often used for preparing semisynthetic penicillin derivatives, whereas its application in the process of peptide synthesis is less common. We described the synthesis of deamino(8-L-lysine)vasopressin [1]; the amino group of lysine was temporarily protected by phenylacetyl group (Pac) which was subsequently removed by penicillin amidohydrolase. During the preparation of new analogues of human insulin, Pac groups were often used for protecting amino groups in the side chains of L-lysine or L-ornithine in position B29. The semi-synthetic approach to the preparation of new analogues of human insulin is based on the condensation of a peptide with the carboxyl group of B22 arginine carried out by tryptic catalysis in partially non-aqueous medium. After the condensation of the octapeptide with desoctapeptide insulin, Pac group was split off by penicillin amidohydohydrolase. Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys(Pac)-Thr penicillin amidohydrolase Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Thr + Phenylacetic acid We prepared 13 different analogues of the terminal octapeptide B23-B30 with the Pac group on lysine or ornithine. With the aim of labeling the amino group of the side chain of the B29 amino acid, we also prepared two analogues with the Pac group on glycine in position B23 of the octapeptide. The protection of amino groups with Pac groups was used in the synthesis of Dalargin and several smaller peptides that could serve as ligands for the preparation of antibodies or for affinity chromatography.

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M2–008P
Structure-biological activity relationship of GnRH-III and its dimer derivatives

G. Mezõ[1], **A. Czajlik**[2], A. Jakab[1], A. Bodor[3], V. Farkas[4], E. Vass[4], Z. Majer[4], B. Kapuvári[5], B. Vincze[5], O. Csuka[5], M. Kovács[6], A. Perczel[4] and F. Hudecz[1,4]
[1]Research Group of Peptide Chemistry, Hungarian Academy of Sciences-Eötvös Loránd University, Budapest, Hungary, [2]Protein Modeling Group, Hungarian Academy of Sciences-Eötvös Loránd University, Budapest, Hungary, [3]Department of Theoretical Chemistry, Eötvös Loránd University, Budapest, Hungary, [4]Department of Organic Chemistry, Eötvös Loránd University, Budapest, Hungary, [5]National Institute of Oncology, Budapest, Hungary, [6]Department of Anatomy, University of Medical School of Pécs, Pécs, Hungary. E-mail: czajlik@para.chem.elte.hu

GnRH-III (EHWSHDWKPG-NH2) isolated from sea lamprey is a naturally occurring GnRH analogue, which suppresses the proliferation of GnRH receptor positive breast cancer cells [1]. However, it did not exert significant endocrine activity suggesting selective anti-tumor activity of GnRH-III [2]. To increase the anti-tumor effect of GnRH-III, disulfide bond containing dimer analogues of GnRH-III were synthesized ([EHWSHDWK(H-C)PG-NH2]2, [EHWSHDWK(Ac-C)PG-NH2]2). Receptor binding affinity and anti-proliferative effect of GnRH-III and of its derivatives were tested on GnRH receptor positive human breast (MDA-MB-231, MCF-7) and colon (HT-29) carcinoma cell lines. Some significant differences in activities were detected. The [EHWSHDWK(Ac-C)PG-NH2]2 peptide showed less activity in releasing of LH from superfused rat pituitary cells than GnRH-III itself. However, it gave the highest anti-tumor activity on colon carcinoma cell lines. For explanation of the differences in biological activity, the solution structure of monomer and dimer derivatives was studied by NMR, CD and FT-IR spectroscopy. Comparing the NMR structure of ([EHWSHDWK(H-C)PG-NH2]2 and [EHWSHDWK(Ac-C)PG-NH2]2 no significant conformational differences were observed. The solution structure of the GnRH-III can only be described in form of a NMR ensemble, while the disulfide bond containing dimer analogues of GnRH-III adopt a single well-defined conformer.

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M2–009P
Phosphorylation and O-glycosylation sites identification in peptides by Ba-hydroxide catalyzed β-elimination/propanethiol addition and mass spectrometric analyses

H.-C. Chen[1], G. Wang[1], P. S. Backlund[2] and R. A. Boykins[3]
[1]Endo/Repro Res. Br., NICHD-NIH, Bethesda, MD, USA, [2]Laboratory of Cell and Molecular. Biophysics, NICHD-NIH, Bethesda, MD, USA, [3]Laboratory of Biophysics, CBER-FDA, Bethesda, MD, USA. E-mail: chenha@mail.nih.gov

Mass spectrometry (MS) methods are the key proteomics tools in the identification of phosphorylation (P-sites) and glycosylation sites in protein modifications for monitoring cell functions. A problem hampering these analyses are their proton sequestration properties by phosphate and glycan, resulting in ionization suppression in the positive ion mode MS. Furthermore, MS/MS identification of P-sites directly using P-peptides is complicated by the loss of PO4-moiety during low energy CID in most cases. We have studied Ba hydoxide catalyzed β-elimination of phosphate and glycan group on Ser and Thr in peptides followed by alkanethiols addition. The MALDI-TOF MS was used to determine the reaction products at the picomole scale. We have developed conditions with minimum alkalinity and side reactions but capable of modifying P-Ser and P-Thr to near completion. The reaction conditions that produced the best results for two model peptides were 20 mM Ba hydroxide, 30% 1-propanol, and 0.5 M alkanethiols and incubation for 24 h at 25̊C. The conversion was carried out at 1μM concentration of the peptide. We found the resulted single n-propylthio and n-butylthio derivatives not only were stable during CID but also yielded at least seven times higher ionization than the parent molecules. Furthermore, abundant y and b fragment ions were easily identifiable under general conditions for ESI tandem MS/MS. We were for the first time able to identify directly four clustered P-Ser residues in a 3.1 kDa betacasein peptide: RELEELNVPGEIVESLSSSEESITR and unequivocal identifications of multiple O-glycosylation sites in κappa-casein peptides using an ESI-quadrupole ion trap MS.

M2–010P
Structural investigations of α-Conotoxin SI chimeras containing epitopes from Herpes Simplex Virus and cancer-related Mucin proteins reveal notable conformational differences

R. W. Janes[1], F. O'Boyle[1], G. Mező[2] and F. Hudecz[2,3]
[1]School of Biological Sciences, Queen Mary, University of London, London, UK, [2]Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös L. University, Budapest, Hungary, [3]Department of Organic Chemistry, Eötvös L. University, Budapest, Hungary. E-mail: RWJanes@qmul.ac.uk

The neuromuscular α-conotoxins are small polypeptides, around 13 residues in length that block muscle endplate nicotinic acetylcholine receptors. All structural studies to date have shown that they have the same backbone conformation, constrained by two disulphide bonds, and known as the a3/5-conotoxin fold, so-called because of the numbers of residues in the loops between the constraining cysteine residues. α-Conotoxin SI (SI), has the sequence ICCNPACGPKYSC* (where * is an amidated C-terminal). Because of the known conformational stability of the structure, four residues, PKYS, two of which are responsible for toxicity, were replaced by epitopes DPVG from glycoprotein D (gD) from Herpes Simplex Virus, and PDTR from the cancer-related protein Mucin-1, to form chimeras known as SI-HSV, and SI-MUC, respectively. These chimeras were designed to generate antibodies that would then recognize their respective wild-type proteins. It was found that antibodies generated by SI-HSV recognized the HSV gD protein far better than those generated by SI-MUC recognized Mucin-1. Solution NMR structure determination, and Synchrotron Radiation Circular Dichroism (SRCD) studies of these two chimeras, relating them to the wild-type SI conformation in each case, were undertaken to determine the reasons behind these different antigenic properties. The alterations in the C-terminal sequences were found to have created critical structural differences both between the chimeras, and from the SI structure. These different conformations successfully accounted for the differences in antigenic properties due to notable changes in surface accessibility of the epitope side chains. Additionally, residues other than the disulphides were shown to be critically important for maintaining the a3/5-conotoxin fold.

M2–011P
Suitability of peptide conjugates containing formyl-peptide residue for chemotactic drug-targeting (CDT)

O. Láng[1], J. Birinyi[1], K. Bai[2], G. Mező[2], F. Hudecz[2] and L. Kőhidai[1]
[1]*Chemotaxis Research Group, Department of Genetics, Cell and Immunobiology, Semmelweis University, Budapest, Hungary,* [2]*Research Group of Peptide Chemistry, Eötvös Loránd University of Sciences, Budapest, Hungary. E-mail: lanors@dgci.sote.hu*

Introduction: Chemotactic drug targeting (CDT) is a new technique developed by us for delivery bioactive substances. For this purpose CDT deals with conjugates, built up by chemotactic ligand, carrier molecule and the drug. Chemotactic moieties of the conjugate provides the selective delivery of the drug: chemoattractant components promote to achieve a rapid, targeted effect in the chemotactically positive responder cells, while the chemorepellent character shields the molecule from the fast degradation done by the non-target cells.
Aims : (i) To investigate the structure–function relationship of the chemotactic ligands and the carrier molecules for CDT; (ii) to characterize cell physiological properties of the new conjugates and (iii) to describe the ability for CDT of the conjugate containing the cytostatic drug, methotrexate.
Materials and Methods: In the experiments THP-1 monocytes were applied as model-cells. Eight peptide conjugates were used containing polylysine (EAK and SAK) and oligotuftsin (T20) as carriers. The chemotactic ligands were fMLF, fNleLF, fMMM. The chemotactic ability of the cells was determined in NeuroProbe® chamber. Internalization of fluorescently labelled conjugates was analysed by FACS. Significance of PI3K pathway was tested by wortmannin.
Results: (i) Conjugation of the carriers with the most effective chemo-attractant fMLF resulted an increased chemotactic ability. (ii) The EAK-fMLF has a strong chemo-attractant moiety (10^{-17}–10^{-16} M) while the SAK conjugate was inactive. (iii) Conjugation of T20 with the three formyl peptides resulted in an increased chemotactic ability, T20-fMLF was the most effective. (iv) The molecular integrity of T20 carrier seems to be crucial, while application of a cleavage sequence [GLFG] had no influence. (v) Investigations of other cell-physiological parameters demonstrated also significant diversities of the native carriers and the conjugates. (vi) However, incorporation of methotrexate had minor modificator effects, the basic chemotactic abilities were not influenced.
Conclusion: Results summarized above provide more structural and functional evidences for CDT.

