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ABSTRACTS

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Is phosphorylation signaling the missing link to explain anti-tumoral activities in antimicrobial peptides (AMPs)? Insights from Lunatins, a peptide family from *Hadruroides lunatus* scorpion venom

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We have previously identified a family of peptides in the venom of the Peruvian scorpion *Hadruroides lunatus*, named Lunatin 1–3. Their primary structures range from 13 to 18 amino acids and show some similarities with bacterial enzymes and other proteins able to bind phosphate groups (e.g., kinases, phosphatases, active transporters). In addition to their observed antimicrobial activity (e.g., Lunatin 1 has MIC values ranging from 1.49 to 5.95 μM against some Gram-positive bacteria), Lunatin 1 also showed an important anti-tumor activity against a variety of tumor cell lines, notably HL60 and MCF7. Lunatin 2 is a potent inhibitor of YopH—a protein tyrosine phosphatase (PTP) from the pathogenic bacteria *Yersinia enterocolitica*—with a calculated IC50 value of 1.37 μM and a 12-fold selectivity in favor of YopH against PTP-PEST, LYP and PTP1B, which are human PTPs. Lunatin 2 was identified as mixed inhibitor of YopH, with Ki value of 1.41 μM and Ki′ of 6.16 μM. Lunatin 1 and Lunatin 2 had their structures determined by NMR spectroscopy and show interesting features that differ from those usually observed for other antimicrobial peptides. They have an alpha helical region that spans six to eight amino acid residues at their N-terminal portions. However, they are unstructured at their C-terminal portions. Synthetic analogs were designed and their structural and functional features were also assessed envisaging further structure–activity relationship studies. The results suggest the discovery of a new class of potent peptide inhibitors (ca 1 kDa) of enzymes that recognize protein phosphates, which may provide a starting point for further development of new inhibitors for these enzymes.

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Effects of TsHpt-I (*Tityus serrulatus* Hypotensin-I)-derived peptides on sponge- induced fibrovascular tissue in mice

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We have previously described a 25mer anti-hypertensive peptide, named TsHpt-I (*Tityus serrulatus* Hypotensin-I), isolated from the Brazilian yellow scorpion (*Tityus serrulatus*) venom. TsHpt-I displayed two different modes of action: one being a bradikinin (BK)-dependent potentiation activity andthe other a BK-independent activity, which was further detailed as an agonist binding to the B2 kinin receptor (B2R) that culminates in nitric oxide (NO) release. By using a structural minimization strategy of synthetic peptides, we identified the C-terminal region of TsHpt-I as being responsible for the described anti-hypertensive effects. We have also highlighted the key amino acids responsible for each of these actions. It is worth mentioning that both B2R activation and NO release are known to be involved in angiogenic and inflammatory processes. In the present study, we investigated whether TsHpt-I-derived peptides could modulate key events (inflammatory cell recruitment, neovascularization and extracellular matrix deposition) of the proliferative fibrovascular tissue induced by polyether-polyurethane sponge implants in mice. Sponges were implanted in the dorsum of 8-week-old C57Bl/6 male mice that received daily intrasponge treatment with either TsHpt-I[17–25] (10 nmol/sponge/day), dipeptide Lys-Pro (10 nmol/sponge/day) or PBS (10 μl/sponge/day) and were assessed on day 7 after surgery. Hemoglobin content (μg/mg wet tissue) and blood flow measurements (laser Doppler perfusion imaging) in the implants, used as indices of vascularization, indicated that the peptides stimulated angiogenesis in the implants relative to the PBS-treated group. Interestingly, the peptides inhibited neutrophil, although stimulated macrophage infiltration, into the implants, as determined by myeloperoxidase (MPO) and *N*-acetyl-*β*-D-glucosaminidase (NAG) activities, respectively. Regarding the fibrogenic component (evaluated by soluble collagen content), we observed that TsHpt-I[17–25] inhibited, whereas the dipeptide Lys-Pro stimulated, collagen deposition in the implants. Overall, our results suggest that TsHpt-I[17–25] and the dipeptide Lys-Pro have pro-angiogenic and anti-inflammatory activities. These effects may indicate the therapeutical potential of these peptides in conditions where inflammation and angiogenesis are associated, such as wound healing and inflammatory diseases.

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Identification and characterization of peptides and proteins from *Tityus serrulatus* venom by mass spectrometry

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Scorpion stings are considered the most common accidents involving venomous animals in Brazil, and *Tityus serrulatus* is responsible for the highest number of these accidents. *T. serrulatus* venom (Tsv) presents a great variety of molecules, such as enzymes (proteinases and hyaluronidases), nucleotides, biogenic amines, oligopeptides, protease inhibitors, histamine releasers, several low molecular mass proteins with neurotoxic activity, and other organic components. In this work, Tsv was fractionated through two different approaches. The reversed-phase fast protein liquid chromatography (RP-FPLC) of Tsv on a C18 column resulted in 67 fractions, whereas 18 fractions were obtained by CM-Cellulose-52 chromatography. Each fraction was

analyzed by a combination of mass spectrometry (matrix-assisted laser desorption ionization–time-of-flight) and N-terminal sequencing by Edman degradation. These analyses allowed the detection of toxins from the venom already described in the literature and the identification of new components, revealing the complexity of its composition. The comparison of the chromatographic methods showed that the isolation of Tsv components was much more effective by RP-FPLC, although this procedure promotes the inactivation of metalloproteinases. Additionally, it was possible to identify some toxins (e.g., Ts1, Ts3, Ts19) in different phases of maturation, indicating that post-translational modifications are relevant in this venom. The results highlight the diversity of the *T. serrulatus* venom, which contains numerous components not studied yet, and many of them may present biotechnological potential, since Tsv is a rich source of molecules acting on ion channels.

Keywords: *Tityus serrulatus*, Scorpion venom, Proteome, Neurotoxin, Mass spectrometry, Venomic.

Proteome of *Crotalus durissus collilineatus* venoms: analysis of individual variations

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Snake venoms present a wide variety of compounds, mostly, protein toxins. The composition of the snake venoms may present a high degree of variability, which influences the venom toxicity. In Brazil, snakes of the genus *Crotalus*, represented by the species *Crotalus durissus*, are able to cause severe envenoming. These snakes inhabit various regions of the country and their venoms can present variability, which can reduce the effectiveness of antivenom therapy. It is important to know the composition of the venom and its variability in a single species to optimize the production of antivenom serum. Therefore, this study purposes to perform a comparative proteomic analysis of the venom from 22 specimens of *Crotalus durissus collilineatus* (Cdc), seeking to identify the individual variations of these venoms. The reversed-phase fast protein liquid chromatography (RP-FPLC) on a C-18 column of the Cdc venom resulted in 25 peaks, which were analysed by electrophoresis (SDS-PAGE) and mass spectrometry (MALDI-TOF/TOF). These venoms from Cdc ($n = 22$) showed a similar chromatographic profile; however, several components presented significant quantitative differences (e.g. LAAO). Interestingly, crotamine was identified in only one of the venoms, representing one of its main components. Additionally, several known components were identified during mass spectrometry analysis such as crotoxin and serine proteinases gyroxin-like. Further, we identified ions with different masses that have not been reported. Our results support that the current immunization protocols need to be revised to produce an improved anti-crotalic serum.

Keywords: *Crotalus durissus collilineatus*, Snake venoms, Proteome, Venomic, Individual variations, Crotamine.

Inhibition of xanthine oxidase by alkyl phenol compounds

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Abstract

Backgrounds: Xanthine oxidase (EC 1.1.3.22) is formed from xanthine dehydrogenase (EC 1.1.1.204) under oxidative conditions. The structure of xanthine dehydrogenase and the oxidase was analyzed by X-ray crystallographic method. Each enzyme contains 1 molybdenum–pterin, 2 iron–sulfurs and 1 FAD. In the case of xanthine oxidase, as enzyme catalyzes the reaction of two substrates, xanthine and oxygen, the reaction progresses as a ping-pong mechanism. At first, we noticed that phenol compounds did not affect xanthine oxidase activity, but some alkyl phenol compounds inhibited it. To understand the reasons, we choose alkyl phenol compounds in natural products as models and examine their inhibitory property.

Methods: Alkyl phenol model compounds were isolated from natural sources. The other related compounds were chemically synthesized. Xanthine oxidase reaction of xanthine with oxygen, which was present in the reaction mixture, was examined in the presence of these compounds. The effect of these compounds on xanthine oxidase reaction rates was analyzed. The scavenging activity of these compounds was also examined.

Results: Anacardic acids (2-hydroxy-6-alkenyl benzoic acids) sigmoidally inhibited uric acid and superoxide anion generation, but salicylic acid did not. Gallic acid and alkyl gallates strongly inhibit superoxide anion generation, but did not scavenge superoxide anon. Gallic acid and alkyl gallates having short chain (<C6) did not inhibit uric acid formation, but alkyl gallates having long chain (C6≤) did. Caffeic acid (3,4-dihydroxycinnamic acid) and alkyl caffeates strongly inhibited superoxide anion generation, and the inhibition of uric acid formation by alkyl caffeates increased by increasing the alky chain length. Cardols (5-alkenyl resorcinol) did not inhibit uric acid formation, but cardols having alkyl chains (C10≤) sigmoidally inhibited superoxide anion generation.

Conclusions: Alkyl phenol inhibitors are divided into three kinds of groups. The first group consists of uric acid formation inhibitors, the second consists of superoxide anion generation inhibitors and the third consists of reducing agents of the enzyme molecule. The enzyme-reducing ability was estimated using the DPPH radical scavenging activity and the activity was attributed to conjugated endiol structures in inhibitors. Though xanthine oxidase was reduced when xanthine was oxidized to uric acid, we deuced that xanthine oxidase was further reduced by the reducing activity of inhibitors and superoxide anion generation was suppressed. As cardols did not reduce xanthine oxidase molecule and inhibited superoxide anion generation, cardols were the specific inhibitors and classified into the second group.

Property of angiotensin-I converting enzyme inhibitor in Japanese burdock root

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Abstract

Backgrounds: The root of the Japanese burdock plant, *Artium lappa*, is edible in Korea and Japan. The root contains a high amount of dietary fibers, with low amounts of fat and protein with the rest comprising water, and is a low calorie food. It was also reported that

burdock root extract inhibited angiotensin I-converting enzyme (ACE) [EC 3.4.15.1], but the structure of the active compound was not clear.
Methods: ACE Activity was assayed by a modified method of Cushman and Cheung (1971). Japanese burdock roots were extracted with aqueous methanol solution. The active compound was purified by using the extraction method and column chromatography and was analyzed. The active compound in the related products was also examined.
Results: The active compound is not extracted with ethyl acetate and *n*-butanol, but with water. The purified compound is an amino acid and is identified as nicotianamine (NA), (2*S*, 3′*S*,3″*S*)-*N*-[*N*-(3-amino-3-carboxypropyl)-3-amino-3-carboxypropyl]azetidine-2-carboxylic acid. Kinetic analysis of purified NA exhibited a mixed-type inhibition, and the kinetic parameters were determined as Ki = 0.40 and Kis = 1.56 μM. The content was the highest in dry burdock root and was found to mildly decrease by roasting, frying or fermentation.
Conclusions: The active compound was identified as NA. The contents of NA in burdock roots and related food products were determined and compared to soy beans. Data revealed that burdock root is a good source of NA.

Characterization of D-amino acid dehydrogenase in a deep-sea piezophilic bacterium, *Shewanella violacea* DSS12

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Shewanella violacea DSS12 (*S. violacea*) is a psychrophilic and facultatively piezophilic bacterium which was isolated from the mud of the Ryukyu Trench. D-Amino acid dehydrogenase (DAD) is a membrane-bound enzyme that catalyzes dehydrogenation of D-amino acids without oxygen. We have detected an activity of DAD in *S. violacea.* In this study, we investigated the substrate specificity, induction by amino acids and effect of pressure of DAD of *S. violacea*, and an electron transfer from the enzyme to cytochromes.
S. violacea was cultivated in Marine Broth 2216 under aerobic or microaerobic condition. The harvested cells were disrupted by ultrasonication and centrifuged to yield a cell-free extract. DAD activity was spectrophotometrically measured with 2,6-dichlorophenolindophenol as an electron acceptor. In both culture conditions, the cell-free extracts highly exhibited DAD activity against D-Pro and D-Ser. When D-Ser was added to the medium in the aerobic condition, the growth of *S. violacea* was inhibited, and DAD activity was inducted to a certain level. DAD was assayed under various pressures (0.1–50 MPa) using D-Pro and D-Ser as substrates. DAD activity was retained (30–50 %) under 50 MPa, indicating that DAD from *S. violacea* is more tolerant to high pressure than DAD from organisms in atmospheric pressure habitats.
Addition of D-Pro into the cell-free extract caused a spectral change of cytochromes indicating that an electron withdrawn from the amino-acid substrate was transferred to cytochromes. This observation suggests that DAD of *S. violacea* plays a role in ATP production.

The effect of lipoic acid and its reduced form on the activity of aldehyde dehydrogenase and glycerylaldehyde-3-phosphate dehydrogenase

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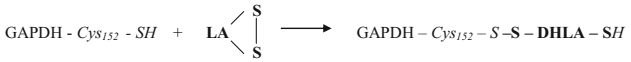
Introduction: Aldehyde dehydrogenases (ALDHs) are a group of enzymes that catalyse irreversible oxidation of a wide spectrum of aldehydes to the corresponding carboxylic acids in the presence of $NAD(P)^+$ and water. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyses the oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3-bis-phosphoglycerate using NAD^+ as a cofactor.
The aim of this study was investigate the effect of lipoic acid and dihydrolipoic acid on the activity of ALDH and GAPDH in vitro.
Materials and methods: The studies were performed using yeast ALDH and GAPDH from rabbit muscle. The activity of both enzymes was monitored by following the increase in absorbance at 340 nm due to NADH formation during propionaldehyde and glyceraldehyde-3-phosphate dehydrogenation, respectively.
Results and conclusions: The present study showed that LA, like DHLA, did not change ALDH activity, but inhibited GAPDH activity by approximately 50 % compared to the LA-free control.
It can be hypothesized that LA causes S-lipoilation of GAPDH according to the following scheme:



Simultaneously, the results suggest that ALDH is not modified by the analogical S-lipoilation process, which was manifested as the lack of any effect of LA on the ALDH activity.
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Murine carnosine synthase—heterologous expression, enzyme assay and expression pattern

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Carnosine is a dipeptide consisting of β-alanine and L-histidine and was first isolated in 1900. High concentrations of carnosine were detected in skeletal muscle and the primary olfactory pathway of mammals. The physiological role of carnosine is not clear, although a number of functions have been proposed. It may act as a buffer substance, anti-oxidant, or a natural protein glycation inhibitor. Carnosine and homocarnosine, a related dipeptide consisting of GABA and L-histidine, are synthesized by the ATP-dependent carnosine synthase, which belongs to the ATP grasp domain protein family and has recently been identified at the molecular level. Compared to other dipeptide synthases, this enzyme contains a large N-terminal domain of unknown function.

To further characterize the carnosine synthase, N- and C-terminal parts of the murine enzyme were expressed separately in *E.coli* to generate antibodies and the full-length enzyme was expressed in insect cells with baculovirus system to affinity purify the antibodies against the native protein. In addition, a nonradioactive, colorimetric enzyme assay was established with full-length carnosine synthase from insect cells, and Vmax and Km values were determined for ATP, β-alanine and L-histidine.

Western blot analyses of murine tissues showed the expression of carnosine synthase in skeletal muscle and at high levels in the primary olfactory pathway, according to the occurrence of the dipeptide, which could be confirmed by thin-layer chromatography. Moreover, age-dependent Western blot and TLC analyses of murine tissues showed an increase of carnosine synthase expression in olfactory bulb along with increasing carnosine content. Furthermore, preliminary results of in situ hybridization and immunofluorescence microscopy indicated the expression of carnosine synthase in receptor neurons of olfactory epithelium, but not in the olfactory bulb, suggesting that the enzyme reaches the olfactory bulb by axonal transport, since the axons of olfactory receptor neurons terminate in the glomerular layer of the olfactory bulb.

Insight into substrate-specific recognition and heat resistance of *N*α-acetyltransferase SsArd1

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*N*α-Acetyltransferases (Nats) possess a wide range of important biological functions. The structure of Nats can vary according to the first two residues of their substrate. However, the mechanisms of substrate recognition of Nats are elusive. SsArd1 from thermophilic Achaea *Sulfolobus solfataricus*, belonging to the NatA family with preference of Ser residues, exhibits the greatest activity of acetylation at optimal temperature of 65 °C. The crystal structure of SsArd1 in complex with the peptide substrate was determined to be 1.84 Å. Structural comparison of SsArd1 with human Naa50p (NatE) reveals significant differences in key residues of enzymes near the first amino acid position of the substrate peptide (Glu35 for SsArd1 and Val29 for Naa50p). The biochemical data revealed that the substrate specificity of SsArd1 could alter the substrate of NatE by a range of Glu35 mutants.

Additionally, crystal structures of SsArd1 in different space group indicated the loop region between β3 and β4 to contain multiple conformations and forming hydrogen bond networks via two Ser residues. Compared with wild-type SsArd1, the variants substituted with Ala (S75A, S82A and S75/S82A) and with loop deletion had almost identical folds. Strikingly, two single-point mutants showed $\sim$3 °C decrease in melting temperature, while two other variants showed even $\sim$7 °C decrease in melting temperature, which correlated to the seriously reducing enzymatic activity. Taken together, the crystallographic studies combining spectroscopic and biochemical characterizations provide a detailed molecular basis for not only understanding the substrate-specific recognition, but also elucidating the mechanism of heat resistance of the ancient archaeal SsArd1.

Small-molecule FRET reporters for real-time visualization of cell-surface proteolytic enzyme functions

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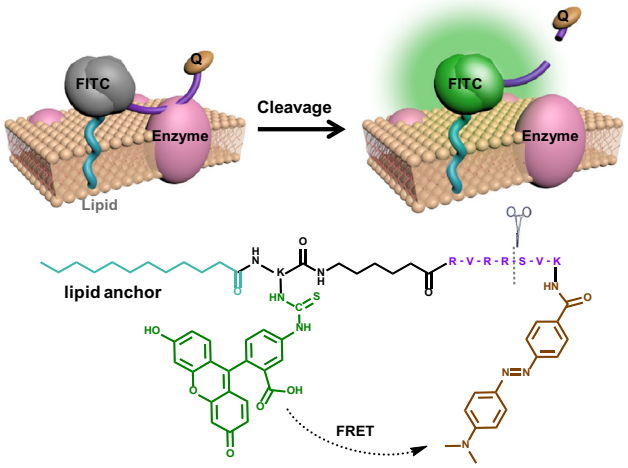
Abstract: Cell surface proteolytic enzymes play critical roles in physiological and pathological processes ranging from extracellular matrix processing, growth factors and receptors' activation to microbial invasion, etc. [1]. These proteolytic events have not only been implicated in the site-specific cleavage of bioactive proteins or peptide substrates within trans-membrane domains, thus performing various biological functions, they are also involved in the progression of various degenerative diseases including cancer, atherosclerosis and neurological disorder [1]. Therefore, diagnostic targeting and regulating of proteolysis becomes one promising approach for understanding the basic pathological pathways and even for the treatment of cancer and other diseases [2].

Furin, a membrane localized proteolytic processing enzyme that belongs to the proprotein convertase (PC) family, is ubiquitously expressed and functions within secretory and endocytic pathways and at the cell surface. Normally, these furin-like convertases can activate a variety of protein precursors in intracellular membrane and cell surface systems, and process them into biologically functional peptides and proteins [3]. For example, furin has been well known to be involved in the intra-membrane processing of several kinds of matrix metalloproteinases (MMPs), which were found to be elevated in several types of human cancers [4]. The activation of α- and β-secretases which are two key enzymes in the processing of toxic amyloidal peptides' generation during the development of Alzheimer's disease (AD) was also mediated by the proteolytic activities of furin [5]. Moreover, apart from the activities contributed to numerous chronic pathological conditions, cell-surface associated furin or furin-like proprotein processing was also highly relevant to the maturation of bacterial toxins and the propagation of many non-enveloped or lipid-enveloped viral pathogens, which are prerequisite processes to mediate bacterial or viral invasion into host cells [6]. In terms of such multiple roles of furin in human pathophysiology, effective strategies to real-time visualize cell-surface associated furin activities will be therefore rather important, which will pave the way for elucidating cell membrane functions and understanding furin-dependent dynamic processes in living cells, thus making them as potential targets for the development of new therapeutic agents. Recently, researchers have introduced fluorescent bio-reporters such as green fluorescence protein (GFP) labeling or genetically encoded fluorescent protein variants to image the extracellular furin activities [7]. Similar non-invasive investigation of cell-surface proteolytic furin activity was also achieved in single cells based on the reengineered anthrax toxin-β-lactamase protein mutants [7c]. Although all these approaches were successful in principle, these genetic manipulations may potentially perturb the cell's physiology and cause unexpected biological responses [7]. As contrast, small-molecule-based probing technology with desirable properties (e.g. easy preparation and manipulation) may offer an attractive alternative to compensate the shortcomings of genetic manipulation [8]. Unfortunately, because of the rapid diffusion and poor immobilizing ability on the cell surface, most of the

existing small-molecule fluorescent reporters for furin-like assays (e.g. Boc-RVRR-AMC etc.) can be mainly applied in fixed cells or cell lysates systems [9]. Rational design of simple and reliable small-molecule probes with unique tractability and immobilizing properties for real-time imaging of specific cell-membrane associated furin activity in living cells or tissues is highly of interest and significance, and to the best of our knowledge, such highly specific and activatable cell surface anchored reporters have not been well exploited yet.

Herein, we present a simple and small-molecule-based reporter to effectively visualize furin-like enzyme activities on the cell surface. This well designed membrane-anchored and furin-responsive probe (MFP) molecule could efficiently confine the probe into the lipid compartments of cell membranes, thereby increasing the effective probe–enzyme interactions. Moreover, such unique reporter may also provide great opportunity to real-time visualization of specific extracellular proteolytic enzyme activities in living cells and tissues through both one-photon and two-photon imaging facilities with minimum autofluorescence and cytotoxic side effect.



Scheme 1 Illustration of the structure of membrane-anchored and furin-responsive probe (MFP) and surface associated furin cleavage on the cell membranes

Scheme 1 illustrates the small molecule reporter toward imaging the process of specific furin cleavage on cell membrane. Basically, the consensus peptide sequences for preferential furin cleavage, (K/R)–$(X)_n$–(K/R)↓, were chosen as our basic moieties (where n = 0, 2, 4 or 6 and X is any amino acid, respectively) [3a,10], which were further integrated with the principles of passive exogenous insertion and Fŏrster resonance energy transfer (FRET) for the purpose of specific imaging of furin activity [11]. In our study, the defined peptide sequence of RXRR was selected as the main binding pocket, which was subsequently conjugated with small hydrophilic residues (e.g. serine) and hydrophobic residues (e.g. valine) at C-terminus, respectively, to achieve enhanced enzyme activatable properties [10a]. To conduct real-time cell imaging, the enzyme responsive RVRRSVK peptide was flanked with activatable FRET pair consisting of fluorescein (FITC) and an effective FITC quencher Dabcyl, attributed to their spectral overlap. The introduction of FITC through a 6-aminohexanoic acid linker was mainly due to its optimal fluorescence quantum yield at the physiological environment and its promising two-photon properties [12]. More importantly, to immobilize the enzyme responsive fluorescent probe onto cell surface [13], the fatty acid lipid moieties with different length of carbon chains were carefully screened which were further conjugated with furin-responsive peptide probe. The optimized lipid moiety could serve as an efficient targeting vector to anchor the fluorescent probe to the cell surface, mostly to the lipid compartments in the cell membranes, to enhance the specific enzyme interaction.

After obtaining MFP probe, we first examined the enzyme hydrolysis of the developed MFP probe by measuring the changes in fluorescence emission upon the addition of furin in HEPES buffer. As shown in Figure 1A, the MFP probe itself was almost non-fluorescent due to efficient FRET quenching. After incubation with enzyme for 2 h, intense fluorescent enhancement (~12-folds) was observed at a maximum wavelength of 525 nm, corresponding to the connected FITC. Similar enzyme hydrolysis with standard inhibitor (Dec-RVKR-cmk) demonstrated limited fluorescence enhancement after 2 h incubation [14], suggesting that the specific furin interaction would split FRET pair by releasing the quencher Dabcyl. Further analysis of hydrolysis kinetics of MFP probe by furin in HEPES buffer revealed the catalytic constants, $K_{cat} = 2.78 \pm 0.51\ s^{-1}$, and Michaelis constants, $K_m = 25.5 \pm 2.1\ \mu M$. The enzyme catalytic efficiency (K_{cat}/K_m) for furin was $1.09 \times 10^5\ M^{-1}\ s^{-1}$. Moreover, the enzyme cleavage effect was examined by HPLC analysis (Figure 1B). A new peak with retention time of 10 min was observed to increase after incubation with furin for 60 min, which was the enzyme cleavage fragment (S-V-K (Dabcyl)) confirmed by LC–MS analysis (Figure 1C). The retention time of enzymatic reaction mixtures incubated with furin inhibitor was the same as that of MFP probe alone, indicating that the developed MFP probe could be specifically cleaved by furin. This was consistent with the results observed in fluorescent measurement.

With the desirable enzyme-responsive properties in hand, we tested the applicability of MFP toward furin-dependent cell surface imaging. In this study, human neuronal glioblastoma U251 cells were selected as our target mainly due to the expression of high levels of furin [15]. Meanwhile, the furin-deficient Lovo cells were chosen as negative control [16]. Typically, U251 and Lovo cells were incubated with MFP probe for 15 min. As contrast, cell membranes were also tracked with commercially available CellMask Deep Red to monitor the status of membrane staining [8d]. As shown in Figure 2, after 15 min incubation, the strong green fluorescence on the U251 cells surface was observed. Moreover, the time-lapse imaging further confirmed the dynamic enzymatic processes on the cell surface, and strong membrane staining could be still detected even after the prolonged incubation to 1.5 h. As control, there was no obvious fluorescence detected in the same cells upon the treatment with furin inhibitor (Dec-RVKR-cmk) [14] or in the negatively controlled furin-deficient Lovo cells even under high concentration probe incubation (Figure 2). Such promising imaging results demonstrated that MFP probe could work as an effective tracer to report the cell-surface associated furin activity in living cells.

To further validate the varying levels of furin expression between U251 and Lovo cells are responsible for the observed fluorescence on the cell surface, the cell lysate-based furin assay was conducted by incubation of MFP probe with lysated cell samples. In this study, we keep the same level of the total protein in cell lysates using the standard Bradford method [17]. As shown in Figure 3, there was substantially low fluorescence detected in furin-deficient Lovo cells, whereas significant fluorescence enhancement was observed in furin

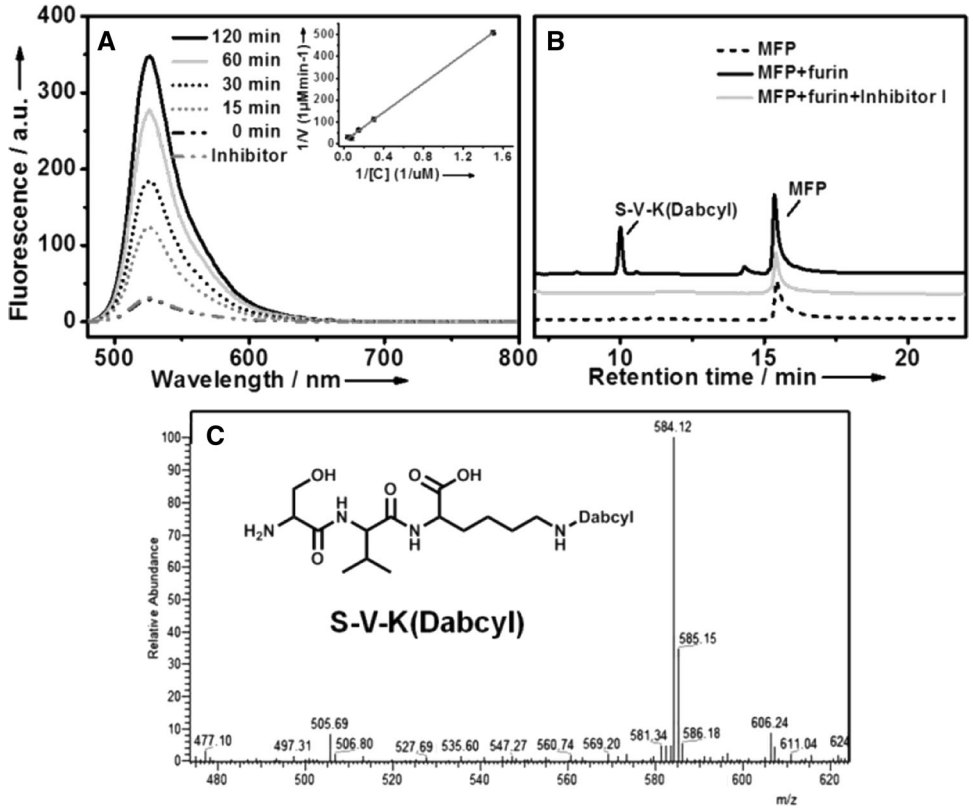


Fig. 1 **a** Fluorescence enhancement of MFP (6.5 μM, in HEPES buffer, pH 7.4) when treated with furin with or without inhibitor Dec-RVKR-cmk at different times (0–120 min, $\lambda_{ex} = 450$ nm) at 37 °C. Dec-RVKR-cmk (100 μM) was preincubated with enzyme for 30 min prior to addition of probe. *Inset* the enzymatic kinetics of furin (2U) with MFP. **b** HPLC analysis of MFP alone, MFP with furin or MFP incubated with inhibitor before addition of furin for 60 min. **c** The LC-Mass spectroscopy of the cleavage product S-V-K (Dabcyl)

expressed U251 cell lysates over all the incubation time. Moreover, the furin cleavage in U251 cell lysates was significantly suppressed with the addition of furin inhibitor, suggesting that the developed MFP could serve as reliable tracer to quantitatively evaluate specific furin activities in complex environment (Figure 3A). Similar enzyme cleavage was also confirmed by HPLC and standard fluorescent furin substrate Boc-RVRR-AMC [9]. To further verify the specific expression level of furin enzyme in different cells, both cell lysated samples were analyzed by immunoblotting with anti-furin antibodies. For quantitative analysis, the expression levels of furin were normalized to internal control of β-tubulin. The chemiluminescence signals in Figure 3B confirmed the highly expressed furin in U251 cells, whereas the same enzyme expression was greatly reduced in Lovo cells [15]. The different levels of furin expression in U251 and Lovo cells verified by immunoblotting further confirmed that our developed MFP probe could be used to reliably evaluate furin activities in different cells. Finally, we also evaluated the potential cytotoxicity of MFP probe using standard MTT assay. There was no obvious cytotoxicity of MFP probe to both U251 and Lovo cells even after long-time incubation, suggesting that MFP probe could supply the possibility for real-time visualization of furin activities without affecting physiological processes.

One significant feature of our developed MFP probe was its great feasibility to specifically stain the cell membranes when upon the specific reaction with surface associated furin enzyme. To confirm if the hydrophobic lipid anchor could facilitate the membrane insertion, the similar fluorescent probe (FP) but without fatty acid moiety was also prepared for live cell imaging. Although enzymatic hydrolysis result clearly indicated that FP could be cleaved by furin in HEPES buffer, the cell imaging study based on FP alone revealed a poor membrane staining when compared to results observed using MFP. This result clearly showed that the lipid anchor would be essential for the localization of MFP probe on the cell surface. To further screen the possibility of chain length in lipid moiety to affect cell membrane staining, the similar MFP peptide sequence but no Dabcyl quencher connection was used to conjugate with different fatty acids (e.g. Caproic acid, Lauric acid and Stearic acid, respectively). The as-prepared FITC-peptide fatty acid conjugates, abbreviated as C6-F, C12-F (same carbon chain with MFP) and C18-F, respectively, were incubated with HEK 293 cells for different time intervals. As shown in Figure 4, there was almost no fluorescence observed in C6-F incubated HEK 293 cells compared to cells treated with C12-F and C18-F. The results confirmed that the increased length of carbon chain could indeed enhance the immobilization of the probe onto cell surface.

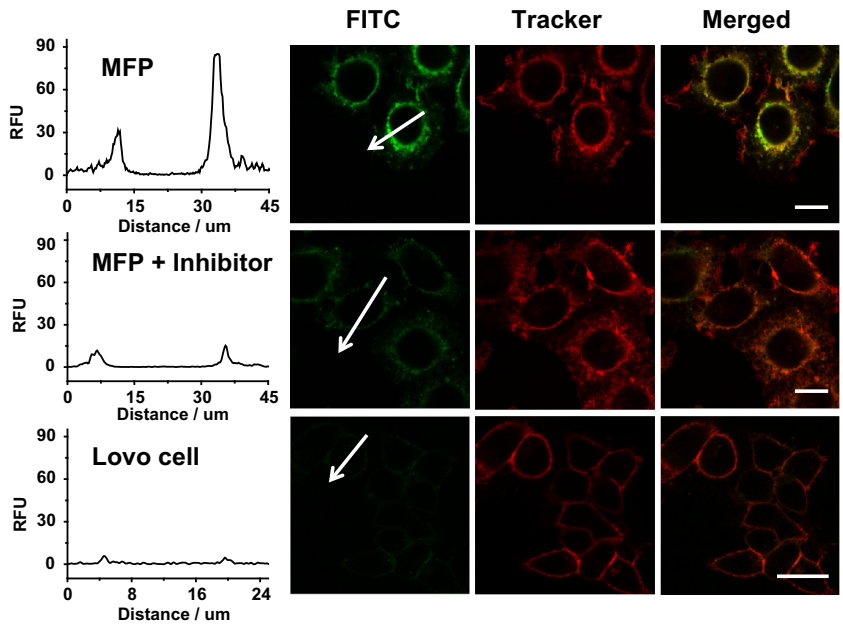


Fig. 2 Fluorescence imaging of U251 cells by MFP. Cells were incubated with MFP alone (130 nM), MFP and inhibitor (40 μM) for 15 min at 37 °C. CellMask *Deep Red* (2.5 μg/mL) were was used as standard to image the membrane. Fluorescence intensity was plotted as a function of distance along with *arrows*. Lovo cells incubated with MFP as control. (*Scale bar* 20 μm)

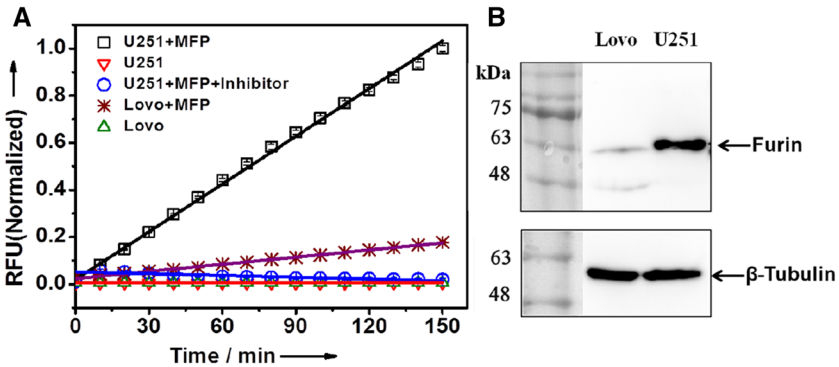


Fig. 3 **a** Fluorescence signals of MFP (10 μM) for quantifying furin activity in U251 and Lovo cell lysates (10 μg total protein was incubated with MFP at 37 °C). The inhibitor Dec-RVKR-cmk (100 μM) was added in U251 cell lysates for 30 min prior to the probe addition; **b** analysis of furin expression by western blot in U251 and Lovo cells and *β*-tubulin was included as internal control

Unlike the status of C12-F, although the C18-F conjugate indeed indicated enhanced tracking ability on cell membrane, the obvious internalization into lysosome would be hard to avoid after prolonged incubation (e.g. 2 h) (Figure 4). Therefore, the MFP with dodecanoyl moiety would be the ideal tracer to specifically localize the probe onto cell membrane, and thus significantly facilitate long-time trackable visualization of surface associated furin in living cells. Such long-time membrane anchoring would provide great potentials to monitor dynamic processes of cell-surface enzyme activities, which is normally difficult for most of existing cell permeable small-molecule probes.

In terms of two-photon characteristic of FITC [12,18], we also investigated the possibility of two-photon imaging based on the specific interactions between the developed MFP probe and cell-surface associated furin enzyme in living cells or tissues. As proof of concept, U251 cells were incubated with MFP probe (1.0 μM) at 37 °C for 30 min, and two-photon imaging was carried out upon the excitation at wavelength of 800 nm. As shown in Figure 5, strong fluorescent staining on the surface of U251 was observed. Similar

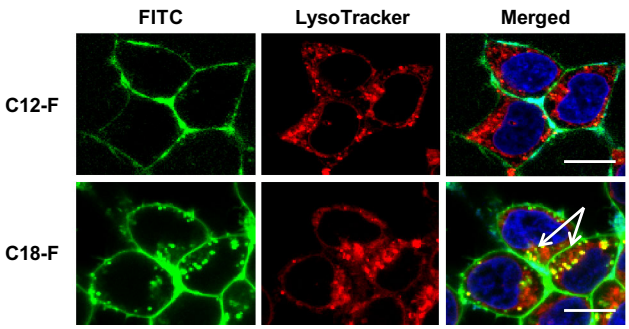


Fig. 4 Fluorescence imaging of live HEK 293 cells after incubation with FITC-peptide conjugates (C6-F, C12-F, and C18-F) (1 μM) for 2 h at 37 °C. (FITC: *green*; Lysotracker, *red*; H33258, *blue*). The *arrow* shows the co-localization of C18-F and LysoTracker (*yellow*). *Scale bar* 15 μm

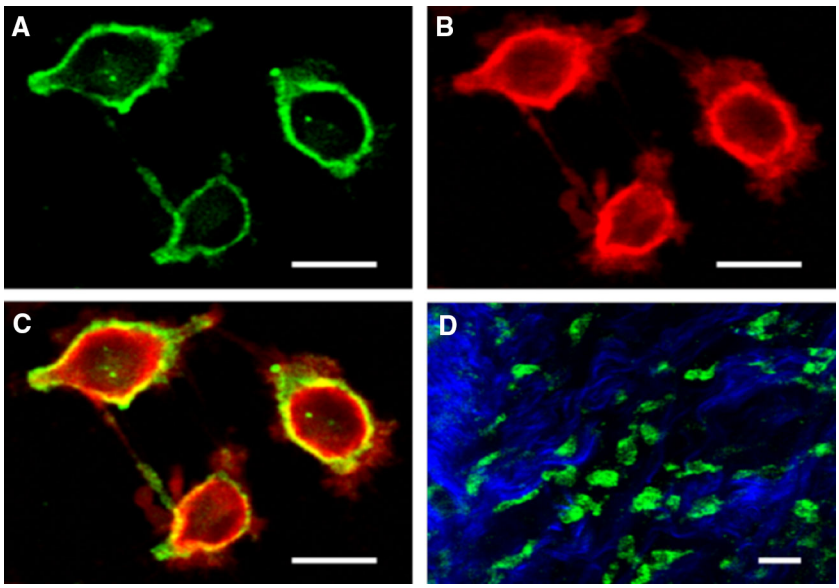


Fig. 5 Two-photon imaging in U251 cell lines and mouse ear tissue after incubation with MFP. Channel: FITC (**a**), Membrane tracker CellMask Deep Red (**b**), Merged (**c**). MFP signals (*green*) in tissues and second harmonic generation from collagen (*blue*) (**d**). *Scale bar* 20 μm

two-photon imaging was also conducted by incubation of MFP with mouse ear tissues and the images were monitored at depths of ~40 μm. The bright fluorescence observed clearly indicated that the developed probe molecule could work properly to monitor furin enzyme activities in living tissues.

In conclusion, the specific localization of our developed MFP probe on the plasma membrane first represents a simple and novel small molecule-based method for direct visualization of furin-like enzyme activity on the cell surface. In this study, the carefully optimized lipid moiety in the molecule structure can efficiently facilitate the immobilization of the probe on the extracellular plasma membrane for a relative long time. Upon the specific enzyme hydrolysis, a strong fluorescence turn-on effect can be observed which shows the great promising real-time imaging of surface-localized furin activities in living cells and tissues using one-photon and two-photon microscopy. Such rational design could be also expanded as a reliable and general method to visualize other membrane-associated protelytic enzymes in their functions and biological roles without the need of invasive procedures. Furthermore, optimization of this type of probe to trace the enzyme dynamics and location in specific subcellular organelles will provide deeper insight into mechanisms of viral or pathogen invasion, which is currently under the process. More importantly, such a unique MFP probe can also be used to screen potential proteolytic enzyme inhibitors to selectively block furin-dependent cell-surface processing without interfering with the normal functions, which will definitely facilitate the new discovery of specific probes or drug molecules towards the ranostics of furin related diseases.

References

[1] a) M. S. Brown, J. Ye, R. B. Rawson, J. L. Goldstein, *Cell* **2000,** *100,* 391; b) A. Weihofen, B. Martoglio, *Trends. Cell. Biol* **2003,** *13,* 71; c) S. Urban, M. Freeman, *Cure. Opin. Genet. Dev.* **2002,** *12,* 512.
[2] a) A. J. Barrett, J. F. Woessner, N. D. Rawlings, *Handbook of proteolytic enzymes, Vol. 1*, Elsevier, **2004**; b) M. S. Wolfe, *Chem. Rev.* **2009**, *109*, 1599; c) T. Jiang, E. S. Olson, Q. T. Nguyen, M. Roy, P. A. Jennings, R. Y. Tsien, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 17867.
[3] a) G. Thomas, *Nat. Rev. Mol. Cell. Bio.* **2002**, *3*, 753; b) N. G. Seidah, A. Prat, *Nat. Rev. Drug. Discov.* **2012**, *11*, 367.
[4] a) P. Stawowy, C. Margeta, H. Kallisch, N. G. Seidah, M. Chrétien, E. Fleck, K. Graf, *Cardiovasc. Res.* **2004**, *63*, 87; b) M. Roomi, J. Monterrey, T. Kalinovsky, M. Rath, A. Niedzwiecki, *Oncol. Rep.* **2009**, *21*, 1323.
[5] E. Hwang, S. Kim, J. Sohn, J. Lee, Y. Kim, Y. Kim, I. Mook-Jung, *Biochem. Bioph. Res. Co.* **2006**, *349*, 654.
[6] a) S. A. Shiryaev, A. G. Remacle, B. I. Ratnikov, N. A. Nelson, A. Y. Savinov, G. Wei, M. Bottini, M. F. Rega, A. Parent, R. Desjardins, *J. Biol. Chem.* **2007**, *282*, 20847; b) T. Matsuzawa, A. Fukui, T. Kashimoto, K. Nagao, K. Oka, M. Miyake, Y. Horiguchi, *J. Biol. Chem.* **2004**, *279*, 2866; c) T. Komiyama, J. M. Coppola, M. J. Larsen, M. E. V. Dort, B. D. Ross, R. Day, A. Rehemtulla, R. S. Fuller, *J. Biol. Chem.* **2009**, *284*, 15729.
[7] a) D. Mesnard, D. B. Constam, *J. Cell Biol.* **2010**, *191*, 129; b) K. Gawlik, A. G. Remacle, S. A. Shiryaev, V. S. Golubkov, M. Ouyang, Y. Wang, A. Y. Strongin, *PLoS One* **2010**, *5*, e11305; c) J. P. Hobson, S. Liu, B. Rønø, S. H. Leppla, T. H. Bugge, *Nat. Methods* **2006**, *3*, 259; d) Y. Yano, K. Matsuzaki, *(BBA)-Biomembranes* **2009**, *1788*, 2124.
[8] a) D. S. Folk, J. C. Torosian, S. Hwang, D. G. McCafferty, K. J. Franz, *Angew. Chem. Int. Ed.* **2012**, *124*, 10953; b) M. H. Lee, H. M. Jeon, J. H. Han, N. Park, C. Kang, J. L. Sessler, J. S. Kim, *J. Am. Chem. Soc.* **2014,** *136*, 8430; c) S. Mizukami, Y. Hori, K. Kikuchi, *Acc. Chem. Res.* **2014**, *47*, 247; d) L. Li, X. Shen, Q. Xu, S. Yao, *Angew. Chem. Int. Ed.* **2013**, *52*, 424; e) T. Kowada, J. Kikuta, A. Kubo, M. Ishii, H. Maeda, S. Mizukami, K. Kikuchi, *J. Am. Chem. Soc.* **2011**, *133*, 17772; f) J. Lovell, T. Liu, J. Chen, G. Zheng, *Chem. Rev.* **2010**, *110*, 2839; g) L. E. Edgington, M. Verdoes, M. Bogyo, *Curr. Opin. Chem. Biol.* **2011**, *15*, 798; h) E. A. Lemke, C. Schultz, *Nat Chem Biol* **2011**, *7*, 480; i) B. E. Cravatt, A. T. Wright, J.

W. Kozarich, *Annu. Rev. Biochem*. **2008**, *77*, 383; j) L. Bu, X. Ma, Y. Tu, B. Shen, Z. Cheng, *Curr. Pharm. Biotech*. **2014**, *14*, 723.
[9] a) S. Molloy, P. Bresnahan, S. H. Leppla, K. Klimpel, G. Thomas, *J. Biol. Chem.* **1992**, *267*, 16396; b) S. M. Cork, B. Kaur, N. S. Devi, L. Cooper, J. H. Saltz, E. M. Sandberg, S. Kaluz, E.G. Van Meir, *Oncogene* **2012**, *31*, 5144.
[10] a) N. C. Rockwell, D. J. Krysan, T. Komiyama, R. S. Fuller, *Chem Rev* **2002**, *102*, 4525; b) A. Dragulescu-Andrasi, S.-R. Kothapalli, G. A. Tikhomirov, J. Rao, S. S. Gambhir, *J. Am. Chem. Soc.* **2013**, *135*, 11015; c) A. Dragulescu-Andrasi, G. Liang, J. Rao, *Bioconjug. Chem.* **2010**, *20,* 1660.
[11] a) D. Rabuka, M. B. Forstner, J. T. Groves, C. R. Bertozzi, *J. Am. Chem. Soc.* **2008**, *130*, 5947; b) H. Kim, B. Kim, H. Choo, Y. Ko, S. Jeon, C. Kim, T. Joo, B. Cho, *ChemBioChem* **2008**, *9*, 2830; c) K. E. Sapsford, L. Berti, I. L. Medintz, *Angew. Chem. Int. Ed.* **2006**, *45*, 4562.
[12] K. Samuelsson, C. Simonsson, C. A. Jonsson, G. Westman, M. B. Ericson, A. T. Karlberg, *Contact Dermatitis* **2009**, *61*, 91.
[13] a) A. Cobos-Correa, J. B. Trojanek, S. Diemer, M. A. Mall, C. Schultz, *Nat. Chem. Biol.* **2009**, *5*, 628; b) S. Gehrig, M. A. Mall, C. Schultz, *Angew. Chem. Int. Ed.* **2012**, *51*, 6258; c) L. Rajendran, A. Schneider, G. Schlechtingen, S. Weidlich, J. Ries, T. Braxmeier, P. Schwille, J. B. Schulz, C. Schroeder, M. Simons, G. Jennings, H. Knolker, K. Simons, *Science* **2008**, *320*, 520; d) Y. Hu, D. Vats, M. Vizovisek, L. Kramer, C. Germanier, K. U. Wendt, M. Rudin, B. Turk, O. Plettenburg, C. Schultz, *Angew. Chem. Int. Ed.* **2014**, ***53,*** 7669; e) D. lingwood, K. Simons, *Science* **2010**, *327*, 46.
[14] S. Henrich, A. Cameron, G. P. Bourenkov, R. Kiefersauer, R. Huber, I. Lindberg, W. Bode, M. Than, *Nat. Struct. Mol. Biol.* **2003**, *10*, 520.
[15] a) A. G. Remacle, A. V. Chekanov, V. S. Golubkov, A. Y. Savinov, D. V. Rozanov, A. Y. Strongin, *J. Biol. Chem.* **2006**, *281*, 16897; b) S. A. Shiryaev, A. V. Chernov, V. S. Golubkov, E. R. Thomsen, E. Chudin, M. S. Chee, I. A. Kozlov, A. Y. Strongin, P. Cieplak, *Plos One* **2013**, 8, e54290.
[16] S. Takahashi, T. Nakagawa, K. Kasai, T. Banno, S. J. Duguay, W. J. Van de Ven, K. Murakami, K. Nakayama, *J. Biol. Chem.* **1995**, *270*, 26565.
[17] a) G. L. Bourne, D. J. Grainger, *J. Immunol. Methods* **2011**, *364*, 101; b) N. J. Kruger, in Basic protein and peptide protocols, *Springer*, **1994**, p 9.
[18] a) M. Pawlicki, H. A. Collins, R. G. Denning, H. L. Anderson, *Angew. Chem. Int. Ed.* **2009**, *48*, 3244; b) G. Egawa, S. Nakamizo, Y. Natsuaki, H. Doi, Y. Miyachi, K. Kabashima, *Sci Rep*. **2013**, *3*, 1.