M2–012P
Microheterogeneity of human transferrin in newborns with unclear neurological symptomatology

A. S. Maslak, D. N. Tokarev, A. I. Shevtsova and I. U. Pismenetskaya
Biochemistry, Biochemistry and General Chemistry, Medical Academy, Dnepropetrovsk, Ukraine. E-mail: maslak_anna@inbox.ru

Some neurological disorders with unclear symptomatology in newborns are considered to be a result of glycosylation defects. Congenital disorders of glycosylation (CDG) are metabolic defects in biosynthesis of glycans which lead to severe mental and psychomotor retardation. One of the major biochemical features of CDG is abnormal serum transferrin pattern. A significant decrease of sialic acid enriched tetrasialotransferrin (S4) and increase in sialic acid deficient di- (S2), mono-(S1) and asialotransferrins (S0) were shown. Using isoelectrofocusing and immunoblotting we investigated a microheterogeneity of blood transferrin in newborns with neurological disorders with unclear clinical picture ($n = 10$) and in healthy individuals ($n = 6$). We found atypical transferrin patterns in three newborns. In one of them with a syndrome of reduced nervous excitability developed after prenatal hypoxia, transferrin profile contained three isoforms: S0 (pI = 5,9), S1 (pI = 5,8), S2 (pI = 5,7). The clinical picture of the second newborn included intrauterine retardation, stigmas of disemryogenesis and convulsive syndrome. The increase in S2 transferrin isoform was detected in plasma of this infant. The newborn 3 presented a slow-down in psychomotor development known as a Dandy-Walker malformation. In plasma of this newborn fractions of S0 and S1 transferrin have been detected. These data suggest that these three newborns may have congenital defects of glycosylation.

M2–013P
Oligoarginine for delivering daunomycin using squaric acid linker

Z. Miklán[1], A. Sum[2], J. Reményi[1], F. Sztaricskai[2], G. Schlosser[1] and F. Hudecz[1,3]
[1]*Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös Loránd University, Budapest, H-1518 Hungary,* [2]*Research Group for Antibiotics of the Hungarian Academy of Sciences and Department of Pharmaceutical, University of Debrecen, Debrecen, H-4010 Hungary,* [3]*Department of Organic Chemistry, Eötvös Loránd University, Budapest, H-1518 Hungary. E-mail: seram@bolyai.elte.hu*

The group of Arg-based oligopeptides derived from the Arg-rich Tat protein domain is considered as one of the most efficient delivery agents for intracellular transport of covalently attached entities like peptide, protein, PNA and drugs [1]. In order to study the effect of linker moiety between the oligoarginine and daunomycin on delivery potential, we have synthesized various amino acid daunomycin conjugate using squaric acid as linker. First we have produced asymmetric amides from daunomycin-squaric acid with diethyl amine, glycine, diglycine, triglycine, L-leucine, L-leucyl-glycin and L-arginine as model compounds. We studied the stability of these compounds under different circumstances using reversed phase high performance liquid chromatography (RP-HPLC). We found that the diamides are sensitive to strong alkaline and acidic conditions but stable at neutral aqueous solution. The conjugation with oligoarginine, monitored with RP-HPLC, was a slow reaction. The crude daunomycin-(Arg_8) conjugate was purified also by RP-HPLC and were identified by mass spectrometry. The biological activity of model compounds and conjugates was evaluated *in vitro* on sensitive and resistant human leukemia (HL-60) cell lines.
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M2–014P
Characterization of small acidic peptides isolated from wheat sprout chromatin and involved in the control of cell growth

I. Calzuola[1,2], G. L. Gianfranceschi[2] and **V. Marsili**[1,2]
[1]*Department of Cellular and Environmental Biology, University of Perugia, Perugia, Italy,* [2]*Cemin Centro di Eccellenza per i Materiali Innovativi e Nanostrutturati, University of Perugia, Perugia, Italy. E-mail: vmarsili@unipg.it*

A family of small acidic peptides, associated with chromatin DNA, were isolated by Gianfranceschi et al., in the seventies, from many eukaryotic and prokaryotic cells. Their biological

activity is related to the control of cell growth and gene expression. Synthetic peptides, designed on the basis of the biochemical and mass spectrometry analysis, are able to reproduce some of the biological effects shown by native peptides; however the effect exerted by these peptides on the control of cell growth is quite low. Here we report the results of the molecular characterization of the peptides isolated from wheat sprout powder, a good source of chromatin peptides. The isolated peptide fraction shows a sharp activity on the control of cell proliferation. Infrared spectroscopy and mass spectrometry have been utilized to characterize the wheat sprout peptides in the attempt to recognize the peptide sequence involved in the control of cell growth. The quantitative presence of a peptide with MH + = 572 appears proportional to the cell growth inhibition activity. This compound has been subjected to extensive mass spectrometry analysis. The automatic computational analysis indicates a peptide sequence, AcHis-Asp-Ser-Glu-ethanolamine. We will synthesize the peptide-ethanolamine complex to check the potential role of the ethanolamine in the biological activity of the chromatin peptides. Moreover, comparing wheat sprout peptides mass spectra with those obtained from other sources, we demonstrated that some sequences of the wheat sprout peptide family are present in the peptide fractions isolated from several other tissues, thus supporting the hypothesis of ubiquitous regulatory peptides.

M2–015P
Immobilization techniques of macromolecules and small analytes onto silica surfaces for the development of optical (OWLS) immunosensors

A. Székács[1], **E. Maloschik**[1], I. Levkovets[1,2], N. Adanyi[3], M. Varadi[3] and I. Szendrõ[4]
[1]*Department of Ecotoxicology and Environmental Analysis, Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary,* [2]*Palladin Institute of Biochemistry, Ukrainian National Academy of Sciences, Kiev, Ukraine,* [3]*Central Food Research Institute, Budapest, Hungary,* [4]*Microvacuum Ltd., Budapest, Hungary. E-mail: emal@julia-nki.hu*

Optical waveguide lightmode spectroscopy (OWLS) sensors offer label-free, real-time qualitative and quantitative macromolecular interaction assays by detecting binding between various biomolecules on the sensor surface. Various functional groups were introduced onto the sensor surface allowing simple covalent immobilization of bioconjugates for regenerable OWLS immunosensors. As the surface of the SiO_2–TiO_2 waveguide contains mainly hydroxyl groups improper for covalent immobilization of biomolecules, the waveguide was modified with functionalized silane reagents. Amino groups created on the chip surface were further derivatized by homobifunctional reagents and by formation of carboxyl groups and subsequent reaction to activated esters, capable to bind proteins to the surface as amides. In optimized immobilization processes, OWLS sensors were developed for the detection of model compounds, including two proteins (bovine serum albumine and bovine cerebral heat-shock protein-70), as well as a pesticide active ingredient (trifluralin). In the case of the small molecule analyte trifluralin, OWLS detection has been validated by gas chromatography – mass spectrometry analysis using electron impact and chemical ionization. Using various immobilization protocols, in each case each component of the antibody–antigen complex could be covalently immobilized on the sensor surface, allowing non-competitive or competitive detection of the analytes.

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M2–016P
Optimal design and production of genetically modified soybean glycinin A1aB1b subunit containing the hypocholesterolemic peptide IIAEK

K. Prak, Y. Maruyama, N. Maruyama and S. Utsumi
Laboratory of Food Quality Design and Development, Graduate School of Agriculture, Kyoto University, Uji, Kyoto, Japan. E-mail: krisna@kais.kyoto-u.ac.jp

IIAEK derived from β-lactoglobulin is a peptide comparable to the medicine, β-sitosterol, known for its hypocholesterolemic activity. To produce this valuable peptide in soybean, we introduced nucleotide sequences encoding the peptide into DNA regions corresponding to five variable regions of the soybean glycinin A1aB1b subunit, and expressed the constructs in *Escherichia coli*. The expression level and solubility of the five mutants, each containing four IIAEK in each variable region, were compared. Overall, the expression level and solubility of the mutant with four IIAEK at the variable region IV was the best. Further introduction of a fifth IIAEK at this site did not decrease expression level and solubility. Increasing the number of IIAEK to seven and ten slightly decreased expression level, but the solubilities went to as low as 40 and 1%, respectively. We combined various mutations from the five mutants to get a mutant having the highest amount of IIAEK possible. Some of the resulting mutants were expressed in the soluble form. The mutant containing eight IIAEK from the combination of variable regions IV and V (IV + V) showed the best expression level and solubility, followed by the combination of variable regions II and III (II + III). The soluble fractions of these mutants were purified by hydrophobic, gel filtration and ion exchange column chromatography. Yields of IIAEK peptide released by *in vitro* digestion with trypsin were around 80%. This is the first report that a large amount of the physiologically active peptide could be introduced into soybean proglycinin, expressed in soluble form and released in a high yield of peptide (IIAEK) after digestion with trypsin.

M2–017P
In vitro anti-tumor effect and localization of daunomycin-polypeptide conjugate in sensitive and resistant cell lines

J. Reményi[1], T. Hegedûs[2], P. Kovács[3], G. Csík[4], B. Sarkadi[2] and F. Hudecz[1]
[1]*Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös L. University, Budapest, Hungary,* [2]*National Institute of Haematology and Immunology, Budapest, Hungary,* [3]*Department of Genetics, Cell and Immunobiology, Semmelweis Medical University, Budapest, Hungary,* [4]*Department of Biophysics and Radiobiology, Semmelweis Medical University, Budapest, Hungary. E-mail: remenyi@szerves.chem.elte.hu*

We have prepared conjugates (cAD-EAK, cAD-SAK) containing daunomycin as a drug, amphoteric and polycationic branched chain polypeptides (EAK, SAK) as carrier and cis-aconytil spacer. The macromolecular carrier can alter the biodistribution and pharmacokinetics of the drug, so can eliminate the side effects (e.g. immunosuppression, cardiotoxicity) and multidrug resistance developed during the treatment. Previous results suggest that the conjugate enters not only the HL-60/sensitive and L1210/sensitive, but two HL-60/resitant cell lines (HL-60/MDR1, HL-60/MRP1) and L1210/resistant cell line. The aim of the present work is to clarify the mechanism of *in vitro* anti-tumor effect and localization of the conjugate. *In vitro* anti-tumor effect was studied against HL-60/sensitive and HL-60/resistant cell lines using MTT assay. The localization of these compounds was

examined by confocal laser microscopy. The fluorescence characteristics of the conjugate and daunomycin were investigated with or without DNA at various pH, mimicking the intracellular milieu of the conjugate. Based on these data we have analyzed the effect of the charge characteristics of the polymer on antitumor activity *in vitro*. Data show that the IC_{50} value of the conjugate was low on HL-60/sensitive cell line. In the case of HL-60/MDR1 and HL-60/MRP1 cells, this value was higher. The fluorescence spectrum of the conjugate and daunomycin was similar, but the fluorescence intensity of the conjugate was significantly lower. Localization of the daunomycin was demonstrated mainly in the nucleus, while the cAD-EAK conjugate is present in the cytoplasm of HL-60/sensitive cells.

M2–018P
Study on binding affinity of polycyclic aromatic hydrocarbons to human albumin.

K. Skupiñska[1], I. Misiewicz[1] and T. Kasprzycka - Guttman[1,2]
[1]*Confocal Microscopy Laboratory, National Institute of Public Health, Warsaw, Poland,* [2]*Department of Chemistry, Warsaw University, Warsaw, Poland. E-mail: skupinska@il.waw.pl*

Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutant and some of them are carcinogenic toward humans. Due to assess exposure risk to PAHs, various biomarkers are used. Widely used are: albumin - PAH adducts since albumin is the most abundant protein in blood. In the study, the binding affinity of 9 PAHs to albumin was determined: anthracene and its 8 oxygen-containing derivates – antraquinone, 9-anthracenemethanol, 9-anthraldehyde, 9-anthracenecarboxylic acid, 1,4-dihydroxyantraquinone, 1,5-dihydroxyanthraquinone, 1,8-dihydroxyantraquinone and 2,6–dihydroxyantraquinone. The fluorescence quenching of albumin was a method used to measure the binding affinity. The corrections regarding PAHs fluorescence and inner filter effect were applied. The aim of this study was to establish if not substituted PAHs can bind to albumin, and how a type, amount and a site of substitution influence the binding affinity. Anthracene and antraquinone failed to quench the albumin fluorescence. 9-anthracenecarboxylic acid showed the highest binding affinity. 9-anthracenemethanol, 9-anthraldehyde showed the weakest albumin binding affinity. It indicates that the type of constituent plays a significant role in PAH-albumin adducts formation. The affinity constants of four dihydroxyantraquinones varied what suggest that a site of substitution in anthracene molecule influence the binding constant. Since anthracene did not interact with albumin it can be supposed that the metabolic activation is an essential condition for PAHs interactions with biological molecules. However, our results can also indicated that oxy-PAHs present in environment can immediately create adducts with albumin and the type of constituent influence the binding affinity.

M2–019P
The role of the scavenger receptor-A in the internalization of branched polypeptides with poly(L-Lys) backbone by bone-marrow derived murine macrophages

R. Szabó[1], L. Peiser[2], A. Plüddemann[2], S. Bõsze[1], S. Gordon[2] and F. Hudecz[1,3]
[1]*Research group of Peptide Chemistry, Hungarian Academy of Sciences, Budapest, Hungary,* [2]*Sir William Dunn School of Pathology, University of Oxford, Oxford, UK,* [3]*Department of Organic Chemistry, Eötvös L. University, Budapest, Hungary. E-mail: pancimanci@yahoo.com*

Selective delivery of anti-parasitic or antibacterial drugs into infected macrophages could be a promising approach for improved therapies. Methotrexate conjugate with branched chain polypeptides exhibited pronounced anti-Leishmania activity *in vitro* and *in vivo*. In order to identify structural requirements for efficient uptake of branched polypeptides we have performed a comparative study on murine bone marrow derived macrophages (BMM) from 129/ICR mice. Here we report on the translocation characteristics of structurally closely related compounds labelled with 5(6)-carboxyfluorescein. We found that this process is dependent on experimental conditions (e.g. polypeptide concentration, incubation time and temperature). Using scavenger receptor inhibitors (poly(I) and scavenger receptor A (SR-A) specific monoclonal antibody) as well as macrophage cells from wild type and SR-A knockout (SR-A –/–) mice we have demonstrated that SR-A is involved in the uptake of polypeptides but this is dependent on their charge. This uptake could be blocked by non-labelled polypeptide, by SR-A inhibitor and also by the monoclonal antibody. Results also suggest that polyanionic polypeptide poly[Lys(Succ-$Glu_{1.0}$-DL-$Ala_{3.8}$)] (SuccEAK) with high charge density translocates more efficiently than poly[Lys(Ac-$Glu_{1.0}$-DL-$Ala_{3.8}$)] (AcEAK), which has lower anionic charge density. Based on experimental data presented, SuccEAK can be considered as potential candidate for the design of a macromolecular carrier for specific drug delivery of bioactive entities into macrophages.

M2–020P
Generation of a fusion protein containing DNA-like peptide and a single chain antibody

Z. Szekeres[1], A. Isaák[1], J. Prechl[2] and A. Erdei[1,2]
[1]*Laboratory of Molecular Biology, Department of Immunology, Eötvös Loránd University, Budapest, Hungary,* [2]*Laboratory of Molecular Biology, Immunology Research Group, Hungarian Academy of Sciences, Budapest, Hungary.*
E-mail: p3259416@elte.hu

Autoantibodies against dsDNA are the most characteristic serological feature of Systemic Lupus Erythematosus (SLE). These antibodies may play an important role in disease pathogenesis: they can bind to various renal antigens, which leads to tissue damage and glomerulonephritis. We are interested in the preparation of a construct which may influence the activity of autoreactive B cells in SLE by crosslinking BCR and CR1/CR2. For this purpose we used 7g6 single chain antibody (scFv) specific for mouse CR1/CR2 and the dsDNA mimotope DWEYSVWLSN decapeptide. We investigated how the mimicking feature of DNA-like peptide changes in recombinant fusion protein form. For building the construct we digested pET-11d vector containing 7g6 scFv with NcoI restriction endonuclease and after that we inserted an NcoI-sticky-ended nucleotide sequence of decapeptide containing a linker region into this vector. On the DNA level, the existing of 7g6 scFv-DNA-like peptide construct was demonstrated by PCR and digestion with restriction endonuclease. Then, on the protein level, we used SDS-PAGE, Western blot and mass spectometry to confirm the fusion of the peptide. We studied the changing of the two member's function after fusion by ELISA and cytofluorimetry. By ELISA test we used anti-DNA antibodies to investigate the DNA-mimicking feature of the fusion protein and 7g6 scFv, as a control. Using cytofluorimetry we examined the binding of the fusion protein and 7g6 scFv to B cells in different dilution. We produced a DNA-like peptide-7g6 scFv construct by genetic engineering. The cytofluorimeter's data showed, that the fusion has not influenced the binding of 7g6 scFv to mCR1/CR2. According to the ELISA test, the anti-DNA antibodies can recognize the DNA-like decapeptide in the construct, so the recombinant form of this mimotope peptide retained its function.

M2–021P
IgG Fc binding peptide chimeras

K. Uray[1], Á. Bartos[1], G. Sármay[2] and F. Hudecz[1,3]
[1]*Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös Loránd University, Budapest, Hungary,* [2]*Research Group of the Hungarian Academy of Science, Department of Immunology, Eötvös Loránd University, Budapest, Hungary,* [3]*Department of Organic Chemistry, Eötvös Loránd University, Budapest, Hungary. E-mail: uray@szerves.chem.elte.hu*

The IgG binding Fc(gamma) receptors (Fc(gamma)Rs) play a key role in defence against pathogens by linking humoral and cell-mediated responses. The Fc(gamma)RI, IIa and III are activating receptors, while Fc(gamma)RIIb is responsible for the immune complex mediated inhibition of B cell activation. Impaired expression and function of the Fc(gamma)Rs may result in the development of pathological autoimmunity. Previously we have found three peptides capable to bind Fc(gamma)R [Uray et al *J Mol Recognition* 2004; **17**: 95–105), and one of these exhibited functional activity as well [Medgyesi et al, *Eur J Immunol* 2004; **34**: 1127–1135). Based on our earlier studies and on the known crystal structure of the IgG Fc – Fc(gamma)R complex, new chimeric peptides were designed in which the sequentially distant but sterically close Fc(gamma)R binding peptide sequences were chemically ligated to mimic the discontinuous receptor binding sites of the CH2 domain of IgG Fc. The individual chains of the peptide chimeras were prepared with solid phase synthesis method, applying both Fmoc and Boc chemistry with orthogonal protecting groups. The peptides were cleaved from the resin with TFA or liquid HF, purified with HPLC, and characterized by MS and amino acid analysis. Certain peptides were cyclized to achieve a conformation similar to that observed in the IgG Fc – Fc(gamma)R complex. The peptide chains were ligated via amide or thioether bond. In this contribution the synthesis of the chimeric IgG peptides will be described. We expect that these chimeras will show enhanced binding towards Fc(gamma)R and will exhibit strong functional activity, and may become parts of future immunomodulatory drugs.

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M2–022P
New approach for creation of drugs endowed with prolonged action in the basis of human serum transport protein

E. A. Zelepuga[1], G. N. Likhatskaya[2], E. V. Trifonov[3] and E. A. Nurminsky[3]
[1]*Laboratory of Proteins and Peptides Chemistry, Pacific Institute of Bioorganic Chemistry, Far Eastern Branch of the Russian Academy of Sciences, Vladivostok, Russian Federation,* [2]*Laboratory of Bioassay and Investigation Mechanism of Action, Pacific Institute of Bioorganic Chemistry, Far Eastern Branch of the Russian Academy of Sciences, Vladivostok, Russian Federation,* [3]*Laboratory of Supercomputing Technologies, The Institute for Automation and Control Processes, Vladivostok, Russian Federation. E-mail: zel@piboc.dvo.ru*

To obtain high active antiviral enzymatic medicine with prolonged action, we have developed a method for conjugating bovine pancreatic ribonuclease (RNase A) to ligand-free human serum albumin (LFHSA). RNase A conjugated to LFHSA, apparently acquires new properties: resistance to proteolysis and inhibitor of RNases (RI), which allows to increase their half-life by 300-fold as against the native enzyme during *in vivo* testing. As crystal structures of conjugates were not determined, to elucidate molecular mechanisms of alteration of RNase A biological properties in the LFHSA-conjugates the protein–protein docking with GRAMM program was carried out. Complex formation energy was estimated by SPDBV program. The analysis of the predicted structures has revealed the existence of several binding sites on the surface of albumin molecule, which involve the basic drug-binding sites ('Sudlow I' and 'Sudlow II') of HSA. The active centers of enzymes in the complex remain accessible to substrate. The removing of ligands bound to HSA was shown to influence the complexation essentially and probably result in nearly 10-fold increasing of the conjugates activity. The participation of proteolysis-labile and immunoreactive RNase A region (residues 32–43) in complex formation was detected. RI interaction with LFHSA-RNase complex was shown to interfere not in enzyme's active center. Theoretical data were in a good agreement with experimental observations. New approach to the creation of enzymatic drugs endowed with prolonged action has been proposed. It includes the use of LFHSA as an enzyme carrier, theoretical prediction of the physio-chemical and biological properties for enzyme–protein complexes, co-condensation of enzyme–LFHSA complexes with glutaraldehyde and isolation of produced conjugates. The approach was approved for another enzyme – binase.

M3 – Role of Peptides in Neuroprotection and Neurodegeneration

M3-001
Non-fibrillar beta-amyloid arrests spike-timing-dependent LTP induction at excitatory synapses in layer 2/3 of the neocortex: involvement of AMPA receptors

Y. Zilberter[1], H. Tanila[2], N. Burnashev[3] and **T. Harkany**[4]
[1]*Department of Neuroscience, Karolinska Institute, Stockholm, Sweden,* [2]*2Department of Neuroscience and Neurology, University of Kuopio, Kuopio, Finland,* [3]*Department of Experimental Neurophysiology, Vrije University Amsterdam, Amsterdam, the Netherlands,* [4]*Laboratory of Molecular Neurobiology, Department of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm, Sweden. E-mail: tibor.harkany@mbb.ki.se*

In the neocortex, information is processed by synaptically connected pyramidal cells under the control of inhibitory interneurons. Recent findings show that spike-timing-dependent plasticity (STDP) monitors the precise timing between pre- and post-synaptic cell spikes and converts it to a change in synaptic efficacy. The progressive cognitive decline in Alzheimer's disease suggests a causal relationship between synaptic dysfunction, impaired synaptic plasticity, and deterioration of learning and memory functions. Therefore, we studied whether non-fibrillar -amyloid (Aâ) fragments can affect STDP at unitary excitatory connections between pyramidal cells in layer 2/3 of the neocortex. We show that acute Aâ pre-treatment (< 500 nM) ablates the induction of spike-timing-dependent LTP (tLTP). Significantly reduced AMPA/NMDA receptor current ratio underscored the Aâ-induced loss of tLTP initiation. Analysis of AMPA and NMDA receptor currents in nucleated patches excised from layer 2/3 pyramids demonstrated a selective decline in AMPA receptor currents while NMDA receptors were not affected. In APP/PS1dE9

mice, an age-dependent reduction of STDP, as indicated by the gradual decline of tLTP induction, correlated with the cortical Aâ plaque load, and reduced tLTP induction was associated with a significantly decreased AMPA/NMDA receptor current ratio. We performed Ca^{2+} imaging in dendritic spines and shaft segments to understand whether Aâ-induced STDP depression was due to a change in intracellular (Ca^{2+}). Acute Aâ application induced only marginal changes in the amplitude of Ca^{2+} transients in synaptically active dendritic spines. In contrast, the basal spine Ca^{2+} level significantly increased. Concordantly, acute enhancement of AMPA receptor currents by cyclothiazide did not recover STDP in APP/PS1dE9 mice. In conclusion, our data show high susceptibility of STDP to Aâ toxicity.