The activity of cholesteryl ester transfer protein with discoidal high-density lipoproteins is determined by cholesterol and cholesteryl ester distribution in the phospholipid matrix

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The activity of cholesteryl ester transfer protein (CETP) with high-density lipoproteins (HDL) determines the efficiency of reverse cholesterol transport as a major anti-atherogenic pathway. The structural determinants of CETP activity with nascent discoidal HDL (dHDL) remain unknown. To investigate the influence of lipid unsaturation and neutral lipid on the maturation of high-density lipoproteins, the reconstituted dHDL with apoA-I, phosphatidylcholine and cholesteryl ester were prepared. Saturated dipalmitoylphosphatidylcholine (DPPC) and unsaturated palmitoyllinoleoylphosphatidylcholine (PLPC), palmitoyloleoylphosphatidylcholine (POPC), and fluorescent probe cholesteryl 1-pyrenedecanoate (CPD) that forms in a diffusion- and concentration-dependent manner short-lived dimer of unexcited and excited molecules (excimer) were used. Probe dynamics fulfilled the quenching sphere-of-action model. The apoA-I/DPPC/CPD complexes were heterogeneous by size, composition and probe location. CPD molecules incorporated more efficiently into larger complexes and accumulated in the central part of the discs. The apoA-I/POPC(PLPC)/CPD were also heterogeneous; however, probe molecules distributed preferentially into smaller complexes and accumulated at the disc periphery. The binding of both cholesteryl ester and cholesterol to apoA-I and CETP was analyzed by a search of cholesterol-binding sites in these proteins. Six cholesterol recognition putative motifs in apoA-I structure are suggested to interact with cholesterol. Cholesterol is a ligand for L101, F104 and W108 residues in a low-resolution structure of HDL-bound apoA-I (Wu et al. 2009). Five putative cholesterol-binding regions were predicted from the CETP primary structure. Amino acid residues in three motifs are suggested to interact with two ligand cholesteryl ester molecules in a crystal structure of CETP (Qiu et al. 2007). CPD transfer by CETP between donor dHDL with CPD and acceptor dHDL with cholesteryl laurate was followed by excimer disappearance. The cholesteryl ester exchange between donor and acceptor particles was characterized by a heterogeneous kinetics; the fast exchanging CPD pool was much higher in the case of POPC compared to DPPC complexes. Probe fraction accessible to CETP increased with temperature, suggesting a more homogeneous probe distribution. Noncompetitive inhibition of probe transfer by acceptor particles was observed. The values of V_{max} and catalytic rate constant k_{cat} together with a similarity of K_m and K_I values for POPC-containing dHDL suggest the efficient cholesteryl ester transfer between nascent HDL with unsaturated phosphatidylcholine in vivo. The phospholipid matrix in dHDL may underlie CETP activity through the self-association, diffusivity and location of cholesteryl ester in the bilayer, the accessibility of cholesteryl ester to cholesterol-binding site in apoA-I structure and the binding of cholesteryl ester, positionable by apoA-I, to CETP. In turn, apoA-I influences the CETP activity modulating the properties of apolipoprotein–phospholipid interface. This may include cholesteryl ester accumulation in the boundary lipid in unsaturated phosphatidylcholine and cluster formation in the bulk bilayer in saturated phosphatidylcholine. Cholesterol may competitively inhibit cholesteryl ester binding to both apoA-I and CETP molecules, thus modulating the transfer of neutral lipid. We are grateful to Dr. A. Tall (Columbia University, New York, USA) for providing the recombinant CETP.

Plasma amino acid profile in relation to mitochondrial dysfunction upon circulation disorders

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Life-threatening consequences of chronic circulatory disorders of any origin are implemented by the progressive development of mitochondrial dysfunction (MD), which is manifested by the

formation of stable shifts in a large number of metabolites into the circulation. Such metabolites include amino acids (AA) because many of them are metabolized in the mitochondria. The aim of our study was to compare the plasma AA profiles of healthy individuals and patients with circulatory disorders in relation to MD, verified by elevated levels of lactic acid and cytochrome C (CytC). Plasma samples of 47 patients (23 men and 24 women, 61(51–64) years old) were analyzed. All patients were characterized by circulation disorders. The diagnosis of aortic stenosis and aortic dilatation was verified by the results of transthoracic echocardiography studies. 40 patients (85 %) had mild signs of heart failure (NYHA functional class 1 and 2). The patients had no renal dysfunction, and their routine biochemical tests were near the reference values. The comparison group included 20 healthy individuals aged 57 (56–60) years. AA concentrations were measured by HPLC with fluorometric detection analysis, lactic and pyruvic acids spectrophotometrically, and CytC by ELISA. Nonparametric methods were used for statistical treatment. With respect to the comparison group, patients were characterized by decreased levels of Asp ($p < 0.0001$), Leu ($p = 0.021$), Phe ($p = 0.025$), Thr ($p = 0.0012$) and increased levels of Ser ($p = 0.0018$) and Lys ($p = 0.0001$). 22 patients (47 %) revealed signs of MD, which manifested by lactic acidemia above 2.2 mM and/or high CytC. In this subgroup, as compared to other patients ($n = 25$), we have found a relative increase in the concentration of Ala, according to Ala/(Phe + Tyr) and Ala/Lys ratios ($p = 0.0059$ and 0.0042, respectively), as well as increased level of citrulline ($p = 0.035$) combined with a reduction in the concentration of Arg ($p = 0.0071$). Thus, the MD developed upon circulation disorders manifested in the AA profile by increasing concentrations of Ala and Ser, which is most likely due to the decreased activity of the pyruvate dehydrogenase complex. The increased citrulline/Arg ratio in patients with chronic circulatory disorders most likely reflects the activation of the NO-synthase pathway. This study was supported by the Russian Foundation for Basic Research No. 15-04-02480.

Arginine transport in human primary granulocytes

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Polymorphonuclear cells (PMNs) are the crucial first-line defense combating microbes by phagocytosis and with a variety of oxidative and nonoxidative effector mechanisms. In addition, PMNs also serve as immunoregulatory cells interacting with other immune cells to coordinate a proper immune response. One central, potentially immunosuppressive effector molecule constitutively expressed in human PMNs is the arginine hydrolyzing enzyme arginase I, located within granules. Local or systemic arginine deficiency mediated by degranulation of PMN is often encountered in cancer patients or during chronic inflammation and can profoundly suppress the adaptive immune response by inhibiting T cell proliferation and cytokine synthesis. In contrast, arginine depletion may also have an anti-cancer effect when tumor cells are unable to resynthesize their own arginine. Interestingly, absence of extracellular arginine has no influence on PMN viability and key effector functions. We thus ask if primary human granulocytes take up arginine at all. In unstimulated cells, we monitored arginine uptake both by HPLC and using radioactive tracers. Among the amino acid transporters that accept arginine as a substrate, we found low mRNA expression for cationic amino acid transporter 1 (CAT-1), y^+LAT1, $b^{0,+}$AT and $ATB^{0,+}$ in these cells. CAT-1 expression was upregulated when the cells were stimulated with lipopolysaccharide and interferon γ. We are now determining the ratio of intracellular arginine to the arginase product ornithine to find out if intracellular arginine is hydrolysed by arginase or protected from arginase situated in granules by its cytoplasmatic location. These data will provide important information if even intact PMNs may deplete extracellular arginine under certain situations.

Estradiol and ethinyl-estradiol decrease proline uptake and transport in intestinal Caco-2 cells

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Background: The proton-coupled amino acid transporter PAT1 has been shown to facilitate the intestinal absorption of GABA-mimetics drug substances such as gaboxadol and vigabatrin in animal models (1–3). Recently, it was suggested that estradiol acts as a blocker of PAT1-mediated glycine transport, when expressed in *X. Laevis* oocytes (4).
Aim: The aim of the present study was to investigate if estradiol and ethinyl-estradiol alter cellular PAT1-mediated uptake and affect transcellular transport of the PAT1-substrate L-proline.
Materials and methods: The uptake of L-[2,3,4,5-^{3}H]-proline (1 mCi/mL) or [^{14}C]-Gly-Sar (1 mCi/mL) was measured in Caco-2 cells cultured for 13 days at the bottom of tissue culture plates or on transwell filter inserts (1.12 cm^2). The uptake and transport were measured in HBSS buffer. Studies were conducted in at least three cell passages (n) (average ± SEM).
Results: The uptake of ^{3}H-Pro (13.3 nM) in Caco-2 cells was decreased in the presence of increasing estradiol and ethinyl-estradiol concentrations with IC_{50}-values of 8.3 ± 1.3 and 30.9 ± 1.1 μM ($n = 4$), respectively. The uptake of ^{14}C-Gly-Sar (17.9 μM), which is a substrate of PEPT1, was unaffected by 100 μM estradiol or ethinyl-estradiol. The transepithelial transport of 1 mM proline (P_{app} $15.9 \times 10^{-6} \pm 5.81 \times 10^{-7}$ cm s^{-1}) decreased in the presence of 100 μM estradiol to $9.6 \times 10^{-6} \pm 4.6 \times 10^{-7}$ cm s^{-1} or to $7.7 \times 10^{-6} \pm 3.2 \times 10^{-7}$ cm s^{-1} in the presence of 100 μM ethinyl-estradiol. However, no effect on the transepithelial transport of ^{14}C-Gly-Sar (17.9 μM) was observed.
Conclusion: Estradiol and ethinyl-estradiol decrease PAT1-mediated uptake of proline in Caco-2 cells and decrease the transepithelial transport of proline.

1. Broberg ML, Holm R, Tonsberg H, Frolund S, Ewon KB, Nielsen AL, Brodin B, Jensen A, Kall MA, Christensen KV, Nielsen CU (2012) Function and expression of the proton-coupled amino acid transporter Slc36a1 along the rat gastrointestinal tract: Implications for intestinal absorption of gaboxadol. Br J Pharmacol.

2. Larsen M, Holm R, Jensen KG, Brodin B, Nielsen CU (2009) Intestinal gaboxadol absorption via PAT1(SLC36A1): modified absorption in vivo following co-administration of L-tryptophan. Br J Pharmacol. 157:1380–1389.

3. Nohr MK, Juul RV, Thale ZI, Holm R, Kreilgaard M, Nielsen CU (2015) Is oral absorption of vigabatrin carrier-mediated? Eur J Pharm Sci. 69:10–18.

4. Shan L, Yang Y, Wang J, Zuo J, Dong X, Li C, Li D (2012) Estradiol inhibits the activity of proton-coupled amino acid transporter PAT1 expressed in Xenopus oocytes. Eur J Pharmacol 695:34–39.

Blood brain barrier amino acid transporters regulation of brain interstitial fluid amino acid homeostasis

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Brain interstitial fluid (BIF) is the direct environment for different cells in the brain; however, very little is known about its qualitative and quantitative content, particularly in terms of amino acids (AA). The present study focuses on the potential role of blood brain barrier (BBB) amino acid transporters (AAT) in BIF AA homeostasis, mainly L-glutamine (Gln), which is a precursor for major excitatory (Glu) and inhibitory (GABA) neurotransmitters.

To estimate BIF AA concentration, we performed in vivo microdialysis (mD) in freely moving mice. Interestingly, all 14 measured standard AA were ~tenfold less concentrated in BIF than in CSF. To follow the potential transfer of AAs from the periphery into BIF, we administered single AAs i.p. and measured their presence in plasma and BIF. Valine injection led to a rapid increase in its concentration in plasma and BIF, while heavy labeled Gln (hGln) administration caused its rapid appearance in BIF without an increase in total BIF Gln. Thus, both AAs are transported across the BBB. Competitive inhibition of Lat1 (Slc7a5) by BCH introduced into the BIF via mD probe caused a stable fourfold increase in basal BIF Gln levels. Finally, simultaneous perfusion of BCH into BIF with hGln i.p. administration strongly increased the influx of hGln into BIF, suggesting that BCH transstimulted Lat1-mediated Gln uptake.

Taken together, our data suggest that Lat1 plays a major role for setting BIF Gln levels. Future experiments using an inducible endothelial-specific Snat3 knockout mouse model will clarify the role of this Gln transporter.

Arginine availability: a key parameter of cancer immunology

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The availability of arginine influences the outcome of antitumoral immune responses and cancer growth in various ways. (1) Arginine transport: we could show that activation via CD3 and CD28 induces arginine transport into primary human T cells via specific upregulation of the human cationic amino acid transporter-1 (hCAT-1). Upon knockdown of hCAT-1, arginine uptake and proliferation are inhibited in primary human T cells. We are currently characterising regulation, expression and physiological importance of arginine transporters in immune and cancer cells under varying arginine conditions. (2) Arginine degradation: the enzyme arginase, which hydrolyzes arginine to ornithine and urea, is constitutively present in human neutrophil granulocytes and myeloid derived suppressor cells (MDSC), which expand in the context of cancer. MDSC-expressed arginase is a determining factor of tumor immune escape, since arginine withdrawal shuts down human T cell proliferation and selectively suppresses cytokine synthesis. Cancer cells themselves also depend on arginine availability for growth and viability. Depletion of arginine very efficiently induces cell cycle arrest and finally death in tumor cells. (3) Arginine resynthesis: cancer cells might lack expression of the enzyme argininosuccinate synthase (ASS), which is necessary for the endogenous synthesis of arginine from citrulline. Enzymatic depletion of exogenous arginine has been tested already in phase III clinical trials for arginine-auxotrophic cancer entities. Activated human T cells upregulate ASS under arginine limitation, take up citrulline and resume proliferation. Targeting arginine availability on the levels of transport, degradation and synthesis is a promising novel therapeutic strategy of cancer immunology.

The anti-epileptic drug substance vigabatrin inhibits transport via the taurine transporter (TauT, SLC6A6) in SKPT cells

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Background: γ-Aminobutyric acid (GABA) is a substrate of the taurine transporter (TauT, SLC6A6) [1]. Vigabatrin, a GABA analog, has been shown to inhibit the uptake of taurine in Caco-2 and MDCK1 cells [2]. The aim of the study was to investigate the interaction between vigabatrin and TauT in SKPT cells cultured under hyperosmotic conditions.
Materials and methods: TauT expression was investigated in SKPT cells cultured for 72 h under normal (300 mOsm) or hyperosmotic conditions (500 mOsm, last 24 h) using raffinose. The expression of TauT was measured using a 5 min uptake study of ^{3}H-taurine (0.5 μCi/ml) or by qPCR. ^{3}H-Taurine (1 μCi/ml) uptake in the presence of GABA or vigabatrin was measured in hyperosmotic treated SKPT cell. All experiments were performed in three cell passages (mean ± SEM).
Results: The expression of TauT was increased in SKPT cells cultured under hyperosmotic conditions. The uptake rate of ^{3}H-taurine (22 nM) was 0.92 ± 0.03 fmol/(min cm^2) in control cells and 10.8 ± 1.5 fmol/(min cm^2) in cells cultured under hyperosmotic conditions. Furthermore, *TauT* mRNA expression was up-regulated 15.2 ± 3.4 times in cells cultured under hyperosmotic conditions. In raffinose-treated SKPT cells, GABA and vigabatrin were able to concentration dependently decrease the uptake of ^{3}H-taurine (44 nM) giving IC_{50} values of 1 mM ($\log IC_{50} = 0.07 \pm 0.07$) and 34 mM ($\log IC_{50} = 1.5 \pm 0.05$ mM), respectively.
Conclusion: Hyperosmotic culture conditions up-regulate the expression of TauT in SKPT cells at the molecular and functional level. Taurine uptake is concentration-dependently decreased by vigabatrin. Vigabatrin might be either a substrate or inhibitor of TauT.
1. Tomi M et al (2008) Function of taurine transporter (Slc6a6/TauT) as a GABA transporting protein and its relevance to GABA transport in rat retinal capillary endothelial cells. Biochim Biophys Acta 1778(10):2138–2142.

2. Plum J et al (2014) The anti-epileptic drug substance vigabatrin inhibits taurine transport in intestinal and renal cell culture models. Int J Pharm 473(1–2):395–397.

Protein diet content influences L-glutamate metabolism and transport in the exocrine pancreas and intestine

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The exocrine pancreas also secrets free amino acids into the pancreatic juice (PJ), with L-glutamate (Glu) being the most concentrated. The accumulation of Glu in acinar cells is dependent on the transport and metabolism of its precursor glutamine (Gln). Furthermore, the inhibition of glutaminase 2, an enzyme involved in the synthesis of Glu from Gln, decreased the accumulation of Glu in acinar cells. The pancreas of animals under a protein-free diet (PFD) has reduced volume and protein synthesis, but unchanged Glu secretion. The accumulation of Gln was unchanged, while the expression of glutaminase 2 increased. These results suggest that the metabolism of Gln in acinar cells is modified under PFD.

In the enterocytes, Glu is used as a source of energy and is also suggested to stimulate intestinal epithelial cell proliferation and maintenance of the epithelial barrier function. Under PFD, the expression of EAAT3 (Slc1a1), the only apical Glu transporter known to be expressed in enterocytes, was increased. The expression of glutamate dehydrogenase, the limiting step to Glu entering the Krebs cycle and the tight junction elements ZO1 and occludin 1, which are frequently decreased with loss of epithelial barrier in enterocytes, was unchanged. Similarly, the transport of Glu attributed to EAAT3 remained unchanged.

Together, our results suggest that Glu is synthesized in the exocrine pancreas from Gln. The Glu secreted in the PJ is taken up by the small intestine. It may support the proliferation and maintenance of the epithelial structure and play an important role in intestinal homeostasis under protein starvation.

Immune-related disorders in the LPI mouse

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Lysinuric protein intolerance (LPI, MIM 222700) is an inherited aminoaciduria caused by mutations in the SLC7A7 gene, which encodes for y + LAT1. The protein is a cationic amino acid (CAA) transporter, mainly expressed in the epithelial cells of intestine, kidney and placenta, and also in spleen, macrophages and lymphocytes from peripheral blood. Typically, symptoms begin after weaning with refusal of protein feeding, vomiting, diarrhea and consequent failure to thrive. Other recurrent features are hyperammonemia, osteoporosis, hematological anomalies and different immune-related disorders, such as: pulmonary alveolar proteinosis (PAP), chronic renal disease and erytroblastophagy. Because *null Slc7a7 mouse* is lethal, we generated the conditional tamoxifen-inducible ablation of *Slc7a7* in mouse. LPI patients' treatment, low-protein diet and citrulline supplementation are needed to maintain the $Slc7a7^{-/-}$ mouse alive. Under these circumstances, the model presents all hallmarks of human LPI: intestinal phenotype [malabsorption of cationic amino acids (CAA)] and renal phenotype (hyperexcretion of CAA) that results in a low CAA in plasma that reduces urea cycle substrates' availability and causes impairment in urea cycle (hyperammonemia, glutaminemia and hyperexcretion of orotic acid). Related to the more life-threatening and unknown immune-related phenotype, $Slc7a7^{-/-}$ mice present clear imbalance between lymphocyte and granulocyte blood cell populations, development of PAP (approximately, 30 % of animals) and suffer a drastic histological alterations in spleen. Preliminary results show increased siderophages in spleen, suggesting macrophage activation and loss of proliferation nuclei in the B cell population, which could be related to an impaired clonal expansion. In summary, we present the first viable LPI animal model to study the mechanisms of pathophysiology of the immune-related disorders of LPI.

Slc5a8 is a conditional tumor suppressor in colon linked to dietary fiber content

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SLC5A8 was first identified as a putative tumor suppressor in colon; it is silenced in colon cancer, and ectopic expression of the transporter in colon cancer cell lines leads to cell death. It functions as an Na^+-coupled high-affinity transporter for short-chain fatty acids, which are produced in colonic lumen by fermentation of dietary fiber by gut microbiota. Butyrate and propionate, two of these short-chain fatty acids, are inhibitors of histone deacetylases. Thus, the concentrative uptake of these HDAC inhibitors into colonic epithelial cells via SLC5A8 provides the molecular basis for the transporter's tumor-suppressive function. Despite strong in vitro data in support of this rationale, in vivo studies with *Slc5a8*-null mice failed to support the tumor-suppressive function of the transporter. We now have data showing that this transporter is a conditional tumor suppressor in colon linked to dietary fiber content. When mice were fed a diet containing optimal fiber content, there were no differences in the incidence and progression of colonic inflammation or colon carcinogenesis between wild-type mice and *Slc5a8*-null mice in experimental model systems. However, when mice were fed a fiber-free diet, the incidence and progression of colitis and colon cancer were accelerated in *Slc5a8*-null mice compared to wild-type mice. We also found that Slc5a8 is critical for the maintenance of symbiotic relationship between colonic bacteria and the host. Mammalian colon harbors trillions of bacteria under normal conditions with no overt activation of the mucosal immune system. The mechanisms underlying this tolerogenic phenomenon remain poorly understood. Among various immune cells in colon, dendritic cells (DCs) are important to induce tolerogenic phenotype in T cells. We found that DCs exposed to butyrate and propionate, but not acetate, expressed the immunosuppressive enzymes indoleamine 2,3-dioxygenase 1 (IDO1) and aldehyde dehydrogenase 1A2 (Aldh1a2), converted naïve T cells to Foxp3+ Tregs, and suppressed conversion of naïve T cells into IFN-γ-producing cells. HDAC inhibition mediated these processes. *Slc5a8*-null DCs did not induce IDO1 and Aldh1a2 and did not generate Tregs or suppress IFN-γ-producing T cells in response to butyrate/propionate, thus demonstrating the obligatory role of this transporter in host–bacteria symbiosis in normal colon.

Reduced intestinal amino acid absorption increases levels of FGF21 and GLP-1 and improves glycaemic control

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Objective: Type 2 diabetes arises from insulin resistance of peripheral tissues followed by dysfunction of β-cells in the pancreas due to metabolic stress. Both depletion and supplementation of neutral amino acids have been discussed as strategies to improve insulin sensitivity. Here, we characterise mice lacking the intestinal and renal neutral amino acid transporter B^0AT1 (*Slc6a19*) as a model to study the consequences of selective depletion of neutral amino acids.
Methods: Metabolic tests, analysis of metabolite levels and signalling pathways were used to characterise mice lacking the intestinal and renal neutral amino acid transporter B^0AT1 (*Slc6a19*).
Results: Reduced uptake of neutral amino acids in the intestine and loss of neutral amino acids in the urine cause an overload of amino acids in the lumen of the intestine and reduced systemic amino acid availability. As a result, higher levels of glucagon-like peptide 1 (GLP-1) are produced by the intestine after a meal, while the liver releases the starvation hormone fibroblast growth factor 21 (FGF21). The combination of these hormones generates a metabolic phenotype that is characterised by efficient removal of glucose, particularly by the heart, reduced adipose tissue mass, browning of subcutaneous white adipose tissue, enhanced production of ketone bodies and reduced hepatic glucose output.
Conclusions: Reduced neutral amino acid availability improves glycaemic control. The epithelial neutral amino acid transporter B^0AT1 could be a suitable target to treat type 2 diabetes.

Induction of the glutamate transporter EAAT3/EAAC1 in oligodendroglial cells: possible implications for cytoprotection in demyelinating conditions

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Abstract: Although the pharmacological therapies for multiple sclerosis (MS) are focused on immunosuppression, additional approaches, aimed at counteracting oligodendrocyte injury, are gaining much interest. One of the most important mechanisms involved in MS-associated oligodendrocyte damage is glutamate-dependent excitotoxicity, which leads to oxidative stress and apoptosis. The glutamate transporter EAAT3 is typically neuronal, but its expression has been demonstrated in both oligodendrocytes (OL) and their precursors (OPC). In neurons, EAAT3 activity is also crucial for the intracellular supply of cysteine, required for GSH synthesis, and is induced under conditions of oxidative stress. However, EAAT3 regulation in OL and OPC is unknown, and its expression in MS lesions not yet ascertained. Some years ago, we found that in rat C6 glioma cells retinoids induce both *Slc1a1* mRNA and EAAC1 protein expression through a RARβ-dependent mechanism leading to a massive increase of transport activity. Here, we show that in rat OPC isolated from the optic nerve of normal P7 animals, EAAC1 is induced by all-*trans* retinoic acid (ATRA). Consistently, EAAT3 expression is stimulated by both ATRA and the synthetic RAR-agonist Am80 in the white matter of organotypic cultures of rat cerebellum, exhibiting co-expression with the oligodendrocytic markers MBP and OLIG2, but not with the astroglial marker GFAP. Moreover, EAAT3/EAAC1 is expressed by Olig2-positive cells in remyelinating MS plaques and in spinal cord lesions of mice affected by experimental autoimmune encephalomyelitis, the animal model of MS. In conclusion, our study shows that EAAT3/EAAC1 is expressed in cells of oligodendroglial lineage in remyelinating lesions in humans and mice and is induced by retinoids in rat OPC and OL. Interestingly, preliminary results indicate that also fumarates, recently authorized for the cure of the relapsing–remitting (RR) form of MS, induce EAAT3 protein expression. Thus, although the protective role of EAAT3/EAAC1 against excitotoxic/oxidative stress in OL and OPC needs to be ascertained yet, these data suggest that retinoids may be novel pharmacological tools for increasing oligodendrocyte protection.

Functional activity of organic cation transporters (OCTs AND OCTNs) in human Airwai epithelial cells

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SLC22A transporter family, composed by the electrogenic transporters OCT1/SLC22A1, OCT2/SLC22A2, OCT3/SLC22A3 and the pH-dependent novel transporters OCTN1/SLC22A4 and OCTN2/SLC22A5, mediates the transport of organic cations including inhaled drugs across epithelial cells. Here we define the functional activity of OCTs and OCTNs in human airway epithelial cells: alveolar A549, bronchial Calu-3, distal-lung NClH441 carcinoma cells and normal bronchial BEAS-2B cells. The OCTs and OCTNs activities were determined by measuring [3H]1-methyl-4-phenylpyridinium (MPP^+) and [3H]Lcarnitine uptake, respectively. Quinidine, prostaglandine E2 and corticosterone were used as preferential inhibitors of OCT1, OCT2, OCT3, respectively, while betaine and ergothioneine of OCTN2 and OCTN1, respectively. In A549 cells MPP^+ uptake is mediated by one high-affinity component identifiable with OCT3. Only corticosterone, indeed, completely inhibits MPP^+ transport and SLC22A3/OCT3 siRNA lowers MPP+ uptake. NCl-H441 shows a modest saturable MPP^+ uptake mediated by OCT1. Calu-3 and BEAS-2B exhibit both an high and a low affinity component, referable to OCT3 and OCT1, respectively. In A549 and BEAS-2B cells a single high affinity transport component for L-carnitine is present and identifiable with OCTN2. Conversely, in Calu-3 and in NCl-H441 are active two transporters: OCTN2 is the high affinity component and $ATB0^+$, a Na^+ and Cl^- coupled transporter for amino acids, represents the low affinity component for L-carnitine uptake. These results are of relevance for the definition of the operative features of organic cation

transporters in organotypic in vitro models of human respiratory epithelium and for the identification of proper in vitro model for researches in the field of drug absorption and disposition.

Loss of function mutation of the Slc38a3 glutamine transporter reveals its critical role for amino acid metabolism in liver, brain and kidney

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Glutamine, the most abundant amino acid in mammals, is critical for cell and organ functions. Its metabolism depends on the ability of cells to take up or release glutamine by transporters located in the plasma membrane. Several solute carrier (SLC) families transport glutamine, but the SLC38 family has been thought to be mostly responsible for glutamine transport. We demonstrate that despite the large number of glutamine transporters, loss of the SNAT3/Slc38a3 glutamine transporter has a major impact on the function of organs expressing Slc38a3/Snat3. *Snat3* mutant mice were generated by ENU mutageneses which resulted in a C to T mutation that led to a premature stop codon. *Snat3Q263X* mice showed stunted growth, altered organ and plasma amino acid levels, hypoglycemia, and died around 20 days after birth. Hepatic concentrations of glutamine, glutamate, leucine, phenylalanine, and tryptophan were highly reduced, paralleled by downregulation of the mTOR pathway possibly linking reduced amino acid availability to impaired growth and glucose homeostasis. *Snat3*-deficient mice had altered urea levels paralleled by dysregulation of the urea cycle, gluconeogenesis, and glutamine synthesis. Mice were ataxic with higher glutamine, but reduced glutamate and GABA levels in brain consistent with a major role of SNAT3 in the glutamine–glutamate cycle. Renal ammonium excretion was lower and expression of enzymes and amino acid transporters involved in ammoniagenesis were altered. Thus, SNAT3 is a glutamine transporter required for amino acid homeostasis and determines critical functions in various organs. Despite the large number of glutamine transporters, loss of Snat3 cannot be compensated suggesting that this transporter is a major route of glutamine transport in liver, brain, and kidney.

High ASCT2 activity as a marker of extracellular glutamine dependence in ammoniagenic multiple myeloma cell lines

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Glutamine (Gln), the most abundant plasma amino acid, is required by many types of cancer cells, where it is needed to balance uncontrolled growth and energy production. The Na^+-dependent ASCT2 transporter, which mediates the exchange of Gln and several other neutral amino acids, is overexpressed in different cancer models, such as AML, prostate adenocarcinoma and breast carcinoma. In these tumors, ASCT2 inhibition or silencing hinders cell proliferation in vitro and in vivo. In this study, enzymes and transporters involved in glutamine metabolism are investigated in human multiple myeloma (MM), a cancer in which Gln role has not been studied yet. In a cohort of 30 patients, bone marrow NH_4^+ levels were significantly increased during myelomagenesis. NH_4^+ increase correlated with decreased glutamine and increased glutamate, pointing to the contribution of glutaminolysis. Neoplastic plasma cells ($CD138^+$), but not other bone marrow cells ($CD138^-$), purified form MM patients, exhibited Gln-dependent NH_4^+ production. Gln-dependent ammoniagenesis was also observed in human myeloma cell lines (HMCLs), but not in acute lymphoblastic leukemia (ALL) 697 cells. All the HMCLs tested expressed glutaminase, but not glutamine synthetase, and were extremely sensitive to Gln starvation. HMCLs expressed SNAT1, LAT1 and ASCT2 transporters at higher levels than ALL cells. Gln transport (100 μM in saline solution) in RPMI-8226 myeloma cells was Na^+ dependent by more than 80 % and markedly inhibited (77 %) by the ASCT2 inhibitor GPNA (1 mM), while MeAIB (SNAT1 inhibition) BCH (LAT1 inhibition) did not show significant effects. To study Gln transport under more physiological conditions, we measured Gln uptake at 0.6 mM (plasma [Gln]) in the culture medium. While the strongest inhibition of Gln uptake was still obtained with GPNA, Gln uptake was also inhibited by MeAIB and BCH. However, GPNA and BCH, but not MeAIB, significantly lowered RPMI-8226 cell viability after 72 h of incubation. These data suggest that MM cells depend upon extracellular Gln and that ASCT2 plays an indispensable role in Gln fuelling. The inhibition of Gln uptake may thus constitute a novel approach for MM control.

Training the aromatic amino acid transporter MCT10 to transport thyroxine: structure-based targeted mutations in MCT10

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Thyroid hormones (TH) (thyroxine, T4 and triiodothyronine, T3) are charged molecules and therefore depend on specific transporters to cross the cell membrane and exert their biological function intracellularly. The monocarboxylate transporter (MCT)-8 and MCT10 are specific TH transporters, whose physiological importance for TH homeostasis has been demonstrated in various studies. Among all members of the monocarboxylate family, MCT8 and MCT10 are most closely related, with an amino acid identity of nearly 50 %. Despite this high homology, both transporters differ in their substrate specificity: both proteins are capable of T3 transport, but only MCT8 accepts T4 as a substrate, whereas MCT10 is an aromatic amino acid transporter (a.k.a. TAT1) capable of tryptophan transport. We believe that the amino acids are responsible for their substrate specificity, making the MCT8/MCT10 couple an ideal model system to investigate the structure substrate specificity relations of TH transporters.

Guided by our MCT8 homology model, which is based on the bacterial glycerol-3-phosphate transporter (glpT), we identified eight candidate amino acids determining the substrate specificity and performed structure-based targeted mutagenesis in MCT10.

The resulting $MCT10^{MCT8}$ chimeras, stable transfected in Madin-Darby canine kidney (MDCK)-1 cells, were tested in 125I-T4 uptake assays and showed a switch in substrate specificity, gaining the ability to transport T4. Furthermore, we could identify one single amino acid important for tryptophan transport.

Given the importance of TH transporters for thyroid hormone effects and the lack of a solved structure of any TH transporter, our findings contribute to a deeper insight into the structure–function relationship of MCT10 and prove the feasibility of structure-based targeted mutations guided by our MCT8 homology model.

Acute regulation of human multidrug and toxin extrusion protein 1 (hMATE1)-mediated influx and efflux

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Vectorial transport of organic cations (OCs) in hepatocytes and proximal tubules is mediated by the sequential action of OC transporters (OCTs) and hMATE1, expressed in the basolateral and luminal plasma membranes, respectively. Cytotoxic drugs such as cisplatin are secreted by this pathway. Sequence analysis of hMATE1 reveals the presence of intracellular phosphorylation sites for different kinases [PKA, PKC, calcium/calmodulin (CaM), $p56^{lck}$ tyrosine kinase ($p56^{lck}$) and caseine kinase II (CKII)]. Therefore, we characterized the acute regulation (10 min) of hMATE1 in its influx and efflux configuration. Influx and efflux of the fluorescent substrate 4-(4-(dimethylamino)styryl)-*N*-methylpyridinium (ASP+) by hMATE1 was analyzed using a microtiter plate-based fluorescence reader. In hMATE1 stably transfected HEK293 cells, the affinity of hMATE1 for ASP+ was higher in the efflux than the influx orientation (1.3 versus 14 μM). Activation of PKA or PKC with 5 μM forskolin or 1 μM DOG, respectively, or inhibition of PI3K with 0.1 μM wortmannin did not significantly change the influx of ASP+, but stimulated its efflux by 35 ± 6 % ($n = 23$), 48 ± 9 % ($n = 7$) and 60 ± 6 % ($n = 24$), respectively. In contrast, inhibition of CaM by 25 μM calmidazolium and of p56lck by 5 μM aminogenistein decreased hMATE1-mediated influx by 18 ± 2 % ($n = 39$) and 15 ± 2 % ($n = 35$), respectively. Also, the inhibition of CaM stimulated hMATE1-mediated efflux by 49 ± 24 % ($n = 7$), while inhibition of the p56lck was without effect. Furthermore, ASP+ influx and efflux were stimulated by inhibition of CKII with 10 μM 4,5,6,7-tetrabromobenzimidazole (14 ± 4 %, $n = 44$ and 32 ± 3 %, $n = 6$, respectively).

Affinities of hMAT1 for ASP+ differ in the inward and outward direction. Like OCTs, hMATE1 shows a complex pattern of kinase-mediated regulation, whereby the effects in the inward-directed mode differ significantly from those in the outward direction of this antiporter. Since PKC activation down-regulates OCT-mediated uptake of OC via the basolateral membrane of proximal tubules, we suggest that PKC stimulates OC renal secretion and inhibits their uptake. Interference with this pathway could be important as a protective approach against drug toxicity (supported by IZKF Münster Cia2/013/13).

Gliadin modulates epithelial permeability by stimulating arginine metabolism in macrophages

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Celiac disease (CD), a chronic small intestinal enteropathy precipitated by exposure to dietary gluten in genetically predisposed individuals, is characterized by a complex interplay between adaptive and innate responses against gluten peptides. Here, we address the effects of gluten on arginine transport and intracellular metabolism in murine macrophages, as well as the effects of arginine metabolites, produced by treated macrophages, on the permeability of intestinal epithelial cells.

RAW264.7 macrophages were incubated for 24 h with 1 mg/ml PTG (obtained through enzymatic digestion of gliadin with pepsin and trypsin) plus 10 ng/ml IFNγ (P + I), to mimic the in vivo inflammatory condition typical of CD. The expression and activity of arginine transporters were then evaluated. The activation of iNOS was monitored through measurements of nitrite (stable derivatives of NO) in the extracellular medium and the arginase pathway through the production/release of polyamines by UHPLC-MS/MS analysis. After the treatment, aliquots of macrophages incubation medium were added to the basolateral side of polarized Caco-2 intestinal epithelial monolayers; the integrity of these latter was monitored after 24 h, by measuring the transepithelial electrical resistance (TEER).

The treatment of macrophages with P + I caused a significant reduction of arginine efflux through system y^+L, referable to the decreased expression of Slc7a6/y+LAT2. An induction of both iNOS and arginase expression was also observed, as well as an increase of ornithine decarboxylase (Odc) mRNA. Consistently, a marked accumulation of nitrite occurred in the extracellular medium of treated RAW264.7, along with an evident production/release of the polyamine putrescine that was completely prevented by the Odc inhibitor 2-(difluoromethyl)ornithine (DFMO) and by the use of arginine-free medium. The addition of P + I-treated macrophages incubation medium on intestinal epithelial monolayers caused a significant decrease in TEER. Since the presence of DFMO or the lack of arginine efficiently reduced this effect, we conclude that the P + I-dependent secretion of polyamines by macrophages may modulate intestinal permeability in vitro. Whether these events also occur in vivo deserves to be further investigated, as well as the existence of differences between healthy and CD monocytes/macrophages that may ultimately produce the well-known gluten-driven intestinal alterations typical of CD patients.

Signaling via protein kinase B/Akt is involved in activation-induced expression of the cationic amino acid transporter hCAT-1 in human primary T lymphocytes

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Proliferation, activation and efficient function of human T lymphocytes are strongly dependent on the presence of the semi-essential amino acid L-arginine. L-arginine deprivation, a condition found in tumor microenvironment and inflamed tissue, can lead to a dramatic reduction in proliferation and INF-γ secretion of local T cells. In primary human T lymphocytes, we identified the human cationic amino acid transporter 1 (hCAT-1) as the major L-arginine transporter. We thus asked which signaling pathways are involved in the regulation of hCAT-1 gene expression.

In earlier studies, we have demonstrated that hCAT-1 expression increases significantly after 6 h on RNA level in stimulated primary human T lymphocytes. Under arginine starvation, we detected persistent hCAT-1 mRNA induction as well as hyperphosphorylation of the protein kinase B (PKB)/Akt in activated T lymphocytes. We could show a complete inhibition of hCAT-1 induction by inhibiting Akt, while pharmacological inhibition of mammalian target of rapamycin C1 (mTORC1) or ERK-Mnk pathway had no significant effect on hCAT-1 induction. We thus hypothesized that phosphorylation of Akt and resultant activation of the kinase probably plays a central role in regulation of hCAT-1 expression.

In further experiments, we addressed the potential role of upstream and downstream elements of Akt in the activation cascade leading to hCAT-1. Using the inhibitors wortmannin or Ly294002, we identified phosphatidylinositol-3-kinase (PI3K) as an activator of Akt under L-arginine starvation. Furthermore, we analyzed the protein expression and phosphorylation of the downstream transcription factors forkhead box protein O (FoxO) 1, 3a and 4. We observed a strong increase of phosphorylation of FoxO1 (Thr24) and FoxO3a (Thr32) already after 1 h of stimulation, before hCAT-1 mRNA induction was detected. At later time points, FoxO phosphorylation—like Akt phosphorylation—persisted specifically upon T cell stimulation in the absence of arginine. Akt inhibition suppressed FoxO phosphorylation at all time points and led to an increased expression of FoxO1 and FoxO3 protein after 24 h of stimulation. These results are in agreement with a causal involvement of FoxO transcription factors in the regulation of hCAT-1 gene expression. Based on these data we currently analyze the promoter elements which are involved in the transcriptional regulation of hCAT-1.

Pathophysiological role of the thyroid hormone transporters Mct8 and Oatp1c1

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Thyroid hormone (TH) actions and metabolism are intracellular events that require the transport of TH across the plasma membrane. This process is facilitated by TH transporters of which the monocarboxylate transporter 8 (MCT8), encoded by the Slc16a2 gene, has been most intensively analyzed. In humans, inactivating mutations in the X-linked *MCT8* gene are associated with a severe form of psychomotor retardation in combination with abnormal serum TH parameters. The clinical picture (also known as Allan–Herndon–Dudley syndrome) clearly underscores the significance of MCT8 for proper brain development as well as normal TH metabolism and signaling. In mice, however, Mct8 deficiency does not grossly affect brain development, whereas the endocrine abnormalities of the patients are fully replicated.

Our studies revealed that in the mouse CNS, another TH transporter is present that can partially compensate for the absence of Mct8. Whereas Mct8 plays a prominent role in facilitating the uptake of the active hormone T3 into the brain, the organic anion transporting peptide Oatp1c1 (Slco1c1) mediates the transport of T4 across the blood–brain barrier. Consequently, mice deficient in both transporters (Mct8/Oatp1c1 dko mice) exhibit a pronounced hypothyroid situation in the CNS, whereas peripheral organs are in a thyrotoxic state.

A first phenotypic description of Mct8/Oatp1c1 dko mice revealed distinct deficits in neuronal differentiation as well as pronounced locomotor deficiencies. The latter phenotype may be explained by a reduced myelination, a retarded cerebellar development and cortical as well as striatal abnormalities. We also studied Mct8 and Oatp1c1 expression in skeletal muscle tissue and found that both transporters strongly expressed in activated satellite cells. Moreover, Mct8/Oatp1c1-deficient muscle tissues showed in vivo as well as in vitro decreased regeneration capacity upon injury. These data point to a hitherto unknown role of Mct8 and Oatp1c1 in timed muscle stem cell differentiation. In addition, our results suggest a possible contribution of an altered myogenic repair program to the neuromuscular abnormalities in muscle injury-prone, quadriplegic MCT8 patients.

The impact of phosphorylation site mutations on human amino acid uniporter LAT4 expression, localization and function in *Xenopus laevis* oocytes

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System L–amino acid transporter LAT4 (SLC43A2) is a sodium-independent uniporter that transports branched chain and few other essential amino acids. It localizes to the basolateral membrane of kidney epithelial cells in the distal tubule, collecting duct, proximal tubule and thick ascending limb of Henle's loop and also to small intestine epithelial cells and several other tissues. Proteomic studies indicate several possible phosphorylation sites on LAT4. We hypothesize that LAT4 surface expression and/or function might be regulated by phosphorylation.

To test this hypothesis, we replaced the serine residues S274, S274 + S278 and S297 of human LAT4 with alanine (A) or glutamic acid (E) to mimic non-phosphorylated and phosphorylated states, respectively, and expressed these mutants in *X. laevis* oocytes. Western blot analysis indicated a reduced overall expression level for all mutants, especially for alanine ones. Immunofluorescence experiments showed changes in subcellular localization—compared to wild-type hLAT4, all glutamic acid mutants appeared to localize more intracellularly. Alanine mutants localized mostly near or at the oocyte surface. Measurements of phenylalanine uptake indicated that the apparent affinity of the glutamate mutants was unchanged, but their maximal transport velocity (V_{max}) was reduced. Both single alanine mutations, S274A and S297A, appeared to slightly increase the apparent affinity

of the transporter. However, S297A mutation reduced V_{max}, whereas S274A did not impact on V_{max}. The double mutant S274A + S278A displayed the most similar kinetics to the wild-type hLAT4.

These results suggest that the abundance, surface localization and transport kinetics of hLAT4 might be regulated by phosphorylation.

Short-term regulation of amino acid transporters by amino acids

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To ensure body homeostasis, amino acids (AA) are (re)absorbed by epithelial cells of the intestinal mucosa and renal proximal tubules. This sequential transepithelial transport includes an apical uptake followed by a basolateral efflux. The luminal uptake is mediated by different AA transporters, but most neutral AAs are transported by the luminal Broad neutral Amino acid Transporter B^0AT1 (SLC6A19).

The potential short-term regulation of this transporter by its substrates has not been investigated. We hypothesized that the expression of B^0AT1 might be repressed if costs of its synthesis and expression exceed its benefits and thus it would be upregulated by AAs to absorb them efficiently when available. To investigate the possible short-term regulation of B^0AT1 by AAs in vivo, we assessed in rats whether after a fast of 4 or 16 h, intragastric AA application upregulates B^0AT1 expression in intestinal brush border membranes. The time point of analysis after AA gavage was determined based on microcomputer tomography measurements showing when the AA mix reached the small intestine. However, functional analysis of AA absorption in isolated ex vivo intestinal rings showed no significant difference between rats having received a water or an AA gavage. In addition, protein expression levels of B^0AT1 in the intestinal brush border did not reveal a difference between rats having received a water or AA gavage. In conclusion, in the selected experimental conditions, the expression and function of the apical AA transporter B^0AT1 was not regulated in the short term by the preceding application of its substrate AAs.

Two amino acid transporters are responsible for amino acid homeostasis and reproduction in PEPT1-deficient *Caenorhabditis elegans*

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Intestinal protein degradation releases short chain peptides and free amino acids. Absorption of amino acids occurs in a group-specific manner by various transporters, whereas the absorption of di- and tripeptides is mediated by the peptide transporter PEPT1. In *Caenorhabditis elegans* a loss of PEPT1 impairs growth and post-embryonal development and reduces reproduction, the latter can partially be rescued by supplementation with a mixture of all proteinogenic amino acids. We here describe studies assessing which amino acids are crucial for the reproduction deficit. Silencing of individual genes such as AAT-6 or Y4C6B.2 encoding amino acid transporters induced no major phenotypic alterations in wild-type *C. elegans*. In worms lacking PEPT1, the mRNÁs coding for heteromeric (HAT) and proton-dependent amino acid transporters (PAT) are highly expressed. In particular, the HAT light subunit homologue AAT-6 and the PAT homologue Y4C6B.2 were found to be of highest importance in amino acid homeostasis of *pept-1 C. elegans*, as their loss caused sterility and impaired larval growth. These phenotypic changes could not be compensated by supplementing free amino acids, emphasizing the crucial functions with the distinct substrate specificity of both transporter proteins in intestinal amino acid absorption.

Regulation and physiological function of cationic amino acid transporters

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Cationic amino acids (CAA), such as arginine, lysine, and ornithine, share the same transport proteins. Most mammalian cells express two types of CAA transport proteins, mediating activities of the so-called systems y^+ (specific for CAA) and y^+L (CAA and neutral AA), respectively. Although only distantly related, the two transporter types belong to the same gene family: SLC7. The family members SLC7A1–A3 correspond to the CAT proteins (CAT for CAA transporter) mediating system y^+ activity. They seem to be the major entry path for CAA in most cells. SLC7A4 and A14 have also been attributed to the CAT subfamily. Their function remains, however, elusive to date. Besides providing cells with CAA for protein synthesis and energy supply, CATs seem to be involved in important signal pathways such as nitric oxide, mTor and neurotransmission. CAT-1 and CAT-2 are extensively regulated on the level of both, transcription and translation. In addition, protein kinase C and small G proteins regulate the location of these transporters in the plasma membrane. SLC7A6 and 7 encode for system y^+L transporter y^+LAT2 and 1, respectively. They catalyze the exchange of CAA against NAA plus Na^+ and thus seem to be rather CAA exporters under physiological conditions. One of their functions in endothelial cells may be the export of CAA derivatives such as the nitric oxide synthase (NOS) inhibitor asymmetrical dimethyl arginine (ADMA). ADMA is an independent risk factor for cardiovascular disease. There is increasing evidence that accumulation of ADMA leads to endothelial dysfunction through inhibition of endothelial NOS. Reduced activity of y^+LATs may thus contribute to endothelial dysfunction.

Arginine transport in human primary granulocytes

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Polymorphonuclear cells (PMNs) are the crucial first-line defense combating microbes by phagocytosis and with a variety of oxidative and nonoxidative effector mechanisms. In addition, PMNs also serve as immunoregulatory cells interacting with other immune cells to coordinate a proper immune response. One central, potentially immunosuppressive effector molecule constitutively expressed in human PMNs is the arginine hydrolyzing enzyme arginase I, located within granules. Local or systemic arginine deficiency mediated by degranulation of PMN is often encountered in cancer patients or during chronic inflammation and can profoundly suppress the adaptive immune response by inhibiting T cell proliferation and cytokine synthesis. In contrast, arginine depletion may also have an anti-cancer effect when tumor cells are unable to resynthesize their own arginine. Interestingly, absence of extracellular arginine has no influence on

PMN viability and key effector functions. We thus asked if primary human granulocytes take up arginine at all. In unstimulated cells, we monitored arginine uptake both by HPLC and using radioactive tracers. Among the amino acid transporters that accept arginine as a substrate, we found low mRNA expression for cationic amino acid transporter 1 (CAT-1), y^+LAT1, $b^{0,+}$AT and $ATB^{0,+}$ in these cells. CAT-1 expression was upregulated when the cells were stimulated with lipopolysaccharide and interferon γ. We are now determining the ratio of intracellular arginine to the arginase product ornithine to find out if intracellular arginine is hydrolysed by arginase or if it is protected from arginase situated in granules by its cytoplasmatic location. These data will provide important information if even intact PMNs may deplete extracellular arginine under certain situations.