M3-002
Aβ in lipid homeostasis and Alzheimer's disease

T. Hartmann
ZMBH, University of Heidelberg, Heidelberg, Germany.
E-mail: tobias.hartmann@zmbh.uni-heidelberg.de

Amyloid beta peptide (Aβ) has a key role in the pathological process of Alzheimer's disease (AD). The physiological function of Aβ and that of the Aβ precursor protein (APP) remained unknown since its discovery two decades ago and whether Aβ has any true physiological function after all remained very much in the open. The expression pattern of APP as well as the ubiquitous production of Aβ would predict, that – if such a function exists at all – this function would most likely be equally ubiquitous. Recent evidence revealed an astonishing correlation between cellular lipid levels and Aβ production, indicating that a physiological function may be related to lipid homeostasis. We will report here on the fascinating molecular and cell biological events linking Aβ with lipids and show based on *in vivo*, cell culture and cell free data, that the Aβ generating enzyme β-secretase is intrinsically necessary for cholesterol and sphingolipid homeostasis, that this involves bi-directional regulatory cycles in which β-secretase activity responds to altered lipid levels and inversely, that the proteolytic activity of this enzyme actively alters cellular lipid levels. This behaviour highlights the critical importance of the Aβ generating machinery in lipid homeostasis and importantly reveals on a mechanistic level how lipids interfere with Aβ production in Alzheimer's disease.

M3-003
Amyloid-beta: neurotoxic mechanisms and neuroprotective approaches

P. G. Luiten[1], T. Harkany[1], I. Granic[1], C. Nyakas[1] and B. Penke[2]
[1]*Department of Molecular Neurobiology, University of Groningen, Haren, the Netherlands,* [2]*Department of Medical Chemistry, Szent Györgyi Medical University, Szeged, Hungary.*
E-mail: p.g.m.luiten@rug.nl

Deposits of the aggregated amyloid β peptide (Aβ) in senile plaques are a characteristic neuropathologic feature of Alzheimer's disease (AD). Due to its neurotoxic properties, Aβ polymers are considered a major component of the neuropathogenic process of AD. With *in vivo* experiments we explored the nature of Aâ-zinduced neuronal injury. Aβ peptides were microinjected into basal forebrain cholinergic cell groups of rats. Neuronal damage was assessed by loss of cholinergic cells and their cortical projections. Localized Aβ injections in cholinergic cell groups initiated cell death, which was accompanied by cognitive dysfunctions. Aβ infused by microdialysis with simultaneous transmitter measurement triggered a high release of glutamate followed by excitotoxic cell death, which could be prevented by the NMDA receptor blocker MK-801. Further evidence for an Aβ-induced excitotoxic cascade came from accumulation of radioactive calcium in the Aβ injection area. Current neuroprotection experiments against Aβ are now carried out with a number of calcium blocking agents. A second major approach was to establish anti-Aβ potential of tetra- or pentapeptides derived from Aβ sequences. Recent experimental data come from forebrain slices of transgenic APPS,L/PS1 mice with amyloid plaques exposed to pentapeptides. Individual plaques stained with thioflavine-S were incubated for 18 h and plaque density measured every hour by CLSM. An almost linear decrease of plaque fluorescence was observed indicating sheet solving properties of Aβ derived pentapeptides, revealing promising potential of such peptide approaches that combat Aβ induced neurodegeneration in an early phase of the disease. Acknowledgment: The authors acknowledge the support of the Hersenstichting Nederland to PGML and TH.

M3-004
Regulated access of peripheral cytokines to the injured CNS

W. Pan, C. M. Cain, Y. Yu, C. Xie and A. J. Kastin
Blood-Brain Barrier Laboratory, Pennington Biomedical Research Center, Louisiana State University, Baton Rouge, LA, United States of America. E-mail: weihong.pan@pbrc.edu

Cytokines such as tumor necrosis factor alpha (TNF) and leukemia inhibitory factor play important roles in neurotrauma and regeneration of the central nervous system (CNS). Ensuring adequate concentrations of cytokines at the right time and place is one of the strategies to promote functional recovery after spinal cord injury (SCI). We have shown that the blood-brain and blood-spinal cord barrier (BBB) transport TNF and LIF by specific, receptor-mediated transport systems. Although the BBB is partially disrupted after SCI leading to an increase in the nonspecific permeation of blood-borne proteins, the transport systems are up regulated at particular time intervals. This talk will focus on the mechanisms and functional implications of such regulated access to spinal cord regeneration. SCI was generated in adult mice by a standardized computer-driven weight-drop contusion device or bilateral compression at the level of T10. The extent of BBB disruption, evaluated in brain and spinal cord sections by extravasation of fluorescin and Evan's blue albumin, was most pronounced at the injury site even a week after injury. At this time, there were changes in the subcellular distribution of the cytokine receptors (TNFR1, TNFR2, LIFR, and gp130), as determined by immunofluorescence. There also was an increase in the mRNA for some of the receptors, as shown by real-time PCR. Correspondingly, the uptake of radioactively labeled TNF and LIF was significantly increased as compared with the laminectomy controls. Excess non-radioactively labeled TNF and LIF, as well as blocking antibodies for the cytokine receptors, significantly dampened the increase. Thus, enhanced expression of the receptors involved in transport is associated with enhanced transport activity. Further functional assays are underway to determine the impact of increased cytokine permeation on spinal cord regeneration.

M3-005
α_v integrins interacting peptides are neuroprotective after an excitotoxic lesion to the immature brain

A. Aris[1], H. Peluffo[2], P. Gonzalez[2], L. Acarin[2], B. Castellano[2], A. Villaverde[1] and B. Gonzalez[2]
[1]*Laboratori de Microbiologia Aplicada, Institut de Biotecnologia i de Biomedicina, Universitat Autonoma de Barcelona, Bellaterra, Spain,* [2]*Unitat d'Histologia, Departament de Biologia Cel·lular, Fisiologia i Immunologia, Universitat Autonoma de Barcelona, Bellaterra, Spain. E-mail: anna.aris@uab.es*

Integrins are cell surface receptors composed of one α and one β subunit. Integrin-mediated cell adhesion leads to signaling events affecting cell activation, cell motility, cell proliferation and apoptosis of polymorphonuclear leukocytes (PMNs) and macrophage/microglia, which are the major cellular effectors of inflammation and tissue injury. In this study we have analysed the effect of peptides that interact with α_v family integrins, through specific RGD motifs, in the central nervous system after an excitotoxic insult. Two hours after an NMDA-mediated excitotoxic lesion to the immature rat brain, we injected intracerebrally the synthetic peptide GPenGRGDSPCA (Gibco, BRL) and the chimeric β-galactosidase NLSCt that presents in its surface the cell-attachment RGD-containing peptide from foot and mouth disease virus [1]. Three days after both treatments, animals showed a significant lesion volume reduction up to 32%, which was in addition dose dependent in the case of the GPenGRGDSPCA peptide (0.007–1 mM) administration. These results overall suggest that these RGD containing peptides interacting with α_v integrins can exert neuroprotection. In this work we will discuss the possible mechanisms by which RGD containing peptides induce neuroprotection, such as the blocking of the PMNs and macrophage/microglia recruitment, trans-endothelial migration, activation and phagocytosis, the reduction of astroglial reactivity and the direct activating integrin outside-in signalling events.

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M3-006
Prion peptide interactions with neuroblastoma cells and liposomes: a potential cytotoxicity mechanism

I. Dupiereux[1], W. Zorzi[1], L. Lins[2], R. Brasseur[2], P. Colson[3], E. Heinen[1] and B. ElMoulaij[1]
[1]*CRPP, Department of Human Histology, University of Liège, Liège, Belgium,* [2]*Centre de Biophysique Moléculaire Numérique, Faculté Universitaire de Gembloux, Gembloux, Belgium,* [3]*Biospectrocopy and Physical Chemistry Unit, Department of Chemistry and Natural and Synthetic Drogues Research Center, University of Liège, Liège, Belgium. E-mail: idupiereux@ulg.ac.be*

Prion diseases are fatal neurodegenerative disorders characterized by the accumulation in the brain of an abnormally mis-folded, protease-resistant and beta-sheet rich pathogenic isoform (PrPsc) of the cellular prion protein (PrPc). Currently, the relationship between accumulation and neurotoxicity of PrPsc remains unclear. In the present work, we were interested to study neurotoxicity, peptide folding and the mode of prion proteins interaction with the membrane using the prion peptides as model. This synthetic sequence corresponds to the amyloidogenic region of the prion protein and is useful for *in vitro* studies of prion-induced neuronal cell death as it retains many PrPsc characteristics: the ability to trigger neurotoxicity and the high b-sheet content. We show that the peptides induce alterations in the human neuroblastoma SHSY-5Y cell line. In order to understand the mechanism of prion peptides neurotoxicity, the potential of such peptides to induce fusion of small unilamellar lipid vesicles was investigated. Here, we demonstrated for the first time by lipid-mixing assay and by the liposome vesicle leakage test that prion peptides induces liposome fusion, thus a cell membrane destabilization. By circular dichroism (CD) analysis we showed that the fusogenic property of the peptide in the presence of liposome is associated with a predominantly b-sheet structure. These data suggest that the fusogenic property associated with a predominant b-sheet structure exhibited by the prion peptides might contribute by destabilizing cellular membranes to the neurotoxicity of these peptides. The latter might be attached at the membrane surface in a parallel orientation as shown molecular modeling.