Regulation of mTOR by nutrient availability: the role of SLC38A9 in the lysosomal amino acid sensing machinery

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Cell growth and proliferation are tightly linked to nutrient availability. The mechanistic target of rapamycin complex 1 (mTORC1) integrates the presence of growth factors, energy levels, glucose and amino acids to modulate metabolic status and cellular responses. Amino acids are essential for mTORC1 activity, as growth factors cannot efficiently activate mTOR in their absence. mTORC1 is activated at the surface of lysosomes by the RAG GTPases and the Ragulator complex through a not fully understood mechanism monitoring amino acid availability and involving the vacuolar H^+-ATPase. Here, we describe the uncharacterized member 9 of the solute carrier family 38 (SLC38A9) as a lysosomal membrane-resident protein competent in amino acid transport. Extensive functional proteomic analysis established SLC38A9 as an integral part of the Ragulator/RAG GTPases machinery. Binding to this complex occurs through the cytoplasmic domain of SLC38A9 and is sensitive to amino acid levels. Gain of SLC38A9 function rendered cells resistant to amino acid withdrawal, while loss of SLC38A9 expression impaired amino acid-induced mTORC1 activation. Thus, SLC38A9 is a physical and functional component of the amino acid sensing machinery that controls the activation of mTOR. Considering the physical and amino acid-sensitive association of SLC38A9 with the Ragulator/RAG GTPase complex, we propose that SLC38A9 can act as a transceptor in which amino acid engagement is used for allosteric signal transduction rather than mere transport.

Regulation of amino acid uptake coordinates adaptive cancer cell metabolism

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Solid tumours activate angiogenic signals to ensure an adequate blood supply. In parallel, amino acid transporters on the cell surface are also increased so as to provide nutrients for the higher metabolic and growth demands of cancers. We are studying a number of amino acid transporters, including L-type amino acid transporters (LAT1 and LAT3) and alanine-serine-cysteine transporter 2 (ASCT2) that mediate uptake of essential amino acids including leucine and glutamine. Leucine and glutamine are critical for the activity of mTORC1, which regulates protein translation and cell growth, as well as contributing to cellular energy and as carbon and nitrogen donors. Therefore, increased amino acid transport in cancer permits mTORC1 signaling and promotes unrestrained cellular proliferation.

Our results have demonstrated that prostate cancer cells coordinate the expression of LAT1 and LAT3 during disease progression, thereby maintaining sufficient leucine to promote mTORC1 signaling and cell growth. Similarly, we have shown that both LAT1 and ASCT2 are highly upregulated in melanoma. Unlike prostate cancer cells, melanoma cells are tolerant to leucine deprivation, while exhibiting sensitivity to glutamine deprivation through ASCT2 chemical or shRNA-mediated inhibition. Interestingly, our data also show that prostate cancer cells are sensitive to both leucine and glutamine deprivation.

In summary, cancer cells respond to the demand for amino acids through integrated pathways that lead to increased amino acid transporter expression and cell growth. Furthermore, ASCT2, LAT3 and LAT1 may provide novel therapeutic targets in early- and late-stage prostate cancer as well as other solid tumours such as melanoma.

Tamoxifen-Cre induced knockout of the LAT1 (Slc7a5) amino acid exchanger in adult mice promotes stress signalling associated with reduction in the mass of peripheral tissues

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This study investigates the importance of large neutral amino acid (LNAA) delivery by LAT1 for control of mammalian tissue growth in vivo through its relationship to activation of the mTORC1 signalling pathway.

We bypassed the difficulty of LAT1-/- embryonic lethality by using tamoxifen-inducible LAT1 deletion in adult mice (Rosa26-CreERT2). This procedure produced >90 % reduction in LAT1 mRNA expression in peripheral tissues (spleen, lung, skeletal muscle, adipose) and a progressive loss of ~17 % body weight over 10 days (food intake unchanged). Reduced mass of skeletal muscle (~10 %) and white adipose tissue (~70 % in abdominal depots) contributed substantially to this weight loss. Stress proteins ATF4 and REDD1/2 (negative regulators of mTORC1 signalling) were abnormally upregulated in tamoxifen-induced $LAT1^{-/-}$ skeletal muscle (gastrocnemius) and adipose tissue, although only marginal reductions in basal mTORC1-S6K signal (p-Thr^{389} S6K) were detected.

To evaluate the role of mTORC1 in animal response to changes in amino acid availability, the effects of fasting (8 h overnight) or leucine injection (40 μg/g *I.P.*) were investigated. LAT1 deletion impaired activation of the mTORC1 growth signalling axis in fat tissue after leucine injection. Surprisingly, fasting resulted in relative retention of intramuscular LNAA in $LAT1^{-/-}$ compared to +/+genotype revealing a role for LAT1 in LNAA efflux from tissues (notably when plasma LNAA concentrations decrease acutely).

Our results indicate that LAT1 is a necessary component of mechanisms linking AA availability and maintenance/growth of mammalian peripheral tissues in vivo and LAT1 has unexpected importance for maintenance of body fat mass.

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Organic cation/carnitine transporter OCTN2 is indirectly regulated by protein kinase C affecting transporter interacting partners

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L-Carnitine, a hydroxyl amino acid with a quaternary amino group, plays an important physiological role in transfer of acyl moieties to mitochondria for their oxidation. Carnitine is transported to the cell by an amino acid transporter $B^{0,+}$ (SLC6A14) and organic cation/carnitine transporter OCTN2 (SLC22A5). We showed increased OCTN2 surface presence upon activation of protein kinase C (PKC); however, OCTN2 itself was not phosphorylated. The present study was aimed at finding a link between PKC activation and OCTN2 regulation, focused on OCTN2 interacting partners in astrocytes. Out of OCTN2 proteome (156 proteins) identified by mass spectrometry, we selected proteins involved in signaling pathways and proteins containing a PDZ motif. OCTN2 co-precipitates with protein phosphatase PP2A catalytic and structural subunits, as well as with its regulatory subunits: striatin and SG2NA. The amount of co-precipitating SG2NA decreased after PKC activation. Since OCTN2 and SG2NA co-localize in vesicular structures in the cytoplasm, we postulate that PP2A arrests OCTN2 in dephosphorylated state in cytoplasm, while SG2NA release facilitates transporter trafficking to the plasma membrane. Moreover, upon PKC activation, OCTN2 co-precipitates with *zonula occludens* ZO-1 protein, phosphorylated under the same conditions. Deletion of a PDZ-binding domain of *octn2* heterologously expressed in HEK293 cells did not affect transporter presence in plasma membrane. Our results indicate a possibility of OCTN2 regulation by other proteins phosphorylated by PKC, out of which PP2A and ZO-1 are novel interacting partners of the transporter.

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Generation of high titer antisera against integral membrane proteins

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Amino acid transporters are integral membrane proteins that often exhibit few antigenic epitopes. Standard immunization procedures using short peptide sequences often fail to raise utilizable antibodies against these proteins. Previously, we have been successful in generating antibodies against different members of the SLC7 family by immunization with fusion proteins between *E. coli* tryptophane E (trpE) and the C-terminus of the respective transporter. These antisera had however only a low titer and needed to be affinity purified to detect endogenously expressed transporters.

In a new approach, we immunized rabbits twice with fusion proteins between glutathione S-transferases (GSTs) and the C-terminus of human cationic amino acid transporter 1 (hCAT-1), expressed in *E. coli*, followed by six boosts with a synthetic peptide containing the entire C-terminus. This resulted in an antiserum that can be used in 1:10,000 dilution to detect endogenous hCAT-1 with high sensitivity. Encouraged by this result, we used a similar strategy for three other SLC7 members, giving rise to similar high titer antisera. Immunization with a long peptide alone was successful in one, but not in two other cases. Because of the high costs of immunization programs, we were not able to test systematically if initial immunization with a GST fusion containing the C-terminal peptide followed by immunization with the same C-terminal peptide is in fact advantageous over using either the GST fusion protein or the long C-terminal peptide for all immunizations, respectively. However, our results show that our immunization scheme has a high probability to produce high titer antisera against transporter proteins.

Impact of uninephrectomy on body L-arginine homeostasis and blood pressure control in wild-type and arginase II knockout mice

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L-Arginine plays an important role as precursor for a variety of physiologically important substances including citrulline, ornithine and nitric oxide. The kidney is a major site for L-arginine metabolism. Uninephrectomy (UNX) is observed to cause an increase in the size of the remnant kidney and to some degree compensation of the glomerular filtration rate. We used UNX or sham-operated C57B/6 female and male mice to test the hypothesis that renal mass reduction impacts arginine metabolism and possibly thereby affects blood pressure control.

Our results show that mice that underwent UNX displayed an increased systolic blood pressure. This effect was more pronounced in females than males. Plasma levels of asymmetric dimethyl arginine (ADMA), an inhibitor of NOS considered to be a good marker for renal disease, were increased in UNX animals, whereas the level of none of the proteinogenic amino acids was changed significantly. There were also no changes in the mRNA expression levels of arginine transporters and enzymes involved in arginine metabolism. Our observations suggest that UNX affects blood pressure and the effects are less pronounced in males, possibly due to a more important remnant kidney compensatory growth.

In addition to experiments in wild-type mice, we have also recently performed experiments in arginase II knock-out mice. Arginase II plays a role in maintaining arginine homeostasis and is involved in the urea cycle where it breaks down arginine to ornithine. Experiments to measure blood pressure show that these mice do not show an increase in blood pressure post-UNX.

FRET sensors to investigate amino acid transport

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The concentration of free amino acids in cells and in the extracellular space (milieu interieur) depends on their transport across plasma membranes and their metabolism. To measure intracellular amino acid concentration changes in real time, we used genetically encoded FRET-based biosensors for fluorescent imaging. Specifically we have

improved an L-tryptophan (Trp) biosensor and developed a new sensor for L-arginine (Arg).

Using the Trp biosensor, we tested the import of this essential amino acid in HeLa cells. Upon stimulation with interferon γ, these cancer cells express intracellular indole 2,3-dioxygenase (IDO) and have been suggested to express an additional transport machinery besides system L to allow Trp uptake even at low extracellular concentration and in the presence of competing amino acids. Using this sensor, we have identified an interferon γ-induced gene product that together with an endogenous amino acid transporter enables HeLa cells to take up Trp even in the presence of an excess of the system L competitive inhibitor BCH. It is proposed that the competition-resistant Trp transport allows cancer cells to deplete extracellular Trp and thereby to escape immune response. A novel Arg FRET biosensor was constructed by placing the Arg binding protein encoded by the *ahrC* gene from *Bacillus subtilis* between the fluorescent proteins CPF and YFP. Using this sensor we visualized intracellular Arg concentration changes mediated by different CAT transporters (SLC7A1-4) expressed in HEK293T cells. We thereby showed the differential transport properties of these uniporters, the absence of major competition from methylated Arg forms and the impact of the membrane potential on Arg transport and steady state intracellular concentration. Taken together, genetically encoded amino acid FRET-based sensors can be used to monitor intracellular amino acid concentration changes in real time and thereby characterize and/or identify amino acid transporters in cultured cells and potentially also in vivo.

Amino acid transporters and enzymes involved in glutathione synthesis are altered in pancreatic acinar cells during acute pancreatitis

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Acute pancreatitis (AP) is a serious inflammatory disease of the exocrine pancreas. During its pathogenesis, reactive oxygen species (ROS) have deleterious effects and they can be neutralised by glutathione (GSH), an intracellular antioxidant. GSH can be synthesised from glutamate, glycine and cysteine, but amino acid transport into acinar cells remains poorly understood. It is our aim to characterise the transport of glycine, glutamate and cysteine (as well as their precursors glutamine and cystine) for the production of GSH during AP in pancreatic acinar cells. Therefore, AP was induced in C57BL6/J mice using supraphysiological doses of cerulein and acinar cells isolated after 12, 36 and 72 h.

Glutamate, glycine and cysteine concentrations are rapidly depleted in acinar cells during AP. Concurrently, gene expression levels of enzymes necessary for GSH synthesis are increased. Levels of free GSH are initially diminished, but they are increased during the regeneration phase. Gene expression levels of the acinar glutamine transporters (LAT1 (slc7a5), LAT2 (slc7a8), SNAT2 (slc38a2), SNAT3 (slc38a3) and SNAT5 (slc38a5) as well as the glycine transporter GlyT1 (slc6a9) are impaired during AP, while the cystine/glutamate exchanger xCT (slc7a11) is increased during the early phase. Immunofluorescence analysis revealed xCT expression on pancreatic duct cells in the healthy state, whereas xCT is also expressed on acinar cells during AP. Our data suggest that acinar cells adapt amino acid transport and enzymes necessary for GSH synthesis during AP. Specifically, xCT expression is up-regulated in acinar cells, presumably to enhance cystine import, the rate-limiting factor of GSH synthesis.

Cooperation of basolateral epithelial amino acid transporters TAT1 and LAT2 investigated in a double knockout mouse model

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Basolateral efflux is a crucial step for amino acid (AA) (re)absorption across the small intestine and kidney proximal tubule epithelia mediated by various transporters. There are uniporters that mediate the facilitated diffusion of essential AAs, as does for instance aromatic AA transporter TAT1 (Slc16a10) and also antiporters such as LAT2-4F2hc (SLC7A8–SLC3A2) that exchanges neutral AAs. To test the hypothesis that the recycling of aromatic AAs via TAT1 allows the vectorial efflux of other AAs via obligatory exchanger LAT2-4F2hc, LAT2$^{-/-}$ TAT1$^{-/-}$ double knockout (dKO) mice were generated. These mice have a reduced body weight (~ -20 % at 3 months), but no other overt phenotypic alteration. Under normal protein diet, they excrete in the urine, compared to LAT2$^{-/-}$ and TAT1$^{-/-}$ knockout mice, higher amounts of aromatic AAs and of some other AAs that are not substrates of TAT1. The amino aciduria was further increased under high protein diet and involved all proteinogenic AAs and some charged ones. Preliminary transport experiments with small intestine gut sacs confirm the alteration of transepithelial transport. These observations support the hypothesis that the basolateral efflux of LAT2 substrates depends on the recycling of aromatic AAs via TAT1.

Fast organo-cation chromatography of amino acids, nucleobases and nucleosides, and oligopeptides using a novel polyfunctional low-capacity cation-exchange column

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It is scientifically important and significant to directly separate amino acids in various matrices without any pre-column derivatization. This paper presents fast and precise liquid chromatography methods for the analyses of biogenic cations, such as amino acids, nucleobases and nucleosides, and oligopeptides, using a novel polyfunctional cation-exchange column (75 mm × 4.6 mm I.D.) packed with a low-capacity sulfo-butylated macroreticular poly(styrene–divinylbenzene) co-polymer (a commercially available packing material for GPC) [1].

The binary gradient HPLC system consisted of a Shimadzu (Kyoto, Japan) DGU-20A3 degasser, two LC-20AD solvent delivery pumps with a static mixer, a CTO-20A column oven equipped with a Rheodyne (Cotati, CA) 7725i syringe-loading sample injector, an SPD-20A UV–VIS spectrophotometric detector, and a Waters (Milford, MA) 474 scanning fluorescence detector. The chromatographic data were acquired and processed using a Runtime Instruments (Sagamihara, Japan) Chromato-Pro, a set of interfacing device and Windows® software.

In the chromatography of amino acids with post-column OPA/N-acetyl-L-cysteine fluorescence detection, the gradient elution was

optimized by programing the delivery rations of two liquids: (A) 2 mM H_3PO_4 and (B) 20 mM NaH_2PO_4/10 % (v/v) CH_3CN. The column temperature was 50 °C and the flow rate was 0.5 mL/min. Twenty-two underivatized amino acids were separated in 20 min with resolutions more than $R = 1.0$ of the critical peak pair observed in a standard chromatogram. The chromatographic cycle time was approximately 25 min. The column regeneration time was considerably reduced compared to those required in the traditional cation-exchange amino acid analyzers. This is probably owing to the low ion-exchange capacity and may be a technically big merit. Several validation data obtained were very good and led to the method significance. The intra-day and inter-day reproducibility of retention times and area intensity were within acceptable levels with RSDs less than 1 and 6 %, respectively. The linear calibration lines ranged between 0.2 and 100 μmol/L (20-μL injections) with determination coefficients (R^2) between 1.000 and 0.9875. The method was applied to the determination of amino acids in foods such as fruits and wines. When using a UV 210-nm detection, the method was applicable to the determination of urinary metabolites such as creatinine and phenylalanine, a diagnostic marker of PKU (an inborn error of amino acid metabolism). In the chromatography of nucleic acid-related compounds with UV detection at 260 nm, the gradient elution using (A) 2 mM H_3PO_4/1 % (v/v) CH_3CN and (B) 20 mM NaH_2PO_4/10 % (v/v) CH_3CN was optimized. Seven nucleobases and their corresponding seven nucleosides were separated in 21 min with good resolutions. The excellent reproducibility data were also obtained for retention times less than 0.8 % RSDs and integration intensities less than 1.3 % RSDs; and linear regression data between 2 and 100 μmol/L were obtained with R^2 more than 0.9990. The method was applicable to the determination of purine and pyrimidine ingredients in beer and beer-like beverages. In addition to this, the chromatograms of origopeptides such as enkephalins and angiotensins will appear on the poster board.

1. Yokoyama Y, Yokokawa A, Niguchi K, Tanabe T (2013) Polyfunctional low-capacity cation-exchange packing material for the separation of underivatized amino acids. Talanta 103: 245–251.

Use of multi-response optimization for the separation of tryptophan and fifteen related compounds by ultrahigh performance liquid chromatography (UPLC)

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A Box Behnken design (BBD) in conjunction with multiple response optimizations was performed to develop the simultaneous separation of tryptophan and 15 related compounds in rice grains by ultrahigh performance liquid chromatography (UPLC) coupled with photodiode array (PDA) detector. The gradient compositions of mobile phase and flow rate were optimized with respect to the resolutions of severely overlapping chromatographic peaks between the phenolic compounds in addition to the total analysis time. Furthermore, two different stationary phases were also evaluated (Cortex C_{18} and BEH C_{18}). A gradient program of only 4 min analysis run using Cortex C18 was found to provide the best resolution for the simultaneous separation of the 16 studied compounds. The method was validated resulting in an adequate sensitivity in the range of 1–100 μg L-1 ($R^2 = 0.9993$–0.9999) with high precisions (CV lower than 2 % on average for both repeatability and intermediate precisions). The suitability of the method was checked by application to the analysis of pigmented and non-pigmented rice samples. Tryptophan and several related compounds were identified and quantified in the tested samples.

Keywords: Tryptophan, Rice, UPLC, Box Behnken design

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Novel analytical detection system suitable for detection of neuronal-specific biomarkers in femto–atto gram level in different body fluids

Classical methods of gene product analysis such as binding assays (e.g. ELISA, Protein chip technology, etc.) are generally time-consuming, laboratory intensive, less sensitive and lack high-throughput capacity. In addition, all existing methods used to measure proteins necessitate multiple divisions of the original sample and individual tests carried out for each substance, with an associated cost for each test. Together with a small biotech company, we developed a novel analytical detection system based on chip technologies. Our system facilitates the development of multiplexed assays that simultaneously measure many different analytes such as selected biomarker panels in a small sample volume at the same time with a much higher sensitivity compared to common binding assay systems. Recent advances of the application of our novel detection system in combination of the binding assay technologies (biochip assay) combine the power of miniaturization, micro fluidics, higher sensitivity and faster quantification. The power of our technology offers great promise for point-of-care clinical testing and monitoring of many important analytes such as neuronal-specific biomarkers in femto to atto gram level in different human body fluids such as CSF, blood, serum or plasma.

Highly efficient chiral resolution of DL-arginine and DL-phenylalanine by cocrystal formation followed by recrystallization under preferential enrichment conditions

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Abstract: In 1993, we discovered a unique, symmetry-breaking spontaneous enantiomeric resolution phenomenon for a certain kind of organic racemic crystals, which does not need any external chiral element, and thereafter referred to this phenomenon as "preferential enrichment (PE)". The mechanism of PE has been elucidated by studies using the first-generation chiral organic compounds (onium sulfonate salts) showing PE, namely, (1) by X-ray crystallographic, powder X-ray diffraction and thermal analyses of the deposited crystals, (2) by temperature-controlled video microscopic and in situ ATR-IR spectroscopic studies on the crystallization process, and (3) by molecular weight measurement using vapor pressure osmometry and molecular dynamic simulations concerning preferential homochiral or heterochiral supramolecular structures in solution, PE has been revealed to consist of several successive processes in which the pivotal process involves the solvent-assisted solid-to-solid transformation of an incipient metastable polymorphic form into a thermodynamically

more stable one during crystallization from the highly supersaturated (five to tenfold) solution of the racemic crystals. Recently, we have demonstrated that PE is applicable to common racemic crystals such as amino acids and drugs if the five requirements based on this mechanism are satisfied. In this presentation, I talk about (1) the concept, properties, category, history, mechanism and requirements with respect to PE, (2) the thermodynamic origin of spontaneous resolution in PE, and (3) the practical application of PE to the racemic two-component cocrystals of arginine or phenylalanine with achiral fumaric acid; PE was not feasible for both free arginine and phenylalanine.

TG2: a credible therapeutic target in fibrotic disease due to its multifunctional roles

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The role of TG2 in fibrosis is reported to be related to two important effects. The first in mediating the deposition and accumulation of the fibrotic extracellular matrix (ECM) via its cross-linking of proteins like fibronectin, collagen I and collagen III; and the second, the activation of latent matrix bound TGFβ1. We report here that the role of TG2 in fibrosis progression can be much more complex.

We also report a new family of TG2-specific inhibitors that can not only inhibit protein cross-linking, but also regulate other functions of TG2, thus increasing their potency which can be demonstrated by their effectiveness in inhibiting fibrosis in two different fibrotic in vivo models.

Significance of evolutionary changes in human transglutaminases

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Transglutaminases catalyze the posttranslational modification of proteins by transamidation of available glutamine residues. Transglutaminase 2 (TGM2) is a unique protein of the eight-member family with multiple enzymatic activities and interacting partners, but its full physiological and pathological role is far from understood. We collected data from large public datasets and found that TGM2 non-synonymus SNVs are rare compared to other transglutaminase family members, they coincide with intrinsically disordered regions and short linear motifs, many of them are highly destabilizing in both open and closed conformations of the enzyme and have high probability of causing recessive diseases. Comparative genomics study between mouse and human as well as primate sequences of transglutaminase family members revealed the appearance of a short stretch of amino acid changes in humans and primates. These linear sequences are mainly in regions of TGM2 outside of so far identified functional sites, suggesting gain of function during evolution to humans. By using bioinformatic and structural analytic tools, we have revealed potential influence of these amino acid clusters on protein stability, secondary structure, short linear motifs, IDRs, phosphorylation and N-glycosylation sites. These data might shed new light on the functional differences between transglutaminases of these species and their relevance to human physiology and pathology.

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A new fluorescence anisotropy-based assay for activity determination of tissue transglutaminase

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Considerable evidence for the implication of tissue transglutaminase (TGase 2) in a variety of pathological processes, such as neurodegenerative diseases, disorders related to autoimmunity and inflammation as well as tumor progression, has been revealed over the recent years. This renders TGase 2 attractive for developing agents which allow the enzyme's targeting for both therapeutic and imaging purposes. The development of such molecules requires the establishment of reliable methods to assess the interaction with TGase 2, which can be done most conveniently in continuous kinetic assays.

Several assays have been published over the last decades to determine TGase 2 activity, with only very few using the method of fluorescence anisotropy. Measurement of fluorescence anisotropy offers a better signal to noise ratio than other techniques, such as those based solely on fluorescence emission and does not need washing or separation of unbound fluorescent substance.

Here, we report a fluorescence anisotropy-based approach for the determination of TGase 2's transamidase activity, established and validated by using fluorescein- and rhodamine B-labeled cadaverines as acyl acceptor substrates. The synthesis of the cadaverine derivatives has been accomplished in a solid-phase approach. To allow efficient conjugation of the rhodamine B moiety, different linkers providing secondary amine functions have been introduced between the cadaverine and xanthenyl entities.

The increase in fluorescence anisotropy resulting from covalent binding of the relatively small cadaverine derivatives to the much larger acyl donor substrate *N*,*N*-dimethylated casein was followed over time and enzyme activities were derived thereof. The assay was found to be highly reproducible and shows no background signal in the absence of the enzyme for all synthesized cadaverine derivatives. After characterization of the enzyme–substrate interaction by determination of the Michaelis constants, K_m, and the maximum velocities of substrate conversion, V_{max}, the assay was validated for screening of non-covalent and covalent inhibitors by using the literature-known substances GTP and iodoacetamide, respectively, as well as a recently reported L-lysine acrylamide derivative.

Synthesis, ^{18}F-labeling and radiopharmacological characterization of a 30mer peptide as potential radiotracer for PET imaging of claudin-4

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The cell surface receptor claudin-4 (Cld-4) represents a single-chain protein containing four transmembrane domains and constitutes cell–

cell contacts of the tight-junction type by engaging in homophilic interactions. Cld-4 is upregulated in various tumors and represents a promising target for both diagnosis and treatment of solid tumors of epithelial origin. Therefore, the development of agents that allow imaging of Cld-4 in vivo such as ^{18}F-labeled compounds for positron emission tomography (PET) appears to be attractive. A suitable ligand to target Cld-4 in vivo seems to be the C-terminal peptidic fragment of the C-terminal domain of the *Clostridium perfringens* enterotoxin cCPE(290-319). This fragment is of 30 amino acids in length and has the sequence SLDAGQYVLVMKANSSYSGNYPYSILFQKF corresponding to positions 290-319 of cCPE.

The synthesis of cCPE(290–319) and analogues derived thereof, such as N-terminally modified derivatives (fluorobenzoylated and FITC-conjugated) and variants in which critical amino acids (Tyr 306 and Leu 315) have been replaced, was envisaged to be accomplished by solid-phase peptide synthesis (SPPS). Among several approaches, sequential SPPS using three pseudoproline-dipeptide building blocks revealed to be the most efficient one to afford cCPE(290–319) and its derivatives. The affinity of the furnished peptides to a soluble protein construct that contains both extracellular loops of Cld-4 was studied by surface plasmon resonance (SPR), which allowed determining a K_d value of 1.4 μM for the N-terminally fluorobenzoylated cCPE(290-319). Labeling of cCPE(290–319) with fluorine-18 was achieved on solid phase using *N*-succinimidyl-4-[^{18}F]fluorobenzoate ([^{18}F]SFB) and 4-[^{18}F]fluorobenzoyl chloride as ^{18}F-acylating agents. Most advantageous results were obtained when [^{18}F]SFB was reacted with resin-bound cCPE(290–319) containing an N-terminal 6-aminohexanoic spacer. Stability assays in cell supernatants and plasma indicated no degradation of the resulting radiotracer in these physiological media. Cell binding of ^{18}F-labeled cCPE(290–319) was investigated using the HT29, A375 and A431 tumor cell lines. Time-dependent binding of the radiolabeled peptide to the Cld-4-positive A375 and A431 cells was observed, which was stronger than for the Cld-4-negative HT29 cell line. These findings are in accordance with results of confocal microscopy studies using FITC-conjugated cCPE(290–319) and A431 cells. The in vivo behavior of ^{18}F-labeled cCPE(290–319) was studied in NMRI nu/nu mice and Wistar rats by dynamic PET imaging and radiometabolite analyses, respectively. These investigations have shown that ^{18}F-labeled cCPE(290–319) is subject to substantial liver uptake and rapid metabolic degradation in vivo.

In conclusion, the synthesis and ^{18}F-labeling of cCPE(290-319) were successfully established. Its binding to Cld-4 in vitro and *in cellulo* has been demonstrated. Initial radiopharmacological studies suggest the limited suitability of this peptide in its current non-stabilized form to target Cld-4 in vivo.

Solid-phase synthesis of selectively mono-fluorobenzoylated polyamines for targeting of transglutaminases and polyamine transporters in tumours

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Transglutaminases and polyamine transporters are promising targets for functional imaging of tumours. Therefore, our aim is to synthesise polyamine-based radiotracers that allow the in vivo imaging of the aforementioned targets by positron emission tomography (PET). Labelling with the radionuclide fluorine-18 can be accomplished via attaching a [^{18}F]fluorobenzoyl group with the prosthetic labelling reagent *N*-succinimidyl-4-[^{18}F]fluorobenzoate ([^{18}F]SFB). To access the required non- radioactive analogues, a solid-phase synthesis was developed that enables selective fluorobenzoylation at distinct amino groups of various polyamines (e.g. cadaverine, spermidine, spermine) on the basis of a recently described synthetic concept for the selective functionalisation of polyamines. The established route can be directly applied to synthesise the 18F-labelled analogues.

The mono-fluorobenzoylated polyamines were obtained by solid-phase synthesis of the corresponding oxopolyamines and subsequent reduction of the amide bond with BH3-THF. By applying Dde and Boc as orthogonal protecting groups and taking advantage of the selective reaction of 2-acetyldimedone with primary amino groups in the presence of secondary amines, the selective fluorobenzoylation (FBz) of different amino groups becomes possible. Additionally, the selective mono-fluorobenzylation (FBn) of selected diamines by reaction with 4-fluorobenzaldehyde and subsequent reduction of the resulting imine using sodium triacetoxyborohydride was performed. Based on the established methodology, the following compounds among others were obtained in good yields: *N*-FBz-cadaverine, *N*-FBn-cadaverine, N^1-FBz- spermidine, N^4-FBz-spermidine, N^8-FBz-spermidine and N^1-FBz-spermine. Furthermore, the naturally occurring diamine cadaverine was conjugated to different reporter groups such as biotin. The identity of the compounds was confirmed by NMR spectroscopy and mass spectrometry. The kinetic parameters towards transglutaminase 2-catalysed acyl transfer were determined for selected compounds with an in-house fluorimetric assay using the fluorogenic acyl donor Cbz–Glu(HMC)–Gly–OH.

Transglutaminase 2 is involved in the inflammatory response through mechanisms linked to NF-κB/HIF-1α pathways

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Transglutaminase 2 (TG2) plays an important role in the inflammatory response. However, its underlying mechanisms are not fully understood. High levels of TG2 have been associated with the constitutive activation of nuclear factor kappa B (NF-κB), considered the main regulator of inflammation (1,2). In this context, the receptor activator of NF-κB ligand (RANKL) and receptor activator of NF-κB have extensive functions in the regulation of cytokine secretion associated with different pathological conditions. Here, we demonstrate that TG2 up-regulation and alterations of RANKL/OPG ratio occur in cultured human periodontal fibroblasts under inflammatory conditions. We also found a positive correlation between RANKL/OPG ratio and TG2 mRNA levels. To further characterize underlying mechanisms of interaction between TG2 and NF-κB pathway we used monocytes differentiated to macrophages by tetradecanoyl phorbol acetate (PMA) treatment and provided evidence that TG2 inhibition reduced both NF-κB activation and RANKL expression in THP-1 cells.

As hypoxia-inducible factor-1 (HIF-1) is a critical determinant response to hypoxia and inflammation, we also detected HIF-1α expression in PMA-stimulated THP-1 cells. The PMA-induced increase in HIF-1α was associated with NF-κB activation and TG2 up-regulation. Treatment with TG2 inhibitors, such as R283, significantly suppressed the activation of HIF-1α.

Taken together, these results suggest that inflammatory response, associated with up-regulation of HIF-1α through NF-κB pathway, may be modulated by TG2 increases.

References

1. Ientile R, Caccamo D, Griffin M (2007) Tissue transglutaminase and the stress response. Amino Acids 33:385–394.
2. Mehta K, Kumar A, Kim HI (2010) Transglutaminase 2: a multi-tasking protein in the complex circuitry of inflammation and cancer. Biochemical Pharmacology 80:1921–1929.

Type II transglutaminase regulates epidermal cancer cell epithelial–mesenchymal transition

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Type II transglutaminase (TG2) is a member of the transglutaminase family of proteins. TG2 is a multifunctional protein that possesses calcium-dependent protein cross-linking and GTP-binding/G-protein activities. TG2 has been described as a master regulator of cancer stem cell survival and levels are highly elevated in advanced disease. The cancer stem cell phenotype has been tied to pathways regulating epithelial–mesenchymal transition (EMT). The idea that cancer stem cells possess self-renewal suggests that may have superior tumor-forming and metastatic potential. In the present study, we demonstrate that TG2 is necessary for survival of epidermal cancer stem cells (ECS cells). We show that TG2 is highly enriched in ECS cells and expression is associated with high levels of other known stem cell markers including Oct4 and Sox2. Expression is also associated with the expression of EMT markers including Snail, Twist, Slug, Vimentin and N-cadherin. Knockdown of TG2 reduces ECS cell survival, ability to form spheroids, migration, invasion and expression of stem cell and mesenchymal markers. Treatment with NC9, a TG2 specific inhibitor, also reduces spheroid formation and ECS cell migration. Of particular interest, TG2 is capable of performing these functions independent of NFκB, a known regulator of EMT. Treatment with TG2-shRNA or NC9 does not result in a reduction of NFκB expression, yet EMT and stem cell markers are still reduced. These studies identify TG2 as an important cancer therapy target in ECS cells.

Reactive oxygen species generation mediated by tissue transglutaminase during uremic toxins induced endothelial injury

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In chronic kidney disease (CKD), metabolic changes and an impaired urinary excretion of metabolites lead to the accumulation of uremic toxins in the bodies. Among these, indoxyl sulfate (IS) and *p*-cresyl sulfate (PCS) are two of the most studied uremic toxins that are responsible for renal fibrotic response. However, the biological function and mechanism by which these toxins exert their toxicity are just beginning to be understood. In addition, CKD patients have a higher risk of overall and cardiovascular (CV) mortality than the general population and the underlying mechanism(s) is just beginning to be understood. In renal tubular cells, data suggest that PCS contributes to the cellular injury by increasing the oxidative stress through the generation of reactive oxygen species (ROS). In endothelial cells, IS was found to have more effects in inducing ROS production and decreasing glutathione levels, whereas PCS had little effects. As tissue transglutaminase (TGM-2) is inducible by TGFβ and inflammatory cytokines which are frequently observed in CKD patients, and was postulated to be involved in the activation of NOX-2 NADPH oxidase system, one of the major pathways in generating intracellular ROS in endothelial cells, we investigated the role of TGM-2 in uremic toxins-induced injury using human umbilical vascular endothelial cells (HUVEC) as the model system. In this model, the cell viability and expression of TGM-2 was not significantly changed after being exposed up to 500 μM of IS or PCS for 24 h. However, ROS generation was increased by twofold when exposed to ≥500 μM IS in as little as 3 h, while similar concentrations of PCS had no effects even after being exposed up to 24 h. When specific and non-specific TGM-2 inhibitors including Z-DON and cystamine at concentrations of 40 and 50 μM, respectively, were used during IS treatment, ROS generation was reduced to a level that was 1.2-fold of untreated control cells. The data indicated that TGM-2 is responsible for 80 % of ROS generation during IS-induced injury. Most importantly, the generation of ROS after exposure to IS was correlated with the activation of in situ transglutaminase activity of TGM-2 which can be observed using western blot assay using streptavidin-HRP to detect the incorporation of biotinylated pentylamine (BP), the substrate of TGM-2. The data demonstrated a role of TGM-2 during IS-induced ROS generation in endothelial cells. This study suggest that inhibition of TGM-2's activity may protect endothelial cells from IS-induced endothelial injury and the inhibitors of TGM-2 might be useful in reducing the uremic toxins-induced cardiovascular risk in CKD patients.

Homoarginine concentrations in human and mouse plasma samples measured by LC–MS/MS and ELISA: a method comparison

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Abstract

Background: Homoarginine is a naturally occurring amino acid that is derived from lysine and glycine, synthesized by L-arginine: glycine amidinotransferase (AGAT). Several studies indicate an association between low circulating homoarginine concentrations and cardiovascular (CV) disease, CV mortality, and all-cause mortality. Homoarginine in biological samples is quantified by high-performance liquid chromatography, gas chromatography, and liquid chromatography, coupled to mass spectrometry, or by an enzyme-linked immunosorbent assay (ELISA).

Methods/aim: The aim of this study was to compare homoarginine quantification by liquid chromatography–tandem mass spectrometry (LC–MS/MS) with ELISA (DLD Diagnostika GmbH, Hamburg, Germany). For LC–MS/MS quantification, a previously reported and validated method was applied (Atzler et al. J Chromatogr B 2011). Pearson's regression coefficients and Bland–Altman plots were calculated for comparison of methods.
Results: Plasma concentrations measured by LC–MS/MS were on average 29 % higher than values obtained from ELISA. We investigated N^6-trimethyllysine as a potential source for the higher homoarginine concentrations, but evaluation of mass spectra indicated no interference of N^6-trimethyllysine with homoarginine in our LC–MS/MS setup. However, both methods show a linear correlation ($r^2 = 0.78$) for human plasma samples. Modifications of both assays enabled a more sensitive quantification in mouse plasma samples with a similar correlation between LC–MS/MS and ELISA ($r^2 = 0.76$). The applicability of both quantification methods is shown in (1) a case–control study of acute coronary syndrome and (2) AGAT-deficient mice.
Conclusion: LC–MS/MS as well as ELISA is suitable for human and mouse plasma, but different reference values for each method need to be considered.

Homoarginine and basic amino acid derivatives in patients with pathology of the left ventricular outflow tract

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Homoarginine (Harg) forms mainly as a by-product in the creatine biosynthesis pathway in arginine:glycine amidinotransferase (AGAT) catalytic step or in the reactions of urea cycle. Reduced level of Harg, elevated contents of asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA) in blood are very often found in groups of patients with high levels of mortality from cardiovascular disease. Pathology of the left ventricular outflow tract (thoracic aortic aneurysm) is one of the main causes of sudden death occurring without marked manifestations of atherosclerosis and metabolic abnormalities of carbohydrate and lipid metabolism. The aim of this study was to determine the levels of Harg, ADMA, SDMA, and ε-*N*-trimethyllysine (TML) in patients with aneurysm $N = 64$, 60 (40–66) years; median (10th–90th percentile) or aortic stenosis $N = 47$, 62 (49–73) years; and in the group donors $N = 30$, 55 (39–61) years. Pyruvate and lactate concentrations were determined in plasma to test the mitochondrial dysfunction. Other functional and routine biochemical tests were applied to monitor dyslipidemia, obesity, diabetes, and coronary heart disease. The concentrations of ADMA and SDMA ($p < 0.0001$ for all) were higher in patients than in healthy individuals. According to obtained data, there is a strong association between ascending aorta diameter and plasma ADMA level. The levels HR and TML in patients were 1.60 (0.86–2.31) and 0.28 (0.17–0.51) μM, significantly lower ($p < 0.001$) than in donors: 2.58 (1.33–4.17) and 0.49 (0.32–0.78) μM, respectively. Since TML is a key metabolite formed in the mitochondria for subsequent synthesis of carnitine, its plasma concentration may reflect the rate of carnitine biosynthesis and the transportation of fatty acids in tissues. Reduction of the TML level in the total patients' group by 1.75 times can be considered as one of the indicators of systemic mitochondrial dysfunction, as well as the degree of methylation of lysine-rich proteins (hypomethylation). Harg levels also dropped in the examined patients by 1.61 times, probably indicating a decrease of AGAT activity in tissues. Characterization of patients with impaired circulation not only by endothelial dysfunction testing, but also by markers of mitochondrial dysfunction is advisable to identify the levels of Harg and possibly TML. This study was supported by the Russian Foundation for Basic Research No. 15-04-02480.

Antimicrobial evaluation and microwave-assisted synthesis of some Schiff's bases from isatin with amino acid conjugates of nalidixic acid

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Abstract: Nalidixic acid is a good Gram-negative antibacterial drug, but it showed resistance toward Gram-positive bacteria which in most cases is a big problem. So any modification of the nalidixic acid molecule by conjugation with other molecules that had been known to have Gram-positive antibacterial activity may result in a new candidate that has activity against both Gram-negative and –positive bacteria. Isatin is an endogenous molecule that has Gram-positive and Gram-negative antibacterial and antifungal activity. This was done by coupling of nalidixic acid with some amino acid esters, namely, valine, leucine, isoleucine and phenyl alanine by the mixed anhydride method. Hydrazinolysis of the amino acid ester conjugates give the corresponding haydrazide, and then microwave-assisted condensation with the isatin derivatives to give the corresponding Schiff bases in short time, with less solvent, less cost, high yield and high purity. All the compound structures were confirmed with elemental analysis and different spectroscopic tools. Antimicrobial activity was evaluated showing some promising candidates.
Keywods: Nalidixic acid, Amino acid conjugates, Isatin, Microwave, Schiff bases and Gram-negative and -positive bacteria.

Epimerization of isoleucine in vanadium Schiff base complexes: a new method for transformation of stereoisomers of isoleucine

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Isoleucine belongs to the branched-chain amino acids and possesses two stereogenic centers on alpha- and beta-carbon atoms resulting in four possible stereoisomers. While L-isoleucine is dominating in biological systems and is proteinogenic; D-isoleucine, L-*allo*-isoleucine and D-*allo*-isoleucine were found in organisms (including humans) in trace amounts and their biological effects are being investigated. The

formation mechanism of the latter three stereoisomers remains unclear. Several recent studies proposed that the carbon skeleton of D-*allo*-isoleucine originates from L-isoleucine and that the transformation may be catalyzed by an amino acid racemase, isoleucine 2-epimerase, found in *Lactobacillus* species. The artificial transformation of L-isoleucine into D-*allo*-isoleucine can be achieved by several procedures involving multistep derivatization of the amino acid and subsequent separation of the diastereomers. In addition, the transformation of stereoisomers of isoleucine takes place spontaneously in nature on millions of years time scale, allowing geological dating. Here, we present a novel vanadium-catalyzed non-enzymatic transformation of stereoisomers of isoleucine by epimerization on the alpha-carbon position involving vanadium Schiff base complexes with the general formula $NBu_4[VO_2(N\text{-salicylidene-isoleucinato})]$. The full conversion of a single stereoisomer to the 1:1 mixture of the corresponding epimers is achieved by refluxing the crystallization solution of the chosen complex in a slightly basic solution within 2 h. Subsequent separation of the diastereomers may be driven by different solubility of the complexes derived from the canonical and the *allo* forms. Although the separation of the epimers needs to be improved for isolating pure stereoisomers, this reaction expands significantly the potential of vanadium-catalyzed asymmetric reactions.

Synthesis of indolyl quinazoline compounds using L-tryptophan hydrazide in the presence of SBA-15-Pr-SO3H and investigation of their biological activities

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Tryptophan is one of the essential amino acids in the human diet. Many indole alkaloids and related natural substances are derived from tryptophan. There are also some quinazolie alkaloids which have indole moieties in their structure with various biological activities, especially on the central nervous system (CNS). In addition, (−)-asperlicin **1**, (−)-fumiquinazoline **2** and (−)-fiscalin B **3** are some of the natural antibiotics-based indolyl quinazoline moieties (Fig. 1).

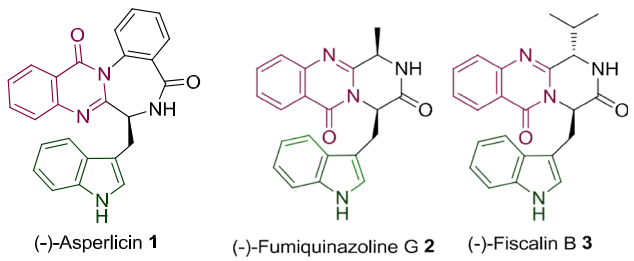
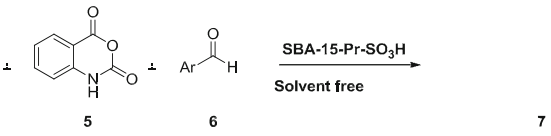


Figure 1.

Combination of biologically active heterocyclic skeleton with amino acids, peptides and/or pseudo peptides has a significant effect on their biological properties. Herein, we used *N*-protected L-tryptophan hydrazide **4** in multicomponent reaction with isatoic anhydride **5** and aromatic aldehydes **6** to obtain indolyl quinazoline compound **7** (Scheme 1). The latter might have beneficial effects on CNS, since it has serotonin-like structure with quinazoline moiety spontaneously. In this synthesis, sulfonic acid functionalized SBA-15 (SBA-15-Pr-SO3H), a hexagonal mesoporous silica, was used as an efficient solid acid catalyst to gain the products in high yield and extra purity. In the conference, the result of synthesis and their biological activities will be presented.



Scheme 1

Synthesis of chiral amino alcohols and aldehydes and their application for the synthesis of bioactive peptides

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Peptide C-terminal aldehydes (PAs) are of interest due to their extended biological activities.

The functionalization of peptides and proteins by aldehyde groups has become the subject of intensive research because of inhibition properties of peptide aldehydes towards various enzymes. Furthermore, peptide aldehydes are of great interest for peptide backbone modification or ligation reactions and they could also be used for the synthesis of functionalized peptides. There are different methods to access peptide aldehydes, but conversion of protected α-amino acids to their corresponding *N*-protected amides and their reduction to α-amino aldehydes is a usual pproach.

In continuation of our research in the synthesis of biologically active opioid peptides, we report the synthesis of *N*-protected *C*-terminal enkephalin aldehydes through synthesis of Leu-, Met-enkephalins and their conversion to the desired Leu-, and Met-enkephalin aldehydes. In the second approach, the peptides were synthesized using Leu-CHO or Met-CHO. The structures of the desired peptides were confirmed based on HR-MS–ESI. The opioid activity and interaction of the peptides with enzymes were investigated.

In the conference, the details about the synthesis of the target peptides and also their enzyme activity results will be explained.

Asymmetric synthesis of new enantiomerically enriched α-amino acids

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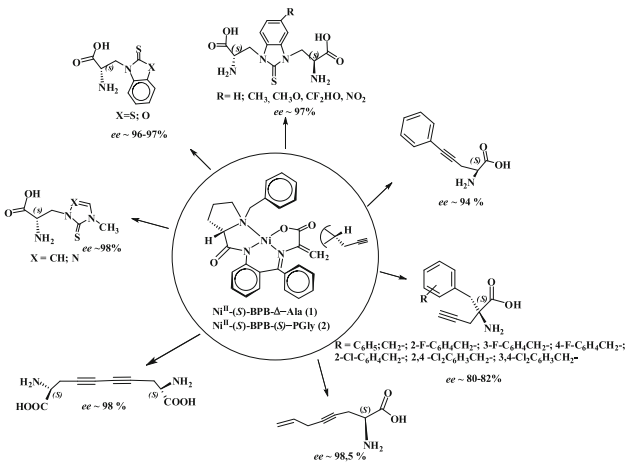
In the last 20 years, the use of enantiomerically enriched non-protein α-amino acids containing unusual groups in the side chain, in various

areas of medicine, pharmacy, biology, chemistry and biotechnology has been actively developing [1].

This work is devoted to the development of asymmetric synthesis effective methods for enantiomerically enriched (*S*)-α-amino acids, not described in literature, containing different heterocyclic and acetylenic groups in the side chain (Scheme).



Ni^{II} complexes of Schiff's bases of dehydroalanine or propargylglycine and chiral auxiliary (*S*)-2-N-(*N*′-benzylprolyl)aminobenzophenone were used as sources of chiral amino acids and dehydroamino acids synthones(**1,2**).

As a result, effective methods of asymmetric synthesis for novel enantiomerically enriched derivatives of (*S*)-alanine ($ee > 97$ %) and (*S*)-propargylglycine ($ee > 80$ %) were developed [2,3].

Literature

1. Saghyan AS (2010) Enantiomerically pure non-protein amino acids. Methods for obtaining, M, Nauka, p. 235

2. Saghyan AS, Mkrtchyan GM, Dadayan AS, Petrosyan SG, Geolchanyan AV, Mkrtchyan AF, Mkrtchyan S, Gevorgyan A, Iaroshenko VO, Langer P (2013) Tetrahedron: Asymmetry 24:229–232

3. Saghyan AS, Simonyan HM, Petrosyan SG, Mkrtchyan AF, Khachatryan LV, Geolchanyan AV, Samvelyan MA, Ghochikyan TV, Kelzhanova N, Saginayev AT (2014) Peter Langer Z. Naturforsch. B 69b:451–460.

Pitfalls in the synthesis of fluorescent methotrexate-containing oligopeptide conjugates

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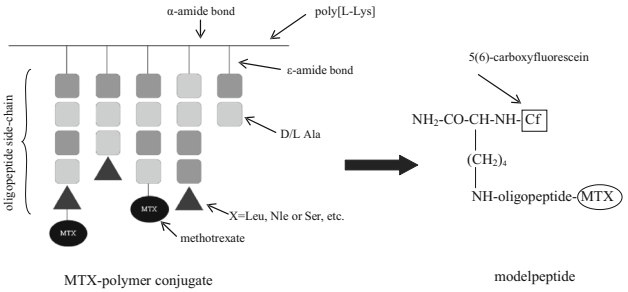
Several methotrexate (MTX)-branched chain polypeptide conjugates with poly[L-lysine] backbone were synthesised and studied for anti-cancer and/or anti-parasitic properties in the recent years in our research group. These polymeric polypeptides contain short oligopeptide side chains connected to the ε-amino groups of the poly[L-lysine] backbone. MTX was coupled covalently through its glutamic acid moiety. Here, we report on the preparation of a model peptide family for structure–activity experiments (e.g. biodistribution, chemical stability, enzymatic degradation). These peptides, correspond to the branched structure of the conjugates containing also 5(6)-carboxyfluorescein (Cf) for fluorescent labelling.



The peptides were synthesized with solid-phase methodology on Rink-Amide MBHA resin, with Fmoc/^{t}Bu or Boc/Bzl strategy. In some cases, Fmoc/Dde protection scheme or solution-phase coupling was the only effective method for the preparation. Several side reactions were identified:

(a) Unexpected cyclopeptide formation with the lactone-carboxylic group of the Cf was demonstrated, when Cf was attached to the α-amino group of the Lys residue. (b) During HF cleavage, MTX was demonstrated to form adducts with anisole and *para*-cresol scavengers. (c) TMSOTf cleavage methodology was found inadequate due to the large number of side products formed.