M3-007P
Neuroprotective effect of cobra venom on the spinal cord of trauma-injured rats: a morpho-functional study of PRP-1 and acid phosphatase activity

S. Abrahamyan[1], I. Meliksetyan[2], E. Chavushyan[2], J. Sarkissian[2] and A. Galoyan[1]
[1]*H. Buniatian Institute of Biochemistry NAS RA, Yerevan, Armenia,* [2]*L. Orbeli Institute of Physiology, NAS RA, Yerevan, Armenia. E-mail: silva@dolphin.am*

Electrophysiological, histochemical (method of acid phosphatase activity detection) and immunohistochemical (ABC immunohistochemical method) studies demonstrate the action of *Naja Naja Oxiana* snake venom on the morpho-functional state of the spinal cord (SC) of trauma-injured rats. On the next day after SC lateral hemisection on the L2-L3 level the animals were i/m injected with the snake venom every day in dose 0.05 LD50 (LD50 – 1mg/kg i/ab). The localization of proline-rich-peptide-1-immunoreactivity (PRP-1-IR) was studied in various SC structures, with and without venom treatment. Hypothalamic PRP-1 (a fragment of neurophysin vasopressin associated glycoprotein, isolated in 1996 by A.A.Galoyan and coworkers from bovine neurohypophyseal neurosecretory granules) has been suggested to play the role of a universal neuroprotector and neuromodulator. Treatment of the trauma-injured rats with Cobra venom prevented formation of the glial scar after SC hemisection and resulted in the recovery of SC motoneurons and appearance of PRP-1-immunoreactive nerve fibers on the injured place. The glial cells observed at a great distance from the injured place were immunohistochemicaly found to be fibrillar astrogliocytes and oligodendrocytes positive to PRP-1 and had the nuclei with activated acid phosphatase. Electrophysiological study has also demonstrated the protective effect of cobra venom. The results obtained assume a potential application of snake venom *Naja Naja Oxiana* in clinical practice for the prevention of chronic traumatic neurodegeneration of central origin and involvement of the above-mentioned substances in the neurodegenerative mechanism.

M3-008P
Tau hyperphosphorylation in a double-transfected hGSK-3β/hTau-ECR cell line. Flow cytometry-based immunocytochemical study

A. Boros, D. Kurkó, M. Sárvári, P. Dezső, J. Nagy and G. Szendrei
Pharmacological and Drug Safety Research, Gedeon Richter. Ltd, Budapest, Hungary. E-mail: a.boros@richter.hu

Alzheimer's disease associated with the neuropathological feature of the presence of intracellular neurofibrillary tangles (NT) in defined regions of the brain. NT consists of paired helical

filaments, which contain mainly a hyperphosphorylated form of microtubule-associated protein Tau. Glycogen synthase kinase-3β (GSK-3β) plays pivotal role in the regulation of Tau phosphorilation. We developed a flow cytometry-based immunocytochemical method to quantify the effect of GSK-3β inhibitors on Tau phosphorylation. Stably transfected human (h) Tau and hGSK-3β ECR cell line was generated to obtain a robust *in vitro* cell-based assay to monitor changes in Tau phosphorylation. EcR-293 as control and hGSK/hTau-transfected cells were induced with 1 μM muristeron-A for 48 h. Cells then were harvested, fixated (1% paraformaldehide) and further processed for immunocytochemistry. Dual color analysis was carried out on FACScan (Becton Dickinson) flow cytometer. Primary antibodies were rabbit anti-human-phospho-Tau (pSer396 and pSer202) (Sigma, 1:100) and monoclonal mouse anti-Tau (Sigma, 1:100). Secondary antibodies were FITC-conjugated anti-rabbit IgG and PE-conjugated anti-mouse IgG (Sigma, 1:100). Mean fluorescence values (arbitrary fluorescence unit) were obtained from gated populations and ratio of phosphorylated Tau and pan-Tau was calculated. As a result of the induced gene expression, pSer396 site-specific immunofluorescence values rose five times of the basal value. This elevation in phosphorylation enables us to evaluate the potential inhibitory effect of GSK-3β inhibitors on Tau phosphorylation at Ser396 site. We have measured Ser202 site-specific phosphorylation as well, but it proved to be less extensive (three times elevation). The selective GSK-3β inhibitor SB-415286 dose dependently inhibited GSK3β overexpression-induced Tau phosphorylation. There was an approximately 300 times difference between the IC50 values for Ser396 site and for the Ser202 site. The marked difference between the effectiveness of the reference compound points to the difference between the sensitivity of the two specific phosphorylation sites. This new methodological approach was also validated by the conventional Western blot analysis.

M3-009P
The roles of cytokines in an experimental peripheral nerve ischemia-reperfusion model

O. T. Bagdatoglu[1], G. Polat[1], C. Bagdatoglu[2] and U. Atýk[1]
[1]*Department of Biochemistry, Faculty of Medicine, University of Mersin, Mersin, Turkey,* [2]*Department of Neurosurgery, Faculty of Medicine, University of Mersin, Mersin, Turkey.*
E-mail: otbagdatoglu@mersin.edu.tr

Although the neuropathology of ischemic nerve fiber degeneration is relatively well known, its pathogenesis is poorly understood. One of the presumed mechanisms is the breakdown of blood-nerve barrier, causing the oxidative stress and lipid peroxidation. Local growth factors called as cytokines, that have neuroprotective effects on inflammation and repair, participates the process by undefined mechanisms. Ischemia and reperfusion injury of sciatic nerve was rendered by clamping the femoral artery and vein for 3 h and followed by varying duration of reperfusion. And than, Activin A, Transforming growth factor β1 (TGF- β1) and Transforming growth factor β2 (TGF- β2) levels have measured by using the serum samples of the rats. All biochemical parameters were found to be increased in ischemia groups when compared with control group ($p < 0.05$). After the reperfusion, a significant difference between the experimental groups was determined causing the different duration of reperfusion ($p < 0.05$). Also some correlations were established between the biochemical parameters in the same group depending on the varying reperfusion time ($r > 0.50$). The ischemia causes some important changes in biochemical parameters, and the nerve injury continues for a while according to the reperfusion time. As a result of this model, ischemia-reperfusion injury of peripheral nerve caused by several reasons, effects on the levels of cytokines. Also these data indicate that all these molecules interact with each other and take part in the process during the injury or/and repair of the peripheral nerve.

M3-010P
Neuronal degeneration and glial cell activation in the hippocampus following exposure of mice to neurotoxin: possible role of oligodendroglia progenitors

A. Fiedorowicz, **K. Dzwonek**, I. Figiel, M. Zaremba and B. Oderfeld-Nowak
Laboratory of Mechanisms of Neurodegeneration and Neuroprotection, Department of Molecular and Cellular Neurobiology, Nencki Institute of Experimental Biology, Warsaw, Poland. E-mail: kdzwonek@nencki.gov.pl

We have employed a chemical-induced murine model of hippocampal damage, injecting neurotoxicant – trimethyltin (TMT). In this model the blood-brain barrier remains intact. In our previous studies we have reported that in mice TMT evoked selective apoptosis of dentate gyrus granule neurons, accompanied by activation of astrocytes and microglia. In the present study we are reporting that yet another glia class – oligodendrocyte progenitor cells (OPCs), identified by antibody against NG2 proteoglycan, became activated in the hippocampus. The strongest activation of OPCs was observed 3 days after TMT intoxication, at the peak of neuronal apoptosis. At that time activated NG2 positive cells, around degenerating granule neurons, displayed ameboid morphology, expressed a specific marker for proliferating cells – PCNA and, most interestingly, showed a colocalization of immunoreactivities of NG2 and OX42/ED1, the markers of microglia/macrophages. OPCs were found to express nestin – an embrionic protein, transiently expressed by precursors of neurons and glia during brain development. Some NG2 positive glial cells expressed also APC, a marker of mature oligodendroglia. Our findings suggest that after hippocampal injury the neurodegeneration and glia activation are mutually interrelated. Oligodendroglia progenitors become activated in response to neurodegeneration and may influence its course in multiple ways. They may release the active substances, give rise to a population of mature oligodendroglia and, as cells bearing the features of neural progenitors, could influence neurogenesis of granule cells, known to occur in injury conditions. On the other hand, these cells acquiring microglia features, can be involved in the inflammatory processes.

M3-011P
Anti-neuroinflammatory activity of melanocortin receptor ligands

M. Dambrova[1], L. Zvejniece[2], L. Baumane[1], R. Muceniece[2] and J. E. Wikberg[3]
[1]*Latvian Institute of Organic Synthesis, Riga, Latvia,* [2]*Latvian University, Riga, Latvia,* [3]*Uppsala University, Uppsala, Sweden.*
E-mail: md@biomed.lu.lv

The melanocortine peptides possess strong anti-neuroinflammatory effects acting via mechanisms that are not fully understood. In our previous studies we demonstrated the anti-inflammatory effects of α-, β- and γ-MSH using an experimental mice brain inflammation model, where β-MSH was found to be the most effective agent. Moreover, we investigated the molecular mechanisms for the β-MSH-induced suppression of brain inflammation and found that β-MSH inhibits LPS-induced nuclear translocation of the NF -κB, as well as expression of inducible nitric synthase, and the following nitric oxide overproduction in the brain, *in vivo*. The MC4 receptor selective antagonist HS014

blocked completely the β-MSH effects. Even though β-MSH binds to the MC3 and MC4 receptors with some preference for the MC3, and HS014 is a quite non-selective antagonist showing also antagonistic activity at the MC3 receptor, it was not possible to state if the effect of β-MSH observed in the central nervous system is exerted via the MC3 or MC4 receptor. Therefore, we evaluated further the role of different melanocortin receptor subtypes in neuroinflammation by using MC3/MC4 receptor subtype selective peptides and synthesized novel analogues of the α-MSH and β-MSH in the radioligand binding and nitric oxide production assays. We found that the test substances dose dependently inhibited LPS-induced nitric oxide production in the mice forebrain in the manner that resembled the order of MC3 receptor binding potency. In conclusion, our results suggest that MC3 receptor is involved in mediating the anti-inflammatory activity effect of MCs and synthetic analogues in mice brain inflammation.

M3-012P
Selenium attenuates oxidative stress responses through modulation of selenium-containing proteins in microglial cells

L. Dalla Puppa[1], N. E. Savaskan[2], A. U. Brauer[3], D. Behne[1] and A. Kyriakopoulos[1]

[1]*Department of Molecular Trace Element Research in the Life Sciences, Hahn Meitner Institute, Berlin, Germany,* [2]*Division of Cellular Biochemistry, the Netherlands Cancer Institute, Amsterdam, the Netherlands,* [3]*Institute of Cell Biology and Neurobiology, Charité-University Medical School Berlin, Berlin, Germany.*
E-mail: dallapuppa@hmi.de

Primary neuronal destruction due to oxidative stress, such as in excitotoxicity and stroke, is potentiated by late-occurring secondary cell death. This secondary response is mediated primarily by activated microglial cells. Since selenium is known to play a role in antioxidative protection, we investigated the effects of selenium supplementation on the microglial cells. Selenium is an essential nutrient for the immune system and overall body function. The functions of selenium are believed to be carried out by selenoproteins, in which selenium is specifically incorporated as the 21st amino acid, selenocysteine. Selenium, added as selenite, protects microglial cells from oxidative stress via the expression of specific selenoproteins. In order to identify these selenoproteins, we have applied (^{75}Se)-selenite labelling as a suitable method in order to assess the expression of selenoproteins in the microglial cells. Furthermore we used loss-of-function (siRNA knock down) approaches to identify candidate neuroprotective selenoproteins in microglial cells. Our results indicate the importance of the nutritionally essential trace element selenium for the prevention of neuronal cell death. Thus, selenium is not only beneficial for neurons but also inhibits microglial activation and thereby reduces secondary cell death.

M3-013P
Influence of flanking and cyclization of a β-amyloid peptide derived B-cell epitope on the antibody recognition and enzymatic stability

K. Horváti[1], M. Manea[2], S. Bősze[1], G. Mező[1], M. Przybylski[2] and F. Hudecz[1]

[1]*Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös L. University, Budapest, Hungary,* [2]*Laboratory of Analytical Chemistry and Biopolymer Structure Analysis, Department of Chemistry, University of Konstanz, Konstanz, Germany.*
E-mail: khorvati@elte.hu

To increase immunogenicity of synthetic peptide epitope several chemical modifications have been described in the literature. A peptide FRHDSGY (Aβ 4–10) derived from β-amyloid is the predominant B-cell epitope, [1] which has a key role in Alzheimer's disease. We studied the influence of its elongation with non-native flanking regions [oligo-(Ala/β-Ala)], blocking the N- and C-terminus, reversed sequence epitope or cyclization on antibody binding and enzymatic stability. The peptides were synthesized on solid phase by Fmoc/tBu strategy. Peptides elongated by cysteine were cyclized via disulfide bond. All peptides were characterized by analytical HPLC, amino acid analysis, MALDI-TOF- and MALDI-FTICR-MS. Binding studies of the peptide derivatives to mouse anti-Aβ 1–17 monoclonal antibody were performed by direct ELISA. Enzymatic stability experiments were carried out in the presence of trypsin and monitored by MS. We found that the antigenicity of peptides with oligo-(Ala/β-Ala) flanking regions was increased significantly. The mouse anti-Aβ 1–17 monoclonal antibody did not recognize the Aβ 4–10 peptide with reversed sequence. Cyclic derivatives were the best substrates of trypsin and degraded quickly. In contrast blocking the N- and C-terminus prevented the tryptic digestion.
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M3-014P
Effects of amyloid beta peptides and of cerebrosterol on cholesterol-depleted membranes from rat hippocampus

Z. Kristofikova

Alzheimer's disease Centre, Prague Psychiatric Centre, Prague, Czech Republic. E-mail: kristofikova@pcp.lf3.cuni.cz

It is suggested that amyloid beta peptides (Abeta) and brain cholesterol play an important role in the pathogenesis of Alzheimer disease (AD). Conversion of cholesterol to its polar metabolite 24(S)hydroxycholesterol (cerebrosterol) is a major pathway for elimination of brain cholesterol and maintenance of cholesterol homeostasis. It seems that high concentrations of cerebrosterol can be neurotoxic and involved in the pathogenesis of AD, too. The increased ratio cerebrosterol/cholesterol was found among others in patients with AD. In this study, effects of non-aggregated and aggregated Abeta fragments 1–40 or 1–42 and of cerebrosterol are evaluated on cholesterol-depleted synaptosomal membranes isolated from rat hippocampi. The measurements of the high-affinity choline transport using (3H) choline and of membrane fluidity using 1,6-diphenyl-1,3,5-hexatriene indicate that: (i) depletion of membrane cholesterol influences the function of membrane-bound choline carriers via alterations in membrane fluidity, (ii) the effects of aggregated Abeta on choline carriers are enhanced especially on cholesterol-depleted membranes, however, membrane fluidity is not changed via actions of Abeta at low concentrations and during short pre-incubations, (iii) the effects of cerebrosterol on choline carriers are enhanced on cholesterol-depleted membranes but cerebrosterol does not penetrate into membranes, and finally (iv) the effects of Abeta are enhanced on membranes pre-treated with cerebrosterol. Our results support data in literature that membrane cholesterol protects neurons against the toxic effects of Abeta. Our experiments suggest also the possible mechanism of toxic actions of cerebrosterol on membrane-bound proteins.
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M3-015P
A synthetic human proline-rich-polypeptide enhances hydroxyl radical generation and fails to protect dopaminergic neurons against MPTP-induced toxicity in mice

V. H. Knaryan[1], S. Samantaray[2], A. A. Galoyan[1] and K. P. Mohanakumar[2]
[1]*Department of Neurohormones Biochemistry, H. Buniatian Institute of Biochemistry, National Academy of Sciences of the Republic of Armenia, Yerevan, Armenia,* [2]*Division of Neurosciences, Indian Institute of Chemical Biology, Calcutta, India.*
E-mail: vknaryan@yahoo.com

Some of the proline-rich-polypeptides (PRPs), containing 15 amino acid residues isolated from bovine neurohypophysis (b-PRP) have been demonstrated to possess neuromodulatory and neuroprotective effects. Recently in our laboratory another PRP has been isolated from human hypothalamus (h-PRP), and its synthetic form is now available [1]. We hypothesized that this endogenous neuropeptide may also exert neuroprotective effects in a neurodegenerative model of Parkinson's disease (PD). A synthetic h-PRP has been examined for its potency to protect against dopaminergic neuronal damage caused by the parkinsonian neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Generation of hydroxyl radicals ($^{\bullet}$OH) in dopaminergic neurons following MPTP administration is known to be one of the major mechanisms for its neurotoxicity. We tested the effect of h-PRP on $^{\bullet}$OH production in a tissue-free system employing Fenton-like reaction and for striatal dopamine (DA) recovery in the MPTP-induced PD. Balb/c mice treated twice with MPTP or h-PRP showed significant loss of striatal DA as assayed by HPLC with electrochemical detection. Pre-treatment with h-PRP failed to attenuate MPTP-induced striatal DA depletion, and h-PRP at 1–10 μM concentration caused a significant increase in the formation of $^{\bullet}$OH in Fenton-like reaction. h-PRP alone could significantly reduce DA levels in the striatum, and it failed to protect against MPTP-induced DA loss. A dose-dependent increase in the generation of $^{\bullet}$OH by h-PRP suggests its pro-oxidant action, and explains its failure to protect against MPTP-induced parkinsonism in mice. A dose equivalent to 0.1 μM or less, which does not have $^{\bullet}$OH generating capacity, need to be tested in this model, since b-PRP is well known to protect neurons *in vivo* in extremely small doses.

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M3-016P
Identification of proteome altered by co-culturing of neuroblastoma SH-SY5Y and astrocytoma U87

C. Kang[1], M. Suh[2], M. H. Kim[1] and J. H. Han[1]
[1]*Proteomics, Neuroscience, Kyung Hee, Yong-In, Kyung-ki-do South Korea,* [2]*Biophysical Chemistry, Chemistry, Sung Kyun Kwan, Suwon, Kyung-ki-do South Korea.*
E-mail: kangch@khu.ac.kr

Communication between neurons and glia is important in central nervous system. It may involve intercellular diffusion of chemicals and extracellular signaling molecules in part. In order to identify extracellular signaling molecules and their effects on cellular proteomes, we studied alteration of proteomes of astrocytes, neuroblastoma and media from a transwell co-culture system. Proteomes of two cell lines (SH-SY5Y & U87) and serum-free media were investigated using 2-dimensional gel electrophoresis visualized by CBB staining followed by MALDI-TOF post-gel analysis. Approximately, 150 spots were visualized on a mini-gel and 11 spots were increased for U87 cell line by co-culturing. And five among 200 spots were enhanced for SH-SY5Y cell line in the same condition. The enhanced spots include enolase1, glyceraldehyde-3-phosphate dehydrogenase and annexin A2, and calreticulin for U87 and SH-SY5Y cell lines respectively. The proteins in the media, presumably secreted from the cells did not appear to be altered by co-culturing and close to those from culture of astrocytoma alone. Five protein spots from the media proteome were identified as valosin-containing protein, urokinase-type plasminogen activator receptor and etc. The exact roles of these changes in intercellular communication are under investigation. A part of proteomes possibly involved in communication between neuroblastoma and astrocytoma are presented in this study.

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M3-017P
Generation and characterization of recombinant hGSK-3β and hTau over-expressing EcR-293 cells

D. Kurkó, A. Boros, P. Dezső, Z. Urbányi, J. Nagy and G. I. Szendrei
Pharmacological and Drug Safety Research Department of Molecular Cell Biology, Gedeon Richter Ltd., Budapest, Hungary.
E-mail: d.kurko@richter.hu

Neurofibrillary tangles are characteristic histopathological markers of Alzheimer's disease. They contain a hyperphosphorylated (HP) form of the microtubule-associated protein Tau. HP Tau has a reduced affinity for microtubules leading to the instability of the cytoskeleton that may trigger neuronal degeneration. *In vitro* studies have shown that glycogen synthase kinase-3β (GSK-3β) plays an important role in the regulation of this process. To examine the function of GSK-3β in Tau hyperphosphorylation we developed a cell-based assay using EcR-293 cell line. ECR-293 cells were co-transfected with human GSK-3β and human tau cDNAs (vectors: pIND/Hygro and pIND/Neo). Cell colonies resistant for the selecting agents were tested for hGSK-3β and hTau mRNA as well as protein expression using quantitative RT-PCR and flow cytometry based immunocytochemistry respectively. A good correlation was found between mRNA levels and protein immuno-reactivity. Protein expression was 3–5 fold over the basal level in the induced clones. According to these results, clone H11 was chosen for investigation of tau phosphorylation using phosphorylation-site specific anti-tau antibodies. In H11 cells pre-treated with the inducing agent muristerone A (MuA), a significant increase in tau phosphorylation at specific GSK-3β-dependent phosphorylation sites (Ser 202 and Ser 396) was detected. Flow cytometry analysis revealed a dose-dependent up regulation of tau hyperphosphorylation with increasing concentration of MuA. In addition, the GSK-3 selective inhibitor LiCl reduced tau phosphorylation in a concentration dependent manner. Thus, the established cell line stably and inducibly co-expressing hGSKβ and hTau is a useful tool to investigate the effect of GSK-3β inhibitors on Tau phosphorylation.

M3-018P
Channel formation of Alzheimer amyloid Aβ1–42 in planar phospholipid bilayer membranes

S. Micelli[1], F. F. Roberto[1], D. Meleleo[1], L. Lastella[1], V. Picciarelli[2] and E. Gallucci[1]
[1]*Department of Physiology, Farmaco-Biologico, Università degli Studi di Bari, Bari, Italy,* [2]*Department of Physics, Interateneo di Fisica, Università degli Studi di Bari, Bari, Italy.*
E-mail: micelli@farmbiol.uniba.it

Neuronal degeneration in Alzheimer's disease (AD) is associated with amyloid b-peptides 1–40 and 1–42. However, due to its propensity to form b-sheet structures, Aβ 1–42 is considered one of the major components of plaques. Recent studies indicate cell membrane perturbation as a "primum movens" of Aβ 1–42 toxicity. In fact, both Aβ 1–40 and 1–42 have been shown to form ion channels in planar bilayer membranes.[1, 2] Besides, Aβ 1–42 has been shown to perturb and increase the permeability of liposome membranes.[3, 4] This study evaluates Aβ 1–42's ability to form ion channels in Phosphatidylserine:Phosphatidylethanolamine (1:1) membranes at 5*10–8 M and pH 7.4. At this concentration, Aβ 1–42 formed voltage-independent channels in the range of (120, –20 mV) with a mean occurrence of 4.23 ± 0.63 and a distribution of open times with a single- or two-exponential function (mean lifetime 1.89 ± 0.65 and 4.01 ± 0.11 sec respectively). These channels have a lower mean conductance than Aβ 1–40. However, Hirakura et al. (1999) found that Aβ1–42 incorporated into synthetic 1-palmitoyl-2-oleoyl-phosphatidylethanolamine:1-palmitoyl-2-oleoyl-phosphatidylglycerol (1:1) planar bilayers at at 22*10–6 M and pH 7.4 forms voltage-independent channels in only 47% of experiments with conductances similar to those of Aβ 1–40 channels. Taken together, these results indicate that membrane composition could influence Aβ 1–42 channel formation.