In addition, we observed that peptides with Leu were insoluble either in aqueous media or in acetic acid, if both Cf and MTX were present. Critical synthetic pitfalls identified improved the production strategy of these novel, complex peptide conjugates.

Acknowledgments: This work was supported by grant from the Hungarian National Research Fund (OTKA K104385). Mónika Sebestyén is grateful for the support of the Pázmány-Eötvös Foundation for the travel grant.

Sex peptide and the post-mating response in female *Drosophila suzukii*

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Drosophila suzukii is a highly invasive species that causes extensive damage to a wide range of berry and stone fruits. We have investigated the locomotor behaviour of *D. suzukii* in a semi-natural environment resembling a typical English summer and show that adult female *D. suzukii* are at least fourfold more active during daylight hours than adult males. This result was reproduced in several laboratory environments and was shown to be a robust feature of mated, but not virgin female flies. Both males and virgin females experience an afternoon sleep period (siesta) between morning and evening periods of activity; however in mated females the elevated daylight activity disrupts their siesta, but not nighttime sleep. The mated female responds to the loss of the siesta by reducing sleep latency. This response of females to mating is similar to that elicited in female *D. melanogaster* by the male sex peptide (SP). We used MALDI-mass spectrometry to identify the SP of *D. suszukii* and to show that this peptide is transferred to the female reproductive tract during copulation. We propose that the siesta experienced by male and virgin female *D. suzukii* is an adaptation to avoid unnecessary exposure to the afternoon sun, but that post-mated females faced with the challenge of obtaining resources for egg

production and finding oviposition sites take greater risks and that the change in female behaviour is induced by the male SP.

This work was supported by the European Union's seventh framework programme for research, technological development and demonstration under grant agreement number 613678 (DROPSA).

Insect neuropeptides: a basis for the design of a novel group of insect control agents: the PK/PBAN family as a case study

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Insect neuropeptides (Nps) are prime targets in the search for novel insecticides, since they regulate many physiological and behavioral processes. Their blockers (antagonists) may disrupt and interfere with the normal growth, development and behavior of insects, and can yield, therefore, receptor-selective, insect-specific control agents. The chemical nature of Nps enables them to be used as the basis for the design of a generic group of non-toxic environment-friendly insect control agents—an approach that has been applied to human Nps in the pharmaceutical industry.

In the course of our research, we have developed a novel generic strategy which enables development of simple and cost-effective Np antagonist-based insecticide prototypes. The strategy, which is based on a rational design approach of small molecule antagonists based on a known Np agonist, was applied to one of the major insect Np families the pyrokinin (PK)/pheromone biosynthesis activation neuropeptide (PBAN) family, known to regulate many key functions in insects (associated with mating, feeding, development and defense). Currently, we have highly potent molecules which inhibit PK/PBAN-mediated functions and are metabolically stable, highly bio-available (through the cuticle and by feeding), environmentally stable and, most important, simple in structure and cost-effective. In addition, two receptors that mediate the PK/PBAN bioactivity have been cloned, stably expressed in an insect cell line and characterized structurally and functionally. The expressed receptors can be used as high-throughput assay for discovery of insect control agents from various libraries. Our achievements on the above issue will be presented.

Substrate and inhibitor specificity of kynurenine monooxygenase and kynureninase

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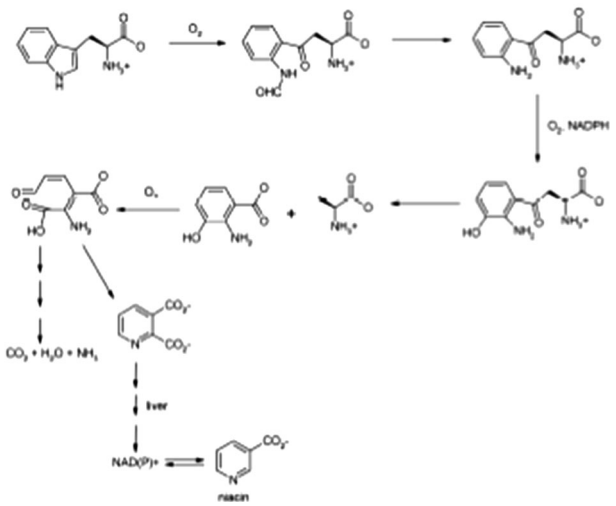
The kynurenine pathway is the major route of tryptophan metabolism in eukaryotes and some bacteria (Figure 1). Tryptophan is first cleaved in the pyrrole ring by a heme-containing dioxygenase to give N-formylkynurenine. Hydrolysis of the formyl group gives kynurenine, which undergoes hydroxylation at the 3-position catalyzed by the flavoenzyme, kynurenine monooxygenase (KMO). 3-Hydroxykynurenine is then cleaved to 3-hydroxyanthranilate and L-alanine by kynureninase, a pyridoxal-5′-phosphate-dependent enzyme. Further metabolism of 3-hydroxyanthranilate by a non-heme dioxygenase gives 2-amino-3-carboxymuconate semialdehyde, which can be catabolized further to CO_2 and NH_3. However, a small portion cyclizes to quinolinate, a precursor for NAD(P).

We have studied the substrate specificity of human kynureninase and KMO from *Cytophaga hutchisonii*. The 3-OH of the substrate forms an H-bond with Asn-333 in the active site of human kynureninase (1). Substitution of the 3-OH with a halogen gives a substrate for kynureninase (2), but an inhibitor for KMO. However, kynurenines substituted at C-5 are good substrates for both kynureninase (2) and KMO. These results may be useful in the design of specific inhibitors of kynureninase and/or KMO that may be useful in the treatment of neurodegenerative diseases (Figure 1. Shows the kynurenine pathway).

Figure 1. The kynurenine pathway



1. Lima S, Kumar S, Gawandi V, Momany C, Phillips RS (2009) Crystal structure of *Homo sapiens* kynureninase-3-hydroxyhippuric acid complex: the molecular basis of kynureninase substrate specificity. J Med Chem 52:389–396.
2. Maitrani C, Phillips RS (2013) Substituents effects on activity of kynureninase from *Homo sapiens* and *Pseudomonas fluorescens*. Bioorg Med Chem 21:4670–4677.

Development of mimetic analogs of pyrokinin-like neuropeptides to disrupt pest insect physiology/behavior

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Pyrokinin (FXPRLamide) neuropeptides regulate a variety of critical processes and behaviors in insects, though they are unsuitable as tools to arthropod endocrinologists and/or as pest management

agents due to sub-optimal biostability and/or bioavailability characteristics. Peptidomimetic analogs can overcome these limitations and can either over-activate or block critical neuropeptide-regulated functions. Diapause hormone (DH), a sub-family of the pyrokinins, terminates diapause in pupae of heliothine insects, important crop pests. Insects enter diapause to escape the debilitating effects of harsh climatic conditions (i.e., winter). Mimetic analogs of DH prevent diapause in pupae that develop from larvae injected with these agents, effectively inducing the pest insects to commit a form of 'ecological suicide'. Amphiphilic analogs of DH containing different hydrocarbon motifs proved to be topically active. Evaluation of a series of pyrokinin analogs on an expressed DH receptor (*HzDHr*) cloned from *Helicoverpa zea* led to the identification of several superagonists, with greater efficacy than the native peptide; as well as several novel analogs with antagonist activity. A series of five PRXamide pyrokinin-like receptors from the red flour beetle (*Tribolium castaneum*), including two that show strong affinity to DH, were subjected to ligand–receptor specificity analysis using a series of peptidomimetic ligands. The study identified biostable mimetic analogs that demonstrate agonist properties selective for one DH-like receptor over another, as well as selective antagonism of the CAP2b receptor. The pyrokinin-like CAP2b class of peptides demonstrates diuretic activity in insects, and mimetic agonists and antagonists can disrupt the internal water balance critical to survival of pest insects.

Neuropropeptide Semax activates adaptive immunity after brain focal ischemia in rats

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One of the most efficient drugs in cerebral stroke therapy is a synthetic peptide Semax (Met-Glu-His-Phe-Pro-Gly-Pro), consisting of a fragment of ACTH (4–7) and the C-terminal tripeptide Pro-Gly-Pro. The molecular mechanisms of its neuroprotective action remain still unknown. Transcriptome response of ischemized brain cortex tissues to Semax in the male Wistar rats was investigated. Focal cerebral ischemia was induced by direct permanent middle cerebral artery occlusion (pMCAO). Gene expression alteration was analyzed in 24 h groups versus 3 h groups in pMCAO and "pMCAO + Semax" groups with Illumina RatRef-12 Expression BeadChip. Data were analyzed by Ingenuity iReport web-server. The changes in the expression levels for hundreds of genes were observed. Semax treatment resulted in the increased expression of 17 genes that belong to the major histocompatibility complex. Also, the neuropeptide treatment was associated with altered expression of genes related to the regulation of immunoglobulin quantity. Totally, 30 genes related to the immunoglobulin processes exhibited altered expression in response to stroke, whereas the expression of 27 genes changed in response to Semax treatment. Totally, 15 genes among two animal groups overlapped and four of them (*Cd74*, *Cxcl9*, *Pycard*, *Tyrobp*) were modulated by Semax more intensively than under ischemia only. Thus, in conditions of rat brain focal ischemia, the action of Semax influenced the expression of genes implicated in immune processes. The data assume the involment of Semax in immune response during ischemia.

Linking chimpanzee social bonds and cooperative behaviour during stressors and non-stressors with urinary oxytocin and glucocorticoid levels

Catherine Crockford, Tobias Deschner, Toni Ziegler, Klaus Zuberbuehler, Roman Wittig

Social mammals can form close and enduring social bonds, where dyads with close social bonds are those that engage in high rates of cooperative behaviours. Maintaining social bonds leads to greater reproductive success, health and longevity. However, underlying mechanisms promoting this effect are still ambiguous, especially for non-kin, non-sexual bonds. One possibility is that direct benefits are derived from cooperative exchange, which may be emotionally rather than cognitively mediated through oxytocin, a key neuropeptide in social bonding. Another is that bond partners buffer the negative effects of physical and psychological stress, where prolonged cortisol release is associated with reduced fertility, health and longevity. We investigated these possibilities in chimpanzees, known for their close social bonds with non-kin. We examined their urinary glucocorticoid and oxytocin levels following several cooperative events, including those considered to be stressors (potentially dangerous encounters with neighbouring chimpanzee communities or food competition contexts), or relaxers (grooming interactions), and contrasted them with socially neutral (resting, social feeding) situations. Our results suggested that generally the combination of engaging in cooperative behaviours with bond partners compared with non-bond partners, rather than either factor alone, was associated with hormonal profiles likely to facilitate both cooperation and stress reduction.

The vasopressin-deficient Brattleboro rat as a genetic model of schizophrenia

Dóra Zelena

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Besides a role in saltwater homeostasis, vasopressin plays a key role in several brain functions like complex social behavior and memory. As these are core symptoms of schizophrenia, it is not surprising that low vasopressin levels can be found in schizophrenic subjects.

We aimed to confirm the role of vasopressin in schizophrenia using a wide range of schizophrenia-like behavioral tests in spontaneous vasopressin-deficient Brattleboro strain and restoring the alterations with antipsychotics.

The vasopressin-deficient animals revealed social deficits in social avoidance and social discrimination tests and a memory decline not only in social discrimination, but also in object recognition task. Reduced prepulse inhibition was also detectable. Already during the early postnatal period (7- to 8-day-old rats) the maternal separation-induced ultrasound vocalization (30–50 kHz) was also reduced in them as a sign of diminished communication. All of these disturbances was normalized after antipsychotic treatment (clozapine, olanzapine, risperidone and aripiprazole), which confirmed the connection with schizophrenia. As a possible background mechanism, we found some alteration in histone acetylation (epigenetic landmark) in the prefrontal brain region and hippocampus of vasopressin-deficient animals.

All in all, vasopressin-deficient rat seems to be an appropriate model of schizophrenia with prominent behavioral alterations. Influencing the ultrasound vocalization in pups requires a small amount of drug, which makes this model preferable for preclinical testing.

Epigenetic changes due to lifelong vasopressin deficit may contribute to the development of symptoms.

STAT3 controls IL6-dependent regulation of serotonin transporter function and depression-like behavior

Eryan Kong, Sonja Sucic, Francisco J. Monje1, Giorgia Savalli, Weifei Diao, Deeba Khan, Marianne Ronovsky, Maureen Cabatic, Florian Koban, Michael Freissmuth and Daniela D. Pollak

Type of presentation: oral presentation

Experimental evidence suggests a role for the immune system in the pathophysiology of depression. A specific involvement of the proinflammatory cytokine interleukin 6 (IL6) in both, patients suffering from the disease and pertinent animal models, has been proposed. However, it is not clear how IL6 impinges on neurotransmission and thus contributes to depression. Here, we tested the hypothesis that IL6-induced modulation of serotonergic neurotransmission through the STAT3 signaling pathway contributes to the role of IL6 in depression. The addition of IL6 to JAR cells, endogenously expressing SERT, reduced SERT activity and downregulated SERT mRNA and protein levels. Similarly, SERT expression was reduced upon IL6 treatment in the mouse hippocampus. Conversely, hippocampal tissue of IL6-KO mice contained elevated levels of SERT and IL6-KO mice displayed a reduction in depression-like behavior and blunted response to acute antidepressant treatment. STAT3 IL6 dependently associated with the SERT promoter and inhibition of STAT3 blocked the effect of IL6 in vitro and modulated depression-like behavior in vivo. These observations demonstrate that IL6 directly controls SERT levels and consequently serotonin reuptake and identify STAT3-dependent regulation of SERT as conceivable neurobiological substrate for the involvement of IL6 in depression.

Glutamatergic neurotransmission and memory reactivation in the rat

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Conditioned odor aversion is the avoidance of a tasteless odored solution (conditioned stimulus, CS) that was previously paired with visceral malaise (unconditioned stimulus, US). This phenomenon has been consistently observed in long-term memory (LTM) retention tests (i.e., 48 h after acquisition) when the interstimuli interval (ISI), i.e., CS–US delay, is shorter than 15 min. However, we recently found a robust odor aversion in rats trained with longer ISI (up to 60 min), when tested in the short term (i.e., 4 h after acquisition), indicating that rats are able to associate both stimuli and to form a short-term aversive memory, but unable to consolidate it into an LTM. Here, we report the presence of a short-term memory (STM) in an additional condition in which no aversion had been detected in LTM tests (i.e., low doses of LiCl). We also extended our research on the effects of the short-term retrieval test upon the persistence of the aversive memory in the long term for both conditions: long ISI and low doses of LiCl. For comparison, groups trained with the same doses of LiCl and long ISI were tested only in the long term. In the STM test, a clear aversion was found in animals trained with LiCl (0.15 M), 1.0 and 0.5 % BW, i.p., as well as using 5, 15, 30, and 60 min ISI. In the LTM tests, aversion was only detected using 2 % BW LiCl or 5 and 15 min ISI. However, equally robust aversion was found in the long-term test of those groups that were tested for STM 4 h after training. We concluded that short-term retrieval enhances LTM consolidation by a reactivation-like effect of conditioned odor aversion. Additionally, systemic administration of the NMDA antagonist MK-801either before or after STM retrieval testing did not have consistent effects on this memory reactivation. This suggests that unlike acquisition of conditioned odor aversion, its memory reactivation depends less on glutamatergic neurotransmission.

There is more to vasopressin than meets the eye

Mike Ludwig

Centre for Integrative Physiology, University of Edinburgh, Edinburgh, UK,

The retina of mammals contains intrinsically photosensitive retinal ganglion cells (RGCs) which mediate non-image-forming visual functions such as pupillary light reflex and circadian photo entrainment. Here, we show a population of vasopressin (VP) expressing retinal ganglion cells (VP-RGCs) which is photosensitive, but does not express either melanopsin or PACAP. Intravitreal injections of an rAAV-expressing Venus (under the control of the VP promoter) show that the vast majority of these cells project to the ventro-lateral core part of the suprachiasmatic nucleus (SCN). Microdialysis data show an increase in intra-SCN VP concentration in response to electrical stimulation of the optic nerve or light stimulation of the eye. Furthermore, in response to these stimuli some SCN neurons showed an excitatory response which was blocked by icv injection of a VP V1 receptor antagonist. Light-induced expression of the immediate early gene product c-Fos during the dark period was strongly reduced by prior administration of a VP V1 receptor antagonist or ablation of the VP-RGCs. Our data suggest that activation of VP-RGC may mediate light cues by releasing VP into the SCN to excite SCN neurons, altering SCN activity and thus regulating neuroendocrine hormonal rhythms.

Role of thyroid hormone on the brain protein synthesis in rats fed the ornithine

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The purpose of the present study was to determine whether the regulation of brain protein synthesis is mediated through changes in the plasma concentration of thyroid hormone (T_3) when dietary ornithine treatment is manipulated in the PTU (6-propyl-2-thiouracil, thyroid inhibitor)-treated or control rats. Experiment was done on four groups of PTU-treated and control (24-week-old) male rats given 0 or 0.7 % ornithine–HCl added to a 20 % casein diet. The fractional rates of protein synthesis in the brains increased significantly with the 20 % casein +0.7 % ornithine compared with the 20 % casein diet alone in both PTU-treated and control groups. The ornithine supplementation to the basal diet did not affect the plasma concentration of T_3. In the cerebral cortex and cerebellum, the RNA activity [g protein synthesized/(g RNA·d)] significantly correlated with the fractional rates of

protein synthesis. The RNA concentration (mg RNA/g protein) was the same in each test group of brain regions. The results suggest that the treatment with ornithine is likely to increase the rate of brain protein synthesis not only in the control rats, but also in the PTU-treated rats, and that the ornithine-induced increase in the brain protein synthesis may not be mediated through changes in the thyroid hormone. The RNA activity is at least partly related to the fractional rate of brain protein synthesis.

Neuropeptides and maternal responsiveness

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Neuropeptides are released from synaptic vesicles in response to high-frequency stimulation and exert long-lasting modulation of neurotransmission. Therefore, they are ideal regulators of slow reproductive events including maternal adaptation of the central nervous system. Indeed, oxytocin has long been suggested to initiate maternal responsiveness independent of its secretion into the blood as a hormone for milk ejection. The medial preoptic area is known to control maternal behaviors as its lesion eliminates maternal care in rodents. Recently, galanin neurons in the medial preoptic area were shown to govern maternal responsiveness. In our previous microarray study, we identified neuropeptides as a group of proteins overrepresented among the changing genes in the maternal preoptic area. Some neuropeptides including melanin-concentrating hormone increases in parallel with the metabolic load exerted by lactation suggesting its function in the elevated food-intake of mothers. In turn, the expression of kisspeptin reduces as part of the regulatory network of lactational anestrous. We also identified a peptide, amylin, which had previously not been known to be present in the brain. Indeed, amylin shows a 25 times increase in the maternal preoptic area and is not expressed anywhere else in the brain as demonstrated by three independent techniques, RT-PCR, in situ hybridization histochemistry, and immunolabeling. Amylin level is minimal in the last day of pregnancy, increases abruptly at parturition, and remains elevated until weaning. In addition, amylin is also induced in virgin female rats as they become maternal during a sensitization procedure without lactation, suggesting that amylin plays a role in maternal behavior or emotional adaptation. To reveal the neuronal inputs received by amylin neurons, we injected the retrograde tracer cholera toxin beta subunit into the medial preoptic area of mother rats. Among ascending projections, we identified the posterior intralaminar complex (PIL) of the thalamus, which contains tuberoinfundibular peptide of 39 residues (TIP39) expressing neurons. Interestingly, TIP39 was also markedly elevated in rat dams in this site, but not in the other thalamic site of its expression, the periventricular gray of the thalamus. Furthermore, TIP39 neurons from the PIL projected to and closely apposed amylin neurons, as well as galanin and oxytocin neurons in the medial preoptic area. We actually demonstrated that using electron microscopy, TIP39 terminals innervate galanin and oxytocin neurons by forming synapses on their cell bodies and proximal dendrites. Injecting a virus encoding a peptide antagonist of the receptor of TIP39 eliminated pup–associates preference in a conditioned place preference test. These data suggest that TIP39, amylin, oxytocin and galanin as well as the neurons that express them may all contribute to the maintenance of maternal responsiveness in the postpartum period providing a leading example of the role of neuropeptides in behavioral adaptation of the brain.

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Relationship of PHF6 hexapeptide motif and abnormal sticky behaviour: complication in tau protein analysis at peptide level

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Tau protein belongs to intrinsically disordered proteins. In Alzheimer disease, tau undergoes modifications leading to the misfolding and formation of paired helical filaments (PHF).

Our aim was to optimize the epitope extraction protocol enabling identification of linear immunodominant epitopes using tau digested by trypsin. Within the eluted fragments containing epitopes specifically reactive with antibodies bound to solid phase, additionally fragment 299-HVPGGGSVQIVYKPVDLSK-317 was detected by mass spectrometry (MS). This fragment comprising minimal interaction motif responsible for the PHF formation, hexapeptide PHF6 (306-VQIVYK-311), distinctly complicated the MS spectra interpretation. The conventional approaches to suppress the nonspecific sorption were not effective to preclude or at least to reduce the "sticky" fragment sorption.

Therefore we carried out protein cleavage by two immobilized enzymes. Chymotrypsin was selected as an additional protease due to the specific cleavage site between Y310 and K311. This newly introduced approach led to suppression of nonspecific sorption without affecting specific epitope–antibody interactions in the epitope extraction protocol.

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Dietary requirements of synthesizable amino acids by animals

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Based on growth or nitrogen balance studies, amino acids had been traditionally classified as nutritionally essential or nonessential for mammals, birds and fish. Nutritionally essential AA (EAA) are defined as either those AA whose carbon skeletons cannot be synthesized de novo in animal cells or those that normally are insufficiently synthesized de novo by the animal organism relative to its needs for maintenance, growth, development, and health and which must be provided in the diet to meet the requirements. In contrast, nutritionally nonessential AA (NEAA) are those AA which

can be synthesized de novo in adequate amounts by the animal organism to meet the requirements for maintenance, growth, development, and health and, therefore, need not be provided in the diet. It was assumed that all the NEAA were synthesized sufficiently in the body to meet the needs for maximal growth and optimal health. However, careful analysis of the scientific literature reveals no compelling evidence to support this assumption. NEAA (e.g., glutamine, glutamate, proline, glycine, and arginine) play important roles in regulating gene expression, cell signaling, metabolic pathways, antioxidative responses, fertility, neurotransmission, and immunity. Additionally, glutamate, glutamine, and aspartate are major metabolic fuels for the small intestine to maintain its digestive function and protect the integrity of the intestinal mucosa. Furthermore, rates of NEAA metabolisms are dependent on species, developmental age, and the environment. Emerging evidence shows that diets for animals and humans must contain all NEAA for optimal survival, growth, development, reproduction, immunity, and health, as well as optimal efficiency of nutrient utilization. (The study was supported by NSFC and USDA).

Effect of dietary L-glutamate on growth performance, bacterial burden against *Citrobacter freudii* infection

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This study was conducted to determine the protective role of dietary L-glutamate supplementation in mouse model with *Citrobacter freudii* infection. A total of 120 female ICR mice were randomly allotted to three treatments: (1) all mice were treated with basal diet ($n = 40$); (2) all mice were treated with basal diet with dietary 1.0 % glutamate supplementation ($n = 40$). Except group one, mice were challenged by oral infections with 0.2 ml 5×10^8 CFU/ml *Citrobacter freudii*. Body weight of each mouse was recorded every day. On day 7, 14, 21 and 28, ten mice from each group were killed; colon length, weight and bacterial burden and mortality were calculated from each group. There were no differences in growth performance and colon weight among all the groups. On day 7 and 21 post-infection, dietary glutamate supplementation increased colon length. On day 14 post-infection, both *Citrobacter freudii* infection groups have an increased colon length than the basal diet control group. On all time points, bacterial burden in basal control group was significantly lower than those in the other two infection groups ($P < 0.01$). On day 7, 21 and 28 post-infections, dietary L-glutamate supplementation significantly decreased bacterial burden compared to those in the basal diet infection group ($P < 0.05$). In conclusion, dietary L-glutamate supplementation decreased bacterial burden and improved colon status.

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Effect of dietary tryptophan on the growth performance in early-weaned piglets

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Tryptophan (Trp), as a constitute body protein and an essential amino acid, plays important roles in feed intake, growth performance and immune responses. This study aims at evaluating the effects of different dietary tryptophan level on growth performance and serum amino acids level. 320 piglets, with similar body weight and weaned at 21 day, were assigned randomly to one of the four treatments: (1) basal diet; (2) basal diet +0.25 % Trp; (3) basal diet +0.5 % Trp; (4) basal diet +0.75 % Trp. Each treatment has 8 replicates and 10 piglets per replicate. The piglets were fed for 28 days. Body weight, feed intake and body weight were recorded. On day 28, blood samples were collected into plastic uncoated tubes for amino acid analysis by venipuncture. Compared with basal diet treatment, all Trp treatments had a trend of increasing feed intake ($P > 0.05$). 0.5 % Trp supplementation increased average daily gain and the feed efficiency significantly ($P < 0.05$), while 0.25 and 0.75 % Trp supplementation showed a trend of increasing average daily gain and feed efficiency ($P > 0.05$). These results showed dietary tryptophan supplementation may have a beneficial effect on feed intake and feed efficiency in weaned piglets.

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Dietary supplementation with glycyrrhetinic acid (GA) modulates ornithine decarboxylase activity, serum amino acids level and growth performance in milk-fed piglets

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Abstract: This study was conducted to test the hypothesis that dietary supplementation with glycyrrhetinic acid (GA) may increase ornithine decarboxylase (ODC) activity, serum amino acids level, and growth performance in young pigs. Twenty piglets (Landrace × Yorkshire) with similar BW (2.92 ± 0.10 kg) were obtained from five sows (4

piglets/sow) and weaned at 7 days of age to a liquid milk-replacement diet. After a 2-day period of adaptation, piglets were assigned randomly to one of the four treatment groups, which received supplementation with 0, 0.01, 0.02 or 0.04 % GA to the milk powder for 12 days (1 piglet/pen; 5 pigs/treatment). BW was measured on days 0, 5 and 12 for all treatments. The jejunum and serum samples were obtained on day 12 from the control and 0.02 % GA treatment groups. Dietary supplementation with 0.02 % GA resulted in the highest ADG among all of the treatment groups ($P < 0.05$). Compared with the control, dietary supplementation with 0.02 % GA increased feed intake by 14.8 % ($P > 0.05$), ADG by 20 % ($P < 0.05$), decreased the feed:gain ratio by 3.4 % ($P > 0.05$) and increased the ODC activity in the jejunum on day 12 ($P < 0.05$). Compared with the control group, GA treatment group had an increase in concentrations of threonine ($P > 0.05$), lysine ($P > 0.05$), total measured AA ($P > 0.05$) and arginine ($P < 0.05$), but a decrease in concentrations of serine ($P < 0.05$) at day 12, an increase in concentrations of lysine, methionine, proline and alanine ($P < 0.05$), a decrease in concentrations of phenylalanine, glutamic acid and threonine at day 9 ($P < 0.05$). The results shows a decreasing trend in arginine concentration in the control group and GA treatment group from day 9 to day 12, but compared with the GA treatment group, the control group had a greater decrease, especially at day 12 ($P < 0.05$). These results suggest that GA increases ODC activity, modulates serum amino acids level and enhances growth performance in milk-fed piglets.

Keywords: Glycyrrhetinic acid, Pigs, Ornithine decarboxylase, Small intestine.

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Dietary regulation of intestinal nutrients sensing by the different levels of arginine and glucose in white cashmere goat

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Abstract: Intestinal nutrient sensing is an important mechanism to maintain the body's homeostasis and which can be acted by several nutrient substrates such as arginine and glucose. The objective of this study is to examine the dietary regulation of intestinal nutrients sensing by the different levels of arginine and glucose in white cashmere goat byreal-time PCR, using chamber and western blot. Twenty-seven goats with permanent rumen fistula and duodenal cannulas were assigned randomly into nine groups. The diet for the three groups was: low nitrogen (CP: 10.5 %), low nitrogen +NCG (CP: 10.5 %) and high nitrogen (CP: 13.5 %). The experimental animals of every diet group were perfused three different levels glucose in the duodenum, and three glucose infusion dose was 0 g/d, 20 g/d and 40 g/d respectively. The results showed that with decrease in the diet nitrogen levels, the mRNA expression of SGLT1 and the content of VPG, PUN, citrulline, INS, GLP-1, GLP-2, GIP and CCK declined, but the mRNA expression of SLC7A9 and SLC7A1 increased. Isc and GT in the jejunum increased and the level of phosphorylation of 4EBP1 and PS6k7 in the jejunum decreased. Under the condition of low nitrogen diet, NCG added in the diet could increase SLC7A9 and SLC7A1 mRNA expression and increase the level of phosphorylation of 4EBP1 and PS6k7, Isc and GT in the jejunum. After infusing 20 g/d glucose in the duodenum, the mRNA expression of SGLT1, T1R1, T1R2 and T1R3 in the low nitrogen group was higher than in the high nitrogen group. Collectively, the low nitrogen which was added NCG is more apparently. It means that by properly reducing the N level in diets, and increasing the glucose and NCG, metabolism and intestinal nutrients sensing could be promoted.

Keywords: Arginine, Glucose, Intestinal nutrients sensing, mTOR, White cashmere goat.

Evaluation of chitosan/fructose model as an antioxidant and antimicrobial agent for shelf life extension of beef meat during freezing

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Abstract: In the present study, the combined effect of chitosan/fructose Maillard reaction products (CF-MRPs) with different ratios of fructose (1.0, 2.0 and 4 %) autoclaved for 15, 30 and 45 min at 140 ± 1 °C on shelf life extension of minced beef meat during frozen storage was investigated. Antioxidant and antimicrobial properties of chitosan–fructose complexes were tested. Antioxidant properties were measured by the DPPH, β-carotene and ABTS methods. These three methods showed the same profile of antioxidant activity. Chitosan with 4 % fructose autoclaved for 45 min (CF9) seemed to have the most effective antioxidant activity. It is indicated that browning product exhibited antioxidant activity. For antimicrobial activity, most chitosan–fructose complexes were more effective than chitosan. Thus, MRPs derived from chitosan–sugar model system can be promoted as a novel antioxidant to prevent lipid oxidation in food system. Chitosan–sugar complex could be a potential alternative natural product for synthetic food additive replacement and also meet consumer safety requirement.

Key words: Chitosan/fructose system, Antioxidant activity, Antimicrobial activity, Fresh minced beef meat.

Phosphorus supplementation improved weight gain of rats maintained on low protein diet

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Background and aim: Inadequate dietary protein intake is known to be associated with several adverse health effects including the development of protein energy malnutrition (PEM). PEM contributes to up to 50 % of childhood mortality in developing countries and is

usually associated with electrolyte imbalance and low total body phosphate. Recently, maternal protein restriction in rat was reported to favor the development of metabolic syndrome in the offspring during adulthood. Proteins are known to be important dietary sources of phosphorus (P). However, the involvement of P in the outcomes of low protein diets is not clear. The aim of this study was to dissect the impact of dietary P manipulation of low protein diet on food intake and weight gain of rats. Egg white protein was used for this purpose since it is known to contain negligible amount of P and have all essential amino acids.

Methods: Forty-nine male SpragueDawley (6 weeks old) rats were randomly allocated to five groups and given isocaloric diets. Group 1 (control) with normal protein 20 % (from egg white), and 0.3 % P, and four groups with low protein diet 10 % (from egg white) with different levels of phosphorus: 0.015, 0.05, 0.1, and 0.3 %. The rats were fed ad libitum for 9 weeks and their food intake and body weight were monitored.

Food intake, weight gain and energy efficiency (EE) of rats over the 9-week period.

Results and conclusion: Food intake, weight gain and EE of low protein groups were improved by the addition of P, in which that of the 0.3 % P group resembles that of the control group. Body changes in the low protein groups are mainly related to decreased P rather than protein intakes. Therefore, the role of P in growth and development requires further investigation, especially under conditions of low protein intake.

Keywords: Phosphorus, Low protein diet, Food intake, Body weight, Rats.

Identification of biomarkers associated with excess L-lysine intake in a rat model

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L-Lysine (Lys) is one of the most popular amino acids supplemented in foods and drinks. However, the potential adverse health effects of excess Lys supplementation are not fully understood and the safe upper limits of dietary Lys intake have not been established. The objectives of this study were to examine the effects of dietary supplementation with increasing amounts of Lys on growth, food intake and various hematological and blood biochemical parameters in rats. Male Sprague–Dawley rats, 10 weeks old, were randomly divided into ten groups (8 rats/group) and fed diets containing either 7 % or 20 % casein and supplemented with either 0 % (control), 1.5, 3, 6 % Lys or 6 % Lys+ 3 % arginine for 1 week. All rats were killed for the collection of blood and tissue samples. The results showed that the rats fed 7 % casein with addition of 1.5 % Lys had lower serum albumin (Alb), leptin and higher LDL cholesterol than rats fed the 7 % casein control diet ($p < 0.05$). Addition of 3 % Lys with 7 % casein significantly reduced body weight gain (BWG), food intake, serum Alb, alkaline phosphatase (ALP), prolactin, osteoprotegerin, leptin and HDL cholesterol, and increased mean corpuscular hemoglobin concentration (MCHC), blood urea nitrogen and pancreatic peptide (PP) compared to the 7 % casein control diet ($p < 0.05$). Addition of 6 % Lys with 7 % casein caused remarkable BW loss ($p < 0.001$) and altered an additional 15 biomarkers measured. Significant linear correlations were found between Lys intake and serum ALP ($R = -0.73$), HDL ($R = 0.70$), PP ($R = 0.60$), BUN ($R = 0.59$) and MCHC ($R = 0.52$) ($p < 0.001$). Addition of 6 % Lys in a 20 % casein diet markedly reduced BWG and food consumption and altered 13 biomarkers. Overall, this study demonstrated that the effects of Lys supplementation was modified by dietary protein levels, and adverse effects of excess Lys intake at low dietary protein (7 %) were noted with 1.5 % Lys supplement in rats. The blood levels of MCHC, PP, ALP, HDL and BUN may be used as markers of Lys excess.

	Control n = 10	P (0.015 %) n = 9	P (0.05 %) n = 10	P (0.1 %) n = 10	P (0.3 %) n = 10	Anova *P*value
Food intake (g/day)	22.88 ± 2.163[a]	19.20 ± 2.361[b]	19.70 ± 1.635[b]	23.13 ± 1.339[a]	23.70 ± 2.446[a]	0.000
Weight gain (g/day)	4.758 ± 0.677[a]	1.896 ± 0.578[d]	2.651 ± 0.407[c]	4.036 ± 0.506[b]	4.344 ± 1.261[ab]	0.000
EE (g/100 kcal)	5.073 ± 0.599[a]	2.382 ± 0.875[d]	3.311 ± 0.560[c]	4.361˘ 0.497[b]	4.408 ± 1.139[ab]	0.000

* Results are expressed as mean ± SD. Values that do not share the same superscript are statistically significant

ŒGut microbiota and aminopeptidase activities: the quest for the link between high-fat diets and hypertension

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Abstract

It has been proposed that the type of fat in the diet possesses a modulatory role in cardiovascular function. There is growing evidence for a preventive role of extra virgin olive oil (EVOO) in cardiovascular diseases. However, the involved mechanisms in this effect are not yet totally understood. There is a local renin–angiotensin–aldosterone system in the kidney, which plays a major role in the pathogeny of hypertension. In this system, Ang II is metabolized to Ang III and this peptide to Ang IV by, respectively, glutamyl- (GluAP) and alanyl- (AlaAP) aminopeptidase activities. The AT4 receptor for Ang IV has been identified as the insulin-regulated aminopeptidase (IRAP), which is identical to cystinyl-aminopeptidase (CysAP) and functionally associated with the GLUT4 glucose transporter.

There is an increasing interest in the influence of the gut microbiota on physiological processes such as the control of blood pressure through its modulation of vasoactive peptides. The microbiota may have an influence on these processes through the generation of metabolites, which enter the bloodstream via colonic absorption. Therefore, there is an interaction between gut microbiota and blood

pressure control, with its analysis being of particular interest to search new strategies for the prevention of cardiovascular diseases.

We have recently demonstrated that the type of fat used in the diet differentially affects blood pressure and other physiologic parameters and also modifies selectively the profile of gut microbiota in comparison with a standard diet.

In the present study, we analyzed the influence on gut microbiota, blood pressure levels and kidney aminopeptidase activities of two high-fat diets: one of them enriched with EVOO and the other with butter, which were compared with control mice fed a standard low-fat diet. Results demonstrated that, in comparison with controls, the two high-fat diets modified differently the profile of gut microbiota, but only the diet enriched with butter increased blood pressure. In kidney, while the diet enriched with butter decreased AlaAP and CysAP, the diet enriched with EVOO increased GluAP activity. These results may suggest an interaction between the type of diet, profile of gut microbiota, level of blood pressure and kidney aminopeptidase activities.

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Alpha-ketoglutarate lowers body weight and affects intestinal immunity through intestinal microbiota

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Abstract: Alpha-ketoglutarate (AKG) is a keto acid synthesized by deamination of glutamate and a critical intermediate in the tricarboxylic acid cycle. AKG shows beneficial effects on intestinal growth and integrity. However, the function of AKG in the intestinal immune system and microbiota is unknown. This study explores the effects of AKG supplementation on mouse intestinal immunity and intestinal microbiota. 120 female ICR mice were randomly assigned into four groups: (1) basal diet + normal water, (2) basal diet + AKG supplementation water (10 mg/ml), (3) basal diet + antibiotics-supplemented water (1 g/L ampicillin; 450 mg/L streptomycin), (4) basal diet + water supplemented with antibiotics and AKG (1 g/L ampicillin; 450 mg/L streptomycin; 10 mg/ml). On day 14, all mice were killed for the collection of ileum, luminal contents in the ileum and feces. The water intake, food intake and body weight gain were monitored during the whole experiment. The composition of the intestinal microbiota in feces and liminal content of ileum was analyzed by 16S rDNA sequencing. The expression of the intestinal proinflammatory cytokines and innate immune factors was analyzed by RT- PCR. Compared with group 1, AKG supplementation lowered body weight and influenced intestinal microbiota; however, AKG supplementation had little effect on body weight in antibiotics-treated mice. Group 2 AKG supplication inhibited the expression of *Muc4* in mouse ileum and promoted expression of mammalian defensins of the alpha subfamily (including *Cryptdin1*, *Crydin4,* and *Crytdin5*) compared with group 1. Meanwhile in antibiotic treatments, AKG reduced the expression of *Cryptdin 1* and *Cryptdin 5*. We concluded that AKG affected body weight and intestinal immunity through intestinal microbiota.

Keywords: Alpha-ketoglutarate, Intestinal immunity, Intestinal microbiota.

Impact of prolonged leucine supplementation on protein synthesis and lean growth in neonatal pigs

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Most low birth weight infants experience extrauterine growth failure due to insufficient nutrient intake. Leucine has been shown to have anabolic properties in skeletal muscle. The objective of this study was to determine if prolonged leucine supplementation increases lean growth in neonatal pigs fed a restricted protein diet. Neonatal pigs (5-day-old; $n = 14$–16/trt; 1.9 ± 0.3 kg BW) were fed by gastric catheter a whey-based milk replacement diet with either a high protein (HP; 22.5 g/kg BW/day protein, 2.4 g/kg BW/day leucine) or restricted protein (RP; 11.2 g/kg BW/day protein, 1.2 g/kg BW/day leucine) content or RP supplemented with leucine to the same level as in the HP diet (RPL). Pigs were fed 40 ml/kg BW per meal every 4 h for 21 days. The mass of the longissimus dorsi (LD) and soleus muscles was increased in the HP compared to RP group ($P < 0.05$) with LD in pigs fed the RPL intermediate to the RP- and HP-fed pigs. Protein synthesis was increased in all muscles in response to a meal ($P < 0.05$) and was further increased in RPL and HP compared to the RP group in LD muscle ($P < 0.05$). Feeding also increased phosphorylation of 4EBP1 and S6K1 and formation of the active eIF4G·eIF4E complex in LD ($P < 0.05$) and the activation of these mTOR signaling proteins was further enhanced in the RPL and HP groups ($P < 0.05$). There was no effect of diet on indices of protein degradation signaling. Thus, when protein intake is chronically restricted, the capacity for leucine supplementation to enhance muscle protein accretion in neonatal pigs that are meal-fed protein-based diets is limited. (Supported by NIH AR444474, NIH HD072891, USDA NIFA 2013-67015-20438, and USDA/ARS 6250-51000-055).

Effect of dietary selenium yeast on the growth performance and serum amino acids level in early-weaned piglets

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Selenium (Se), as an essential trace element and a functional component of the antioxidant defense system, is needed for the maintenance of the immune functions. 40 21-day early-weaned piglets with similar body weight were randomly assigned into four treatments, with 10 replicates per treatment and 1 piglets per replicate. The control treatment (T1) was fed with basic diet; the other treatment groups were supplemented with 0.1 % (T2), 0.2 % (T3) and 0.3 % (T4) yeast selenium in basal diets, respectively. On day 21, dietary yeast selenium (T2, T3, and T4) increased ($P < 0.05$) the average daily feed intake (ADFI), average daily gain (ADG), and final body weight significantly, and decreased the feed/gain ratio ($P < 0.05$) significantly compared with T1. Results indicated that compared to the control group, T2 and T3 decreased ($P < 0.05$) the activities of myeloperoxidase (MPO) and the concentration of malondialdehyde (MDA) in the plasma, as well as increased ($P < 0.05$) the activities of catalase (CAT) in the plasma. No significant difference was found in superoxide dismutase (SOD) among all the groups. On day 21, T2 and T3 increased serum concentrations of Ser, Lys and Arg ($P < 0.05$) significantly, whereas T1 had a trend of increasing Ser, Lys and Arg ($P > 0.05$) level. These findings indicate the beneficial effects of dietary yeast selenium on growth performance and antioxidant status.

Impact of dietary protein level on digestive and immune proteomics profiles of piglets' intestine

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Abstract: The objective of this research is to investigate the effect of low dietary CP level with free AA supplementation on digestive and immune proteomics profiles of piglets' intestine. Eighteen piglets (Duroc × Landrace × Yorkshire, female) were weaned at 28 days of age, and the experiment began after a 7 days adaptation period when the piglets were weighed to determine an initial mean body weight (BW) (9.57 ± 0.64 kg, mean ± SD). Pigs were randomly assigned to three different groups (6 pigs/treatment): 20 % CP group (control, NRC 2012 requirements), 17 % CP group and 14 % CP group. Each of the groups were fed diets with different levels of CP (14, 17 and 20 %), but the same level of digestive energy and four amino acids: Lys, Met, Thr, Try, respectively. All of the animals were housed individually in cages and had free access to feed and drinking water at all times throughout the experimental period. Blood and longissimus muscle were collected immediately for experiment after pigs were killed.

Results: Compared with the control (20 % protein level), the average daily feed intake, the average daily gain and gain feed ratio of the 17 % protein level did not decrease ($P > 0.05$), but those of 14 % protein level decreased ($P < 0.05$). KEGG pathway analysis for the proteomics profiles showed that, compared with the control, steroid hormone biosynthesis, ribosome, alcoholism and chemical carcinogenesis were down-regulated, while focal adhesion, PI3K-Akt signaling pathway, phagosome, protein digestion and absorption, ECM–receptor interaction, complement and coagulation cascades were up-regulated in the 17 % protein group. Although the protein digestion and absorption, ECM–receptor interaction and complement and coagulation cascades were up-regulated, the protein export also was up-regulated in the 14 % protein group, while alcoholism, insulin signaling pathway, systemic lupus erythematosus, PPAR signaling pathway, fat digestion and absorption and neuroactive ligand–receptor interaction were down-regulated. Conclusion: the protein level decreased from 20 to 17 %, the growth performance was not affected, the protein digestion and absorption was increased and the immune function was also improved, which implied that 17 % protein level may be of benefit for nutrition absorption in pigs.

Control of cell signaling by *O*-GlcNAcylation, the sweet counterpart of phosphorylation

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Post-translational modifications (PTM) are the ultimate element that perfects the existence and the activity of proteins. Owing to PTM, no fewer than 500 million biological activities arise from approximately 20,000 protein-coding genes in humans. PTM regulate protein functions by promoting or preventing protein–protein interactions (theory of "one partner–one function"). The modification therefore re-localizes the protein, impacts on its expression or regulates enzyme activity as shown recently for PFK1. Glycosylations is a large family of PTM that includes *O*-GlcNAcylation and may be the simplest one from the structural point of view. *O*-GlcNAcylation consists in the reversible addition of a single *N*-acetylglucosamine to the hydroxyl groups of serine and threonine of cytosolic and nuclear proteins. *O*-GlcNAcylation processes are managed by a unique couple of enzymes, namely OGT (*O*-GlcNAc transferase classified to the GT41 family in CAZy) responsible for the transfer of the GlcNAc moiety and OGA (*O*-GlcNAcase classified to the GH84 family in CAZy) that reverses the reaction. Despite appearances, *O*-GlcNAcylation hides a very complex regulation mode, such as phosphorylation with which *O*-GlcNAcylation could either compete or act in concert. In this way, *O*-GlcNAcylation is part of signaling pathways which control the level of the glycosylation and in turn *O*-GlcNAcylation interferes with the regulation of the PI3K and MAPK pathways. Moreover, in response to insulin, OGT is targeted to lipid microdomains in a PI3K-dependent manner. *O*-GlcNAcylation levels intimately correlate with the nutritional status of the cell due to the nature of the nucleotide–sugar, UDP–GlcNAc, from which it comes from. Therefore, *O*-GlcNAcylation relays nutrient and energy status to cell homeostasis. Accordingly, we recently proposed that β-catenin *O*-GlcNAcylation, which reduces its proteasomal susceptibility, is responsible for epithelial tumorigenesis. Our in vivo experiments show that Ob/Ob mice, in comparison with C57Bl6 mice, developed tumors few weeks after injection of azoxymethane (AOM) and that mice fed a high-carbohydrate diet exhibited higher amounts of β-catenin and *O*-GlcNAc compared with mice placed on a standard diet. These results show that the rate of *O*-GlcNAcylation correlates with availability of nutrients and could explain how a nutritional imbalance interferes with colorectal cancer emergence. We next investigated the influence of OGT on cancer cells lines properties, such as proliferation, migration and aggregation. We showed that OGT silencing decreased proliferation, migration and adhesion of cancer cell lines such as HT29 and HCT116, but also of the fetal non-cancer cell line CCD841CoN. In another set of studies, we showed that *O*-GlcNAcylation is crucial for cell cycle progression. OGT is required for quiescent cells to reach the G0/G1 transition, notably glycosyltransferase is needed for cyclinD1 expression, and for *Xenopus laevis* oocytes to mature in a process similar to G2/M transition. On the contrary, OGA activity correlates with G1/S transition. The focus of this talk is to give precise examples in which OGT and *O*-GlcNAcylation are pivotal elements for cell fundamental processes.

O-GlcNAcylation and pre-replication: OGT interacts with the MCM2-7 helicase complex in human somatic cells

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Several works, including those of our laboratory, showed that a disruption of the dynamics of *O*-GlcNAcylation affects cell division. In addition, the overexpression of *O*-GlcNAc transferase (OGT) and the increase of its activity contribute to tumorigenesis by promoting growth and invasion of cancer cells, both in in vitro and in vivo models. We previously studied the importance of *O*-GlcNAcylation during G0/G1 and G1/S transitions in human somatic cells and identified around 60 proteins for which differences in *O*-GlcNAcylation status were observed between G1 and S phases. Among them, we demonstrated that the mini chromosome maintenance proteins MCM2, MCM3, MCM6 and MCM7 involved in the formation of the pre-replicative complex are differentially *O*-GlcNAcylated between the G1 and S phases. The aim of our present work is to decipher the role of *O*-GlcNAcylation and OGT in the formation, the recruitment to the chromatin and the helicase activity of the MCM2–7 complex in human somatic cells. By co-immunoprecipitation approach and in situ proximity ligation assay (PLA), we show that OGT interacts with some of the MCM proteins. Furthermore, the extinction of the expression of either OGT or OGA by RNA interference alters the localization of certain subunits of the MCM2–7 complex between the soluble and chromatin-bound fractions. Finally, we are currently studying the crosstalk between phosphorylation and *O*-GlcNAcylation on the MCM proteins by two-dimensional electrophoresis combined with *Click-chemistry* labelling and western blotting. Overall, this study will bring new elements to understand the role of OGT in the molecular mechanisms involved in the regulation of the initiation of DNA synthesis.

Role of *O*-GlcNAcylation in cell signaling and pathophysiology

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O-GlcNAc glycosylation (*O*-GlcNAcylation) corresponds to the addition of *N*-acetylglucosamine on serine and threonine residues of cytosolic and nuclear proteins. *O*-GlcNAcylation is a dynamic post-translational modification, analogous to phosphorylation, which regulates the stability, the activity or the sub-cellular localisation of target proteins. This reversible modification depends on the availability of glucose and therefore constitutes a powerful mechanism by which cellular activities are regulated according to the nutritional environment of the cell. *O*-GlcNAcylation has been implicated in important human pathologies including Alzheimer disease, type-2 diabetes and cancer. Only two enzymes, OGT and OGA, control the *O*-GlcNAc level on proteins. Therefore, *O*-GlcNAcylations do not organize in signaling cascades as observed for phosphorylations. O-GlcNAcylations should rather be considered as a "rheostat" that controls the intensity of the signals travelling through different pathways according to the nutritional status of the cell. For instance, OGT attenuates insulin signal by *O*-GlcNAcylation of proteins involved in proximal and distal steps in the PI-3 kinase signaling pathway. This negative feedback may be exacerbated when cells are chronically exposed to elevated glucose concentrations and could thereby contribute to alterations observed in diabetic patients. Thus, we previously provided the first evidence that *O*-GlcNAcylation of FoxO1 in liver cells stimulates its transcriptional activity, resulting in increased expression of genes involved in glucose production by the liver. More recently, we observed that *O*-GlcNAcylation of FoxO1 occurs in pancreatic β-cells under hyperglycaemic conditions, and this could contribute to the deleterious effects of hyperglycaemia on β-cell pancreatic function, resulting in worsening of hyperglycaemia (glucotoxicity). In addition, several lines of evidence indicate that *O*-GlcNAcylation in immune cells may also participate in inflammatory processes associated with chronic hyperglycaemia. Therefore, this post-translational modification appears to play crucial roles in chronic diseases associated with modern lifestyle.

Metabolic control of tumor growth by PKM2 *O*-GlcNAcylation

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Metabolism drives all biological processes, dysregulation of which fuels a plethora of human diseases. This is largely achieved by reprogramming gene expression in the metabolic network. However, a major question in the field is how various posttranslational modifications of metabolic enzymes contribute to metabolic alterations in normal and disease states. Tumor cells divert a majority of glucose to anabolic pathways to support rapid cell growth. A fraction of glucose channelled into the hexosamine biosynthesis pathway enables the dynamic post-translational modification of intracellular proteins by O-linked β-*N*-acetylglucosamine (*O*-GlcNAc). *O*-GlcNAcase (OGA or NCOAT) is a bifunctional enzyme harboring *O*-GlcNAc hydrolase and cryptic lysine acetyltransferase activities. Here, we report that OGA drives aerobic glycolysis and tumor growth by inhibiting pyruvate kinase M2 (PKM2). PKM2 is dynamically *O*-GlcNAcylated in response to glucose. Under nutrient-rich conditions, PKM2 is a target of OGA acetyltransferase activity, which facilitates the recruitment of *O*-GlcNAc transferase (OGT) to *O*-GlcNAcylate PKM2. *O*-GlcNAcylation inhibits PKM2 enzymatic activity and promotes tumor growth. These studies define PKM2 *O*-GlcNAcylation as a metabolic rheostat that regulates aerobic glycolysis, and suggest that the crosstalk between various post-translational modifications on metabolic enzymes integrates metabolic cues to control cell survival and proliferation.

Type of presentation: Invited talk.

Use of a cytotoxic HEXIM1 peptide in cancer therapy

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Abstract: Hexamethylene bisacetamide-inducible protein 1 (HEXIM1) is best known as the inhibitor of positive transcription elongation factor b

(P-TEFb) and is recently identified as a novel positive regulator of p53. Our previous studies show that human double minute-2 protein ubiquitinates the basic region (BR) of HEXIM1. The BR is also found to mediate the binding of HEXIM1 to a nucleolar protein, nucleophosmin (NPM), which is critical in ribosome biogenesis. Here, we report that the treatment of cells with a fusion peptide, which comprises BR and a cell-penetrating peptide, and triggers rapid cytotoxic effect independent of p53. When fused with a breast cancer cell-targeting peptide, LTV, the LTV–BR fusion peptide exhibits specific killing of breast cancer cells. Unlike the commonly used cytotoxic peptide, KLA, BR peptide shows minimal or no non-specific cell killing when it is not fused with any cell-penetrating or cancer-targeting peptides. We find that HEXIM1 BR peptide depolarizes mitochondrial membrane potential in minutes and its cell-killing activity is unaffected by caspase inhibition, suggesting that HEXIM1 peptide induces cell toxicity through a non-apoptotic mechanism. Furthermore, we observed an accumulation of the internalized BR peptide in the nucleoli of treated cells and an altered localization of NPM. These results illustrate a novel mechanism by which the BR peptide induces cell death by disrupting mitochondrial membrane potential and sub-cellular localization of NPM, possibly leading to inhibition of ATP generation and ribosome biosynthesis/protein translation, respectively. Taken together, we demonstrate the potential use of HEXIM1 BR peptide as a novel therapeutic peptide against cancer.

Interactions of *Galleria mellonella* cecropin D-like peptide with bacterial cells

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Antimicrobial peptides (AMPs) have been described as evolutionary ancient weapons, which are produced by a wide variety of organisms as part of an innate immune response. The secondary structures of AMPs could allow dividing them into: (1) α-helical, (2) β-stranded due to the presence of two or more disulphide bonds, (3) β-hairpin or loop due to the presence of a single disulphide bond and/or cyclization of the peptide chain, and (4) extended. In hemolymph of the great wax moth *Galleria mellonella* larvae, an impressive set of cationic and anionic peptides differing in biochemical and antimicrobial properties was reported. We purified and characterized eight *G. mellonela* peptides, which appeared in larval hemolymph after immune challenge, among which eas the cecropin D-like peptide. Cecropins are one of the best-known α-helical cationic AMPs isolated from insects; however, cecropin D family members have been described only in Lepidoptera. Cecropins have an ability to kill or neutralize Gram-negative and Gram-positive bacteria, fungi (including yeasts), parasites, cancer cells, and even enveloped viruses like HIV. It is known that α-helical peptides disrupt cell membrane, which finally leads to bacterial death.

In the present study, we demonstrated that a 2.5 μM concentration of *G. mellonella* (Gm) cecropin D-like peptide is enough to perforate *Escherichia coli* JM83 cells. To provide more insight into the antimicrobial action of Gm cecropin D-like peptide, the bacteria incubated without and in the presence of the peptide were imaged using atomic force microscopy (AFM) and transmission electron microscopy (TEM). The cell surfaces of the peptide-treated bacteria became rougher in comparison to the control cells and deep holes were visible. The RMS roughness values of these cells after 60 min incubation with the peptide increased considerably to 2.58 ($\pm$0.65) in comparison with 1.78 ($\pm$0.46) calculated for the control ones. The evident changes in the surface structure were accompanied by changes in the biophysical properties. The analysis of the images suggested that treatment of the cells with the Gm cecropin-D-like peptide decreased the cell turgor pressure, which was confirmed by significantly decreased elasticity, reflected by the DMT (Derjaguin-Muller-Toporov) modulus, ca. 3- and 2.5-fold after 30 and 60 min incubation, respectively. In turn, the adhesive force between the AFM tip and cell surface after 30 min incubation was almost unaffected, but 60 min incubation was sufficient to decrease it ca. 2.6-fold. To examine the direct effect of the Gm defense peptide on *E. coli* cell morphology, the bacterial cells were observed by TEM. Microscopic investigations of the cells treated with the Dm cecropin D-like peptide for 60 min revealed many alterations of the structure of *E. coli* cells, especially cell envelope damage and changes in the cytoplasm appearance. The peptide induced disruption in the cell wall and appearance of an enlarged periplasmatic space. Swollen cells with clear cytoplasm that retained the shape were also observed. Moreover, Fourier transform infrared (FTIR) spectroscopy was used to gain detailed information on the type of conformational changes in Gm cecropin D-like peptide induced by interactions with *E. coli* cells. The results indicated that Gm cecropin D-like peptide binding to components of the bacterial cell envelope is responsible for the anti-*E. coli* JM83 activity of this α-helical AMP.

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Structural characterization and antimicrobial activity of innovative compounds: a novel strategy for in silico peptides design

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Infectious diseases account for approximately 13.3 million deaths worldwide each year. With the increasing emergence of multidrug-resistant microorganisms, the demand for new and more effective antimicrobial agents has never been more urgent and several strategies have been so far used to develop novel antimicrobial peptide agents.

Recently it has been reported that different antimicrobial peptides exhibit an immediate cytotoxic effect against cancer cells, selectively causing a mitochondrial membrane damage and subsequent triggering

apoptosis [1]. Therefore, it can be supposed that mitochondrial targeting peptides could also display antimicrobial activity by binding and disrupting the plasma membrane of bacterial cells.

In this context, two peptides named rNterC and rTM2C including 13 and 15 amino acids, respectively, were projected starting from protein sequences which are known to bind or to be targeted to the outer mitochondrial membrane. By site-directed mutation modeling using wild-type sequences as scaffolds, the peptides were modified to possibly increase antimicrobial potency taking into account several important aspects specifically concerning the appropriate balance among the total net charge, amphipathicity and hydrophobicity [2].

Usually, a large proportion of antimicrobial peptides are unstructured in aqueous solution but they adopt amphipathic α-helices when bound to a phospholipid bilayer. However, in different solvents (such as TFE) these peptides can change conformation if they have a tendency to fold.

Aim of the study was to investigate the structural properties of the two peptides and to evaluate their antimicrobial activity against *Listeria monocytogenes* (NCTC 11994-HPA—London) using different concentrations (10, 50, 100 μg/mL); for the assays we suspended a lenticula *of L. monocytogenes* (4.3×10^3–2.1×10^4 UFC/lenticule disc) and the growth of the microorganism was observed in two different culture media: blood agar (Biolife-Italia) and ALOA medium (Biolife-Italia). Results revealed that the highest concentration produced the best antimicrobial activity for both peptides analyzed. Specifically, at 100 μg/mL concentration, **rTM2C** efficiently inhibited the growth of *L. monocytogenes* by 98.5 or 99.0 %, on blood agar or ALOA medium, respectively, while at the same concentration **rNterC** inhibited the growth of *L. monocytogenes*, by 94.7 or 95.5 %, on the two culture media, respectively.

Finally, circular dichroism (CD) analyses performed in different environment and temperature conditions revealed that **rTM2C** assumed a α-helix structure in TFE 50 % which was stable until 50 °C and a combined α-helix/β-turn structure in 3 and 10 mM SDS which remained stable until 90 °C. In contrast the **rNterC** peptide showed CD spectra typical of a random-coil structure in all experimental conditions investigated.

Moreover, preliminary studies by NMR spectroscopy suggested a large number of inter-residue NH–NH contacts in **rTM2C** and an α-helix structure at the C-terminal region even if the spectra were acquired in a strong denaturing solvent such as DMSO.

1. Constance JE, Lim CS (2012) Targeting malignant mitochondria with therapeutic peptides. Ther Deliv 3(8):961–979.
2. Lira F, Perez PS, Baranauskas JA, Nozawa SR (2013) Prediction of antimicrobial activity of synthetic peptides by a decision tree model. Appl Environ Microbiol 79(10):3156–3159.

Kinases-superparamagnetic beads for hyperphosphorylation of peptides/proteins

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Abstract: Hyperphosphorylated Tau protein preparation, as the marker of Alzheimer disease, was the purpose for the kinases immobilizations. We focused on the immobilization of proline-directed protein kinases: extracellular signal-regulated kinase (ERK2) and glycogen synthase kinase 3β (GSK-3β).
Today, the carriers with immobilized enzymes as recoverable, stable and specific catalysts are routinely applied in many fields of industry and science, e.g. basic biochemical and pharmacological research. Magnetic microparticle carriers bring the advantage of the non-contaminating and very specific and sensitive reaction on their substrates, peptides and proteins. Magnetic microparticles were used for the kinase immobilization: BcMag®-Aldehyde, SeraMag-carboxyl, SIMAG-PGL, SIMAG-IDA-Co^{2+}, and SIMAG-IDA-Ni^{2+}. Relevant methods of covalent immobilization, non-oriented and oriented, were chosen and reaction conditions were optimized. Phosphorylations of low molecular substrates followed by MALDI MS analysis were used as detection system. Operational and storage stabilities of kinase-superparamagnetic beads were observed. Both the soluble and immobilized ERK2 and GSK-3β were applied for recombinant tau protein phosphorylation. Tryptic phosphopeptides enrichment was performed by ion-metal affinity chromatography using TiO_2 magnetic nanoparticles. The level and the position of phosphorylation sites were identified using MALDI-LTQ-Orbitrap MS.

Present results document very well the ability of ERK2- and GSK-3β-superparamagnetic beads to phosphorylate the target peptides or proteins with desired efficiency and specificity. The process of phosphorylation can be better controlled; subsequent purification of phosphorylated Tau can be omitted.
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New synthetic cathelicidin peptides with anti-microbial activity against methicillin-resistant *Staphylococcus aureus*

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The Gram-positive *Staphylococcus aureus* is a major human pathogen [1, 2], and the increasing prevalence of methicillin-resistant *S. aureus* (MRSA) in hospitals and communities, both human and animal has led to a demand for new agents that could be used to decrease the spread of this bacterium.

Anti-microbial peptides (AMPs) are essential components of innate immunity in humans and other higher organisms, contributing to our first line of defense against infection. The AMPs belonging to the cathelicidin family are a large group of peptides ranging from 12 to 80 amino acid residues in length. LL-37 [3] is the 37-residue human cathelicidin, which represents one of the most studied AMP showing a broad-spectrum anti-microbial activity against Gram-negative and Gram-positive bacteria, including *S. aureus* [3]. However, MRSA strains appear to be less sensitive to LL-37 [4], demonstrating the need to identify more effective AMPs. Thus, we have developed two synthetic cathelicidin peptides, **hCath-D** and **NaCath-D**, from human and snake (*Naja atra*), respectively, and tested their anti-microbial activity against MRSA. The peptides were modified to possibly increase antimicrobial potency taking into account the appropriate balance among the total net charge, amphipathicity and hydrophobicity. Interestingly, they showed a significant potential

antimicrobial activity (Boman index: 3.0 and 2.54, GRAVY values: 0.22 and -1.58, total net charge: +5 and +8, for **hCath-D** and **NaCath-D**, respectively), when submitted to the predictive tool available at the Antimicrobial Peptide Database v2.34 (APD2; http://aps.unmc.edu/AP/main.php).

In addition, using different concentrations (25, 50, 100 μM) of both peptides in anti-microbial assays against MRSA (ATCC 33591), it was demonstrated that only **hCath-D** was effective in killing the tested strain using a concentration of 100 μM.

Hence, this peptide can be used in the development of new pharmaceuticals research activities or in the food industry to increase the shelf life of foods products.

1. Menzies BE, Kenoyer A (2005) *Staphylococcus aureus* infection of epidermal keratinocytes promotes expression of innate antimicrobial peptides. Infect Immunity 73(8):5241–5244.

2. Lowy FD (1998) *Staphylococcus aureus* infections. N Engl J Med 339(8):520–532.

3. Gennaro R, Scocchi M, Merluzzi L, Zanetti M (1998) Biological characterization of a novel mammalian antimicrobial peptide. Biochimica et Biophysica Acta 1425(2):361–368.

4. Ouhara K, Komatsuzawa H, Kawai T, Nishi H, Fujiwara T, Fujiue Y, Kuwabara M, Sayama K, Hashimoto K, Sugai M (2008) Increased resistance to cationic antimicrobial peptide LL-37 in methicillin-resistant strains of *Staphylococcus aureus*. J Antimicrob Chemother 61(6):1266–1269.

Chemical modifications of peptide sequences via S-alkylation reaction performed on solid–solid phase

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Chemical derivatization of an individual amino acid is a commonly used approach to manipulate and study protein and peptide therapeutics. In this regard, we have recently described a strategy for performing postsynthetic peptide modifications via selective *S*-alkylation of a peptide sequence, under mild conditions, that requires only the appropriate alkyl halide and activated molecular sieves to catalyze the reaction. This approach was widely applied to various alkyl groups.

The current work focuses on the alkylation of cysteine-containing peptides anchored to the solid support. In other words, we tried to tune the S-alkylation reaction parameters by performing chemical modification on solid–solid phase. Indeed, in analogy with previously reported studies, the solid molecular sieves are employed as base to promote the *S*-alkylation. As we have previously discussed, the molecular sieves, activated by heating at 280 °C for 4 h under vacuum in atmosphere of Ar, are supposed to capture the proton of the sulfhydryl group. A broad range of peptide models were synthesized, all containing one or more cysteine residues protected with the acid-labile methoxytrityl group on their side chain. The alkylation was performed by suspending the peptidyl-resin in DMF, then the appropriate alkyl halide was added and the stirring was kept at room temperature for 12 h. The reached yields were quite high for most of the chosen alkyl groups. As an application of this novel strategy, we have planned to synthesize peptides reproducing natural sequences and containing specific substituents on cysteine residues.

Cyclopropane pipecolic acids as templates for linear and cyclic peptidomimetics

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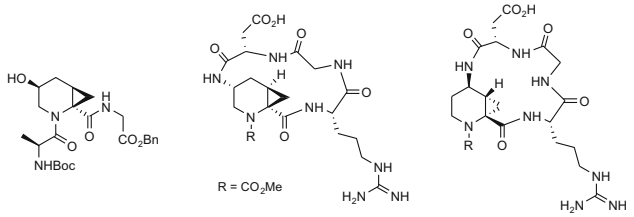
Cyclopropane amino acids have been exploited to reduce conformational mobility in peptidomimetics.[1] However, to our knowledge there are no reports on Cyclopropane Pipecolic Acids (CPA) embodied in peptides.

Employing as key steps Pd-catalyzed methoxycarbonylations and OH-directed cyclopropanations, we managed to synthesize various substituted CPAs[2,3] and to use them to build linear and cyclic peptidomimetics (figure 1)0.[3]



We obtained two cyclic peptidomimetics bearing an RGD amino acid sequence, which displayed nanomolar activity as antagonists for the $\alpha_v\beta_3$ subfamily of integrins.[3] This protein plays a critical role in tumour-induced angiogenesis and metastasis, representing a potential therapeutic target.

Thus, CPAs appear suitable for the generation of novel peptidomimetics for drug discovery.

References:

1. Hanessian S, Auzzas L (2008) Acc Chem Res 41:1241–1251.

2. Occhiato EG, Casini A, Guarna A, Scarpi D (2011) Eur J Org Chem 6544–6552.

3. Sernissi L, Petrović M, Scarpi D, Guarna A, Trabocchi A, Bianchini F, Occhiato EG (2014) Chem Eur J 20:11187–11203.

The purification and characterisation of dipeptidyl peptidase IV from sheep lung

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Abstract: In this study, dipeptidyl peptidase IV from sheep lung was purified 370-fold with a yield of % 60 using ammonium sulfate precipitation followed by DEAE-Sepharose, ConA-Sepharose 4B and

Gly-Pro-EC-HA Sepabeads affinity chromatography. DPPIV identity was confirmed by activity glycoprotein staining of non-denaturating PAGE/SDS-PAGE gels and LC–MS/MS analysis of tryptic peptides. Carbohydrate content of the enzyme was estimated as 18.9 %. The unreactivity of purified DPPIV to CD26 antibodies indicated the CD26 and lung DPPIV is unidentical. Molecular weight of the enzyme purified from sheep lung was estimated to be about 180 kDa by gel filtration and 90 kDa by SDS-PAGE suggesting that the native enzyme is a dimeric form. Isoelectric focusing indicated a pI of approximately 4.8–0.2. The optimum temperature and pH was 45 °C and 7.4, respectively. KM and Vmax values for the hydrolysis of Gly-Pro-pNA were 0.32 mM and 35.9 μmol/min/mg, respectively. Temperature and pH stability of the DPPIV at 0–45 °C and pH 7.0–9.0 were defined as 100 and 94 %, respectively. Furthermore, the enzyme maintained 88 % of its activity after storage at −80 °C for 8 weeks. Lung DPPIV was inhibited by Hg^{+2}, Zn^{+2} and Cd^{+2} ions and activated by Mg^{+2} ions. Diprotin A (0.1 mM) was shown to inhibit the enzyme activity at a ratio of 91 %. Using PMSF and 1–10 fenantroline in the concentration range of 0.1–5.0 mM resulted in enzyme inhibition in a concentration-dependent manner. However, γ-mercaptoethanol and DTT in the same concentration range had a slight effect on enzyme activity. Among serine protease inhibitors, benzamidine and PABA were effective only at high concentrations suggesting the substrate affinity difference of trypsin-like serin proteases. The enzyme hydrolysed natural and synthetic substrates containing proline at a faster rate, while it slowly hydrolysed natural and synthetic substrates containing alanine.

Keywords: Serine protease, Lung, Dipeptidyl peptidase IV, Purification, Characterization.

Interactions of unique *Galleria mellonella* proline-rich defense peptides with bacterial cells

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Antimicrobial peptides are key factors of humoral immune response against invading pathogens in all living organisms, e.g. humans, mammals, and insects. Proline-rich peptides (PRPs), originally isolated from insects, are widely distributed in the animal kingdom. PRPs contain a high proportion of proline residues (25 %) and hydrophobic amino acids (40 %). Insect PRPs are divided into two subgroups, one consisting of relatively short (18–20 amino acid-long) peptides and one consisting of longer (32–34 amino acid-long) peptides. They kill mostly Gram-negative bacteria with high efficiency. These peptides appear to inhibit metabolic processes, such as replication, transcription, protein synthesis or chaperone-assisted protein folding. The hemolymph of bacteria-challenged *Galleria mellonella* (Gm) larvae contains a great repertoire of antimicrobial peptides. Among them, there are two cationic proline-rich peptides 1 and 2 with unique amino acid sequence. Proline-rich peptide 1 (P1) consists of 37 aa and contains 5 proline residues (13.5 %); its molecular mass is 4322.0 Da. Proline-rich peptide 2 (P2) is a unique 42-mer peptide, which has 11 proline residues (26.2 %) and molecular mass 4927.6 Da.

It is assumed that PRPs affect essential cellular processes by interacting with intracellular targets after penetrating across the membrane layer; however, the mode of interaction of proline-rich peptides with bacterial cells is not yet fully clarified. Taking this into account, research on the effect of Gm proline-rich peptides 1 and 2 on Gram-negative bacteria *Escherichia coli* JM83 has been undertaken.

The membrane perforation ability of these peptides was determined by measuring of β-galactosidase activity released from damaged bacterial cells. Both P1 and P2 permeabilized bacterial membrane, but the perforation level was 9 and 49 %, respectively, when the bacteria were incubated with 10 μM concentration of the peptides. To determine if these peptides could bind to *E. coli* JM83, fluorescein isothiocyanate (FITC)-labelled P1 and P2 were used. Fluorescence microscopy imaging of the bacteria after 15 min incubation with FITC-P1 or FITC-P2 revealed the presence of green fluorescing bacterial cells, indicating interaction of the peptides with *E. coli.* Interestingly, P2 bound evenly to the cell surface, whereas increased binding of P1 on the cell poles was detected. Atomic force microscopy (AFM) imaging of *E. coli* treated with the peptides demonstrated cells forming disordered structures in addition to the damaged ones. AFM analysis revealed also alterations of nanomechanical properties of the bacterial cells. Upon P1 treatment, the RMS roughness value and the elasticity of the bacteria decreased in comparison to the control ones. However, adhesion increased after 60 min incubation, but after 45 min incubation of *E. coli* with P1 it was lower than the control cells. The RMS roughness values of the P2-treated bacteria increased compared to the control cells. In contrast, the analysis revealed a decrease in elasticity and adhesion properties of the cells after 45 min incubation with P2, but after 60 min incubation P2 did not influence the adhesion and considerably increased the cell elasticity. Our data suggested different mode of interaction of the two unique *G. mellonella* proline-rich peptides with bacterial cells.

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Synergy between host defense peptides with different mechanisms of action

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Over the last years, a growing number of bacterial species have become resistant to antimicrobial treatment due to improper use of antibiotics. Host defense peptides (HDP) have received widespread attention as possible alternatives to conventional drugs due to their rapid efficacy against a broad range of multiresistant pathogens, the membranolytic mode of action and the consequent low rate of resistance development. However, their comparatively high toxicity against mammalian cells is an obstacle in the way of development of therapeutics. To date a number of studies of the synergy between conventional antibiotics and the peptides have been reported. One major concern regarding the utility of this approach is the tendency of conventional antibiotics to induce resistance when administered in sublethal doses which is common for synergic combinations. In contrast, there have been fewer studies of a synergy between HDPs although this phenomenon contributes to the explanation of the presence of substantial diversity of the peptides in any anatomic site in the host.

In this study, we examined the synergy between HDPs representing three main structural classes found in animals when using their combinations against pathogenic bacteria *Staphylococcus aureus*, *Escherichia coli*, or *Pseudomonas aeruginosa*. We tested a variety of combinations of the peptides with different modes of action including β-hairpin peptides arenicin and tachyplesin, the α-helical acipensin (fragment of the histone H2A), and the extended-structure molecules. The synergy was measured by microtiter dilution assay, with the view of searching for a reduction in the minimum inhibitory concentration (MIC) of each compound in the presence of the other. The most pronounced effect was observed for the tachyplesin–acipensin pair with a 16-fold decrease of the individual MIC values. One possible explanation for this effect is based on increasing the membrane permeability by the β-hairpin peptide that promotes intracellular uptake and DNA binding of the histone-derived acipensin. The obtained results suggest a potential medical application of combinations of HDPs with different modes of action.

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Solid phase synthesis of antifreeze glycopeptides

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Antifreeze Glycopeptides (AFGPs) are a class of biological antifreeze agents found in Arctic and Antarctic species of fish which enable living at temperatures below the freezing point of the body fluids. Antifreeze glycopeptides usually consist of 4–55 tripeptide units alanine-alanine-threonine, glycosylated at the threonine side chains. These unique compounds show the ability to regulate ice nucleation and ice crystal growth. Due to their interesting properties AFGPs have many potential applications (especially in food industry and cryopreservation of various organs, tissues, stem cells). Applying these materials to biomedical and industrial purposes requires significant amounts of AFGPs in a pure form and at reasonable costs. Gaining AFGPs from natural sources is both expensive and labor-intensive. Therefore, it is crucial to develop an efficient synthetic strategy to produce a range of homogeneous and native AFGPs. Here, we present the synthesis of a tripeptide AFGP analogues using solid phase peptide synthesis approach which is a convenient method of obtaining heavily glycosylated peptides.

Synthesis of bioactive peptides through multicomponent reactions

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Multicomponent reactions (MCR) have become important tools in the preparation of structurally diverse chemical libraries of drug-like polyfunctional compounds. There is a continuous need for novel reactions with high efficiency and selectivity in novel reaction media. The importance of peptides as pharmaceuticals has increased significantly and peptide drugs have an essential role in pharmaceutical market. In this way, finding of a suitable way for drug delivery and modification of peptide structure is the subject of the recent researches. Now, some glycopeptides and nucleopeptides are introduced as novel drugs.

During the past 6 years, we have been actively involved in the using of multicomponent reaction to access modified peptides and have developed a series of bioactive compounds. This lecture will highlight some of our contributions to this area which contained:

(a) Ugi-4CR as an approach for the synthesis of some novel GnRH analogues and investigation of their anti-cancer activity.

(b) Design and synthesis of novel fentanyl analogues based on Ugi-4CR, and also functionalization of natural analgesic peptides.

(c) Synthesis of novel cyclopeptides through Ugi ligation/click reaction to construct the cyclopeptides which have a triazole moiety and also lipophilic moieties.

(d) Synthesis of azapeptides and combination of active heterocyclic backbones with peptides.

Synthesis of aza-enkephalins using amino acids hydrazides and comparison of their opioid activities

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Azapeptides are peptide analogues in which the R-CH moiety of one or more amino acid residues in the peptide chain is replaced by a nitrogen atom. This modification has been shown to induce $\alpha\beta$-turn geometry due to electronic repulsion between the adjacent nitrogen lone pairs and/or the formation of intramolecular hydrogen bonds.

Aza-amino acids can thus be used to improve the stability and bioavailability of peptide drugs and also for the designing of prodrugs. Our knowledge in the field of pain and analgesia has significantly improved over the past decades enkephalin analogues, in which the amide bonds were sequentially and systematically replaced by a nitrogen atom.

We present application of synthesized hydrazides and amino acid hydrazides in construction of azapeptide derivatives of enkephalin that they might possess anti-pain activity. These azapeptide derivatives seem to increase blood–brain barrier permeability and improve stability against carboxypeptidase enzyme. According to the experiment, we found the anti-nociceptive properties comparable to the corresponding index drugs. The details will be present in the conference.

Isolation and purification of antitumor and anti-aging peptides from Spirulina platensis

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Abstract: Spirulina platensis is a multicellular edible blue-green alga containing ~60 % of proteins. In this study, *S. platensis*

proteins were extracted by freeze-thawing and ultrasonication methods, enzymatic hydrolysis (trypsin, pepsin, papain, alcalase, chymotrypsin or their combinations) were performed on the proteins; ultrafiltration and gel filtration chromatography were employed to purify bioactive peptides, which were identified by mass spectrometry (MALDI-TOF–MS/MS). Specifically, a fraction from alcalase and papain digests of the proteins, D1, exhibited antitumor activities on MCF-7,HepG-2 and A549 cancer cells, with IC50 values of <31.25, 49.36 and 79.32 μg/mL, respectively, and weak inhibition on normal cells L-02, 33.2 % at 500 μg/mL. In vivo experiment confirmed the inhibitory effects of fraction D1 on HepG-2 cancer cells, ~30 % inhibition (~41 % for 5-FU at 20 mg/d.kg body weight) after 13 days of gavage (200 mg/d.kg body weight) in mice. It is noted that 5-FU remarkably decreased the body weight of mice, but D1 almost had no influence on the body weight. After hydrolysis with trypsin, pepsin and chymotrypsin, an 8-aa peptide Tr1, after solid-phase synthesis, displayed antitumor activity on colon cancer HT-29 cells with IC50 value as 99.88 μg/mL, and little inhibition on normal cells L-02, 5.37 % at 500 μg/mL. Moreover, by enzymatic hydrolysis (trypsin, pepsin or papain) and gel chromatography, 6 peptides (P1-P6) were obtained, which exhibited anti-aging effects. For example, P1, P3 and P6 had ABTS free radical scavenging activities, with IC50 values of 11.11, 11.07 and 8.48 μg/mL, respectively; P1 had DPPH free radical scavenging activity, IC50 = 24.22 μg/mL; P6 displayed positive influences on SOD (Superoxide Dismutase) and LDH (Lactic Dehydrogenase); P5 and P6 remarkably facilitated synthesis of collagen at the concentration of 10–50 μg/mL. These results suggest the potential applications of Spirulina platensis-derived peptides in functional foods, biomedicine and cosmetics.

Keywords: Spirulina platensi, Enzymatic hydrolysis, Peptides, Antitumor, Anti-aging

Possible mechanisms of amyloid formation: determination of the size of folding nuclei of protofibrils from the concentration dependence of the rate and lag-time of their formation

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In this work, a kinetic model of the process of formation of amyloid protofibrils is suggested which allows calculation of the size of the nuclei using only kinetic data. In addition to stage of primary nucleation, which is believed to be present in many protein aggregation processes, the given model includes both linear growth of protofibrils (proceeding only at the cost of attaching of monomers to the ends) and exponential growth of protofibrils at the cost of growth from the surface, branching, and fragmentation with the secondary nuclei. Theoretically, only the exponential growth is compatible with the existence of a pronounced lag-period (which can take much more time than the growth of aggregates themselves). According to our theory, one can distinguish some mechanism of growth on the basis of kinetic data. Thus, the small (<0.2) value of L_{rel} (the lag-time to the growth time ratio) together with independence of L_{rel} on the total concentration $M_{\sum}$ of protein in solution determines applicability of the linear regime of growth, and in case of inapplicability of the latter, consideration of the exponential mechanism of growth is required. For insulin and LysPro insulin we have $L_{rel} \approx 2$–3, which means that it is definitely an exponential growth scenario. One can roughly define three types of possible mechanisms of exponential growth: growth from the surface, bifurcation, and fragmentation. Despite the difference of the processes, in kinetic experiment, fragmentation and bifurcation show a very similar behavior if the size of the secondary nucleus is equal to zero.

Synthesis of novel RGD-containing peptides and aza-peptides and comparison of their activities

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Arginine-glycine-aspartic (RGD), is a cell adhesion motif displayed on many extracellular matrix (ECM) and plasma proteins. RGD plays an important role in cell recognition and also tumor therapy and tissue engineering. Using RGD peptides have some advantages: (1) RGD is much smaller as compared to monoclonal antibodies, and RGD conjugates can have easier access to the tumor tissue; (2) the use of RGD minimizes the risk of immune reactivity or pathogen transfer; (3) simple and inexpensive synthesis, which facilitates translation into the clinic; (4) the applications of RGD are much wider than folic acid.

Among tools for developing peptide mimics, azapeptides are one of the best candidates which could be formed by the replacement of one or more amino acids by aza-amino acid. They could induce turn conformations in peptides with improved pharmacological properties such as duration of action, potency, and/or selectivity has been observed in aza-analogues.

Based on the extended biological activity of RGD, we decided to synthesize novel RGD with the addition of some aza amino acids, active heterocyclic skeletons to obtain novel aza-RGD peptides or addition of some functionalized aza amino acids, D-amino acids, β-amino acids, and we will show the details of the synthesis of the compounds and also their biological activities.

Efficient and selective capture of $\alpha v\beta 3$ integrin-expressing cells on functionalized surfaces

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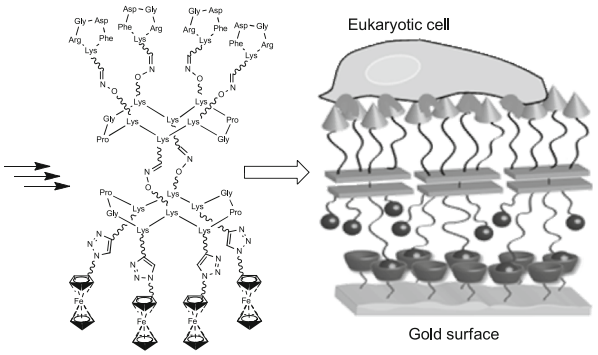
Further progress in tissue engineering requires an accurate knowledge of the cell response to their interactions with their surrounding material. To properly seed cells on synthetic substrates through immobilization of cell adhesion ligands on a surface, the study of the interactions between the ligands and the substrate as well as their influence on cell adhesion is of high interest. Supramolecular chemistry, allowing tunable molecular recognition, is particularly attractive for the attachment of biomolecules to solid surfaces, which is a key issue in nanotechnology.

Our group has developed a regioselectively addressable cyclodecapeptide scaffold featuring, in a spatially controlled manner, two independent functional domains using chemoselective ligations. The structural feature allows a guest domain for the anchoring onto self-

assembled monolayers β-cyclodextrin surface (β-CD SAM) and a domain devoted to cell adhesion (RGD cell-adhesive ligands).[1]

Here, we describe the convergent synthesis of macromolecules featuring both clustered guest motifs and clustered RGD cell-adhesive ligands, achieved using chemoselective ligations (oxime, Huisgen cycloaddition).[2] The strength of the ligand interaction with the surface is modulated by varying the chemical nature of the guest motifs (ferrocenyl or adamantyl) and the valency. The ability of the obtained bioconjugates for selective cell capture is demonstrated by characterization of the supramolecular bioassemblies on β-CD SAM substrates by Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) and complementary optical and fluorescence microscopy.



References

(1) Thakar, D.; Coche-Guérente, L.; Claron, M.; Wenk, C. H. F.; Dejeu, J.; Dumy, P.; Labbé, P.; Boturyn, D. ChemBioChem 2014, 15, 377.

(2) Galibert, M.; Dumy, P.; Boturyn, D. Angewandte Chemie International Edition 2009, 48, 2576.

Syntheses and structural changes of neurodegenerative peptides caused by oxidative stress

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Many physiological disorders including neurodegenerative diseases are influenced by oxidative stress. Nitration of aromatic amino acids can influence the role of important proteins. To better understand the influence of nitration on biophysical functions, we propose selective synthesis of nitrated peptides and proteins. We focused on 3-nitrotyrosine (H–Nit–OH). The use of Boc–Nit(Bzl)–OH, Fmoc–Nit–OH and Fmoc–Nit(Trt)–OH was described in the literature. For Fmoc–Nit(Trt)–OH, we have found that the described procedure does not provide Fmoc–Nit(Trt)–OH but a mixture of Fmoc–Nit–OH and Trt–OH. The tritylation did not lead to completion; attempts of chromatographic purification using mobile phase with various bases led to Trt cleavage. It appears that Nit(Trt) is more labile than Tyr(Trt). We have investigated the synthesis of neurodegenerative peptides using Fmoc–Nit–OH and Fmoc–Nit(Bzl)–OH. We have introduced Nit to fragments of alpha-synuclein and prion proteins. We have studied the system using HPLC, CD, IR, VCD, Raman, and ROA techniques. The nitration influenced many parameters such as solubility, hydrophobicity, and structure. In some cases, the nitration led to higher structural order, whereas in other cases the molecule organization was reduced.

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Preventive effect of ellagic acid on cognitive disorders in two mouse models of oxidative stress (influenza infection and scopolamine-induced dementia)

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Background: Studies from previous years demonstrated that natural polyphenol ellagic acid (EA) combines various pharmacological effects. Our unpublished data reveal significant improving effect of EA on cognitive function of rodents.
Objectives: To study the mechanism of preventive effect of EA on cognitive functions of mice under systemic oxidative stress.
Materials and methods: Male Albino ICR mice (18–20 g) were used in two experimental models of oxidative stress: (1) influenza virus infection (IVI) A/Achi/2/68 H3N2 and (2) Alzheimer's disease dementia (AD) via scopolamine treatment (1 mg kg i.p. 11 days). Animals received EA (400 mg/кg b.w., per os, 11 days). Changes in cognitive functions were studied using Step through test (for learning and memory) and Holeboard test (for exploratory activity). Lipid peroxidation and dopamine content in brain were also studied. Data were analyzed using *t* test of Student–Fisher.
Results: Our experiments demonstrated significant preventive effect of EA on the processes of learning and memory and exploratory activity in mice under condition of experimental oxidative stress (both IVI and AD). In mice with AD model, EA prevention of memory is high (50 %). In IVI model, EA improving effect of EA is combined with decreased lipid peroxidation in blood serum and liver in dose-dependent manner. Brain dopamine content is significantly increased in EA-treated animals.
Conclusion: EA significantly restored cognitive functions of animals in conditions of systemic oxidative stress probably via its antioxidant capacity as well as a model of dopamine metabolism in the brain.
Keywords: Ellagic acid, Oxidative stress, Cognition, Influenza viral infection, Alzheimer's disease.

Preventive effects of the monoterpene Myrtenal on Alzheimer's disease progression on experimental mouse model

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Abstract
Background: Alzheimer's disease (AD) is type of dementia with unclear pathogenesis at the moment. Recent studies revealed neuroprotective potential of some natural products. Monoterpene myrtenal combines in its effects both AO and anti-AChE activity. Our previous unpublished data show its significant improving effect on cognitive function of rodents.
Objectives: To evaluate protective effect of Myrtenal on progression of cognitive disorders in dementia mice.
Materials and methods: Experimental model of dementia from AD type was produced on male Albino ICR mice via scopolamine treatment (1 mg/kg i.p.11 days). Animals were treated simultaneously with myrtenal (20 mg/kgi.p.5 and 11 days). The changes in their cognitive functions were evaluated using Step through test (learning and memory) and Holeboard test (exploratory activity), as well as AchE activity and lipid peroxidation in the brain. Correlations among the results were analyzed in parallel with histological changes in the brain. The effect of myrtenal was evaluated in comparison to referent compounds: galanthamine (anti-AChE agent) and lipoic acid (antioxidant). Data were analyzed using *t* test of Student–Fisher.
Results: Scopolamine-treated animals demonstrated severe memory loss, accompanied by increased AchE activity and lipid peroxidation as well as with degenerative changes in brain. Co-treatment with Myrtenal of dementia mice produced a significant restoration of cognitive function (with 33 %—comparable with the effects of both referent compounds). The correlations among changed parameters (cognitive, biochemical and histological) discover some new details in preventive mechanisms of Myrtenal on AD progression.
Conclusion: Myrtenal can be promising pharmacological agent for AD prevention with complex mechanism.
Keywords: Myrtenal, Alzheimer's disease, Prevention, Lipoic acid

Free D-amino acids as physiologically active molecules in skin

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Free D-amino acids such as D-serine (D-Ser) and D-aspartic acid (D-Asp) have been clarified in recent years to be metabolically synthesized in mammals, and their physiological functions have been reported. In addition, the presence of the other free D-amino acids in mammalian tissues has also been gradually reported. Concerning skin tissues, studies on the presence of free D-amino acids and their physiological functions were rare, despite that cutaneous amino acids are well known to play essential physiological roles such as natural moisturizing factors. Accordingly, we aimed in the present study to clarify enantiomer-distinguished distribution of amino acids in skin tissues as well as physiological functions of free D-amino acids.

Free D/L-amino acids in skin tissues were extracted from human dermis, epidermis and stratum corneum, and the amino acids were subsequently derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole. The amino acid enantiomers were then analyzed using an automated two-dimensional high-performance liquid chromatography system employing a microbore-monolithic ODS column and a narrow bore-enantioselective column[1]. As a result of the distribution study, D-Ser, D-Asp, D-alanine (D-Ala), D-glutamic acid and D-proline (D-Pro) were demonstrated to be present in human dermis and epidermis. In the stratum corneum, the abovementioned D-amino acids except D-Pro were determined, and the amounts of the D-amino acids were shown to decrease with aging. In the meantime, the amount of epidermal D-Ala was 25.9 ± 5.3 nmol/g ($n = 5$, mean $\pm$ SE). This amount is almost equal to that in rat pancreas where specific localization of D-Ala was reported. Therefore, we investigated effects of D-Ala on various protein productions of cultured epidermal keratinocytes. Consequently, D-Ala dose-dependently promoted the production of laminine 332 which is known to accelerate the recovery of basement membrane in skin. The effect of L-Ala was approximately 1/5 compared with that of D-Ala. Regarding the dermis, we focused on D-Asp because dermal $\%D$ of free Asp ($=D/(D + L) \times 100$) was 2.6 ± 0.2 % ($n = 5$, mean $\pm$ SE) which was the highest among those of the intrinsic D-amino acids in the dermis. As a result of studies on physiological functions of D-Asp using cultured dermal fibroblasts (FB), we found that D-Asp promoted type-I collagen production of FB. Further, we demonstrated that D-Asp accelerated construction of the collagen fiber in three-dimensional culture of FB using a second-harmonic-generation microscopy technique (Ex. 800 nm, Em. 390–410 nm). Any effects relating to the collagen production or the fiber construction were not observed in control experiments using L-Asp.

In the present study, free D-amino acids, especially D-Ala and D-Asp, were demonstrated to be physiologically active molecules relating to the maintenance of basement membrane as well as construction of dermal extracellular matrices. Further works such as a supplementation of the D-amino acids to skin are expected for a novel approach to anti-aging of skin.

Reference
[1] Tojo Y., et al., *J. Chromatogr. B*, 875 (2008) 174.

Safety assessment of two antimicrobial peptides with food industry potential application: in vitro study

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Two peptides, rNterC and rTM2C, including 13 and 15 amino acids, respectively, were designed from proteins which are known to bind or to be targeted to outer mitochondrial membrane. These peptides were modified to increase antimicrobial potential considering the appropriate balance among their total net charge, amphipathicity and hydrophobicity.

These peptides were able to efficiently and selectively inhibit *Listeria monocytogenes* (NCTC 11994-HPA—London) growth, an important food-borne pathogen. Therefore, rNterC and rTM2C could be proposed in food industry to increase the shelf life of very valuable foods. In this study, we assessed the toxicity of rNterC and rTM2C in a model of non-tumorigenic intestinal epithelial cells (IEC-6).

Antiproliferative assay, performed at 24, 48 and 72 h, showed a weak antiproliferative effect, in particular for rNterC, however, indicating an $IC_{50} > 40$ μM for both peptides. The intestinal epithelium when injured by various stimuli (e.g. normal digestion, toxic substances, inflammation, oxidative stress) undergoes a wound healing process. rNterC and rTM2C peptides induce a weak reduction in the restitution process both in normal and inflammatory conditions (induced by lipopolysaccharide from E.coli plus interferon γ) in IEC-6 with an $IC_{50} > 40$ μM for both peptides. Our data on IEC-6 cells indicate that rNterC and rTM2C show only a weak antiproliferative activity and a weak effect on migration rate in IEC-6. Between the two peptides, the safer seems to be rTM2C that has the best antimicrobial activity against *L. monocytogenes*, despite the evaluation of other parameters will be necessary to further assess its safety.

Indoxyl sulphate, a tryptophan metabolite, affects astrocyte response during neuroinflammation

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Indoxyl sulfate (IS) is a protein-bound uremic toxin derived from dietary tryptophan metabolism. Tryptophan is metabolized into indole by intestinal bacteria, after intestinal absorption is further converted to IS in the liver and then excreted by kidneys. In patients with impaired kidney function, such as chronic kidney disease (CKD), IS is recognized as a uremic toxin that accumulates in the blood inducing nephrotoxicity and impairing immune response. CKD is characterized by various complications such as neurological dysfunction. Since neuroinflammation has also been recognized to contribute to cognitive complications, and because CKD patients are also affected by immune dysfunction, we evaluated the effect of IS on C6 rat astroglial cell line in inflammatory conditions.

IS, at uremic concentrations (60–15 μM), added for 1 h and then simultaneously with lipopolysaccharide from *E. coli* (LPS; 1 μg/ml) + Interferon γ (IFN; 100U/ml) for 24 h, significantly increased nitric oxide release in C6 cells at all tested concentrations. In the same experimental conditions, IS induced also a significant increase in inducible nitric oxide synthase and cyclooxygenase-2 expression, in tumor necrosis factor-α levels and in nitrotyrosine formation in astrocytes. Moreover, IS induced also nuclear factor-kB activation and aryl hydrocarbons receptor nuclear translocation in C6 cells in presence of LPS + IFN, thus contributing to inflammatory response modulation. In addition, wound healing assay indicated that IS caused also a reduction in astrocytes migration during inflammation. These results indicate that IS significantly affects astrocyte function increasing inflammatory response during neuroinflammation, thus potentially contributing to neurological complications observed in CKD.

Exploring the chemistry and pharmacological potential of bioactive peptides from insects

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The variety of natural compounds still displays one of the largest resources for the discovery of novel pharmaceutical lead compounds. Over the recent years, peptides have attracted much interest in drug development approaches. This is in large part due to advances in analytical and synthetic chemistry that are encouraging to overcome their major limitations such as poor stability and low oral bioavailability. One class of bioactive peptides is antimicrobial peptides (AMP) that have been identified in plants, invertebrates and vertebrates, including human. AMPs have been shown to be able to interact with biological membranes, but have been equally ascribed to modulate immunity via intracellular targets. Their activity against a range of microorganism, including relevant pathogens makes them interesting molecules for the discovery of novel lead compounds.

Considering the sheer numbers of insect species ($\sim$4 million), one of the largest groups of organisms on earth, and the vast variety of ways they interact with their environment through chemistry it is clear that they have significant potential as a source of new drug leads. However, little is known about the peptide content of individual species and its pharmaceutical potential.

Our aims are to discover and characterize novel bioactive peptides, determine their three-dimensional structure and evaluate their pharmacological activity. Further, we aim to synthesize optimized peptide compounds, identify their molecular targets and determine their potential as novel drug leads. To accomplish these goals we are using a peptidomics workflow combining state-of-the-art mass spectrometry together with in silico mining. Identified peptides will then be characterized using solution NMR and solid-phase peptide synthesis will be used to obtain sufficient material for bioactivity studies.

Mechanism of the cholesterol-lowering effect of taurine

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Objective: The aim of the present study was to elucidate the mechanisms involved in the cholesterol-lowering effects of taurine.
Methods: Male Sprague–Dawley rats were fed a high-cholesterol diet containing 0.5 % cholesterol and 0.15 % sodium cholate for 2 weeks with or without 5 % taurine. Serum and hepatic cholesterol levels and biliary bile acid levels were determined. In addition, the activity and mRNA expression of enzymes responsible for cholesterol synthesis and metabolism were determined in the liver.
Results: Dietary taurine significantly reduced serum and hepatic cholesterol levels ($p < 0.01$). The fecal excretion of total bile acids was significantly increased in the taurine-treated rats, compared with that in the untreated rats ($p < 0.05$). Biliary bile acid levels were also increased after taurine treatment. The increase in bile acid synthesis was associated with a twofold increase in the enzymatic activity and threefold increase in the mRNA expression of hepatic cholesterol 7α-hydroxylase (CYP7A1), a rate-limiting enzyme for bile acid synthesis. In addition, taurine supplementation significantly suppressed the activity of two key enzymes, acyl-CoA:cholesterol acyltransferase ($p < 0.01$) and microsomal triglyceride-transfer protein ($p < 0.05$), which are responsible for cholesterol esterification and very low-density lipoprotein (VLDL) particle assembly, respectively, in the liver.
Conclusions: Taurine enhanced the synthesis and excretion of bile acids and stimulated the conversion of cholesterol to bile acid by increasing the expression and activity of CYP7A1. This resulted in

the depletion of regulatory cholesterol pool of the liver and down-regulation of cholesterol esterification and lipoprotein assembly for VLDL secretion, leading to reduction in the serum and hepatic cholesterol levels.