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M3-019P
Interaction of beta-amyloid 1-42 (Abeta-1-42) with synaptosomal proteins as well as pentapeptide derived from Abeta 1-42

B. Penke, V. Szegedi, Z. Datki, Y. Verdier, L. Fülöp, Z. Penke, K. Soós and M. Zarándi
Department of Medical Chemistry, University of Szeged, Szeged, Hungary. E-mail: penke@ovrisc.mdche.u-szeged.hu

β-Amyloid peptides, that are overproduced in Alzheimer's disease rapidly forms fibrils, which are able to interact with various molecular partners. We have identified abundant synaptosomal proteins binding to the fibrillar beta-amyloid 1–42. Protein identification was accomplished (i) by separating the tryptically digested peptides of the protein pellet by one-dimensional reversed-phase high pressure liquid chromatography and analysing them by an ion-trap mass spectrometer with electrospray ionization; (ii) by subjecting the precipitated proteins to gel electrophoretic fractionation, in-gel tryptic digestion and to matrix-assisted laser desorption ionization, time-of-flight mass measurements and post-source decay analysis. Six different synaptosomal proteins co-precipitated with fAβ were identified by both methods: vacuolar proton pump ATP synthase, glyceraldehyde-3-phosphate dehydrogenase, synapsins I and II, β-tubulin and cyclic nucleotide phosphodiesterase. Most of these proteins have already been associated with Alzheimer's disease. Although the precise mechanism of the neurotoxic effect of amyloid peptides has not been discovered, several methods are known for neuroprotection against Aβ 1–42. Short fragments and fragment analogues Aβ 1–42 display a protective effect against Aβ-mediated neurotoxicity. After consideration of our earlier results with *in vitro* bioassay of synthetic Aβ-recognition peptides and toxic fibrillar amyloids, four pentapeptides were selected as putative neuroprotective agents: Phe-Arg-His-Asp-Ser-amide (Aβ 4–8) and Gly-Arg-His-Asp-Ser-amide (an analogue of Aβ 4–8), Leu-Pro-Tyr-Phe-Asp-amide (an analogue of Aβ 17–21) and Arg-Ile-Ile-Gly-Leu-amide (an analogue of Aβ 30–34). *In vitro* electrophysiological experiments on rat brain slices demonstrated that four of these peptides counteracted with the field excitatory post-synaptic potential-attenuating effect of Aβ 1–42. In *in vivo* experiments using extracellular single-unit recordings combined with iontophoresis, all these pentapeptides protected neurons from the NMDA response-enhancing effect of Aβ 1–42 in the hippocampal CA1 region. These results suggest that Aβ recognition sequences may serve as leads for the design of novel neuroprotective compounds.

M3-020P
Temporal and spatial distribution of substance P and its receptor regulated by calcitonin gene-related peptide in the development of airway hyper-responsiveness

X. Qin, H. Wu, Y. Xiang, Y. Ren and C. Guan
Pulmonary, Physiology, Central South University, Xiangya Medicine School, Changsha, Hunan, PR China.
E-mail: xiaoqun1988@xysm.net

The mechanism of airway hyper-responsiveness (AHR) is still unclear, for which more and more of attention is being paid to the hypothesis of neuron origin recently. Substance P (SP), as an important neurotransmitter of sensory C-fiber in lung and one of the most extensively studied members in tachykinin family, probably plays important roles in pulmonary inflammation or in regulation of airway function, is therefore involved in the development of airway hyper-responsiveness. Another important sensory C-fiber neuropeptides, calcitonin gene-related peptide (CGRP), which coexist with SP in same nerve ending, possibly affects SP signal transmission due to their co-existence relation. To understand the time course and spatial distribution of SP with its receptor and the mutual actions in lung between SP and CGRP, a novel animal model of AHR has been created in our laboratory through injuring airway epithelium with ozone exposure. Based on this ozone stressed AHR model, the level and the time course of SP and CGRP in lung were measured with radio-immunoassay in the present research; the spatial distribution of SP and its receptor NK-1 in lung were also observed with immunohistochemistry and *in situ* hybridization respectively; Western Blotting and RT-PCR were used to investigate the regulatory effect of CGRP on NK-1 expression and its relevant signal pathway.

Results: Exposure with ozone, the concentration of substance P in lung homogenate began to rise within 24 h and peaked on day two then decreased slowly; immuno-histochemistry indicated that numbers of SP-immunoreactive cell bodies which distributed widely in lung structure including bronchial smooth muscle, wall of pulmonary blood vessel, basal membrane, alveolar epithelial and interstitial, was increased on ozone-stress day one and maximal at day two, then began decline at day four to six. Meanwhile, *in situ* hybridization showed that SP receptor NK-1 mRNA positive cells were distributed extensively in lung tissues, along with vascular smooth muscles, bronchial smooth muscles, airway epi-

thelium, and mainly in pulmonary interstitial. Hybridization signal increased with the elongation of ozone-stress and peaked on day four, then decreased. With few NK-1 mRNA signal visible in lung on day six, the expression of NK-1 was mainly located in perivascular and peribronchiolar; the time course of CGRP content change in lung was similar to those detected for SP, it raised to climax on day two, then was down slowly, which showed a typical correlation between SP and CGRP; Western Blotting showed that, CGRP up-regulated NK-1 protein expression in a time-dependent manner; and a dose-dependent increase of NK-1 mRNA was also observed in CGRP group with RT-PCR; this effect of CGRP could be diminished by calmodulin inhibitor W7, PKA inhibitor H-89, or TPK inhibitor genistein.

Conclusion: Airway epithelium damage can induce an airway inflammation, and in it , capsaicin sensory C nerve fibers play a crucial role. SP, through binding with receptor NK-1, transfer the stress and inflammation signals and initiate inflammation responses. Through up-regulating NK-1 expression, CGRP could aggregate airway inflammation, which is signal pathway may be participated in by calmodulin , PKA, and TPK.

Key words: airway hyper-responsiveness, Substance P, NK-1 receptor, calcitotin gene-related peptide.

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M3-021P

Salivary flow, sodium renal excretion, urinary volume and arterial blood pressure induced by pilocarpine: influence of nitric oxide

W. A. Saad[1], G. Garcia[3], L. A. Camargo[3], I. F. Guarda[3], W. A. Saad[3], R. S. Guarda[2] and J. A. Rodrigues[3]

[1]Department of Physiology, Basic Institute of Neuroscience, University of Taubaté, Taubaté, São Paulo Brazil, [2]Department of Physiology, Exat and Natural Science, UNIARA, Araraquara, São Paulo Brazil, [3]Department of Physiology, Physiology and Pathology, UNESP, Araraquara, São Paulo Brazil.
E-mail: saad@foar.unesp.br

We investigated the effect of pilocarpine in the central nervous system in the physiological responses. Male Holtzman rats weighting 200–250 g were anesthetized with zoletil 50 mg/Kg into quadriceps muscle and a stainless steel cannula were implanted into their supraoptic nucleus (SON). We investigated the effects of the injection into the supra-optic nucleus (SON) of FK 409, a nitric oxide donor, and NW-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor (NOS), on the salivary secretion, arterial blood pressure (MAP), sodium excretion and urinary volume induced by pilocarpine which was injected into SO. FK 409 and L-NAME were injected at doses of 20μg/0.5μl and 40μg/0.5μl respectively. Injection of pilocarpine (10, 20, 40, 80, 160μg/0.5μl) produced a dose-dependent increase in salivary secretion. L-NAME produced an increase in salivary secretion due to the effect of pilocarpine. FK 409 attenuating the increase in salivary secretion induced by pilocarpine. MAP increase after injections of pilocarpine into the SON. L-NAME injected increased the MAP. FK 409 injected prior to pilocarpine attenuated the effect of pilocarpine on MAP. Pilocarpine (0.5μmol/0.5μl) injected into the SON induced an increase in sodium and urinary excretion. L-NAME injected prior to pilocarpine into the SON increased the urinary sodium excretion and urinary volume induced by pilocarpine. FK 409 injected prior to pilocarpine into the SON decreased the sodium excretion and urinary volume induced by pilocarpine. In summary the present results show: (i) SON is involved in pilocarpine-induced salivation; (ii) that mechanism involves increase in MAP, sodium excretion and urinary volume.

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M3-022P

Exploring the role of the histidine residues in the Alzheimer's disease Amyloid-beta peptide

D. G. Smith[1,3], G. G. Ciccotosto[1,3], C. C. Curtain[1,4], C. L. Masters[1,3], R. Cappai[1,2,3] and K. J. Barnham[1,3]

[1]Department of Pathology, The University of Melbourne, Parkville, VIC, Australia, [2]Centre for Neuroscience, The University of Melbourne, Parkville, VIC, Australia, [3]Mental Health Research Institute of Victoria, Parkville, VIC, Australia, [4]School of Physics and Material Engineering, Monash University, Clayton, VIC, Australia. E-mail: d.smith2@pgrad.unimelb.edu.au

Alzheimer's disease (AD) is a neurodegenerative disease that affects cognitive ability and memory. Amyloid β peptide (Aβ) is the cleavage product of the larger amyloid precursor protein (APP), and is the putative neurotoxic agent in AD. Progression of AD has been found to correlate with the concentration of soluble Aβ oligomers. The presence of the specific transition metals – Cu, Fe and Zn have been shown to be a principal component for the toxicity of these oligomers. Moreover, transition metal chelators have been successful in reducing AD symptoms in a phase 2 human clinical trial as well as reducing amyloid deposits in the brains of APP-transgenic mice. Nevertheless, the specific mechanism of toxicity of Aβ involving these transition metals is not clearly understood. The metal binding site of Aβ is located near the N-terminus and involves the imidazole sidechain nitrogens of histidines 6, 13 and 14, along with an undefined fourth ligand. These histidine ligands have also been found to be potentially involved in a bridging moiety and may play a pivotal role in lipid membrane interactions. In this study we have characterized three single histidine to alanine mutations (AβH6A, AβH13A and AβH14A) and probed the specific role of these residues in Aβ toxicity. By tracking the formation of soluble oligomers and oxidative products together with cell viability assays, we have found histidine 14 to be crucial for Aβ toxicity.