Tripeptide glutathione as redox regulator for development of cancer cell drug resistance

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Tripeptide glutathione (GSH) plays the significant role in maintaining cellular redox status by participating in thiol–disulfide exchange which regulates a number of important cellular functions such as proliferation, differentiation, apoptosis. The ratio of reduced glutathione to its oxidized form (GSH/GSSG) is essential for cell viability largely due to its role in redox-dependent regulation of gene expression. The aim of our study was to determine the role of GSH/GSSG ratio in regulation of expression of redox-dependent genes (isoforms of glutaredoxin, thioredoxin, peroxiredoxin) which are important for antioxidant defense under development of cancer cells resistance to cisplatin (CDDP) possessed pro-oxidant action. Under development of resistance of human erythroleukemia K562 and ovarian carcinoma SKOV-3 cells to CDDP co-ordinative enhanced expression of genes encoding glutathione synthetase (GS), heavy and light subunits of γ-glutamylcysteine synthetase (γ-GCSH, γ-GCSL, γ-GCS, respectively) was found in resistant cells in comparison with wild cells. In addition, the growth of GSH/GSSG ratio as index of cellular redox state, enhanced expression of redox-regulated genes of isoforms of glutaredoxin (*GLRX1, GLRX2)*, thioredoxin (*TRX2)*, peroxiredoxin (*PRDX6)* as well as elevated level of transcription factor Nrf2 were observed in both types of resistant cells. We suggest that mechanism of development of cancer cells resistance to CDDP can include activation of GSH synthesis *de novo* owing to the increase of expression of *γ-GCSH*, *γ-GCSL* and *GS* genes, redox-dependent elevation of expression of *GLRX1*, *GLRX2*, *TRX2*, *PRDX6* which are co-ordinative regulated by redox-dependent transcription factor Nrf2 and elevated level of GSH/GSSG ratio.

Protein–Ligand Interactions: heme binding to proteins with Therapeutic Potential

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Protein–ligand and, particularly, protein–drug interactions are currently considered in many important aspects, such as drug efficacy, drug delivery, protein modifications, purity and safety of protein therapeutics, and concomitant administration, to mention a few. This presentation will focus on some therapeutic and analytical aspects of heme binding to proteins with regards to native plasma proteins, therapeutic proteins and heme-based therapeutics. As a prosthetic group of hemoglobin, myoglobin, and several other hemoproteins, heme is one of the most important and most abundant native ligands, thus enabling various native hemoproteins to perform diverse biological functions. However, in its free form heme is intrinsically toxic in vivo. Increased free heme in blood due to hemoglobin catabolism is typical for hemolytic disorders. Exogenous sources of free heme include stored red blood cell preparations and heme-based therapeutics, such as Panhematin and Normosang that are used for the treatment of porphyrias. Due to its potential toxicity, heme binding to plasma proteins and determination of heme impurities in the protein therapeutics derived from human blood are important safety issues. Various aspects of heme–protein interactions will be discussed in this presentation, including analytical assessment with a special emphasis on protein-induced chirality.

Piezoelectric 3D polymeric micro/nanotube assemblies as potential self-powering drug delivery platforms

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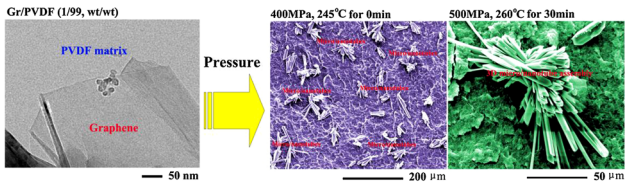
Self-propelled micro/nanoscale machines have demonstrated considerable potential for performing operations and tasks in on-demand and targeted delivery of therapeutic payloads.



Nevertheless, it is still challenging in fabricating the structurally complex biomedical objects of this size and scaling down of the familiar macroscopic principles of powering them in vivo. In this study, graphene (Gr), a two-dimensional (2D) nanostructured carbon material that can be exploited to achieve high drug loading of poorly soluble drugs without compromising potency or efficiency, was well dispersed in a biocompatible polymer, poly (vinylidene fluoride) (PVDF), and then the binary composites were crystallized at pressure with a piston–cylinder apparatus. After removing the amorphous parts of the recovered samples by a delicate etching technique, unique one-dimensional (1D) PVDF micro- and nanotubes with polar piezoelectric crystalline structures were observed. The large-scale growth of such micro/nanotubes was assigned to the synergistic action of graphene and pressure. The size and morphology of the crystalline polymeric micro/nanotubes were found to be very sensitive to the crystallization conditions. By regulating crystallization temperature, pressure and time, PVDF micro/nanotubes with folded- and extended-chain lamellae as substructures were obtained, respectively. Particularly, three-dimensional (3D) micro/nanotube assemblies, totally with polar extended-chain lamellae as their substructures, were formed under specific conditions. Combining the properties of graphene and the in situ-formed piezoelectric nanostructures, the pressure-crystallized hybrid composites may permit niche applications in self-powering drug delivery by scavenging energy from their working environments in living organisms.

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Fullerene and pressure co-mediated growth of novel biodegradable crystalline polymeric scaffolds for controlled and localized delivery of therapeutics

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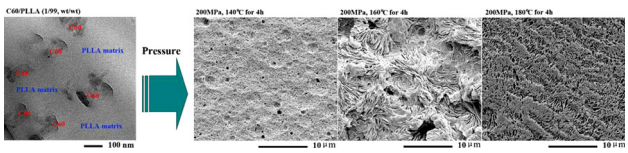
The drug-releasing scaffolds based on biodegradable polymers such as poly (lactic acid) (PLLA) show great promise in tissue engineering.



Nevertheless, major challenges still lie in the design and fabrication of customizable constructs with controllable pore structures, sufficient mechanical properties and predictable degradation rate. In this study, fullerene C60/PLLA composites were fabricated and then crystallized at high pressure. The results showed that controllable growth of 3D resorbable PLLA scaffolds, with crystalline alpha form and with interconnected pores of high porosities, were achieved through a fullerene and pressure co-induced phase transition. The pore size and morphology, porosity and surface area, recognized as important parameters for a tissue engineering scaffold, were easily mediated by varying crystallization temperature, pressure and time, together with the etching by a 1:2 water–methanol mixture containing 0.025 mol/L of sodium hydroxide. Furthermore, the synergistic action of C60 and pressure had a significant influence in crystalline morphology of PLLA, which further mediated not only the macro- and microstructures of the formed scaffolds but also their degradation rate. The in vitro data from a wide range of PH values suggested the introduction of C60 followed by pressure treatment enhanced the hydrolytic degradation of PLLA. With distinguishing features, the as-crystallized PLLA scaffolds may permit niche application in tissues such as muscle, tendon, ligament, intestine, bone and teeth for controlled localized delivery of entrapped small molecule drugs.

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Identification and characterization of SDF-1α Variants with Various Glycosaminoglycan-Binding Properties

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The stromal cell-derived factor 1α (SDF-1) belongs to the CXC chemokines and interacts with its CXC-receptor 4 and 7. It is a regulator during the immune response, influences angiogenesis and recruit tissue specific stem cells. Therefore, SDF-1 is an attractive target for the regeneration of damaged tissue. Recent studies focus on the development of hydrogels, which can be integrated in this type of damaged tissue and improve wound healing. The aim of this study is to generate a stable, long-term chemotactic gradient using immobilized SDF-1 and variants, which leads to the recruitment of stem cells and, therefore, to the regeneration of the affected tissue. The present work focusses on the identification and characterization of SDF-1 variants with different glycosaminoglycan (GAG)-binding properties to recruit different cell types that express the CXCR4.

Specific amino acids of SDF-1α for GAG binding were determined by investigation of the interaction with different native and artificial GAGs by use of ^{1}H-^{15}N-HSQC NMR spectroscopy.

Additionally, single amino acids were exchanged by mutations based on NMR results. The migration and signaling assay demonstrated that the single mutation does not influence the ability of the SDF-1 to recruit specific cells and the G protein-coupled signaling by the phospholipase C is not influenced.

To sum up, additional knowledge on the specific interaction of SDF-1 with different GAGs could be achieved. The influence of the GAG-binding site of the interaction of the SDF-1 with its CXCR4 could be characterized in more detail.

Prolyl-hydroxyproline, a collagen dipeptide, alters gene expression in a co-culture of mouse skin cells

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Introduction: Prolyl-hydroxyproline (Pro-Hyp), a collagen-derived dipeptide, has long been suggested to be the key peptide in the effects of dietary collagen on the skin. Pro-Hyp is highly resistant to intestinal peptidases and can be transported to the skin in its intact

form. It was found that this peptide can stimulate cell growth of mouse skin fibroblasts in vitro. Nevertheless, little is known about its effects on gene expression in the skin. We used a co-culture system of mouse skin cells to address the effects of Pro-Hyp on gene expression changes.

Methods: Co-culture: primary mouse skin keratinocytes were overlaid on top of a dermis equivalent (primary mouse skin fibroblasts in a collagen gel). The co-culture was exposed to an air–liquid interface, and a skin-like layer was formed. After 4 days, the basal medium was replaced with fresh medium containing Pro-Hyp, proline, or hydroxyproline (200 μM) for 24 h. The skin-like layer was then separated from the dermis equivalent. Real-time RT-PCR was used to determine mRNA level changes in the skin-like layer.

Monolayer culture: primary mouse skin keratinocytes were cultured in a fibroblast-conditioned medium on a collagen-coated plate. After four days, the medium was replaced with fresh medium containing Pro-Hyp (200 μM) for 24 h. Real-time RT-PCR was used to determine mRNA level changes.

Results: The addition of Pro-Hyp up-regulated the mRNA levels of Krtap16-7, Krtap15, Krtap14, and Krtap8-2 which code keratin-associated proteins in the skin-like layer of the co-culture. In contrast, the amino acids did not significantly alter the expression levels of these genes. In the monolayer culture, the addition of Pro-Hyp did not induce the same changes as in the co-culture.

Conclusion: Our data demonstrate the regulatory effect of Pro-Hyp in its intact form on gene expression in the co-culture of mouse skin cells. The keratin-associated proteins are components of hair follicles which play an important role in retaining temperature in the skin. These proteins are produced in the growth phase of the hair cycle. Therefore, the up-regulation of the Krtap genes by Pro-Hyp might contribute to the effects of this collagen peptide in the skin as well as in hair follicles.

Reduction of inflammatory state in CKD patients during cholesterol lowering treatment is related to oxidative stress decrease and taurine concentration increase

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Tryptophan (Trp) degradation via indoleamine (2,3)-dioxygenase, with consequent increased in kynurenine (Kyn) concentrations, has been proposed as marker of immune system activation. We hypothesized that cholesterol lowering treatment might directly affect IDO activity in CKD, with consequent changes in the Kyn/Trp ratio. We sought to address whether these effects were associated with changes in markers of oxidative stress malondialdehyde (MDA) and allantoin/uric acid ratio (All/UA) and taurine. Serum concentrations of Trp and Kyn, oxidative stress indices (MDA) and All/UA ratio, and anti-oxidant amino acid taurine were measured in 30 CKD patients. Both Kyn and Kyn/Trp ratio significantly decreased after cholesterol lowering treatment, to values comparable with healthy controls after 1-year treatment (1.67 ± 0.62 vs 1.31 ± 0.51 μmol/L, $p < 0.0001$ and 0.036 ± 0.016 vs 0.028 ± 0.012 $p < 0.0001$, respectively). This was paralleled by a significant decrease in MDA (218 ± 143 vs 176 ± 123 nmol/L, $p < 0.01$) and All/UA ratio (1.47 ± 0.72 vs 1.19 ± 0.51, $p < 0.01$) in CKD patients. Taurine concentration significantly increased all over drug treatment from 51.1 ± 13.3 μmol/L at baseline to 63.1 ± 16.4 μmol/L ($p < 0.001$). The reduction of Kyn concentrations and Kyn/Trp ratio during therapy was significantly associated to the reduction in MDA concentrations ($r = 0.965$ $p = 0.037$ and $r = 0.997$ $p = 0.003$, respectively) and All/AU ratios ($r = 0.964$ $p = 0.036$ and $r = 0.998$ $p = 0.002$ respectively). Amelioration of both oxidative and inflammation status after cholesterol lowering treatment in CKD might be mediated by restoration of antioxidant taurine concentrations during therapy, suggesting that improvement of both oxidative and inflammation status in CKD patients could be explained, at least partly, by the cholesterol-lowering effects.

Incorporation of functionalized amino acids during cell-free protein expression

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Dehydrogenation and hydroxylation of proteins are generally assumed to involve post-translational free-radical modifications, and such processes are typically associated with dysfunctional proteins and disease states. However, using cell-free protein synthesis, it has been found that hydroxy,[1] dehydro and chloro[2] amino acids, prepared using radical processes, are readily mis-incorporated into proteins in place of the twenty normal amino acids. Depending on the modification, it may or may not affect the protein structure and function. These findings not only challenge our understanding of the fidelity of protein biosynthesis, since the biological machinery is sometimes unable to differentiate between the hydroxy, dehydro and chloro amino acids and the normal amino acids, but are also conceptually attractive because they involve replacing the non-reactive side chains of aliphatic amino acids with functionalized derivatives. The functionalized proteins provide new tools for biotechnology, such as latent cleavage sites for peptide synthesis.

Reference

(1) K. Ozawa, M. J. Headlam, D. Mouradov, S. J. Watt, J. L. Beck, K. J. Rodgers, R. T. Dean, T. Huber, G. Otting, N. E. Dixon, Translational incorporation of L-3,4-dihydroxyphenylalanine (DOPA) into proteins, *FEBS J.* **2005,** *272*, 3162–3171.

(2) Z. I. Watts, C. J. Easton, Peculiar stability of amino acids and peptides from a radical perspective, *J. Am. Chem. Soc.* **2009,** *131*, 11323–11325; D. J. Stigers, Z. I. Watts, J. E. Hennessy, H.-K. Kim, R. Martini, M. C. Taylor, K. Ozawa, J. W. Keillor, N. E. Dixon, C. J. Easton, Incorporation of chlorinated analogues of aliphatic amino acids during cell-free protein synthesis, *Chem. Commun.* **2011**, *47*, 1839–1841; I. N. Arthur, J. E. Hennessy, D. Padmakshan, D. J. Stigers, S. Lesturgez, S. A. Fraser, M. Liutkus, G. Otting, J. G. Oakeshott, C. J. Easton, In situ deprotection and incorporation of unnatural amino acids during cell-free protein synthesis, *Chem. Eur. J.* **2013**, *19*, 6824–6830.

Cortico-limbic neuropeptidases after acute restraint stress

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Abstract: Oxytocin, vasopressin as well as enkephalins are neuropeptides involved in the stress response and in memory processes. These neuropeptides are partially regulated by several neuropeptidase activities such as oxytocinase/vasopressinase and enkephalinase activities. The medial prefrontal cortex, amygdala and hippocampus are brain areas involved in the stress response. These areas constitute a circuit in which they interact with each other for such a response. Therefore, in order to investigate the role of these neuropeptidases in the response to stress, we analyzed the profile of regional distribution of these enzymatic activities as well as their patterns of interaction into this circuit after causing an acute restraint stress in adult male rats. With regard to the regional study, while most activities showed a preponderance of the amygdala on hippocampus and medial prefrontal cortex, in both groups of control and stressed animals, enkephalinase activity demonstrated a change after stress increasing in the hippocampus and decreasing in the amygdala. The analysis of the correlation study suggested that neuropeptidase activities are connected with the functional status of this cortico-limbic circuit, changing significantly the pattern observed in controls after acute restraint stress. In controls, the suggested connection implied essentially a positive interaction between medial prefrontal cortex and amygdala, without a clear relationship between the other regions. In marked contrast, after the applied stress, there was a highly significant change in the pattern of interactions, activating a positive correlation between medial prefrontal cortex and hippocampus and between amygdala and hippocampus and diminishing the interaction medial prefrontal cortex versus amygdala observed in controls. The global analysis of interactions between neuropeptidases and areas suggests a differential role for oxytocinase/vasopressinase activity in the medial prefrontal cortex in comparison with the rest of activities and areas. The present results support a possible role for neuropeptidases and consequently for their corresponding neuropeptidergic substrates vasopressin, oxytocin and enkephalin in response to stress of the regions analyzed.
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Abstract: L-Serine is essential for all living organisms because the amino acid is used as precursors for other amino acids, phospholipids and nucleic acids, which play key roles for cell proliferation and maintenance. In plant, two serine biosynthesis pathways are revealed. One is glycolate pathway which is originated from 2 molecules of glycine generated by photorespiration. Another is phosphorylated pathway which is originated from 3-phosphoglycerate, a biosynthetic intermediate from Calvin cycle and glycolysis. The phosphorylated pathway is regulated by a well-known feedback mechanism of a key enzyme 3-phosphoglycerate dehydrogenase (PGDH). Therefore, the regulation mechanism of PGDH activity is crucial for the flux regulation of the pathway. Recently, physiological importance of the phosphorylated pathway in Arabidopsis was revealed. The pathway is strongly related to fixation of carbon dioxide and biosynthesis of indole-3-acetate (IAA) and glucosinolates, secondary metabolites derived mainly from tryptophan and methionine. Based on biochemical analysis using Hill equation, we found that PGDH isozymes are regulated via allosteric activation by aspartate family- and pyruvate family-amino acids in various plants. Furthermore, mutagenesis experiments of PGDH isozymes indicated that these regulatory amino acids bind to different binding sites on PGDH isozymes. Based on these results, we suggest synergetic regulatory mechanisms of PGDH by aspartate family- and pyruvate family-amino acids in plants.

Feedback regulation of methionine biosynthesis mediated by ribosome stalling in *Arabidopsis*

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Cystathionine γ-synthase (CGS) catalyzes the key step of methionine biosynthesis in plants. In *Arabidopsis*, expression of the *CGS1* gene is negatively feedback-regulated by its mRNA degradation in response to *S*-adenosyl-L-methionine (AdoMet), a direct metabolite of methionine. Degradation of *CGS1* mRNA depends on the translation of *CGS1* mRNA itself. This suggested that AdoMet-sensing occurs during the translation of *CGS1* mRNA. By in vitro translation system, we have previously shown that AdoMet induces ribosome stalling at the Ser-94 codon on *CGS1* mRNA, which is followed by mRNA cleavage at the vicinities of the stalled ribosomes.

The amino acid sequence of the MTO1 region, located several codons upstream of the Ser-94 codon, is involved in this regulation by acting in cis. The biochemical analyses of the AdoMet-stalled ribosome-nascent peptide complex showed that conformation changes occur in the nascent peptide/ribosomal exit tunnel region. This suggested that CGS1 nascent peptide including the MTO1 region changes its conformation during AdoMet-induced ribosome stalling. Recent data suggest involvement of the exit tunnel in the AdoMet-induced ribosome stalling. The mechanism of AdoMet-sensing during *CGS1* mRNA translation will be discussed in the context of nascent peptide-mediated ribosome stalling.

Effects of cultivar practices on tryptophan levels during grape ripening

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Different cultivar practices have been applied to vine to promote specific components related to final wine flavor. Some of the cultivar practices are related to vine stress, including leaf removal, grape bunch removal and vegetable cover crops. Some nitrogen and sulfur supplements have been also used directly to leaves during the last stage of the ripening period. Main effects of the cultivar practices are related to the final composition of organic acids, volatiles components and phenolics. However, some effects on amino acids have been also found. This paper specifically deals with the effects on tryptophan level because it has strong effects in the evolution of several other related compounds, including auxins and melatonin. Six different cultivar practices were applied in three replicated forms in the same vineyard. Evolution of tryptophan levels was determined starting just after grape veraison till harvest date. In some cases, some specific treatments were also evaluated after regular harvest. Results point out the specific effect of vegetable cover crops and leaves removal to promote higher levels of tryptophan in grapes.

Keywords: Tryptophan, Grapes, Ripening.

Acyl-CoA-binding proteins mediate stress tolerance in plants

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Acyl-CoA-binding proteins (ACBPs) from the model plant Arabidopsis have been recently reported to mediate stress tolerance. The AtACBP protein family, which shows conservation at the acyl-CoA-binding domain, is encoded by six genes (*AtACBP1* to *AtACBP6*) distributed across four classes in *Arabidopsis thaliana*. In the monocot, rice, six genes also encode ACBPs; however, their distribution across the four classes differs from Arabidopsis. Class I ACBPs consist of small ($\sim$10 kDa) proteins, Class II ACBPs comprise of proteins with ankyrin repeats, Class III ACBPs are large proteins >70 kDa and Class IV ACBPs contain kelch motifs. The ankyrin repeats of Class II ACBPs and the kelch motifs of Class IV ACBPs can potentially facilitate protein–protein interactions. Experiments using *acbp* mutants and transgenic Arabidopsis overexpressing AtACBPs have shown that these proteins can mediate various forms of stress tolerance. Sometimes AtACBPs work together with their protein partners which have also been identified as stress-related proteins. It has been demonstrated that AtACBP1- and AtACBP2-overexpressors can better withstand heavy metal/oxidative stress, AtACBP2-overexpressors tolerate drought stress, AtACBP3-overexpressors are protected against biotic stress and AtACBP6-overexpressors are freezing tolerant. The ability of the acyl-CoA-binding domain of recombinant AtACBPs in binding acyl-CoA esters has been tested using Lipidex assays as well as in isothermal titration calorimetry. The lipid-binding properties of AtACBPs provide explanation, to a certain extent, on their ability to better withstand stress treatments as lipids form important components of cellular membranes. Given that transgenic Arabidopsis overexpressing AtACBPs from the *35SCaMV* promoter are conferred enhanced tolerance to various stresses, these proteins show promise for applications in agriculture and phytoremediation.

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Phosphorylation of monoamine transporters

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The dopamine transporter (DAT) and the serotonin transporter (SERT) mediate reuptake of dopamine (DA) and serotonin, respectively, from the synaptic clefts to terminate monoaminergic signaling. The functions of transporters are regulated by oligomerization, protein–protein interactions and post-translational modification, such as phosphorylation. Previously, we found that phosphorylation at Thr53 in rat DAT is involved in amphetamine-mediated DA efflux[1]. In this study, interaction of protein phosphatase (PP) 1 and PP2A with mouse DAT exclusively from the wild-type, but not knock-out ($DAT^{-/-}$), striatum by mass spectrometry (MS) was investigated. We further confirmed PP1 and PP2A interactions with human DAT in heterologous cells by Co-IP and identified DAT phosphorylation at Thr48 by MS analysis. Thr48 phosphorylation is strongly enhanced by okadaic acid (OA), an inhibitor of PP1/2A, determined by stable isotope labelling method in MS-based quantitative proteomics. In addition, OA augmented phosphorylation in the N-terminus of SERT. These findings suggest that the N-terminal phosphorylation status of transporters is orchestrated by multiple kinases and phosphatases and further its specific implications in modulation of transporters will be examined.

1. Foster JD*, Yang JW*, Moritz AE, Challasivakanaka S, Smith MA, Holy M, Wilebski K, Sitte HH[§], Vaughan RA[§] (2012) Dopamine transporter phosphorylation site threonine 53 regulates substrate reuptake and amphetamine-stimulated efflux. J Biol Chem 287(35):29702–29712

Maternal immune activation epigenetically regulates serotonin transporter levels in adult offspring hippocampus

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Major depressive disorder (MDD) is one of the most debilitating diseases worldwide, yet the pathological mechanisms underlying

this common mental illness are poorly understood. Increasing evidence points towards epigenetic mechanisms as central mediators of the impact of environmental influences on gene expression and consecutively brain structure and function and an involvement in the pathogenesis of multifactorial neuropsychiatric disorders has been proposed. Here we employed maternal immune activation through the administration of Poly (I:C), mimicking gestational infection, to investigate possible epigenetic dysregulations in a mouse model of depression. The effects of MIA on molecular participants of the epigenetic machinery with special focus on the serotonin transporter (SERT) critically involved in the aetiology of MDD and pharmacological antidepressant treatment. Deranged hippocampal SERT expression at the mRNA and protein level, associated with a distinct pattern of histone modifications at the SERT promoter and paralleled by alterations in the global hippocampal histone acetylation profile was observed in MIA offspring. These findings support the notion that epigenetic mechanisms contribute to the environmental programming of brain development and behaviour by embedding the impact of the early life experiences on gene expression. A distinct hippocampal global and gene-specific histone acetylation pattern is suggested to ingrain the effects of MIA on SERT expression and depression-like behaviour later in life.

Functional regulation of amphetamine-induced efflux by CaMKII

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Monoaminergic neurotransmission is terminated by reuptake of extracellular dopamine (DA) or serotonin (5-HT) by the respective high-affinity transporters (DAT, SERT). These transporters are major molecular targets for psychostimulants such as cocaine and amphetamines. Both drugs act as competitive uptake inhibitors when competing with the physiological substrate for the uptake site. However, amphetamines are more complex in their ability to induce transport-mediated efflux. This reverse transport process is not a simple back-diffusion reaction because it involves the activity of different sets of kinases, including the calcium and calmodulin-dependent kinase IIα (αCaMKII). The presentation will focus on the recent developments and discuss the regulation of both DAT and also SERT by αCaMKII. Furthermore, the in vivo importance of αCaMKII modulation for amphetamine action at DAT and SERT will be outlined.

Imaging the effects amphetamine in sensitization and first-episode schizophrenia: a [^{11}C]-(+)-PHNO positron emission tomography study

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Patients with schizophrenia show increased behavioral sensitivity to the effects of dopamine-transporter blocking or dopamine-releasing agents. Repeated administration of amphetamines leads to increased behavioral sensitivity to the drug, an effect that has been termed sensitization. Patients with schizophrenia have thus been conceptualized as being in a state of 'natural' sensitization toward the effects of amphetamines. Release of endogenous dopamine in the living human brain can indirectly be measured by imaging blocking of radioligand binding to dopamine $D_{2/3}$ receptors using positron emission tomography (PET). For this study, healthy subjects undergo repeated administration of a small oral dose of d-amphetamine and repeated PET scans. Patients with first-episode schizophrenia undergo a single intake of d-amphetamine and two PET scans. Binding of the dopamine $D_{2/3}$ receptor agonist radioligand [^{11}C]-(+)-PHNO is analyzed using simplified reference tissue models. Effects of repeated amphetamine administration to healthy subjects on behavior and dopamine release are compared to amphetamine effects in patients with schizophrenia.

Sodium–potassium pump (Na, K-ATPase) is a target of amyloid beta

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Na, K-ATPase provides active transport of Na^+ and K^+ across plasma membrane in all types of animal cells. Onset of the Alzheimer's disease (AD) is accompanied by decreasing Na, K-ATPase activity. Disruption of ionic homeostasis leads to the changes of resting membrane potential and excitable properties of neurons. It has been shown that decrease in activity of Na, K-ATPase in postmortem samples of hippocampus from transgenic mice and in the microsomal fraction of brain tissue is correlated with the presence of beta-amyloid (Aβ), which is

the main component of amyloid plaques formed in AD. These findings support a hypothesis that Aβ can bind directly to Na, K-ATPase. At the first step of the study, we used purified Na, K-ATPase preparation. Applying Isothermal Titration Calorimetry, we have found thermodynamic parameters of Na, K-ATPase interaction with Aβ (1-42). Dissociation constant (K_d) of the complex is equal to 4 μM, and the binding is enthalpy driven. Evaluation of hydrolytic activity showed that incubation of Na, K-ATPase with Aβ (1-42) leads to inhibition of the enzyme. At the next step, we have evaluated the effect of Aβ (1-42) on hydrolytic and transport activity of Na, K-ATPase in the cells of neuroblastoma SH-SY-5Y. The cells were incubated with 10 μM of Aβ (1-42) over one hour. Treatment of cells with Aβ (1-42) reduces both hydrolytic and transport activity of Na, K-ATPase. The latter was estimated from the ouabain-sensitive K^+ (^{86}Rb) influx. Rapid decline in transport activity of Na, K-ATPase in intact cells, suggests that Aβ (1-42) binds to the extracellular part of the enzyme. To check the ability of Aβ (1-42) to enter into the SH-SY-5Y cells, they were treated with 10 μM Aβ (1-42), labelled by fluorescein. Accumulation of Aβ (1-42) in the cells was observed only after 4 h of incubation. This demonstrates that inhibition of Na, K-ATPase activity is associated with the interaction of Aβ (1-42) with the extracellular part of the protein molecule. These findings help to explain early disruption of the action potential of neuronal cells in the pathogenesis of AD.

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The C-terminus as a Folding Checkpoint in SLC6 Transporters

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In mammalian cells, essentially all integral membrane proteins are synthesized in the endoplasmic reticulum (ER) and they have to be exported from the ER via the secretory pathway to reach their final destination. ER export is contingent on formation of COPII-coated vesicles, where the inner coat layer is formed by the SEC23/SEC24 dimer. One of the four isoforms of SEC24 acts as the cargo receptor by binding to short exposed motifs on the cytosolic side of client proteins. Accordingly, there must be a proofreading mechanism, which prevents premature export of unfolded or partially folded client membrane proteins. We proposed a chaperone-COPII-exchange model for transporters of the SLC6 family: this posits that a heat shock protein (HSP) relay operates on the C-terminus of the transporter to assist the folding of the transporter. This shields the SEC24 binding site and precludes the premature recruitment of the SEC24 to the C-terminus. The model makes several predictions, many of which have been verified using the serotonin transporter (SERT, SLC6A4), e.g.: (i) there is HSP70 binding site in the C-terminus of SERT, which is adjacent to the SEC24 binding motif. (ii) HSP701A binds to SERT, this binding is restricted to SERT residing in the ER. (iii) Mutations within the C-terminus impair folding of SERT. Individual mutants are stalled at different stages within the folding trajectory. Accordingly, they are complexed with different chaperones (HSP701A and HSP90β) and co-chaperones (HOP, p23, CHIP). (iv) The folding defect of mutated transporters can be corrected by inhibiting HSP701A and/or HSP90β with pifithrin-μ and 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (DMAG), respectively. The relative efficacy of these two compounds depends on the point, at which their folding trajectory is stalled. (v) The C-terminus interacts with the first intracellular loop via a salt bridge. This allows for sampling the folding trajectory. Thus, the C-terminus can act as a folding sensor and transmit the information to the HSP relay. Anterograde trafficking of SLC6 transporters is of interest, because there are human diseases, which arise from folding-deficient, ER-retained mutants. Notable examples are infantile dystonia/parkinsonism and hyperekplexia/startle disease, which are caused by mutations in the genes encoding the dopamine transporter (DAT, SLC6A3) and the glycine transporter-2 (GlyT2, SLC6A5), respectively. Based on our insights, strategies are conceivable, which restore ER export of the mutated transporters. These are being examined in *Drosophila melanogaster*.

Phosphorylation of voltage-gated Kv7.2 channels regulates their PIP_2 sensitivity

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Kv7 channels are a subfamily of voltage-gated K^+ channels that play a major role in the regulation of neuronal excitability. One important factor in the function of these channels is PIP2, which is required for gating. GPCRs govern Kv7 channels by determining the levels of PIP2, on one hand, and via phosphorylation on the other hand. However, an interaction of these pathways has not been explored. By applying liquid chromatography-coupled mass spectrometry to Kv7.2 immunoprecipitates of rat brain membranes and heterologous cells, we located a cluster of phosphorylation sites in one of the PIP2-binding domains. To evaluate the effect of phosphorylation on PIP2-mediated Kv7.2 current regulation, we generated a quintuple alanine mutant of according serines (S427/436/438/446/455; A5 mutant) to mimic a dephosphorylated state. Activation of the voltage-sensitive phosphatase Dr-VSP was used to reduce PIP2 levels. Perforated patch-clamp recordings showed that the Kv7.2 A5 mutant needed longer VSP activation time for current inhibition than the wildtype (WT) channels. *In vitro* phosphorylation assays with the purified C terminus of Kv7.2 revealed that various kinases are able to phosphorylate these 5 serines. After treatment of cells expressing the WT Kv7.2 with inhibitors of PKA, p38MAPK, CamKII and CDK5, activation of VSP had to be significantly longer than in untreated controls in order to achieve current inhibition. Our results reveal that the phosphorylation status of residues located within the putative PIP2-binding domain determines the phospholipid sensitivity of Kv7 channels.

Polyamine oxidase synthesis by blood lymphocytes as the result of their stimulation of phytohemagglutinin in the cerebral gliomas

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Tumor-associated inflammation stimulates the growth and progression of the brain malignant gliomas. AQPeripheral blood lymphocytes play an important role in tumor-associated inflammation by migrating in inflammation and stimulating tumor cell proliferation and invasion into surrounding healthy tissue. Polyamine oxidation enzymes—diamine oxidase(DAO) and polyamine oxidase (PAO)—are important factors in the regulation of proliferative cell function during inflammation, primarily of lymphocytes. The accumulation of polyamines in the third stage of inflammation (proliferation and differentiation of cells) is governed by these enzymes. One of the regulators of cellular functions, in particular, the migration of blood cells and leukocyte adhesion, is the degree of blood cells aggregation, which can be considered as an indirect indicator of transmembrane potential. In this work, we have used a new method of determining the degree of blood cells aggregation based on the effect of surface plasmon resonance. The aim of this work was to study the DAO and PAO activities in the transformation process of lymphocytes in the blasts when exposed to various concentrations of phytohemagglutinin with the "lymphocytes blasttransformation reaction" test. The blood samples taken on an empty stomach in patients with cerebral gliomas of varying degrees of malignancy were the objects of the study. Phytohemagglutinin was used within a concentration gradient from 10-1 to 10-5 times. Blood cells (without serum) were pre-incubated with different concentrations of the drug 20 min at a temperature of 37 °C. The original solution of phytohemagglutinin was used as the control. Then nutrient medium RPMI, antibiotics and cultured cells according to well-known "lymphocytes blasttransformation reaction" methods were added. A cellular fraction of heparinized venous blood was used to determine the degree of blood cells aggregation as a shift of the minimum of the curve of the surface plasmon resonance in degrees. DAO and PAO activities were determined by a spectrophotometric method. In the group of healthy individuals, indicators of the degree of blood cells aggregation and the DAO and PAO activities are within normal limits with slight deviations depending on the dilution of phytohemagglutinin. There is a significant reduction in the proliferative activity of lymphocytes when the decrease in surface plasmon resonance is observed. The DAO and PAO activities also reduced. There is an increase in the level of DAO and PAO activities in the same time with increasing surface resonance. Blasts remain reduced. The decrease in surface plasmon resonance coincides with a decrease in the number of blasts and DAO and PAO activities when there are gliomas with the II stage of malignancy. The increase of surface plasmon resonance coincides with a decrease in the number of blasts and an increase in DAO and PAO activities in the oxidation of spermine. PAO activity in the oxidation of spermidine, on the contrary, is decreased. The decrease in surface plasmon resonance is accompanied by decreased DAO and PAO activities and increased number of blasts when there are gliomas with III stage of malignancy. Blasts decreases simultaneously with the decrease in DAO activity, and by increasing the PAO activity, the level of performance of surface plasmon resonance raise. The ratio between the level of reducing aggregation of blood cells and the DAO and PAO activities under the influence of various dilutions of phytohemagglutinin emphasizes the important role of transmembrane potential in the proliferative activity of lymphocytes in inflammation and tumor growth.

The antiproliferative and proapoptogenic effects of aniline derivative copper (II) complex that activates catabolism and inhibits the synthesis of polyamines

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As a result of prescreening on a cell-free assay system, a copper (II) complex with aniline derivative 3-(2-fluoroaniline)-1-phenylpropanone-1 was selected. It showed an activating effect on the oxidative deamination of polyamines and inhibitory effect on their synthesis which indicates its potential carcinostatic properties. The aim of the work was to test the selected active agent in vitro on tumor cell cultures, as well as to study its effect on the readiness of the cells for apoptosis. In cellular test system investigation, it was found that its IC50 against melanoma cells MEL7, breast cancer MCF-7, and prostate cancer PC3 was $\leq 10^{-4}$ M. On mononuclear leukocyte cells it has been shown that under the action of the compound, the expression level of the prototype death receptor CD95 increases, which indicates an increased willingness to cell apoptosis as the latest can be provoked by interaction of CD95 with its ligand. Thus, the compound is sufficiently active in regard to its antiproliferative activity. One can assume that one of the probable mechanisms of its antiproliferative action is the start of programmed cell death. For a more accurate conclusion, further detailed study is required.

The correlation between the influence of aniline derivatives on oxidative deamination of polyamines and their ionization constants

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The polyamine exchange being associated with the processes of cell proliferation and differentiation can be used as a target for potential antitumor agents. Reducing the intensity of oxidative deamination of polyamines is in some cases a primary mechanism for polyamine level increasing in the cell about the malignancy. Thus, it can be regarded as an experimental index for the primary selection of potential anticancer agents. The aim of our work was to study the direct influence of 11 aniline derivatives with different substituents on the aromatic ring, for which ionization constants were found by spectrophotometric titration. Regenerating rat liver was used as a cell-free test system. The results showed the following: Only substance A6 activated oxidative deamination putrescine. Increasing of oxidative deamination rate of spermidine was observed on the background of the substances A2, A5, and A6. Oxidative degradation of spermine reliably activated 4 compounds: A9, A3, A4, and A8. The aminoxidase activity value against the action of compounds from the group of aniline derivatives correlates with their ionization constants in the case of putrescine ($R = -0{,}85$, $p = 0{,}002$) and spermine ($R = -0{,}72$, $p = 0{,}019$). This means that the more basic properties have these substances, the greater is the degree of aminoxidase inhibition. Diamine oxidase is a copper-containing enzyme. One of the possible mechanisms of its activation by these structures is coordinating its copper-containing active center with aniline derivatives behaving as ligands. In the case of the spermine catabolism, further research is needed to allow hypothesizing mechanism of inhibition.

Activators of polyamine oxidase as potential antitumor agents

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Our preliminary studies of polyamine metabolism in transplantable hepatomas 27, 22a, 60, 61, 46, 48 and during diethylnitrosamine-

induced hepatocarcinogenesis were aimed to investigate the mechanisms of PA regulation in normal, malignant and regenerating liver. Results demonstrate the difference between PA synthesis and catabolism during the pathological proliferation compared to healthy tissues. Accumulation and increased concentrations of PA observed during cancer growth process are associated mostly with their catabolism suppression but not only with the acceleration of biosynthesis as in regenerating liver. Decreased rate of oxidative deamination of PA in hepatocarcinogenesis is most likely to be the primary process, while the increase of decarboxylation rate may be considered as the result of the transformed hepatocytes' dedifferentiation. Diamine oxidase (DAO) and polyamine oxidases (PAO), which regulate the PA levels, may have direct or mediated effects on the enhanced cell proliferation and tumor growth. Enzyme activity of PAO in the early stages of hepatic malignization can be down-regulated by the high concentrations of PA (substrate inhibition) and vitamin B_6 deficiency (urgent regulation type). In the late phase of neoplastic transformation, the DAO activity may be genetically regulated by the termination of apoenzyme synthesis (chronical regulation type). Elimination of DAO and PAO activities in tumor cells may be the defence against the toxic products of oxidation. Thus, the activators of PA catabolic enzymes would have the cancerostatic potency. So, in both acellular test-systems of tumor and regenerating tissue, bis-(uracil) PA analogs have demonstrated not only inhibition of PA synthesis, but also activation of PA catabolism. This is the probable reason for the proliferation suppression of cancer (CaOv) cells growth in culture. These results indicate that the stimulators of PA oxidative deamination showed antitumor activity and may be used as potential therapeutic agents. The investigations of the aberrant regulation of polyamine metabolism in tumors made it possible to suppose that inhibitors of the polyamines' oxidative deamination could have the carcinogenic properties. In contrast, compounds, which activate the polyamine catabolism, may be potential antineoplastic agents. The aim of our present study was the prediction of carcinogenic and antiproliferative properties of the novel compounds. The objective was to evaluate the influence of benzimidazole, azofluorene, dioxaboreninopyridine and aniline derivatives on the rate of putrescine, spermidine and spermine oxidative deamination in the acellular testing system of the high mitotic index tissue. We studied 32 compounds. To sum up: (1) 7-amino pyrido[1,2-α]benzimidazole and 7-nitro pyrido[1,2-α]benzimidazole demonstrated 1.5-1.7 fold inhibition of amino-oxidase activity in regenerating liver and can be potential cancerogenic. 1-amino-9-phenylamino-4-azo fluorene and 1,4-diazoacetonaphtyleno[1.2-f]-fluorinanten were activators of polyamine catabolism, thus can probably have antiproliferative effects. (2) Aniline derivatives , especially 3-(4-iodine anilino)-1-phenylpropane-1, activated the process of oxidative deamination and may be considered as potential cancerostatics. (3) The repression of amino-oxidase activity by all dioxaborenynopyridine derivatives enables to consider them as potential tumorogenic due to the presence of common 6-Methyl-4,8a-diphenyl perhydro[1,3,2]-dioxaborinino [5,4-c]pyridine core structure.

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The influence of aniline derivatives, their copper complexes and dioxaborininopyridine derivatives on ornithine decarboxylase activity

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The biosynthesis of polyamines is one of mechanisms of their in-cell concentration regulation, which in its turn is a regulating factor in respect of cell growth and differentiation, especially in the case of regenerative processes and tumor growth. The character of the action on it of various substances gives talk about their potential carcinogenic or carcinostatic properties. The effects of aniline derivatives (group A), their copper complexes (group B) and dioxaborininopyridine derivatives (group C) on the activity of the key enzyme of polyamine biosynthesis—ornithine decarboxylase (ODC)—were investigated. The experimental model served as a cell-free test system derived from regenerating rat liver. Complex compounds were prepared according to standard procedures by mixing alcoholic solutions of aniline derivatives with copper chloride solution. All the compounds of group A caused a significant decrease in ODC activity, which is typical for carcinostatics. In the case of copper complexes, no uniformity was observed. Significant inhibition of ODC activity was indicated for the compounds B1, B4–B9. Activation of ODC was observed on the background of B11. All the compounds of the group C, except for the C7 and C8, caused a significant increase in the activity of ODC, which is typical for carcinogens. Thus, in terms of impact on the biosynthesis of polyamines, the substances of the first group showed oncoprotective properties, the substances of the third group had carcinogenic properties. In regard to the second group of substances, it can be said that their biological effects are different. Additional studies are necessary for the explanation of this fact.

Identification of structural targets in Polyamine oxidase active site for the directed synthesis of polyamine catabolism activators

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Polyamine oxidases (PAO), a FAD-containing enzyme is involved in the biodegradation of polyamines, catalyzing their oxidative deamination. Polyamine oxidation products—aminoaldehydes are cytotoxic and have been considered as a cause of apoptotic cell death due to their ability to act as the carbonyl agents, conjugate with proteins and nuclear acids. The catalysis of polyamines by the oxidative deamination is decreased or almost arrested in tumor cells. Thus, the activators of polyamine catabolism are supposed to be potential antineoplastic agents. The docking of polyamine-like compounds with yeast PAO enzyme Fms1 was implemented to find out the key structural parameters for directed chemical synthesis of novel anticancer molecules. The modeling of aniline, azofluorene, benzimidazole, and dioxaboreninopyridine derivatives interaction with PAO was performed using Molegro Virtual Docker software with flexibility in torsion angles of ligands. The lowest energy pose of each compound was described by energies of ligand interaction with different residues of enzyme. Decomposition of total docking score into partial ligand-residue terms was performed using the "energy inspector" tool embedded in MVD. Few critical atoms in the sphere radius 12 Å of the PAO active center were identified: His67, Tyr450, His191, Trp174, Gly487. The in vitro activity of about 40 compounds among aniline, azofluorene, benzimidazole, and dioxaboreninopyridine derivatives on the rate of putrescine, spermidine and spermine oxidative deamination was evaluated in the acellular testing system of the rat regenerating liver. Only azofluorene and aniline compounds were activators of polyamine catabolism, especially 1-amino-9-phenylamino-4-azofluorene, 1-amino-2-bromo-4-azofluorene-9, 3-(4-iodine anilino)-1-phenylpropane-1, 3-(1-phenyl-2-fluoro aniline)-propanone-1. The antiproliferative activity was examined in prostate

cancer cell line PC-3 by vitality test with Alamar Blue. Results enabled to construct a binary classification model for prediction of potential influence of novel compounds on polyamine metabolism and their possible anticancer properties from docking parameters. Determined amino-acid residuals revealed the structural basis for the design and synthesis of novel activators of polyamine catabolism as potential antitumor agents.

Fluoro derivatives of (+)-usnic acid as the potential exchange regulators of polyamines

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Polyamines (PA) can be a convenient experimental target in studying the proliferative activity of cells. On the other hand, (+)-usnic acid is a well-known bioactive substance. This work is devoted to obtaining of fluoro derivatives of (+)-usnic acid. These derivatives are considered as the potential exchange regulators of polyamines. Chemical modification of (+)-usnic acid was conducted according to scheme: **(+)-usnic acid + base + fluorinating agent => fluorinated derivative of (+)-usnic acid**. The influence of different process parameters (e.g., humidity level of solvent) on the character of modifications was examined. The reaction ability of hydroxyl groups of usnic acid was studied. It was indicated that among the three OH groups of (+)-usnic acid, only OH group in position 7 participates in the reactions with perfluoroolefines.

Abstract: Oral Presentation in the Polyamine Section

New insights into novel inhibitors against deoxyhypusine hydroxylase from plasmodium falciparum: compounds with an iron chelating potential

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Abstract: Deoxyhypusine hydroxylase (DOHH) is a dinuclear iron enzyme required for hydroxylation of the aminobutyl side chain of deoxyhypusine in eukaryotic translation initiation factor 5A (eIF-5A), the second step in hypusine biosynthesis. DOHH has been recently identified in *Plasmodium*. Both enzymes have very peculiar features including a diiron centre in their active site. Structural predictions based on the amino acid sequence of the active site in comparison to the human enzyme show that four conserved histidine and glutamate residues provide the coordination sites for chelating the ferrous iron ions. Recently, we showed that *P. vivax* DOHH is inhibited by zileuton (*N*-[1-(1-benzothien-2-yl)ethyl]-*N*-hydroxyurea), a drug that is known for inhibiting human 5-lipoxygenase(5-LOX) by the complexation of ferrous iron. A novel discovery program was launched to identify inhibitors of the *P. falciparum* DOHH from the Malaria Box, consisting of 400 chemical compounds, which are highly active in the erythrocytic stages of Malaria infections. Selection for potential ligands of ferrous iron resulted in three compounds from different scaffold classes namely the diazonapthyl benzimidazole MMV666023 (Malaria Box plate A, position A03), the bis-benzimidazole MMV007384 (plate A, position B08), and a 1,2,5,-oxadiazole MMV 665805 (plate A, position C03). As a proof of principle, a bioanalytical assay was performed and the inhibition of hypusine biosynthesis was determined by GC–MS. MMV 665805 exhibited the strongest inhibitory effect. The results were in accordance with the preliminary quantum–mechanical calculations suggesting the strongest iron complexation capacity for MMV 665805 which might be a novel lead structure for inhibitors of *P. falciparum* DOHH.

Mechanism of mitochondrial permeability transition prevention or induction by spermine in mammalian mitochondria

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Mitochondria permeability transition (MPT) is a phenomenon involved in apoptosis occurrence. MPT is induced by a complex assembly of several proteins belonging to all the mitochondrial compartments, leading to formation of the transition pore. This causes a bioenergetic collapse, redox catastrophe and mitochondrial swelling with outer membrane rupture and release of pro-apoptotic factors. MPT is induced by supraphysiological [Ca^{2+}] and by an oxidative stress inducer. MPT is prevented by cyclosporin A, ADP and reducing agents. Depending on its concentration, spermine is able to directly induce MPT at low concentrations (3–5 μM), or protect at high concentrations (50–100 μM) against MPT induced by other agents. The proposed mechanism is related to the generation of reactive oxygen species (ROS) by spermine or to a ROS scavenging effect exhibited by the polyamine. The above observations have been obtained in isolated mitochondria. Spermine added to tumor cells, in the presence of bovine serum amine oxidase (BSAO), induces apoptosis, by evidencing the presence of strongly swollen damaged "in situ" mitochondria. These results raise the question in what is the

cause and the effect of apoptosis and mitochondrial damage. Mitochondria isolated from hepatoma cells treated with BSAO plus spermine (50–100 μM) undergo MPT induction and release pro-apoptotic factors, thus demonstrating that the primary event leading to apoptosis is pore opening. In conclusion, these results demonstrate the pathophysiological role exhibited by spermine at different concentrations in isolated or "in situ" mitochondria and in particular the possible utilization of spermine together BSAO for therapeutic interventions.

Inhibition of cellular proliferation and differentiation by polyamine depletion

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The polyamines spermidine, spermine and their diamine precursor putrescine are naturally occurring polycations that are essential for cellular proliferation and differentiation. Depletion of cellular polyamines results in growth cessation that can be resumed upon re-addition of polyamines to the growth medium of the arrested cells. The tight association between polyamines and cellular proliferation made the polyamine metabolic pathway an appealing therapeutic target for treating hyperproliferative pathologies including cancer. However, the mechanism by which polyamine depletion inhibits cellular proliferation is mostly unknown. We set out to investigate the molecular mechanism by which polyamine depletion causes growth inhibition by investigating changes in translational and transcriptional activity in the depleted cells. Our results demonstrated that translation is inhibited at the initiation step and that this inhibition is accompanied by transcriptional response that together suggest establishment of stress response that is manifested through activating the PERK arm of the UPR. While we demonstrate phosphorylation of eIF2α in the polyamine depleted cells, we show that polyamine depletion cause growth and translational cessation also in cells harboring a mutant non-phosphorylated allal of eIF2α, suggesting that possible involvement of additional stress mechanisms or the involvement of polyamines in the initiation process. We also demonstrated that polyamine depletion inhibits adipogenic and myogenic differentiation. While polyamine depletion inhibits the expression of key myogenic and adipogenic regulators, polyamines seem to play a key role in the proliferative process of mitotic clonal expansion.

Can polyamines be used to combat cancer? New therapeutic approaches

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MTT assays showed that the cytotoxicity induced by bovine serum amine oxidase (BSAO) and spermine (SPM) was enhanced in colon adenocarcinomas (LoVo) and melanomas (M14) cell lines, by the pre-treatment of tumour cells with anandamide (AEA), a natural endocannabinoid. This compound sensitized both wild-type (WT) and multi-drug resistance (MDR) cells to the subsequent exposure to SPM metabolites. The sensitizing effect was higher on MDR cells than wild-type ones in both cell lines. The results are supported by Annexin V-FITC/PI assay. Pre-treatment with AEA increased the percentage of apoptotic cells on both WT and MDR phenotypes. Flow cytometry analyses showed that BSAO and spermine induced a remarkable appearance of subG1 peak, on both cell lines, that was even more increased when cells were pre-treated with AEA. In order to obtain an unbiased global view on the effect of BSAO in tumor cells, stable isotope labelling of amino acids in cell culture (SILAC) proteomics approach was performed using prostate cancer (LnCaP) cells treated for 1 h with and without BSAO. In total, 721 unique proteins were identified of which 40 were differentially expressed by more than 1.3 folds. The canonical pathways that exhibited the largest differences between BSAO treated and untreated cells in the presence of spermine include the mitochondrial dysfunction and eIF-2 signaling. Interestingly, complex I was up-regulated, while complexes III and IV and V were downregulated in cells treated with BSAO as compared to untreated cells. We conclude that the mechanism of the cytotoxicity of BSAO/SPM is partly related to mitochondrial dysfunction. To increase the stability of the enzyme and the release of cytotoxic products, BSAO was conjugated on a new injectable nanohydrogel (NHs), obtained by derivatizing hyaluronic acid (HA) with cholesterol (CH). The HA-based NHs system is a useful controlled delivery system for future therapeutic enzymes application.

Mapping the transglutaminase-2 interactome in a model of chronic kidney disease

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A significant feature of progressive kidney scarring is the release of Transglutaminase-2 (TG2) by tubular epithelial cells into the surrounding tubulo-interstitium, leading to increased deposition of extracellular matrix (ECM) components, ECM stabilisation and activation of Transforming Growth Factor beta-1. Therefore, controlling TG2 externalisation could be a new strategy to limit fibrosis progression. To identify the molecular partners of TG2 leading to its export, we utilised the unilateral ureteric obstruction (UUO) murine model of chronic kidney disease. TG2 immunoprecipitation from whole kidney membrane preparations of WT and TG2-null diseased mouse kidneys was coupled to quantitative proteomics by SWATH-MS. Differences between WT and TG2-null precipitated proteins were determined by a paired sample z-test. Among the plasma membrane-associated proteins specifically interacting with TG2 ($p \leq 0.05$, $n \geq 4$) and uniquely expressed in the UUO model, a predominance of cell adhesion, actin dynamics, redox regulation and endosome-related proteins was revealed, by searching Gene Ontology terms overrepresented in TG2 partners ($p \leq 0.05$) compared to reference proteomes. Protein interaction network analysis (STRING 9.1) showed that a subnetwork of proteins responsible for endosomal transport were associated with TG2 and formed significant "hubs" post UUO. Furthermore, the heparan sulphate (HS) proteoglycans syndecan-4 and perlecan were specifically associated with TG2 in the

UUO model. Our study shows that UUO induces association of TG2 with several endosomal proteins suggesting their involvement in TG2 trafficking during the progression of fibrosis.

Pathways for synthesis of polyamines and their roles in conceptus development and pregnancy recognition signaling in sheep

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Embryonic mortality, a major constraint to reproductive performance in mammals, occurs during the peri-implantation period of pregnancy in ungulates when conceptuses undergo morphological transitions from spherical to tubular and filamentous forms prior to implantation. Secretions from uterine epithelia and nutrients transported into the uterine lumen include arginine, a nutritionally essential amino acid for conceptus survival and development. Arginine increases 13-fold in the ovine uterine lumen between Days 10 to 15 of pregnancy and is metabolized to nitric oxide (NO) via NO synthase (NOS3) or polyamines via ornithine decarboxylase (ODC1). Morpholino antisense oligonucleotides (MAO) knockdown of translation of ODC1 mRNA in ovine conceptus trophectoderm resulted in half of the conceptuses to be morphologically and functionally normal and one-half being abnormal. Normal conceptuses increased expression of arginine decarboxylase and agmatinase to compensate for loss of ODC1 for synthesis of polyamines. We also determined effects of arginine mediated by NOS and polyamines on ovine trophectoderm cells (oTr1). Arginine (0.2 mM) increased ($P < 0.05$) proliferation, production of interferon tau (IFNT) and cellular protein. IFNT is the pregnancy recognition signal in ruminants. Inhibitors of NOS3 (L-NAME), ODC1 (DFMO) or L-NAME + DFMO decreased ($P < 0.05$) cell proliferation; however, arginine alone increased cell proliferation 1.5-fold ($P < 0.05$) over control medium suggesting that arginine is a growth factor. Putrescine and NO stimulated proliferation via TSC2-MTOR signaling, but only putrescine increased production of IFNT by oTr1 cells. Thus, arginine-derived polyamines stimulate proliferation and IFNT production by oTr1 cells via the TSC2-MTOR signaling pathways to enhance embryonic growth, development and survival.

Erythrocyte polyamine determinations: their clinical impact as specific predictors in cancer and in non-cancer diseases

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After extrusion of its nucleus, the erythroblast becomes an erythrocyte. Macrophages present in the bone marrow will phagocytose this nucleic material, and the erythrocyte becomes a transporter not only of hemoglobin but also of polyamines. The circulating erythrocytes will later be physiologically destroyed in the spleen by macrophages. Interestingly, a partial splenectomy in tumor bearing animals reduces tumor growth and metastases spreading. Erythrocytes contain the major pool of blood polyamines, especially spermidine (Spd) and spermine (Spm). Though intracellular Spd and Spm levels reflect differences in uptake rather than in outward flux across the cell membrane, the values of erythrocyte putrescine (Pt) appear to be the resultant of influx and efflux. During experimental liver regeneration but also in tumor-grafted animals, the erythrocyte Spd levels increase in proportion to the intensity of the hyperplasic phase. In these experimental models, the Spd/Spm ratio may be considered as a circulating index of hyperplasia. Erythrocyte polyamine determinations have revealed their clinical usefulness in patients suffering from hepatocellular carcinoma, malignant glioma, bronchopulmonary cancer, childhood acute lymphoblastic leukaemia, bone marrow transplantation in children, prostatic carcinoma and non-cancer diseases. Recently, the polyamine pathway has been incriminated in the pathogenesis of Parkinson disease. Erythrocytes can no more be considered as small circulating "polyamine bags". The bioamines they transport may be used as clinical "markers" in various pathologies, but also participate in the pathogenesis and in the maintenance of many human diseases.

Biological background of polyamine-induced lifespan extension

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Summary: Heathy foods and healthy dietary patterns have higher concentrations of polyamines (spermine and spermidine). The continuous intake of high-polyamine foods has been shown to increase blood polyamine concentrations in mice and humans. And, high-polyamine chow inhibited aging-associated pathological changes of Jc1:ICR mice and extended their lifespan. Aging is accompanied by decreased DNA methyltransferase (Dnmt) activities, increased expressions of leukocyte function-associated antigen 1 (LFA-1) on immune cells, and an enhanced abnormal methylation status (increased methylation and demethylation) of DNA. Increased LFA-1 expression indicates pro-inflammatory status, and abnormal DNA methylation is one of the major pathogenesis of aging-associated diseases. *In vitro* experiments using Jurkat cells have shown that polyamine deficiency decreased Dnmt activities, increased LFA-1 expressions associated with the increased demethylation of LFA-1 promoter areas (ITGAL), and enhanced abnormal methylation of entire genes, whereas supplementation with spermine (which has powerful biological activities) reverses such changes. Moreover, the abnormal methylation status and the aging-associated increase in LFA-1 expressions observed in aged mice fed normal chow was not observed in age-matched mice fed high-polyamine chow. These findings indicated that the aging-associated enhancement of abnormal DNA methylation is inhibited by polyamines. Unlike lower organisms with short lifespans, mammals have long lifespans and complicated neurological, metabolic, and immunological functions which are mutually related. Polyamines seem to exert their biological activities by affecting the expressions of many genes via the control of the methylation status of promoter areas.

Benextramine derivatives as probes to target human monoamine oxidases

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Benextramine is an "old" tetraamine disulfide acting as irreversible α-adrenergic antagonist, able to recognize additional targets, such as nicotinic and muscarinic receptors and acetylcholinesterase enzyme, all involved in neurodegeneration. Thanks to its disulfide moiety, benextramine can react with Cys residues of receptors and enzymes leading to a covalent bond resulting in irreversible alterations of the biological activity of the protein target. Benextramine also behaves as competitive M_2 muscarinic receptors antagonist and reversible AChE inhibitor. Using benextramine as "template", various polyamine analogues were designed to improve selectivity and activity towards the multiple targets, besides the cholinergic system, involved in neurodegeneration (Melchiorre et al., Med Res Rev. 2003; 23: 200). Monoamine oxidases (MAO) are well-known targets in neurodegenerative diseases and both isoforms contain Cys residues important for their catalytic activity (Hubalek et al., JBC 2003, 278:28612). On these bases, we studied the effect of some benextramine derivatives on these potential novel targets. Most of the tested compounds were foundto be able to inactivate both MAO isoforms. In particular, increasing the length of the methylene chains between the nitrogen atoms of the polyamine scaffold increased the inactivation constant and the presence of the catechol moiety on the terminal nitrogen atoms increased the selectivity towards MAO B. In the case of MAO A, inactivation was found irreversible. Further studies are in progress to elucidate the binding mode of these compounds to MAOs, with the aim to modify their structure in order to improve their affinity and selectivity for potential application in neurodegenerative diseases.

Understanding the role of the hypusine-containing protein eIF5A in translation

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The eukaryotic translation factor 5A (eIF5A) undergoes hypusination, a unique and essential posttranslational modification in which the polyamine spermidine is required. Although eIF5A has been extensively involved in several cellular processes and pathologies, almost all of these functional correlations can be seen as a result of its loss of function in translation. The role of eIF5A in translation was strengthened by the genetic and functional interaction between eIF5A and the eukaryotic elongation factor 2 (eEF2). To further understand this relationship, we have investigated the influence of these factors on their ribosome binding. The data obtained revealed that both factors are not able to bind to the same ribosome at the same time and they can replace one another in the pre-assembled ribosome 80S, suggesting an anti-cooperative ribosome binding effect. Moreover, the addition of eEF2 decreases the affinity of eIF5A to the 80S ribosome. Additionally, conditional eIF5A yeast mutants display reduced binding affinity to the ribosome, and this is probably the cause of their defects in growth and protein synthesis. Finally, to further investigate the cellular function of eIF5A, we have performed high-throughput genetic interaction studies using the Synthetic Genetic Array (SGA) analysis. The results obtained so far revealed interesting synthetic genetic interactions, e.g. with genes encoding proteins involved in cell cycle and cytoskeleton organization, which are in agreement with a role for eIF5A in the translation of a specific mRNA subset.

"Role of Polyamines and EGFR Signaling in *Helicobacter pylori* Infection and Gastric Carcinogenesis: Targets for Intervention"

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H. pylori (*Hp*)-associated gastric cancer is the third leading cause of cancer deaths worldwide. We have implicated in gastric carcinogenesis both ornithine decarboxylase (ODC), the rate-limiting enzyme for polyamine synthesis, and spermine oxidase (SMOX) that generates oxidative stress from metabolism of spermine. Our data show that *Hp*-induced phosphorylation of epidermal growth factor receptor (EGFR) increases SMOX and DNA damage in gastric epithelial cells (GEC) and pro-inflammatory responses in macrophages. Inhibition of ODC with α-difluoromethylornithine (DFMO) or shRNA knockdown reduces *Hp*-induced DNA damage in GEC. We investigated interactions of polyamines and pEGFR during *Hp* infection of Mongolian gerbils for 8 or 12 weeks. Animals received ± DFMO in the drinking water and ± the EGFR inhibitor, gefitinib, in the diet. Gastric cancer was reduced with either treatment, but the effect was stronger with DFMO. There was no additive effect with combined treatment. DFMO or gefitinib reduced levels of γ-H2AX, a marker of DNA damage, in gerbils where carcinoma/dysplasia was prevented. *Hp* output strains from DFMO-treated, but not gefitinib-treated gerbils had decreased ability to translocate the oncogenic virulence factor CagA into GEC, and to induce pro-inflammatory/pro-tumorigenic IL-8. However, NF-κB activation was decreased with strains from either DFMO or gefitinib-treated gerbils. In vitro pretreatment of GEC with DFMO or gefitinib decreased *Hp*-stimulated pro-inflammatory cytokine production. Gefitinib also decreased *Hp*-induced ODC in macrophages. Inhibition of both EGFR signaling and ODC may have a potential role in the chemoprevention of gastric cancer, but depletion of polyamines by DFMO has the additional benefit of attenuating virulence of *Hp*.

RNAseq reveals polyamine signaling in relation to tomato fruit development and ripening

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Signaling between polyamines (PAs) and the classical plant hormones in regulating free and bound forms of hormones was previously summarized [Anwar et al. (2015), In: Kusano and Suzuki (eds) Polyamines, DOI 10.1007/978-4-431-55212-3_22)]. RNAseq analysis of wild-type (WT) fruit and those of transgenic tomato line *SlE8:ySpdSyn,* which accumulates high levels of spermidine (Spd) and spermine (Spm), has further demonstrated that Spd/Spm differentially regulate a wide range of genes including those that encode for the biosynthesis and signaling of plant hormones. The GO evaluation revealed that the differentially expressed genes represented myriad of biochemical and developmental functions. In the transgenic tomato, along with abundance of *mat* and *ACS* transcripts, *ETR* (ethylene receptor) transcripts also increased. Similarly, higher jasmonate-zim-domain protein transcripts accumulated in transgenic fruit, overriding effect of higher AOC and lipoxygenase transcripts. Both IAA and GA effects in fruit appeared to be further potentiated by higher PA levels. PAs likely negate cytokinin signaling. A similar negative trend was seen for ABA—PAs enhancing transcripts of the regulatory component of ABA receptor and PP2C with a decrease in KAT1 transcripts. Accumulation of the BZR1 and TGA (TGA transcription factor) transcripts in transgenic compared to WT fruits suggests a role(s) for PAs in brassinosteroids and salicylic acid signaling pathways. Additional research is needed to establish PA crosstalk with different plant hormones. Implications of these results in plant growth and development, particularly fruit development, will be discussed. Supported by USDA-NIFA 2012-67017-30159 (AKH).

Synthesis of biologically active analogues of spermine and spermidine

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Biogenic polyamines spermine and spermidine are present in mM-microM concentrations in all living cells and involved in the regulation of many vitally important metabolic transformations. Spermine and spermidine analogues are useful tools to study cellular functions of polyamines, as well as the enzymes of their metabolism. Different synthetic strategies being suitable for the synthesis of biologically active polyamine analogues will be discussed. Special attention will be paid to *C*-methylated derivatives of spermine and spermidine; the biological activity can be regulated by moving methyl group along the polyamine backbone and/or changing the stereospecificity of chiral center [1]. A possibility for the introduction of methyl group into the drugs of polyamine nature to regulate/improve their metabolic stability will be discussed.

1. Keinänen TA, Hyvönen MT, Alhonen L, Vepsäläinen J, Khomutov AR (2014) Selective regulation of polyamine metabolism with methylated polyamine analogues. Review. Amino Acids 46(3):605–620.

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Bovine serum amine oxidase and chromate-modified iron oxide nanoparticles for polyamine biosensing

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Surface active maghemite nanoparticles (SAMNs) represent a new class of naked maghemite nanoparticles, characterized by peculiar surface properties. A novel hybrid nanomaterial based on SAMNs was developed by coating nanoparticle surface with bichromate, leading to a SAMN@Cr_2O_7 core–shell nanoconjugate. Electrochemical impedance spectroscopy, cyclic voltammetry, and chronoamperometry showed lower charge transfer resistances, higher capacitive currents, better electrochemical performances, as well as more reversible electrochemical behavior with respect to bare SAMNs. Bichromate shell enhanced SAMN electrocatalytic properties toward hydrogen peroxide reduction at neutral pH. Furthermore, bovine serum amino oxidase (BSAO) was immobilized on the surface of SAMN@Cr_2O_7 by self-assembly, leading to a magnetic drivable nanocatalyst for polyamine oxidation (SAMN@Cr_2O_7-BSAO). The hybrid bio-nanomaterial was kinetically characterized and compared with the native enzyme. Immobilized BSAO showed a catalytic constant of about 10.2 % with respect to the native enzyme, while the Michaelis constant, K_m, was about 3.7 times higher upon enzyme immobilization. Correspondingly, the binding process led to a 2.4 fold decrease of BSAO catalytic efficiency, k_{cat}/K_m. The immobilized enzyme (SAMN@Cr_2O_7-BSAO) was exploited, in a simple carbon paste electrode configuration, for the development of a new reagentless electrochemical biosensor for polyamines.

Keywords: γ-Fe_2O_3, Hybrid nanomaterial, Electrocatalysis, Bovine serum amine oxidase, Bichromate, Polyamine biosensor.

Use of polyamine to synthesize fluorescent carbon quantum dots as bactericide and transfection reagents

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Department of Bioscience and Biotechnology, National Taiwan Ocean University, Keelung, Taiwan

Abstract: We developed two strategies to prepare fluorescent carbon quantum dots from natural polyamines. In the first work, the fluorescent carbon quantum dots (CQDs) were synthesized by pyrolysis of ammonium citrate in the solid state and then modified with

spermidine by a simple heat treatment at different temperatures. We observed that the spermidine–CQDs possess positive surface charge and have the ability to inhibit bacterial growth of many species such as *Escherichia coli, Staphylococcus aureus,* and *Proteusbacillus vulgaris. In vitro* studies and the in vivo studies of the antibacterial activity of Spd–CQDs in media and mouse, respectively, revealed promising outcome that is comparable or better than other commercial antimicrobial agents. In the second work, CQD was directly synthesized by pyrolysis of polyamines at different temperatures. Such kind of CQD also possesses positive surface charge and low cytotoxicity. Further, we found polyamine CQD was able to bind DNA and facilitate DNA transfection into mammalian cells. This study reveals that using natural polyamines as ingredients to synthesize CQD opens up new materials with a wide range of properties for bioimaging, bactericide, and transfection applications.

Design, synthesis, and biological evaluation of lipopolyamines, especially spermine conjugates as non-viral pDNA delivery vectors

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Our research is into the design, synthesis, and biological evaluation of lipopolyamines. In particular, into spermine conjugates for use as non-viral pDNA delivery vectors where we aim to achieve efficient gene delivery and low cellular toxicity in target cells using cationic lipids at minimal ammonium (N)/phosphate (P) ratios. Genes (plasmid DNA) are now available as drugs, but they need efficient vectors for delivery. Barriers to gene therapy, e.g., endocytosis, endosome escape, and transcription and translation, are now largely understood and overcome. The remaining major obstacles in gene delivery are in nuclear trafficking which limits the rational design of efficient non-viral vectors. We use lipopolyamines to package pDNA by self-assembly for cellular uptake by endocytosis, where the DNA is condensed in a packaging mechanism mimicking nuclear histones and spermidine and spermine polyamines. Cholesteryl carbamate and N^4,N^9-di-fatty acid spermine polyamine amide conjugates were prepared, purified, and used to condense pDNA into lipoplexes. Efficient delivery of pEGFP was achieved in hard to transfect human primary skin FEK4 fibroblast cells and in a human cervix carcinoma, HeLa-derived and transformed cell line (HtTA), best achieved with LinOS, an unsymmetrical spermine conjugate of both linoleic and oleic acids which also showed high cell survival (MTT assay). These results show that N^4,N^9-di-fatty acid conjugates of spermine with C18 chain-length are excellent non-viral vectors for pDNA-mediated gene delivery. LinOS more than delivers as an efficient, non-toxic, non-viral vector. Such lipopolyamine vectors will have the potential as medicines for gene therapy in a variety of difficult-to-treat diseases.

Design, synthesis, and biological evaluation of N^4,N^9-di-fatty acid conjugates of spermine as non-viral siRNA delivery vectors

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We aim to achieve quantitative gene silencing by siRNA delivery using fatty acid conjugates of spermine as non-viral vectors formulated for low cellular toxicity. N^4,N^9-Di-fatty acid (erucic, lauric, linoleic, oleic, and stearic) spermine conjugates were prepared, purified, and used to condense siRNA into lipoplexes of particle size (DLS) 132–575 nm with zeta-potentials (electrophoretic mobility) from +28 to +50 mV. After FACS gating a healthy cell population, siRNA delivery did not relate directly to gene silencing efficiency as N^4,N^9-dierucoyl spermine resulted in higher siRNA delivery (ninefold) compared to N^4,N^9-dioleoyl spermine, but stably expressed green fluorescent protein (GFP) was knocked-down quantitatively (HeLa cells, 48 h post transfection) with the latter as the delivery vector in the presence of 10 % fetal calf serum (FCS) in DMEM. Unsaturated fatty acid lipopolyamine conjugates are more efficient delivery vectors than saturated fatty acid amides. The unsymmetrical conjugate LinOS, spermine bearing both a linoleoyl, and an oleoyl side-chain, gave quantitative silencing and 92 % cell survival in the alamarBlue® cell viability assay. These results show that N^4,N^9-di-fatty acid conjugates of spermine with C18 chain-length are excellent non-viral vectors for siRNA-mediated gene silencing. LinOS more than delivers as an efficient, non-toxic, non-viral vector for siRNA gene silencing in HeLa cells stably expressing GFP even in the presence of FCS. Such lipopolyamine non-viral vectors can achieve quantitative gene silencing with sequence-specific gene knock-down that will have potential as medicines in a variety of difficult-to-treat diseases.

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Development and properties of pectin/spermidine hydrocolloid films

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Low-methoxyl pectins (PEC) are able to produce hydrogels as a consequence of carboxyl group interaction with positively charged ions such as Ca^{2+}. PEC are also able to electrostatically bind both low and high molecular weight polycations (i.e. polyamines or positively charged proteins) which are useful in structuring hydrocolloid materials like biodegradable and/or edible films (Perez Espitia et al., *Food Hydrocoll.* 35, 287, 2014). In this respect, we considered the possibility of using spermidine (SPD) as a bridge between the carboxyl groups of PEC chains to modify the properties of PEC-based films. To assess SPD capacity of interaction and define the best complexation experimental conditions useful to generate a PEC/SPD binding, we performed preliminary titration studies of PEC/SPD mixtures by following both pH variation (potentiometric titration) and macromolecular assembly (turbidometric measurement at 600 nm). Starting from a PEC/SPD ratio of 10:1, we observed the typical inflection point of the amino group (pKa about 9.0) and a significant reduction of the tritated carboxyl groups (highlighted by the displacement of inflection point from pH 4.6 to 4.0 and from the reduction of the consumed equivalents) induced by the interactions of PEC carboxyl groups with SPD amino reactive moieties. This phenomenon appeared highest at PEC/SPD ratio of 2:1 (w/w). However, the turbidimetric titration indicated that the whole formation of complexes occurred using a 2:1 PEC/SPD ratio at a complexation pH of about 8.0. Therefore, we prepared and characterized PEC/SPD films obtained at pH 7.5 using different PEC/SPD ratios.

Role of spermine oxidase in modulation of glutamate receptors and transporters during excitotoxic stress

Pietropaoli S[1], Leonetti A[1], Maura G[2], Marcoli M[2], Cervetto C[2], D'Amelio M[3], Berretta N[3], Mariottini P[1], Cervelli M[1]

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Abstract: Discovering molecular mechanisms involved in neurodegeneration is very important for both early diagnosis and proper treatment. Polyamines (PAs) are polycationic molecules essential for cell growth, proliferation, regeneration, and differentiation. They play an important role in cancerous cells proliferation, and the functional role of PAs in normal and diseased brain is under active research. In this study, a Dach-SMO mouse genetic model overexpressing Spermine oxidase (SMO) specifically in neocortex provides novel evidences of the complex and critical functions carried out by SMO and Spermine (Spm) in mammalian brain. SMO is an important enzyme involved in PAs metabolism; it oxidases Spm-producing Spermidine (Spd), hydrogen peroxide, and 3-aminopropanal (3-AP). Physiological and molecular analyses, performed in normal and excitotoxic conditions, showed an increased sensitivity of this transgenic model to epileptic stimuli caused by a complex intercellular pathway triggered by SMO overexpression and its reaction products in neocortical neurons. The production of H_2O_2 and 3-AP, derived from Spm oxidation, together with direct effects of Spm on AMPA and KA receptors, are synergistically involved in ROS increase and ultimately in neuronal degeneration and death. SMO enzyme can be considered one of the most important H_2O_2 producers in the brain, and the transgenic Dach-SMO mice represent a useful genetic model for studying brain pathologies such as epilepsy, Alzheimer's disease, and other forms of dementia. Both neurons and glial cells resulted to be involved in this increased sensitivity model showing different levels of expression and activity in receptors and transporters implicated in glutamate response in synaptic transmission.

Synthesis of polyamines from L-proline in the porcine placenta and neonatal enterocytes

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Polyamines (putrescine, spermidine, and spermine) play crucial roles in regulating gene expression, signal transduction, ion channel function, DNA and protein synthesis, as well as cell proliferation and differentiation. Physiological levels of polyamines are also scavengers of reactive oxygen species, thereby protecting DNA, proteins, and lipids from oxidative damage. Thus, there is much evidence that polyamines are key regulators of angiogenesis, early mammalian embryogenesis, placental and fetal growth, as well as growth and remodeling of the neonatal small intestine. Classic textbooks describe only the arginase pathway for synthesis of polyamines in animals, wherein arginine is hydrolyzed by arginase to form ornithine which is decarboxylated by ornithine decarboxylase (ODC1) to yield putrescine. However, we found that neither porcine placentae throughout the entirety of gestation nor porcine neonatal enterocytes during the suckling period contain arginase activity or produce ornithine from arginine. In contrast, both porcine placentae and porcine enterocytes express high levels of mitochondrial proline oxidase to oxidize proline to generate pyrroline-5-carboxylate which is subsequently converted into ornithine by mitochondrial ornithine aminotransferase. Ornithine is transported out of the mitochondria into the cytoplasm where it is decarboxylated by ODC1 to yield putrescine. Putrescine is then utilized to produce spermidine and spermine by spermidine and spermine synthases, respectively. Collectively, results of our studies demonstrated polyamine synthesis from proline in porcine placentae and enterocytes, both of which lack arginase, but grow rapidly. This new knowledge will help in development of novel means to enhance placental and fetal growth, as well as intestinal growth and adaptation in mammalian neonates.

Mapping the transglutaminase-2 interactome in a model of chronic kidney disease

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Increased synthesis and export of Transglutaminase 2 (TG2) by tubular epithelial cells into the surrounding tubulo-interstitium is a significant feature of progressive kidney scarring. Once outside the cell, TG2 accelerates the deposition of available extracellular matrix (ECM) components and confers ECM-resistance to proteases. Further, TG2 enhances TGF-beta1 activation. Clinical development of anti-TG2 therapy is hampered by limited understanding of its unique unconventional export mechanism which may be targeted to control extracellular TG2. In this study, we report a comprehensive and unbiased analysis of the membrane interactome of TG2 in kidneys subjected to unilateral ureteric obstruction (UUO), a murine model of chronic kidney disease. 12 TG2-null and WT inbred C57BL/6J mice were subjected to UUO of the left kidney or a sham operation, and harvested at 21 days post-surgery. UUO kidneys were positive to alpha-SMA and displayed a significantly higher level of active TGF-beta1. To identify TG2-associated proteins, we combined TG2 immunoprecipitation from whole kidney membrane preparations of WT and TG2-null kidneys (employed as background controls) with quantitative proteomics by SWATH-MS. To avoid bias from individual donors and to achieve generalizable results, we performed 5 independent experiments, each based on a lysate of 2 kidneys, and employed only male mice. Differences between WT and TG2-null precipitated proteins were established by a paired sample z-test. Ribosomal, nuclear, and mitochondrial proteins were subtracted from the protein list. 205 membrane-associated proteins specifically interacting with TG2 were identified ($p \leq 0.05$, $n \geq 4$), of which 95 were specifically expressed in the UUO model. Gene Ontology (GO) terms overrepresented in TG2 partners ($p \leq 0.05$) compared to references proteomes revealed a predominance of cell adhesion, actin dynamics, redox regulation, and a considerable number of endosome-related proteins. Protein interaction network analysis among the TG2 membrane partners were investigated using STRING 9.1 (confidence

0.35). A subnetwork of proteins (Clathrin, Adaptor protein 2, Dynactin, and Sorting Nexin proteins) responsible for endosomal transport was associated with TG2 and formed significant "hubs" post UUO. Furthermore, the heparan sulphate (HS) proteoglycans syndecan-4 and perlecan were specifically associated with TG2 in the UUO model. Our study shows that UUO induces association of TG2 with endosomal trafficking proteins suggesting their involvement in TG2 trafficking during the progression of fibrosis.

Use of polyamine to synthesize flurorescent carbon quantum dots as bactericide and transfection reagents

Han-Jia Lin, Yu-Jia Li and Chih-Ching Huang

Department of Bioscience and Biotechnology, National Taiwan Ocean University, Keelung, Taiwan

Abstract: We developed two strategies to prepare fluorescent carbon quantum dots from natural polyamines. In the first work, the fluorescent carbon quantum dots (CQDs) were synthesized by pyrolysis of ammonium citrate in the solid state and then modified with spermidine by a simple heat treatment at different temperatures. We observed the spermidine-CQDs possess positive surface charge and have the ability to inhibit bacterial growth of many species such as *Escherichia coli, Staphylococcus aureus* and *Proteusbacillus vulgaris. In vitro* and in vivo studies also revealed that the antibacterial activity of our Spd–CQDs is comparable or even better than other commercial antimicrobial agents. In the second work, CQD was directly synthesized by pyrolysis of polyamines at different temperatures. Such kind of CQD also possess positive surface charge and low cytotoxicity. Further, we found polyamine CQD was able to bind DNA and facilitate DNA transfection into mammalian cells. This study showed that using natural polyamines as ingredients to synthesize CQD brings up new materials with a wide range of properties for bioimaging, bactericide and transfection applications

Understanding the role of the hypusine-containing protein eIF5A in translation

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The eukaryotic translation factor 5A (eIF5A) undergoes hypusination, a unique and essential posttranslational modification in which the polyamine spermidine is required. Although eIF5A has been extensively involved in several cellular processes and pathologies, almost all of these functional correlations can be seen as a result of its loss of function in translation. The role of eIF5A in translation was strengthened by the genetic and functional interaction between eIF5A and the eukaryotic elongation factor 2 (eEF2). To further understand this relationship, we have investigated the influence of these factors on their ribosome binding. The data obtained revealed that both factors are not able to bind to the same ribosome at the same time and they can replace one another in the pre-assembled ribosome 80S, suggesting an anti-cooperative ribosome binding effect. Moreover, the addition of eEF2 decreases the affinity of eIF5A to the 80S ribosome. Additionally, conditional eIF5A yeast mutants display reduced binding affinity to the ribosome, and this is probably the cause of their defects in growth and protein synthesis. Finally, to further investigate the cellular function of eIF5A, we have performed high-throughput genetic interaction studies using the Synthetic Genetic Array (SGA) analysis. The results obtained so far revealed interesting synthetic genetic interactions, e.g. with genes encoding proteins involved in cell cycle and cytoskeleton organization, which are in agreement with a role for eIF5A in the translation of a specific mRNA subset.

Pathways for Synthesis of Polyamines and Their Roles in Conceptus Development and Pregnancy Recognition Signaling in Sheep

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Embryonic mortality, a major constraint to reproductive performance in mammals, occurs during the peri-implantation period of pregnancy in ungulates when conceptuses undergo morphological transitions from spherical to tubular and filamentous forms prior to implantation. Secretions from uterine epithelia and nutrients transported into the uterine lumen include arginine, a nutritionally essential amino acid for conceptus survival and development. Arginine increases 13-fold in the ovine uterine lumen between Days 10 and 15 of pregnancy and is metabolized to nitric oxide (NO) via NO synthase (NOS3) or polyamines via ornithine decarboxylase (ODC1). Morpholino antisense oligonucleotides (MAO) knockdown of translation of ODC1 mRNA in ovine conceptus trophectoderm resulted in half of the conceptuses to be morphologically and functionally normal and one-half being abnormal. Normal conceptuses increased expression of arginine decarboxylase and agmatinase to compensate for loss of ODC1 for synthesis of polyamines. We also determined effects of arginine mediated by NOS and polyamines on ovine trophectoderm cells (oTr1). Arginine (0.2 mM) increased ($P < 0.05$) proliferation, production of interferon tau (IFNT) and cellular protein. IFNT is the pregnancy recognition signal in ruminants. Inhibitors of NOS3 (L-NAME), ODC1 (DFMO) or L-NAME + DFMO decreased ($P < 0.05$) cell proliferation; however, arginine alone increased cell proliferation 1.5-fold ($P < 0.05$) over control medium suggesting that arginine is a growth factor. Putrescine and NO stimulated proliferation via TSC2-MTOR signaling, but only putrescine increased production of IFNT by oTr1 cells. Thus, arginine-derived polyamines stimulate proliferation and IFNT production by oTr1 cells via the TSC2-MTOR signaling pathways to enhance embryonic growth, development and survival.

Role of spermine oxidase in modulation of glutamate receptors and transporters during excitotoxic stress

Pietropaoli S[1], Leonetti A[1], Maura G[2], Marcoli M[2], Cervetto C[2], D'Amelio M[3], Berretta N[3], Mariottini P[1], Cervelli M[1]

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this study a Dach-SMO mouse genetic model overexpressing Spermine oxidase (SMO) specifically in neocortex, provides novel evidences of the complex and critical functions carried out by SMO and Spermine (Spm) in mammalian brain. SMO is an important enzyme involved in PAs metabolism; it oxidases Spm producing Spermidine (Spd), hydrogen peroxide and 3-aminopropanal (3-AP). Physiological and molecular analyses, performed in normal and excitotoxic conditions, showed an increased sensitivity of this transgenic model to epileptic stimuli caused by a complex intercellular pathway triggered by SMO overexpression and its reaction products in neocortical neurons. The production of H_2O_2 and 3-AP, derived from Spm oxidation, together with direct effects of Spm on AMPA and KA receptors, are synergistically involved in ROS increase and ultimately in neuronal degeneration and death. SMO enzyme can be considered one of the most important H_2O_2 producers in the brain and the transgenic Dach-SMO mice represent a useful genetic model for studying brain pathologies such as epilepsy, Alzheimer's disease and other forms of dementia. Both neurons and glial cells resulted to be involved in this increased sensitivity model showing different levels of expression and activity in receptors and transporters implicated in glutamate response in synaptic transmission.

Proline oxidase–adipose triglyceride lipase pathway restrains adipose cell death and tissue inflammation

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Increased visceral adiposity during ageing is related to a severe inflammatory state. Expanded visceral adipose tissue (AT) has reduced blood vessels density leading to hypoxia and limited nutrient delivery to resident adipocytes. The lack of an adaptive response of adipocytes to such hostile microenvironment is causative of death and production of inflammatory cytokines. We show that adipocyte adaptation to hypovascularization involves a mitochondrial production of ROS via proline oxidase (POX), which in turn induces the FoxO1-dependent transcription of Adipose Triglyceride Lipase (ATGL) with consequent liberation of fatty acids (FAs) from stored triglycerides. To confirm this, POX lacking adipocytes show abortive response to nutrient shortage displaying a restrained up-regulation of FoxO1/ATGL axis. Triglycerides-derived FAs participate in signal transduction promoting the expression of genes related to mitochondrial oxidative metabolism thus setting a metabolic switch towards lipid utilization and maintenance of adequate ATP levels. Our data provide compelling evidence for a previously unrecognized crosstalk between amino acid and lipid catabolism, with the ROS produced by POX functioning as the upstream signalling molecules impinging the ATGL-mediated expression of oxidative genes thus limiting energetic failure.

Impaired insulin-/IGF1-signaling extends life span by promoting mitochondrial L-proline catabolism

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In mammals, altered insulin and insulin-like growth-factor 1 signaling (IIS) are linked to several chronic diseases, including type 2 diabetes, cancer and neurodegeneration, whereas in the well-characterized model organism *C. elegans*, reduced IIS due to impaired expression of *daf-2*, the orthologue of the mammalian insulin-/IGF-1-receptors, extends life expectancy by more than two-fold. Here we show that acute *daf-2* impairment reduces glucose availability in *C. elegans* which causes a transient energy deficit and a compensatory induction of mitochondrial non-glucose metabolism to promote stress resistance and to increase longevity by generation of a transient production of reactive oxygen species (ROS). By transcriptome analyses of three models of IIS, namely *daf-2*-impaired *C. elegans*, as well as mammalian fibroblasts either homozygously deficient for the insulin receptor substrate 1 (IRS1) or heterozygously deficient for the insulin receptor (IR±), we identified mitochondrial proline catabolism to be uniformly upregulated in these models. Impairment of proline catabolism by *knock-down* of *B0513.5*, the *C. elegans* orthologue of proline dehydrogenase, in nematodes abolishes induction of mitochondrial metabolism as well as ROS production and impairs the life span extending capacity of IIS. In addition, supplementation of proline as well as overexpression of undisclosed enzymes that control proline metabolism extends the life span of *C. elegans*. Together, consistent with the concept of mitochondrial hormesis (mitohormesis), these findings indicate that impaired IIS reduces glucose metabolism and induces proline catabolism in a compensatory manner to induce a transient ROS signal which causes an adaptive response and culminates in increased stress resistance and extended life span.

Proline oxidation plays a sensory–regulatory role during the transition to quiescence in nutrient-starved phytoplankton

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In the context of biological oceanography, we investigate the responses of phytoplankton towards changing environmental parameters to understand the biogeochemical consequences of climate change. It is a common knowledge that nutrient-deprived phytoplankton stop growing and 'wait' for the environmental conditions to become more favorable again. However, little is known about the involved molecular processes that trigger and coordinate this behaviour. We have used microarrays to assess the gene expression of the cosmopolitan microalgae *Emiliania huxleyi* in response to starvation in Nitrogen and Phosphorus. Under nutrient starvation, *Emiliania* shuts down the cellular growth apparatus as well the primary plastid functions, i.e. photosynthesis and nitrate assimilation. The cells apparently readjust their lipid metabolism and mitochondrial traffic to maintain efficient ATP Synthesis at reduced cellular carbon throughput. In both starvation scenarios, we observed strong indications for increased amino acid oxidation, as well as prominent induction of polyamine biosynthetic pathways and proline oxidase. These interacting pathways appear to constitute a sensory–regulatory system that monitors and backs up cellular budgets of Nitrogen. It can be further concluded that the molecular decision processes that regulate cell growth versus quiescence or senescence are highly conserved across wide parts of the eukaryotic 'tree of life'.

Structural and conformational preferences of antifreeze glycopeptides

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Biological antifreezes are a divergent group of proteins and peptides found in arctic and antarctic fish, as well as insects, plants and amphibians. These unique cryoprotectants inhibit the growth of ice, which enables the survival of a variety of organisms in extreme climate conditions, where sub-zero temperatures are encountered on a daily basis. Following this strategy, several fish species produce antifreeze glycopepties (AFGPs), which allows them to endure the harshness of polar and subpolar oceans. Typically, AFGPs are composed of 4–55 tripeptide units (Ala-Ala-Thr), in which the secondary hydroxyl group of threonine is glycosylated with β-D-galactosyl-(1,3)-α-D-*N*-acetylgalactosamine. Despite their discovery in the 1960s, the adsorption mechanism of AFGPs, which underlies their ability to hinder ice growth, is not fully defined. Recently, the growing need for non-toxic and highly effective cryoprotectants for the medical, pharmaceutical, agricultural and food industries has spurred the research in the field of antifreeze glycopeptides. Evidence suggests that the conformational and structural preferences of those molecules may play a pivotal role in their antifreeze activity. In our research, we utilize the nuclear magnetic resonance spectroscopy, as well as computational methods (molecular dynamics, ab initio calculations) in order to thoroughly study those preferences. The results will allow us to create a reliable framework for prediction of antifreeze glycopeptide structure.

Proteomic study to develop biomarkers related to MRI findings of myxofibrosarcoma

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Backgrounds: Myxofibrosarcoma (MFS) is a rare malignancy with a relatively high prevalence in the elderly. MFS is characterized by high invasive activity and high local recurrence rate after surgery. Clinically, high invasive activity of MFS is evaluated by MRI. The surgical operation is the only curative treatments for MFS. However, recurrence after surgery was observed in up to 50–60 % cases, and there is no effective treatment for the recurrent cases. Elucidation of the proteomic backgrounds of the extremely high invasive activity of MFS cells has been required to improve clinical outcomes of MFS.
Methods: This study included 11 MFS cases; 6 cases had invasion on MRI imaging, and 5 did not. The frozen samples of these MFS cases were subjected to two-dimensional difference gel electrophoresis (2D-DIGE), and the proteins associated with invasion were identified by LC–MS/MS. The associations of their expression levels with invasion were confirmed by immunohistochemistry.
Results: The protein expression profiles consisting of 3,453 protein spots were created by 2D-DIGE. We found that the expression levels of 47 unique proteins were associated with invasion. These included cell motility regulators, cytoskeleton organizers, and cell cycle regulators. Literature mining revealed that they were reported in physical events such as the development, and tumor invasion in the other malignancies but not in MFS until our study. Immunohistochemical validation revealed the associations of several proteins with invasion of MFS, suggesting their possible clinical applications.
Conclusion: The proteins for invasion in MFS were identified by proteomics. Further elucidation of the expression of these proteins will lead to novel treatment strategies and improve the prognosis of MFS.

Integrated omics study and meta-analysis for sarcomas; prognostic biomarker candidate, promyelocytic leukemia, in gastrointestinal stromal tumor

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Biomarkers for novel therapeutic strategy have been required in sarcomas. The samples for biomarker study such as frozen tissues are limited in sarcomas, because of their low incidence. To approach to this issue, we employed integrated omics study and meta-analysis for biomarker study. We examined gastrointestinal stromal tumor (GIST), which is sarcomas in gastrointestinal tract. GISTs of the small intestine (I-GISTs) generally have poorer prognosis than those of the stomach (S-GISTs), and the proteins responsible for the malignancy in I-GISTs may be potential prognostic biomarkers. We generated protein expression profiles for 4 cases each of surgically resected I-GISTs and S-GISTs by mass spectrometry. For proteins with differential expressions, meta-analysis of mRNA expression was achieved in 9 I-GISTs and 23 S-GISTs. We found that promyelocytic leukemia (*PML*), a tumor suppressor gene, was significantly down-regulated in I-GISTs at both the protein and mRNA levels. Immunohistochemistry of 254 additional cases showed that PML-negative cases were significantly frequent in the I-GIST group. The 5-year recurrence-free survival rate was significantly lower in the PML-negative than in the PML-positive cases. Multivariate analysis revealed that down-regulation of PML was an independent unfavorable prognostic factor. Our study indicated the potential utility of PML as a prognostic biomarker in GIST patients. Similar omics study and meta-analysis can be performed for the other sarcomas with rare incidence.

Cold vaporization of tissue with a pico-second infrared laser for protein species extraction

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Protein extraction from tissues is a critical step within proteomics workflows. Since cellular compartments are destroyed during *conventional mechanical homogenization,* diverse enzymes are released, which can degrade and convert other proteins. As a result during mechanical homogenization, the original composition of species is changed in vitro giving a false view on the proteome. With the picosecond infrared laser (PIRL), tissue can be vaporized in seconds by *quantum mechanical homogenization*. In the nearly homogenous tissue-aerosol, the proteins are dissolved. We hypothesize that the protein species composition extracted from tissue by PIRL is more close to the original in vivo composition. For proving this hypothesis, we analyzed and compared the protein species composition yielded by classical protein extraction and by PIRL extraction by electrophoretic and mass spectrometric approaches. SDS-PAGE showed similar but not congruent patterns. The pattern of the two-dimensional electrophoresis showed more clearly the significant differences, which can be interpreted as increased proteolysis under classical conditions. In addition, the overall yield of protein species in the PIRL extract is larger. In summary, the data demonstrate that under PIRL extraction, less enzymatic degradation occurs compared to classical extraction. It can be assumed that by PIRL extraction of proteins from tissues, a more reliable view to the original in vivo composition of protein species is possible.

Proteomics I

Proteomic profiling of muscular dystrophy

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X-linked muscular dystrophy is characterized by primary abnormalities in the dystrophin gene and presents the most frequently inherited neuromuscular disease of childhood. An established animal model of Duchenne muscular dystrophy is the mdx mouse. An interesting pathobiochemical feature of the dystrophic mouse is the greatly varying severity of dystrophic changes in different subtypes of muscle despite the same genetic abnormality. Extraocular fibres and certain distal muscle groups are only mildly affected, while the mdx diaphragm and the aged heart exhibit extensive necrosis and fibrosis. In order to better understand the divergent secondary alterations in different muscle types, comparative mass spectrometry-based proteomics was used to determine global changes in soleus, extensor digitorum longus, flexor digitorum brevis, interosseus, extraocular, diaphragm and heart muscle from the mdx mouse model of dystrophinopathy. Individual types of contractile tissues that lack the membrane cytoskeletal protein dystrophin showed considerable differences in their expression patterns of key muscle proteins. Mildly dystrophic fibres exhibited only minor changes. In contrast, the highly necrotic mdx diaphragm was shown to have lower levels of key calcium-regulatory proteins, an increased concentration of collagen and related components of the extracellular matrix and disturbed amounts of proteins involved in the excitation–contraction–relaxation cycle, muscle energy metabolism and the cellular stress response. Findings from proteomic screening studies were verified by immunoblotting surveys and immunofluorescence microscopy. Newly identified proteins with a greatly altered concentration in dystrophic muscles may be useful biomarker candidates for the improved design of predictive, diagnostic, prognostic and therapy monitoring approaches.

Computational analysis of promiscuous drug protein target spectra

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Continuous developments in the field of chemical proteomics provide the scientific community with unprecedented tools to understand drugs. Still, some compounds such as tyrosine kinase inhibitors interact with a rather large number of proteins thus making the interpretation of their target spectra an exciting computational challenge (Rix et al., PLoS One, 2013). In a recent paper (Müllner et al., Mol Syst Biol, 2015), we introduced a new computational method to identify targets likely to induce drug response from chemical proteomic data. This was demonstrated with midostaurin, a kinase inhibitor that we identified as specifically toxic to basal-like cells representing triple-negative breast cancer cells.

Simultaneous determination of electrophoretic and dielectrophoretic mobilities of human red blood cells

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Abstract: Electrophoresis and dielectrophoresis of cells can reveal many distinct cellular properties but are often conducted separately. Herein a simultaneous strategy was proposed, and a simple method was established by making cells migrate through a cross channel under a micro video for real-time observation. The experiment can be performed within 0.044–1 s. In combination with digital calculation based on electromagnetic theory, the method was validated to be applicable to the determination of electrophoretic and dielectrophoretic mobilities, μ_{EP} and μ_{DEP}, of human blood erythrocytes, giving $\mu_{EP} = -(0.87 \pm 0.16) \times 10^{-4}$ cm^2 V^{-1} s^{-1} and $\mu_{DEP} = -(4.5 \pm 1.3) \times 10^{-8}$ cm^4 V^{-2} s^{-1} by vector decomposition, or $\mu_{EP} = -(0.89 \pm 0.14) \times 10^{-4}$ cm^2 V^{-1} s^{-1} and $\mu_{DEP} = -(4.6 \pm 1.2) \times 10^{-8}$ cm^4 V^{-2} s^{-1} by least squares fitting, all agreeing with the published data. Hydrodynamic and electroosmotic flows were eliminated for better measurement. It was found that the location of cells had a serious impact on the measurement precision, and the upstream of the cross channel along the electric field was chosen for precise

measurement. The method is also extendable to the study of other cells and particles.

Cryo-slicing BN-MS: a novel technology for high-resolution complexome profiling

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Abstract: Blue-native gel electrophoresis (BN) is a powerful method for protein separation. Combined with liquid chromatography–tandem mass spectrometry (LC–MS/MS), it enables large-scale identification of protein complexes and their subunits. Current BN–MS approaches, however, are limited in size resolution, comprehensiveness, and quantification. Here we present a new methodology combining defined sub-millimeter slicing of BN gels by a cryo-microtome with high-performance LC–MS/MS and label-free quantification of protein amounts. Application of this cryo-slicing BN–MS approach (csBN-MS) to mitochondria from rat brain demonstrated a high degree of comprehensiveness, accuracy, and size resolution. The technique provided abundance-mass profiles for 774 mitochondrial proteins including all canonical subunits of the oxidative respiratory chain assembled into 13 distinct (super-)complexes. Moreover, the data revealed COX7R as a constitutive subunit of distinct super-complexes and identified novel assemblies of VDACs/porins and TOM proteins. Together, csBN–MS enables quantitative profiling of complexomes with resolution close to the limits of native gel electrophoresis.