M3-023P

Human Tryptophanyl-tRNA synthetase-derived peptides are linked to fibril formation and neurodegeneration

O. S. Sokolova[1] and E. L. Paley[2]

[1]Bioprocess Inc., Moscow, Russian Federation, [2]Expert BioMed Inc., Miami, FL, United States of America.
E-mail: sokolova@bioprocess.ru

Tryptophanyl-tRNA synthetase (TrpRS) is a key enzyme of protein synthesis. TrpRS associated fibrils were found inside the blood vessels as well as in the intra- and extracellular manifestations of Alzheimer's brain. It was shown that tryptamine, a neuromodulator and inhibitor of TrpRS, induces the generation of neurofibrillary tangles (NFT) in human neuronal cells. The tangles contained paired helical filaments (PHF), similar to those detected in Alzheimer's brain. It was demonstrated earlier that the TrpRS is co-localized with the tau-protein, a known component of PHF/NFT. To identify which part of the TrpRS is responsible for fibril formation, we constructed three novel peptides: N-peptide corresponded to the NH2-, C-peptide – to the COOH and M-peptide – to the middle region of the TrpRS. The electron microscopical study of the negative-stained peptides demonstrated that the N-peptide, corresponded to the residues 32–50 of the full-length TrpRS, self-assembles into variety of fibrils with an average diameter of 100Å. Some fibrils exhibit a form of a pronounced helical twist, consisted of a double helix. The C-peptide (residues 414–437) forms few non-helical fibrils, whereas the M-peptide (residues 329–348) is not fibrillogenic at all. We also demonstrated that the N-peptide is highly cytotoxic; on the contrary, the C- and M-peptides stimulated the neuronal cells growth activity. It is

interesting to note, that the N-peptide belongs to the amino-terminal domain of the protein that is normally produced by cleavage/processing of the TrpRS. As a result, we proposed that the TrpRS is linked to neurodegeneration and fibril formation as a target for the tryptamine that induces PHF/NFT formation.

M3-024P
Effect of metals on beta-amyloid: conformation, aggregation and redox properties

K. Trzesniewska, M. Brzyska and D. Elbaum
Laboratory of Bio-Physical Methods, Nencki Institute of Experimental Biology, Polish Academy of Science, Warsaw, Poland. E-mail: kpergol@nencki.gov.pl

Beta amyloid, a 39–43 amino acids residue peptide derived from a larger transmembrane precursor protein (APP) is the major constituent of senile plaques found in brains of Alzheimer's disease patients. Metal ions were shown to play a major role in the processes involving aggregation of beta-amyloid and reactive oxygen species formation. Transformation of beta-amyloid into aggregates as well as oxidative damage has been postulated to be responsible for the peptide neurotoxicity. Our aim was to investigate the ability of various metals to induce alterations in the structure of beta-amyloid (1–40), and correlate the observed results with the status of the peptide molecule in solution and in the early phase of aggregation. We report here that metal ions complex formation with beta-amyloid induce hydrophobicity alterations, measured as bisANS fluorescence changes due to peptide-metal binding. The reactions are concentration and time dependent. The observations are consistent with conformational transitions caused by metal binding. It has been suggested that binding of redox active transition metals by beta-amyloid (especially copper) may have two opposite effects: generation of free radicals or preventing neurons from oxidative damage. In light of the above we investigated the relationship between stoichiometry of Cu(II) binding to beta-amyloid and oxidative properties of the formed complex. At stoichiometric concentration of Cu(II) to beta-amyloid we were unable to demonstrate the metal oxidative properties. However, complexes with higher Cu(II) to amyloid concentration ratios, possessed the redox abilities. Thus our results support pleiotropic behavior of amyloid in the presence of Cu(II).

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M3-025P
Protection against neurodegenerative effects of serum amyloid P component by glycosaminoglycans *in vitro*

Z. Urbányi[1], E. Forrai[2], M. Sárvári[1], I. Likó[1], J. Illés[2] and T. Pázmány[3]
[1]*Laboratory of Molecular Cell Biological Research, G. Richter Ltd, Budapest, Hungary,* [2]*Laboratory of Technological Development II., G. Richter Ltd, Budapest, Hungary,* [3]*G. Richter Ltd, Budapest, Hungary. E-mail: z.urbanyi@richter.hu*

Serum amyloid P component (SAP), a normal plasma glycoprotein, has been suggested to contribute to the progression of neurodegeneration. It binds to β-amyloid (Aβ) fibrils and protects the amyloid deposits against proteolytic degradation. Furthermore we previously demonstrated that SAP induces neuronal apoptosis *in vitro*. Here we show that glycosaminoglycans inhibited both the SAP – Aβ interaction and the neurotoxic effect of SAP. Interestingly different structure-activity relationship was revealed in the case of the two different effects. While the efficacy of the inhibition on the SAP-induced cell death increased with the uronic acid content, the inhibitory activity on the SAP–Aβ interaction decreased with the increasing uronic acid content of glycosaminoglycans. The inhibitory effects of glycosaminoglycans on the interaction between complement component C1q and Aβ showed a similar structure-activity relationship as on the SAP–Aβ interaction. This data suggested that glycosaminoglycans interfered with the binding site on Aβ for SAP and C1q. The functional consequence of the binding data was demonstrated by heparin, which inhibited SAP - Aβ binding and promoted the proteolysis of Aβ by pronase in the presence of SAP. Our results suggest that glycosaminoglycans may have therapeutical potential on the neurodegeneration inhibiting the harmful action of SAP and reducing its progress.

M3-026P
Serotenergic functions in alcoholism subtypes

G. Ucar[1], B. Demir[2], S. Yabanoglu[1] and B. Ulug[2]
[1]*Department of Biochemistry, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey,* [2]*Department of Psychiatry, Faculty of Medicine, Hacettepe University, Ankara, Turkey. E-mail: gulberk@hacettepe.edu.tr*

Alcoholism is associated with alterations in the serotonergic and mono-aminergic systems and serotonin (5-HT) is assumed to play a significant role in the pathophysiology of psychiatric diseases including alcoholism. Since 5-HT was implicated in the regulation of alcohol preference in humans and male alcoholics who start drinking early and exhibit early antisocial behavior was suggested to have a serotonergic defect, present study was undertaken to compare alcoholic subtypes (Type I versus Type II) with regard to platelet 5-HT content and the activity of monoamine oxidase (MAO), an enzyme which is responsible for the deamination of biogenic amines, in order to clarify the possible determinative role of serotonergic system in subtyping of alcoholics. Possible relationships between platelet 5-HT and MAO activity, personality traits and executive functions were also investigated. Seventeen Type I and 16 Type II, male chronic alcoholic patients and 17 healthy, male volunteers as controls were included in the study. The personality traits were investigated by the Minnesota Multiphasic Personality Inventory-2 (MMPI-2). Executive functions were assessed by the Wisconsin Card Sorting Test (WCST). Plasma and platelet MAO activities and 5-HT levels were determined by spectrophotometric and HPLC methods. When compared to the healthy subjects, platelet MAO activity was reduced and platelet serotonin content was increased whereas plasma serotonin content was reduced in both alcoholic groups. Platelet MAO activity and plasma serotonin level of the Type II group were significantly lower and platelet 5-HT content was significantly higher than those of Type I patients. Both groups of alcoholic patients also displayed impairment in executive functions. The comparison of the MMPI-2 scores of the study groups revealed that Type II alcoholics had more severe psychopathology. The results of this study suggest that platelet 5-HT content and MAO activity are useful biochemical measures for the subtyping of alcoholics.

M3-027P
Interaction of some pyrazoline derivatives with tissue semicarbazide sensitive amine oxidase (SSAO)

S. Yabanoglu[1], N. Gokhan[2], G. Ucar[1] and A. Yesilada[3]
[1]*Department of Biochemistry, Hacettepe University, Ankara, Turkey,* [2]*Department of Pharmaceutical Chemistry, Hacettepe University, Ankara, Turkey,* [3]*Department of Basic Pharmaceutical Sciences, Hacettepe University, Ankara, Turkey. E-mail: syabanoglu@yahoo.com*

On the basis of our previous studies, which have been shown that some pyrazoline derivatives containing a thienyl ring had potent

monoamine oxidase (MAO) inhibitory activities, biological interactions of twelve newly synthesized 1-N-substituted thiocarbamoyl-3-phenyl-5-thienyl-2-pyrazoline derivatives (3a–3l) with rat liver semicarbazide sensitive amine oxidase (SSAO) were assessed. Compounds 3a, 3b, 3d–3h which were previously reported to be non-selective MAO inhibitors, were found to be potent SSAO substrates. The compounds 3i–3l which were previously reported to be selective MAO-B inhibitors, also showed selectivity towards SSAO whereas the others were appeared as more selective for MAO-B. The inhibition of SSAO with these compounds were found to be non-competitive and irreversible. Compound 3i, which carries an electron rich substituent such as methoxy group showed the highest inhibitory potency towards SSAO. Data showed that novel SSAO inhibitors may be used to discriminate between Cu-containing (SSAO) and FAD- containing (MAO) amine oxidases as well as to determine the possible roles of SSAO in physiological events and also in some SSAO-related disorders.

M3-028P
Nociceptive activity of melanocyte stimulating hormones (MSH) and HS014 in the formalin and tail flick tests

L. Zvejniece[1], M. Dambrova[2], V. Z. Klusa[1] and R. Muceniece[1]
[1]Faculty of Medicine, University of Latvia, Riga, Latvia, [2]Laboratory of Pharmaceutical pharmacology, Latvian Institute of Organic Synthesis, Riga, Latvia. E-mail: liga@biomed.lu.lv

The aim of the present study was to investigate the nociceptive effect of MC peptides in the two animal pain models such as formalin induced acute long-lasting pain evoked-behaviour model and tail flick test in the mice. Male ICR mice received α, β, γ1 and γ2 MSH and MC3/4 receptor antagonist HS014 at doses 20 nmol/mouse s.c. or indomethacin (5 mg/kg) like reference drug by i.p. injection. In the tail flick test we tested the same dose for MSHs whereas HS014 we administrated at doses 10 and 20 nmol/mouse and indomethacin (5 and 20 mg/kg). Antinociception we observed after 15, 30 and 60 min. Results show that the s.c. administration of all tested MCs (except γ1-MSH) and HS014 in the formalin test caused a decrease in the nociceptive threshold. Interestingly, the effect of α-MSH was present in both phases although apparently more pronounced it was in the second phase (like indomethacin). In the same time, in the tail flick test α-MSH showed algesic activity but HS014 and indomethacin analgesic activity. Other peptides after s.c. administration did not show statistically significant nociceptive activity in the tail flick test. The present studies support the role of MCs in the pain and inflammation control, and demonstrate the possibility of MCs peptides to act after peripheral administration. Comparing the effects of peptides after s.c. administration in the two pain models we suggest that anti-inflammatory activity of the MC receptor agonists prevails their nociceptive effects, and that balance between agonistic and antagonistic activities determines shift from algetic to analgetic status. In summary, the main finding is that targeting of the MCs receptors could generate a novel treatment for the non-specific inflammation including chronic neuralgic pain.