Potential antioxidant peptides released after simulated gastro-intestinal digestion of Stracchino soft cheese

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As life expectation increases, age-related pathologies become more frequent, and thus the search for new compounds, with natural origin, possessing antioxidant and anti-inflammatory is expanding. Numerous evidences have demonstrated that milk proteins are a source of bioactive encrypted peptides. In order to exert their activity, these peptides should survive during digestion; we carried out an in vitro multistep gastro-intestinal digestion of *Stracchino* soft cheese. Considering the importance of oxidative stress conditions in many diseases, the obtained peptides were fractionated by preparative liquid chromatography and subsequently evaluated their antioxidant potential. The effect of each fraction (250–10 μg/ml) was evaluated in inflammatory and oxidative stress conditions in intestinal epithelial cells (IEC-6). The fractions added to IEC-6 cells, 1 h before and simultaneously to pro-inflammatory *stimulus* (Lipopolysaccharide from *E. coli* 10 μg/ml + Interferon γ 10 U/ml) for 24 h, significantly inhibited the release of reactive oxygen species (ROS), evaluated by flow cytometry. Among tested fractions, during inflammatory conditions, fraction number two exerted the highest effect in inhibiting ROS release in IEC-6. Fraction number two showed the highest antioxidant potential also when cells were treated with hydrogen peroxide (1 mM) as pro-oxidant agent. Nrf2 is an important regulator of cellular resistance to oxidants and, among tested fractions, fraction number two showed the stronger effect in activating Nrf2. Antiproliferative assay, evaluated by MTT test, indicated that the tested fractions did not affect IEC-6 proliferation. Peptide sequences of fraction two were determined by LC–MS/MS, identified peptides belong to beta casein, in particular most intense peak possessed sequence EAMAPK.

Thiol-based redox regulation of aldehyde dehydrogenase

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The sulfhydryl group of cysteine in proteins is very susceptible to oxidative modifications, including S-nitrosylation, S-thiolation, and S-sulfhydration. The first reports describing the process of S-sulfhydration came out in 2009 and since that time many proteins have been observed to be modified through this process. Aldehyde dehydrogenase (ALDH) has in its catalytic center three residues of cysteine. The aim of the present study was to examine whether the activity of aldehyde dehydrogenase can be regulated by the S-sulfhydration process. The studies were performed using yeast ALDH. Samples of the enzyme were incubated with various S-sulfhydrating species: Na_2S, GSSH, diallyl trisulfide (DATS), and K_2S_x. The activity of the enzyme was measured immediately after incubation by recording the increase in an absorbance at 340 nm derived from NADH. All tested compounds except for Na_2S alone produced an inhibitory effect on the activity of ALDH. Moreover, dithiothreitolwhen added to the incubation mixture restored the inhibited activity of the yeast enzyme. Similar effects of S-sulfhydrating species on the ALDH activity were observed in the experiment performed on the rat liver homogenate. The obtained results clearly demonstrated that aldehyde dehydrogenase activity could be regulated by S-sulfhydration, and in this case, the addition of a sulfane sulfur atom to the key Cys residue led to the inhibition of the enzyme. The results also suggest that not H_2S but polysulfides (DATS, K_2S_x) or hydropersulfides (GSSH) were the oxidizing species responsible for S-sulfhydration. This is the first study reporting the regulation of ALDH activity via S-sulfhydration.
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Characterisation of peroxynitrous acid mediated damage to extracellular proteins and its consequences

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Background: The extracellular matrix (ECM) is critical to the function and mechanical properties of tissues. Matrix components, including laminin, perlecan, collagen and fibronectin both maintain

tissue structure, and interact with growth factors and proteins to regulate cell adhesion, proliferation and migration. These interactions are perturbed in some pathologies, including cardiovascular disease (atherosclerosis), with this has been postulated to via the formation of oxidants including peroxynitrous acid (ONOOH) by activated white cells.

Hypothesis: The exposure of ECM proteins to oxidants results in changes to structure, function and cell adhesion.

Results: We have demonstrated co-localization of 3-nitrotyrosine (a product of tyrosine damage by ONOOH) and laminin in human atherosclerotic lesions. ONOOH-induced damage to isolated purified murine laminin-111 and ECM extracts was examined. Exposure of laminin-111 to ONOOH resulted in dose-dependent loss of protein tyrosine and tryptophan residues, and formation of 3-nitrotyrosine, 6-nitrotryptophan and the cross-linked material di-tyrosine, as well as formation of aggregates and fragments. This damage was detected with molar ratios of target and oxidant as low as 1:1. This damage was modulated by bicarbonate, a known modifier of ONOOH reactions. Endothelial cell adhesion to laminin-111 and ECM mixtures, exposed to >10 μM ONOOH, was decreased by up to 25 % compared to controls.

Conclusions: These data indicate that laminin is readily modified by ONOOH, with this resulting in structural and functional changes. These modifications, and the resulting compromised cell–matrix interactions, may contribute to endothelial cell dysfunction, a weakening of ECM structure, and an increased propensity of atherosclerotic lesions to rupture.

Redox reactions of thiols in proteins: selective hydrogen transfer mechanisms and formation of D-amino acids

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Protein oxidation is a hallmark of oxidative stress and aging. It is also of increasing concern to the biotechnology industry, where the development of efficacious and safe protein therapeutics requires potent molecules with no or minimal immunogenicity. Frequently, protein oxidation is the result of free radical reactions, where these radicals are generated by enzymes, metabolic processes, environmental stress, or, in the case of protein formulations, the decomposition of formulation constituents. The pathways of protein oxidation by free radical reactions are complex, and often initial target sites on proteins are not the sites where the ultimate damage accrues, i.e., the result of radical transfer processes along the protein. In proteins, thiols represent primary targets of oxidation, generating one- and two-electron oxidation intermediates and products. Specifically, the one-electron oxidation generates thiyl radicals ($RS^{\cdot}$), which are at the origin of a manifold of radical reactions in proteins, and may trigger the ultimate oxidation of neighboring amino acids. We will present detailed mechanistic information on selective radical reactions of amino acids, peptides, and proteins induced by thiyl radicals, the contribution of kinetic and thermodynamic parameters on the selectivity of these reactions, and the effects of sequence, structure, and the environment. Protein examples will include GAPDH, various antibodies, and SERCA1a. Specific emphasis will be placed on the radical-induced formation of D-amino acids in selected sequences, and on the potential consequences for protein structure, activity, and immunogenicity.

4-Hydroxynonenal in Parkinson's disease

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Parkinson's disease (PD) is the second most abundant neurodegenerative movement disorder with a critical role of oxidative stress (OS) in its etiopathogenesis. Clinical symptoms of the disease include rigidity, resting tremor, bradykinesia and postural instability. Pathologically PD is characterized by loss of dopaminergic neurons from substantia nigra with the presence of protein inclusions called Lewy bodies. Oxidative stress and mitochondrial respiratory failure have been implicated as the major contributors to nigral cell death. The brain is rich in phospholipids and polyunsaturated free fatty acids (PUFAs), both of which are highly susceptible to oxidants producing reactive oxygen species (ROS). ROS induce lipid per oxidation generating highly reactive aldehydes witch act as 'toxic second messengers' increasing the injurious of free radicals. One of the most abundant and highly toxic peroxidation products is 4-hydroxynonenal (HNE). HNE is a potent modulator of different cell processes such as oxidative stress signaling, cell proliferation, transformation and cell death. This study aimed at monitoring the HNE concentration changes in PD patients and sex–age-matched healthy control subjects. HNE levels were higher in patients than in the control group. We found a statistically significant increment in HNE of patients with Parkinson's disease on therapy compared to untreated patients. This opens a question whether methyldopa itself generates free radicals. That will be subject of our future investigations.

Serum concentrations of 3-hydroxyisobutyrate and 3-hydroxy-3-methylbutyrate as BCAA metabolites in endurance exercise

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Abstract: In endurance exercise, branched-chain amino acids (BCAA) are catabolized into acetyl-CoA and succinyl-CoA in the mitochondrion of human skeletal muscle for energy production. Therefore, BCAA supplementation has been paid attention for exercise performance. Furthermore, BCAA has also been reported to attenuate exercise-induced muscle damage and pain. In valine catabolism, a small molecule 3-hydroxyisobutyrate (3HIB) is produced and is released from the mitochondrion to extracellular circulating fluid. In addition, approximately 5 % of alpha-ketoisocaproate that is an intermediate of leucine is transferred to the cytoplasm and is metabolized to 3-hydroxy-3-methylbutyrate (3HMB) that has some positive effects on muscle protein synthesis and muscle damage inhibition. Therefore, it is a possibility that 3HIB and 3HMB as valine and leucine metabolites would be biomarkers of the BCAA catabolism in human skeletal muscle. The present study evaluated a hypothesis that blood levels of 3HIB and 3HMB might be increased by endurance exercise with BCAA supplementation. Serum 3HIB and 3HMB concentrations increased significantly immediately after a full-marathon race compared to those before the race, and then, recovered to the respective basal levels after 1 day. Furthermore, BCAA

supplementation increased significantly both 3HIB and 3HMB concentrations in serum immediately after the race and after the 1st day. In the present study, the increased catabolism of BCAAs by endurance exercise and BCAA supplementation could be confirmed in human by measuring of serum levels of 3HIB and 3HMB as BCAA metabolites.

Nitrogen sources affect patterns of amino acid utilization in pig small-intestinal bacteria

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This study was conducted to determine whether patterns of amino acid (AA) utilization in small-intestinal bacteria are affected by nitrogen sources. Freshly luminal contents from the jejunum or ileum of 20 kg Duroc × Landrace × Yorkshire pigs were processed and inoculated into semi-defined media containing 5.5 g/L casein or soy protein hydrolysates as major nitrogen sources. After 24 h of incubation, samples were taken and subsequent subcultures were carried out twice (24 h for each time). Free AA and peptide-bound AA in the cultures were determined to calculate the rates of AA utilization. Results showed that free AA comprised >97 % total AA in casein or soy protein hydrolysates. Concentrations of arginine, histidine, leucine, lysine, methionine, phenylalanine and tryptophan (expressed as nmol/mL in media) were higher in casein hydrolysates than in soy protein hydrolysates, while the concentration of glycine (expressed as nmol/mL in media) was higher in soy protein hydrolysates than in casein hydrolysates. When casein hydrolysates were used as the nitrogen source, bacteria from both the jejunum and ileum utilized more arginine, lysine and tryptophan. Similar observations were obtained after two times of subculture. However, the analysis of peptide-bound (mainly bacterial protein) AA showed that the amounts of arginine, leucine and lysine in the protein of jejunal and ileal bacteria (expressed as nmol/mg bacteria) were higher when cultured with soy protein hydrolysates than with casein protein hydrolysates. Interestingly, the amount of glycine was higher in the protein of jejunal and ileal bacteria (expressed as nmol/mg bacteria) when cultured with casein protein hydrolysates than with soy protein hydrolysates. These results indicate different patterns of AA utilization in pig small-intestinal bacteria incubated with casein hydrolysates versus soy protein hydrolysates as the nitrogen source. Our findings will also help to develop new strategies for optimizing the utilization of dietary AA by pigs fed different sources of protein.

Effects of low dietary protein on the metabolites and microbial communities in the caecum of growing pigs

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Abstract: The experiments were conducted to identify the fermentable metabolites including ammonia N, microbial protein, biogenic amines and short chain fatty acids (SCFAs) and population in the caecum of growing pigs fed with corn–soybean meal diets containing 12 % (LP) and 18 % (NP) protein levels. A total of 12 growing pigs (body weight 30.0 ± 1.0 kg) were randomly assigned to the two groups. Each pig was housed individually. The experiment lasted 30 days until slaughter and fresh digesta and mucosa of caecum were sampled. Results showed that compared with the NP group, the concentrations of ammonia (N) ($P < 0.05$), microbial protein ($P < 0.05$) and acetate ($P < 0.05$) decreased significantly, while putrescine and the ratio of butyrate to total SCFAs increased ($P < 0.05$) significantly in the LP group. DGGE analysis showed that there was no difference on microbial diversity of both digesta and mucosa ($P > 0.05$). The similarity of caecal digesta microbiota between LP and NP groups was only 37 %, while those from caecal mucosa samples were higher than 89.8 %. Meanwhile, the amounts of *Clostridium* cluster IV ($P < 0.05$) were significantly higher in caecal digesta of the LP group, but no significant difference of microbial communities ($P > 0.05$) was observed in mucosa. In conclusion, the caecal fermentable metabolites of protein decreased and microbial communities in digesta were influenced in pigs fed with LP diet, while microbial population were unaffected in mucosa . The low-protein diet showed a positive effect on the health of growing pigs.
Keywords: Low-protein diet, Caecum, Metabolites, Microbial communities.

Gut microbiota and metabolites in response to low-protein diets in growing pigs

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Abstract: Feeding low-protein diet is a sufficient strategy due to the deficiency of feeding protein resources and low ratio of nitrogen utilization in pigs. The microbiota is a vital part of the gut and influences the metabolism of dietary nutrients. However, study on the effect of low-protein diets on gut microbial communities and microbial metabolites in growing pigs is limited. In this study, eighteen growing pigs (9.58 ± 0.60 kg) were allocated to three diets (14, 17 and 20 % crude protein in the diet supplemented with lysine, methionine, threonine and tryptophan to meet the NRC requirement) in a completely randomized design to investigate the effect of reduction of dietary protein content on the metabolites and microbial communities in colon contents. After a 45-day experimental period, the bacterial composition in colon contents was determined by pyrosequencing analysis of the V3–V4 region of bacterial 16S rRNA. The microbial metabolites, such as ammonia nitrogen (NH3-N), volatile fatty acids (VFAs) and biogenic amines were, respectively, measured by indophenol method, gas chromatography and high-performance liquid chromatography. Results showed that a total of 13 phyla were distributed amongst all pigs on three diets. The bacterial communities were dominated by phyla of *Firmicutes*, *Spirochaeta*, *Tenericutes*, *Bacteroidetes* and *Actinobacteria* regardless of dietary protein content. At the genus level, the sequences were assigned to 153 different genera. Reduction of crude protein content linearly increased the relative abundance of *Rhizobium* ($P = 0.002$),

Enhydrobacter ($P = 0.024$), *Micromonospora* ($P = 0.016$) and *Klebsiella* ($P = 0.016$). A significance of quadratic effect of decreasing the dietary protein content was detected for *Marvinbryantia* ($P = 0.002$), *Clostridium* ($P = 0.014$) and *Weissella* ($P = 0.016$). Overall, at the species level, among the total of 781 16S operational taxonomic units (OTUs) detected in this study, 14 OTUs were significantly affected by the decrease of dietary crude protein content. For example, the relative abundance of OTU49 (Family: *Ruminococcaceae*) ($P = 0.012$), OTU124 (*Eubacterium coprostanoligenes*) ($P = 0.019$) and OTU574 (*Clostridium botulinum*) ($P = 0.049$) were linearly decreased, while OTU564 (*Moraxella osloensis*) ($P = 0.024$), OTU622 (*Agrobacterium tumefaciens*) ($P = 0.002$) and OTU763 (*Klebsiella variicola*) ($P = 0.016$) were linearly increased with the decreased dietary protein content. Reduction of protein content of the diets linearly decreased the concentration of putrescine ($P = 0.009$), spermidine ($P = 0.012$) and spermine ($P = 0.004$) in colon contents. The trend towards significance effect of the crude protein content in diets on the concentration of isobutyrate ($P = 0.063$), total branched-chain VFAs ($P = 0.073$), NH3-N ($P = 0.075$) were detected. Further, our results showed that the concentrations of metabolites were significantly related to some certain genera and species of *Bacteroidetes* and *Firmicutes* in the colon contents. Our findings revealed a significant shift in the structure of microbiota and metabolites in response to the decrease of dietary crude protein in growing pigs. It indicates that gut microbiota may exert an important effect on nitrogen utilization in growing pigs which needs further study.

Keywords: Low-protein diets, Microbial communities, Microbial metabolites, Colon, Growing pigs.

High-protein diet alters colonic microbiota composition and metabolism and results in epithelial transcriptomic change in rats

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Abstract: Increase of fecal hazardous compounds and decrease of butyrate-producing bacteria have been implicated to be detrimental to colonic health during weight loss by high-protein low-carbohydrate diet (HPLC) in human. The doubt remains that whether the change of luminal microbiota and metabolites were paralleled with the alteration of colonic function in vivo. Hence we investigated the influence of HPLC diet on microbiota, microbial metabolism, and colonic gene transcription in rats during 6 weeks. The dynamic alterations of the microbiota were reflected by the significant decrease of fecal acetate, propionate, butyrate, *Akkermansia muciniphila,* and increase of *Escherichia coli* after HPLC feeding. The colonic microbiota was characterized by the increased *Escherichia/Shigella*, *Bacteroides*, *Lactobacillus*, *Enterococcus*, *Streptococcus,* and decreased *Ruminococcus* and *Akkermansia*. Several OTUs, including *E. coli/Shigella flexneri*, *Bacteroides vulgatus*, *Enterococcus faecium* increased, while *Ruminococcus bromii* and OTUs within *Roseburia*, *Faecalibacterium* (*Faecalibacterium prausnitzii*-like) decreased in HPLC group. Increase of cadaverine, spermidine, sulfide and decrease of butyrate indicated distinct microbial fermentation pattern in colon of HPLC rats from control group. Transcriptome analysis identified the upregulation of chemotaxis, TNF-α signal process, antigen presentation, apoptosis, glutathione S-transferase activity and downregulation of innate immune, O-linked glycosylation of mucin, oxidative phosphorylation. Correlation of host transcriptomes, microbial metabolites, and microbiota composition revealed associations between *Escherichia/Shigella* and *Akkermansia*, and differential expression of genes involved in chemotaxis and mucin glycosylation. These findings suggested that HPLC diet altered the microbial community, shifted the metabolic profile, and affected the host response in rats, providing further insights into understanding the diet-microbiota-host axis under HPLC diet.

Keywords: High-protein low carbohydrate, Microbiota, Metabolism, Transcriptome, Colon.

Changes in gut microbiota induced by early antibiotic intervention modifies intestinal luminal amino acid metabolism in piglets

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Abstract: Gut microbiota plays an important role in the metabolism of dietary protein and amino acids in the gastrointestinal tract. In this study, we investigated the influence of the intestinal microbiota change induced by an antibiotic treatment on the intestinal luminal metabolome in piglets. Sixteen piglets were fed diets with (Ant) or without antibiotics (Con) from postnatal day 7 to day 42. Jejunal and ileal contents were obtained for microbiota and metabolome analysis. With the bacterial composition altered, the intestinal luminal metabolome measured by gas chromatography–mass spectrometry (GC–MS) were changed by early antibiotic intervention. The patterns of metabolites by principal component analysis showed a remarkable difference in the jejunal and ileal luminal metabolome between Ant and Con pigs. GC–MS indentified 77 metabolites from the intestinal luminal metabolome and 18 metabolites from the jejunum and 15 metabolites from the ileum showed significant differences in the concentrations between Ant and Con pigs ($P < 0.05$). The 18 differential metabolites in the jejunum were mainly classified into amino acid metabolism by pathway analysis, whereas the 15 differential metabolites in the ileum were mainly assigned into lipid and carbohydrate metabolism. The metabolite set enrichment analysis showed that Protein biosynthesis, Ammonia recycling, Glutathione metabolism and Glycine, serine and threonine metabolism were enriched in the jejunum, and Insulin signaling, Alpha linolenic acid and linoleic acid metabolism, Galactose metabolism, and Glucose–alanine cycle were enriched in the ileum induced by antibiotic intervention. These indicate that intestinal microbiota has different effects on the jejunal and ileal luminal metabolome, which influences amino acid metabolism in the jejunum but lipid and carbohydrate metabolism in the ileum. A comprehensive understanding of intestinal luminal metabolome is critical for clarifying host-gut microbiota interactions.

Key words: Amino acid metabolism, Gut microbiota, Antibiotic, Small intestine, Piglets

Effects of early antibiotic intervention on growth performance and fecal fermentation profile in pigs under different protein level diets

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Abstract: This study aimed to determine the effects of early antibiotic intervention on later growth performance and fecal fermentation end-products of pigs under different CP level diets. Eighteen litters of crossbred newborn piglets were randomly allocated into 2 groups each with 9 litters and fed from postnatal d (PND) 7–42 either a commercial creep feed without any antibiotic (Con) or commercial creep feed + antibiotic (Ant). On day 42, the pigs of Con or Ant groups were further randomly assigned to provide a normal or low level of CP diet on the basis of BW, respectively, which generated 4 groups, each with 5 pen replicates and 9 pigs per pen. At PND 77 and 185, 5 pigs per group were slaughtered for organ weight analysis. Results showed that low-CP diet decreased ($P < 0.05$) ADG compared with normal-CP diet during the whole period, but early antibiotic treatment improved ($P < 0.05$) the ADG under low-CP diet. Similarly, antibiotic treatment increased ($P < 0.05$) apparent digestibility of CP under different CP diets at PND 77, whereas low-CP diets decreased ($P < 0.05$) apparent digestibility of CP and ether extract. At PND 77, early antibiotic treatment increased ($P < 0.05$) the concentrations of total protein, albumin, globulin, AST, glucose, triglyceride, HDLC, LDLC, gastrin, growth hormone, glucagon, and insulin under low-CP diet, but only increased AST, glucose, and gastrin under normal-CP diet. Low-CP diet decreased ($P < 0.05$) AST, urea N, LDLC, gastrin, and insulin concentrations. Meanwhile, the concentrations of glucose, urea N, triglyceride, HDLC, growth hormone, and glucagon also decreased ($P < 0.05$) by low-CP diet at PND 185. Pigs previously fed antibioticreduced cadaverine concentration under low- or normal-CP diet at PND 77, but improved ($P < 0.05$) skatole concentration under low-CP diet. At PND 185, early antibiotic usage improved ($P < 0.05$) putrescine concentration under low-CP diet, whereas decreased ($P < 0.05$) under normal-CP diet. The concentration of cadaverine was increased ($P < 0.05$) by antibiotic usage under normal-CP diet. Furthermore, low-CP diets reduced ($P < 0.05$) fecal methylamine, tryptamine, cadaverine, tyramine, *p*-cresol, indole, and skatole concentrations at PND 77, and also decreased ($P < 0.05$) acetate, propionate, total SCFA, mainly amines, and phenol content at PND 185, whereas increased ($P < 0.05$) *p*-cresol concentrations. These results indicate that low-CP diet decreased ADG, serum biochemical indexes, and hormone levels, whereas early antibiotic treatment increased ADG, serum biochemical indexes, and hormone levels under low-CP diet. Meanwhile, the concentrations of potentially harmful fermentation end-products were improved under normal-CP diet, but early antibiotics have different effects on fermentation end-products under different CP level diets.

Keywords: Antibiotic, Low-CP diet, Growth performance, Fermentation end-products, Pigs.

Effects of a low-protein diet on the bacterial composition and fermentation profile in the colon of weaned piglets

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Abstract: The aim of this study was to assess the effects of a low-protein diet on the bacterial composition and fermentation profile in the colon of piglets. Thirty-six weaned piglets were selected and randomly assigned to different diets: (1) a standard protein diet (SPD), of which the crude protein (CP) was 20 %; (2) a low crude protein diet (LPD), of which the CP was 16 %. Six piglets from each dietary treatment were randomly selected to be slaughtered and sampled on day 10, day 25, and day 45, respectively. Colonic digesta was collected and the bacterial community profiles were analyzed by denaturing gradient gel electrophoresis and selected bacterial groups were quantified by real-time PCR. Concentrations of short chain fatty acids (SCFA), ammonia, and microbial crude protein were determined to evaluate the fermentation profile in the colon. Results show that the LPD did not change the abundance of the total bacteria and the Firmicutes, but decreased the abundance of Clostridium IV and the Clostridium XIVa on day 10, enriched the Bacteroidetes on day 25 and day 45. The *Escherichia* spp. was also found to be elevated on day 45. However, the SHANNON index was unaffected by dietary protein, while the band similarity coefficient between the LPD and SPD group was higher on day 25 and day 45 than that on day 10.The microbial crude protein and the concentration of ammonia were both decreased in the LPD group on day 10 and ammonia tended to be lower on day 25. The LPD did not affect the SCFA profiles but increased the ratio of acetate to short chain fatty acids (acetate/SCFA) on day 45. In conclusion, feeding piglets with a low-protein diet ameliorated the protein fermentation in the colon in the initial period by inhibiting the Clostridium IV and Clostridium XIVa.

Keywords: Low-protein diet, Piglets, Colon, Microbiota, Metabolites

Excessive levels of L-cysteine activate endoplasmic reticulum stress in intestinal porcine epithelial cells

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Elevated plasma cysteine is associated with increased risks for multiple disorders, such as preeclampsia, premature delivery, low birth weight and cardiovascular disease, Alzheimer's disease, and Parkinson's disease. However, the underlying mechanism remains elusive. This study was conducted with an intestinal epithelial cell line (IPEC-1), isolated from the jejunum of a newborn piglets, to test the hypothesis that excessive L-cysteine activates endoplasmic reticulum stress signaling.

IPEC-1 cells were cultured with DMEM/F-12 medium in the absence or presence of L-cysteine (2.5, 5.0, or 10 mmol/L). Cell viability, morphologic alterations, mRNA expression for genes involved in ER stress, protein abundances for GRP78, CHOP, eIF2α, ERK1/2, p38 MAPK, and JNK1/2 were determined. Our results showed that high levels of L-cysteine (5–10 mM) resulted in extensive formation of cytoplasmic vacuoles and reduced cell viability in IPEC-1 cells. Excessive L-cysteine promoted mRNA expression of endoplasmic reticulum (ER) stress-related gene (spliced X-box-binding protein 1 (XBP1s), heat shock 70 kDa protein 5 (HSPA5), and DNA damage-inducible transcript 3 (DDIT3)) ($P < 0.05$). In addition, the protein abundance of DDIT3 and the phosphorylation levels of eukaryotic initiation factor 2α (eIF2α) were significantly up-regulated. These findings suggest that excess L-cysteine-induced cell death is associated with activation of ER stress signaling in intestinal porcine epithelial cells.

Glycine inhibits protein degradation in mouse myoblast cell C2C12

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In higher eukaryotes, skeletal muscle is one of the most important sites for the control of intracellular protein turnover, which is an essential process for maintaining tissue integrity, growth, as well as functionality. Our recent study showed that glycine, a conditionally essential amino acid for young pigs, is critical to support their optimum growth and health. It remains elusive whether glycine modulates protein degradation signaling and contributes to the regulation of protein turnover in muscle cells. In this study, mouse myoblast cells (C2C12) isolated from the thigh muscle of the C3H mouse were cultured in the absence or presence of different concentrations of glycine. Cell proliferation and the mRNA levels of genes involved in protein degradation were determined. The data indicated that 0.25 mmol/L glycine promoted ($P < 0.01$) cell growth, as compared with the absence of glycine. The mRNA levels of muscle-specific ubiquitin ligases (Atrogin-1 and MuRF1) were reduced ($P < 0.01$) by glycine. Furthermore, we found that PI3K/AKT inhibitor LY294002 blocked the downregulation of MuRF1, but had no effect on the mRNA level of Atrogin-1. These results suggest that glycine inhibits protein degradation by reducing the mRNA level of genes involved in protein degradation, thus contributing to increases in net protein synthesis and growth of muscle cells.

L-Leucine depletion sensitizes intestinal epithelial cells to tunicamycin-induced cell death

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Intestinal epithelial cells are constantly exposed to food antigens, xenobiotics, toxicants, and intestinal microorganism, which may affect the biological activity of endoplasmic reticulum (ER). Dysfunction of ER activates unfold protein response and contributes to multiple intestinal disorders, including intestinal bowel diseases. Recent studies indicate that branched-chain amino acid (BCAA) can function as a signaling nutrient in various metabolic pathways. It is unknown whether L-leucine deprivation affects ER stress-associated cell death in enterocytes. To test this hypothesis, intestinal epithelial cells (IEC-6) cultured with complete or leucine-deprived medium were treated with tunicamycin (1 μg/mL) to induce ER stress. Cell viability, quantitative real-time PCR, and Western blot analysis were performed to determined cell death, transcriptional expression, and protein levels of genes involved in ER stress. Our results showed that tunicamycin induced cell death as evidenced by increased mRNA levels of LC3-II/LC3-I, increased phosphorylation levels of c-Jun N-terminal kinase (JNK), and decreased protein abundance of Bcl-2. Importantly, the tunicamycin-induced cell death was markedly enhanced by L-leucine deprivation, as compared with the absence of leucine. Collectively, these results indicate that L-leucine depletion sensitize ER stress-induced cell death in intestinal epithelial cells. Provision of sufficient L-Leucine is likely an effective nutritional strategy to maintain intestinal mucosal barrier function by reducing the death of epithelial cells.

N-Acetylcysteine stimulates protein synthesis in enterocytes independently of glutathione availability

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Abstract: *N*-acetylcysteine (NAC) has been used to treat gastrointestinal diseases. However, the underlying mechanisms are not fully understood. This study tested the hypothesis that NAC enhances enterocyte growth and protein synthesis independently of cellular glutathione (GSH, the reduced form of glutathione). Intestinal porcine epithelial cells (IPEC-1) were cultured for 3 days in Dulbecco's modified Eagle medium containing 0 or 100 μM NAC. To determine the role of GSH on regulation of NAC on cell growth and protein synthesis, additional experiments were conducted using different culture media containing 100 μM GSH, 100 μM GSH ethyl ester (GSHee), or/and a GSH-depletion agent (diethylmaleate, DEM; 10 μM), a GSH-synthesis inhibitor (buthionine sulfoximine, BSO; 20 μM). NAC increased ($P < 0.05$) cell proliferation, GSH concentration, and protein synthesis and inhibited ($P < 0.05$) proteolysis. Interestingly, GSHee enhanced ($P < 0.05$) cell proliferation and GSH concentration without affecting protein synthesis and inhibited ($P < 0.05$) proteolysis. Conversely, BSO or DEM reduced ($P < 0.05$) cell proliferation and GSH concentration without affecting protein synthesis but promoted protein degradation. NAC augmented ($P < 0.05$) the abundances of total mTOR, phosphorylated mTOR, and phosphorylated 70S6 kinase proteins in cells. NAC also increased ($P < 0.05$) mRNA levels for mTOR and p70S6 kinase. These results indicate that NAC upregulates expression and activation of mTOR signaling proteins to stimulate protein synthesis in enterocytes independently of GSH. Our novel findings provide a new mechanism for beneficial actions of NAC in cells.

Keywords: *N*-Acetylcysteine, Intestinal cells, Glutathione, mTOR, Protein synthesis.

Effects of dietary supplementation with α-ketoglutarate on cell signaling and energy metabolism in skeletal muscle of lipopolysaccharide-challenged piglets

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Abstract: Growth of skeletal muscle is impaired markedly under inflammatory conditions. The underlying mechanisms remain largely unknown. This study was conducted to determine the effects of dietary supplementation with α-ketoglutarate (AKG) on cell signaling and energy metabolism in skeletal muscle of piglets challenged with lipopolysaccharide (LPS). Twenty-four piglets were assigned randomly to the control, LPS, or LPS + AKG group. Piglets in the control and LPS groups were fed a corn- and soybean meal-based diet, whereas piglets in the LPS + AKG group were fed the basal diet supplemented with 1 % AKG. On days 10, 12, 14, and 16 of the trial, piglets in the LPS and LPS + AKG groups received intraperitoneal administration of LPS (80 μg/kg BW), whereas piglets in the control group received the same volume of saline. On day 16 of the trial, blood samples were collected 3 h after LPS or saline injection. Twenty-four hours post-administration of LPS or saline (On day 17 of the trial), piglets were killed to obtain gastrocnemius samples for analysis. LPS challenge dramatically increased ($P < 0.05$) plasma concentrations of globin and TNF-α, while decreasing ($P < 0.05$) those for glucose, insulin, IGF-I and EGF, as well as the ratio of albumin to globin. LPS challenge also decreased ($P < 0.05$): (1) the concentrations of ATP, ADP, and total adenine nucleotides (TAN), (2) the ratios of phosphorylated mTOR to total mTOR, and (3) the ratio of phosphorylated p70S6K to total p70S6K in gastrocnemius, while increasing ($P < 0.05$) the intramuscular concentration of AMP and the ratio of AMP to ATP. These adverse effects of LPS were ameliorated ($P < 0.05$) by AKG supplementation. Moreover, AKG prevented the LPS-induced increase in the abundance of phosphorylated ACC protein in gastrocnemius. Collectively, these results indicate that dietary supplementation with AKG is beneficial for improving skeletal muscle growth and energy status in LPS-challenged piglets through enhancing mTOR signaling activity and reducing ACC phosphorylation.

Keywords: α-Ketoglutarate, Piglets, Lipopolysaccharide, Gastrocnemius.

Dietary supplementation with *N*-acetylcysteine improves tissue energy status in piglets challenged with lipopolysaccharide

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Abstract: This study was conducted with a lipopolysaccharide (LPS)-challenged piglet model to determine the effects of N-acetylcysteine (NAC) on energy status in the small intestine, liver and skeletal muscle of piglets. Eighteen piglets were assigned randomly into the control, LPS, or LPS + NAC group. The control and LPS groups were fed a corn- and soybean meal-based diet, and the LPS + NAC group was fed the basal diet + 500 mg/kg NAC. On days 10, 13 and 20 of the trial, piglets in the LPS and LPS + NAC groups received intraperitoneal administration of LPS (100 μg/kg body weight), whereas piglets in the control group received the same volume of saline. On day 20 of the trial, blood samples were collected 3 h after LPS or saline injection. On day 21 of the trial, piglets were sacrificed to obtain the intestinal mucosa, liver and gastrocnemius for biochemical analysis. Compared with the control group, LPS challenge reduced ($P < 0.05$): (1) the concentrations of ATP and adenylate energy charge (AEC) in the small-intestinal mucosa, liver and gastrocnemius; (2) the concentrations of ADP in the liver and total adenine nucleotides (TAN) in gastrocnemius, while increasing ($P < 0.05$) the concentrations of AMP and the ratio of AMP to ATP in the small-intestinal mucosa, liver and gastrocnemius. These adverse effects of LPS were attenuated ($P < 0.05$) by NAC supplementation. Moreover, NAC prevented the LPS-induced increase in the ratio of p-AMPK to AMPK (AMP-activated protein kinase) in the jejunal mucosa and gastrocnemius. Collectively, these novel results indicate that dietary NAC supplementation improves energy status in major tissues of protein metabolism in LPS-challenged piglets via inhibiting AMPK activation.

Keywords: *N*-Acetylcysteine, Energy status, Piglets, Lipopolysaccharide.

1. Alanyl-glutamine but not glycyl-glutamine improved the proliferation of enterocytes as glutamine substitution in vitro

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The synthetic dipeptides alanyl-glutamine (Ala-Gln) and glycyl-glutamine (Gly-Gln) are used as glutamine substitution to provide energy source in the gastrointestinal tract due to their high solubility and stability. This study aimed to investigate the effects of Ala-Gln and Gly-Gln on mitochondrial respiration and protein turnover of enterocytes. Intestinal porcine epithelial cells (IPEC-J2) were cultured for 2 days in Gln-free Dulbecco's modified Eagle's-F12 Ham medium (DMEM-F12) containing 2.5 mM Gln, Ala-Gln or Gly-Gln. Results from 5-ethynyl-2′-deoxyuridine (Edu) incorporation and flow cytometry analysis indicated that there were no differences in proliferation between Gln- and Ala-Gln-treated cells ($P > 0.05$), whereas the percentages of Edu-positive cells and cells in the S-phase in Gly-Gln treatment were lower than those of Gln and Ala-Gln treatments ($P < 0.05$). The results of mitochondrial respiration measurement showed that Ala-Gln treatment increased but Gly-Gln

treatment decreased the basal respiration and adenosine triphosphate (ATP) production, compared with Gln treatment ($P < 0.05$). Consistent with the data on cell proliferation, there were no differences in protein turnover between Gln- and Ala-Gln-treated cells ($P > 0.05$), but Gly-Gln treatment reduced protein synthesis and increased protein degradation ($P < 0.05$). In addition, Ala-Gln treatment increased relative protein levels for phosphorylated mTOR and phosphorylated 4EBP1 but Gly-Gln treatment decreased mTOR, phosphorylated mTOR and S6K1 compared with those of Gln treatment ($P < 0.05$). However, the UBE3A mRNA expression in Gly-Gln-treated cells was higher than that of Gln-and Ala-Gln-treated cells ($P < 0.05$). These results indicate that Ala-Gln has the same effect on cell proliferation, mitochondrial bioenergetics and protein turnover as Gln in porcine intestinal epithelial cells in vitro and can be substituted glutamine as energy and protein sources in the gastrointestinal tract.

Keywords Glutamine, Dipeptide, Mitochondrial bioenergetics, Protein turnover, Enterocyte.

2. *N*-acetyl-L-cysteine (NAC) protects the enterocyte against oxidative damage by preserving mitochondrial function

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The intestinal mucosa is vulnerable to oxidative stress in piglets and *N*-acetyl-L-cysteine (NAC) has been widely used to prevent oxidative stress. The objective of this study was to evaluate the protective effects of NAC on H_2O_2-induced oxidative damage in porcine intestinal epithelial cell. Intestinal porcine epithelial cells (IPEC-J2) were cultured for 48 h in the basal medium supplemented with 0 or 800 μM NAC and treated with 0 or 100 μM H_2O_2 for 4 h at the end of 24 h. The DNA synthesis, cell cycle and apoptosis, mitochondrial ROS production, mitochondrial respiration were determined using 5-ethynyl-2′-deoxyuridine (Edu) incorporation, flow cytometry analysis, dihydroethidium probe, and XF-24 Extracellular Flux Analyzer, respectively. The results showed that H_2O_2 inhibited cell proliferation, induced apoptosis and mitochondrial ROS production, and decreased the basal respiration, proton leak, maximal respiration, spare respiratory capacity, and ATP production ($P < 0.05$). However, NAC supplementation remarkably decreased mitochondrial ROS production and increased the percentages of Edu-positive cells, basal respiration, maximal respiration, and ATP production in H_2O_2-treated IPEC-J2 cells ($P < 0.05$). These findings showed that NAC can improve mitochondrial bioenergetics and alleviate oxidative damage in enterocyte of piglets.

Keywords *N*-acetyl-L-cysteine, Mitochondrial bioenergetics, Oxidative stress, Enterocyte

3. Porcine circovirus type 2 affects serum profile of amino acids and intestinal expression of amino acid transporters in mice

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PCV2 is highly pathogenic; however, its effect on serum amino acids profile is unknown. This study was conducted to explore the profile of amino acids in the serum in porcine circovirus type 2 (PCV2)-infected mice. The serum levels of amino acids were detected with isotope dilution liquid chromatography–mass spectrometry methods at 3, 7, 10, and 14 days post infection (DPI). Meanwhile, the expression of seven amino acids transporters (SLC6A14, SLC6A20, SLC7A5, SLC7A6, SLC7A7, SLC7A8, SLC7A9) in the jejunum was analyzed by reverse transcription polymerase chain reaction (RT-PCR) at 3 and 7 DPI. Serum PCV2 load was also analyzed by quantitative PCR at 3, 7, 10, and 14 DPI. Serum levels of most amino acids, such as Pro, Orn, and Met, significantly ($P < 0.05$) increased at 3 DPI. However, most amino acids, including Asp, Sar, Arg, Hyl, Pro, Lys, Val, Ile, and Leu, significantly ($P < 0.05$) decreased at 7 DPI. There was no significant difference for most amino acids at 10 and 14 DPI. PCV2 infection significantly ($P < 0.05$) decreased expression of SLC7A5 and SLC7A6 at 7 DPI. In conclusion, PCV2 infection affects the profile of amino acids in the serum and the expression of amino acids transporters in the intestine.

Keywords Porcine circovirus type 2, Amino acid, Amino acid transporter, Isotope dilution liquid chromatography–mass spectrometry methods.

4. Dietary protein intake affects expression of transporters and receptors of amino acids and peptides in porcine skeletal muscle in a genotype-dependent manner

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This study was conducted to test the hypothesis that dietary protein intake regulates AA profiles in blood and muscle, as well as mRNA levels for key receptors and transporters for AA in skeletal muscle. Forty-eight barrows of both purebred Bama mini-pigs (fatty genotype) and Landrace pigs (lean genotype) were randomly assigned to one of the two dietary treatments (low- or adequate-protein diet), with 24 individually fed pigs per treatment group. Pigs were fed between 5 weeks of age and market weight. During this period, samples of blood and skeletal muscles were collected at nursery, growing, and finishing phases. Our data showed that the majority of free AA decreased in plasma and increased in muscles, and pig-strain interacted with growth stage as regard to AA pool. Dietary protein level affected the mRNA levels of genes related to AA receptors and transporters in *longissimus dorsi* and *biceps femoris* muscles in a genotype-dependent manner. Specifically, low-protein diet upregulated ($P < 0.05$) mRNA levels for T1R1/T1R3, but high-protein diet enhanced ($P < 0.05$) mRNA levels for PAT1, PAT2, and ASCT2, as well as neutral AA uptake in muscle. Bama mini-pigs exhibited elevated ($P < 0.05$) mRNA levels for LAT1, SNAT2, and EAAC1, while decreasing ($P < 0.05$) the mRNA level for PepT1, when compared with the Landrace pigs. Additionally, developmental stage affected mRNA levels for most AA receptors and transporters, as well as concentrations of free AA in muscles. Collectively, these findings indicated that adequate provision of dietary AA plays an important role in regulating the expression of key receptors and transporters of AA in skeletal muscle in a genotype- and tissue-specific manner.

Keywords Bama mini-pig, Dietary protein, Skeletal muscle, AA transporter, AA receptor

5. Roles of dietary amino acids in intestinal B-lymphocyte homing

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Secretory immunoglobulin A (SIgA) is a non-inflammatory antibody that is secreted by plasma B cells. SIgA has a critical role in intestinal health by neutralizing pathogens in the lumen of the intestine to shield intestinal surfaces. The content of SIgA in the intestine depends on the numbers of IgA-producing plasma B cells in the intestine. To home to the intestine, B cells need to express the gut-homing receptors (CCR9 and integrin $\alpha 4\beta 7$) on the cell membrane. Various nutrients (i.e. retinoic acid, a vitamin A metabolite) enhance the expression of integrin $\alpha 4\beta 7$ and CCR9 on B cells. There is growing evidence that dietary supplementation with amino acids (i.e. L-glutamine and L-arginine) enhances the intestinal SIgA contents in various models. Also, it has been found that L-glutamine or L-arginine supplementation increases the number of IgA-producing plasma B cells in the intestine. These compelling findings indicate that dietary L-glutamine or L-arginine, or other amino acids, can regulate the expression of gut-homing receptors and the gut homing of lymphocytes.

Keywords SIgA, Glutamine, Arginine, Gut homing

6. Apparent digestibility of crude protein, gross energy, gross ash and amino acids from four dietary protein sources in young pigs

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This study was conducted to investigate the effects of different dietary protein sources on the dynamic changes in the apparent digestibility of crude protein (CP), gross energy (GE), gross ash (GA) and amino acids (AAs) in different parts of porcine gastrointestinal tract. Twenty-four young pigs (18 kg body weight) were assigned randomly into 4 treatments (6 pigs/treatment), representing supplementation with four types of diets including fish meal (FM), soybean meal (SBM), rapeseed meal (RSM) and cottonseed meal (CSM), respectively. Pigs were slaughtered and the digesta samples were collected for 1 h, 4 h, 8 h after feeding. The results showed that there were no significant differences ($P > 0.05$) in the apparent digestibility of CP, GE and GA in cecun, proximal colon, distal colon at 1, 4, 8 h, but significant differences ($P < 0.05$) in stomach, duodenum, proximal jejunum, distal jejunum and ileum as well as significant differences at 1, 4, 8 h were observed. The digestibilities of CP and GE in CSM-based diet and the digestibility of GA in FM-based diet were the highest ($P < 0.05$). The digestibilities of total AAs significantly influenced by diets, time and gastrointestinal segments and that were increased linearly from the stomach to the ileum along the tract, and the highest in FM treatment and the lowest in RSM treatment ($P < 0.05$); increased from 1 to 4 h then decreased remarkably at 8 h in the four detected segments of tracts. In the proximal jejunum, digestibilities of total AAs were higher in CSM and RSM treatments but lower in FM and SBM treatments which showed an obviously opposite trend in the distal jejunum. These findings suggest that CSM and RSM maybe can be substituted for FM and SBM as a protein source for pigs. Collectively, these findings indicated that the dynamic degestive profile differed among each segment of the gastrointestine response to different dietary protein sources, which should help to explore and utilize unconventional protein sources.

Keywords: Protein Sources, Apparent Digestibility, Crude protein, Gross Energy, Gross Ash, Amino acids.

7. Protective effect of dietary L-arginine against enterotoxigenic *Escherichia coli* infection in mice

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Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of about 300,000-5000 deaths in children under the age of 5 each year and the leading causes of diarrhea in neonatal and young pigs. This study was conducted to evaluate the protective role of dietary arginine supplementation in a mouse model with Enterotoxigenic *E. coli* infection. 100 female ICR (Institute for Cancer Research) mice were randomly divided into five groups: (1) all mice were treated with basal diet ($n = 20$); (2) all mice were treated with basal diet ($n = 20$); (3) all mice were treated with dietary 0.4 % arginine supplementation (0.4 % Arg + basal diet) ($n = 20$); (4) all mice were treated with dietary 0.8 % arginine supplementation (0.8 % Arg + basal diet) ($n = 20$); (5) all mice were treated with dietary 1.5 % arginine supplementation (1.5 % Arg + basal diet) ($n = 20$). At day 7, except group one, mice were challenged by oral infections with 5×10^8 *E. coli* SEC 470. All mice were killed to collect duodenum, jejunum and ileum at 24 h post infection. Bacterial burden, mortality and SIgA were analyzed from each group. In the basal diet group, 6 out of 20 mice died, whereas dietary 0.4 % arginine supplementation excised total protection, resulting in significantly decreased mortality compared to those with basal diet ($P < 0.05$). Meanwhile, 1 out of 20, 2 out of 20 mice died in 0.8, 1.5 % arginine supplementation group, respectively. Compared with basal diet, three different doses of arginine groups significantly ($P < 0.05$) decreased the bacterial burden in duodenum and jejunum. Meanwhile, compared with basal diet group, all arginine groups ETEC infection significantly ($P < 0.05$) increased the expression of SIgA in the jejunum. In conclusion, dietary arginine supplementation protects ETEC-infected mice by modulating the immune response and lowering the bacterial burden.

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Effects of uridine monophosphate feeding on hormone receptors expression in hypothalamus and taste bulb of tongue in weaned piglets

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Abstract: Uridine monophosphate (UMP), also known as 5′-uridylic acid, is a nucleotide that is used as a monomer in RNA, but also a flavor-enhancing compound in diets. However, little was known about the effects of dietary UMP on receptors of appetite-regulating hormone and tongue taste bulbs in pigs. In order to demonstrate whether UMP causes changes in taste bulbs and hormonal receptors expression in hypothalamus in weaned piglets, total of 18 duroc × landrace × big white pig were given diets with 0, 0.07 or 0.14 % of UMP ($n = 6$), ad libitum for 4 weeks. Feed intake and body weight were measured weekly. Wet brain weight was also measured and hypothalamus samples were taken at the end of experiment for qPCR determination. Tongues were isolated and fixed in formaldehyde solution. The results showed that feed intake and body weight was increased in pigs fed 0.07 % UMP on first week. Wet weight of brains were changed by UMP supplement ($P = 0.038$). No effect on wet weight of hippocampus was observed. In hypothalamus, gene expressions of ghrelin precursor (GHRL) and insulin receptor substrate 1 (IRS1) were altered by supplement of 0.07 % UMP ($P < 0.05$). No change on taste bulbs was observed unless increase trend on amount of fungiform bulb in anterior tongue of pigs fed with 0.14 % UMP diet ($P = 0.0586$). Densities of fungiform bulbs in anterior tongue of pigs fed UMP diets were higher, measured by regular paraffin section. From these findings, we conclude that diets supplement with UMP affect the amount of taste bulbs and appetite by regulating hormone receptors gene expression in hypothalamus in weaned piglets.

Keywords: Uridine monophosphate, Weaned piglets, Appetite, Taste bulb.

Effects of dietary supplementation with uridine monophosphate on performance and amino acids of weanling piglets

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Abstract: Uridine monophosphate (UMP) is the richest nucleotide in milk of sows; however, it is limited in stater feeds when compared to sow milk on lactation day 21. To evaluate the effects of adding UMP to starter diets for weanling piglets on growth performance and amino acids contents, 48 piglets (Landrace × Large White × Duroc) were weaned at 21 ± 1 day of age and randomly assigned into 2 groups: The control group and UMP group, and fed one of the following diets respectively for 14 days: a conventional starter diet (CSD) and CSD + UMP (0.07 %). Six piglets from each treatment group were sacrificed on d 14. Growth performance, serum physiological and biochemical indexes, plasma amino acids, and liver amino acids were measured to determine the effects of UMP supplementation. The results showed that there are significant increases in ADFI ($P < 0.05$) and ADG ($P < 0.01$), and F:G ($P < 0.01$) in UMP group was lower than the control group. Meanwhile, UMP diets decreased diarrhea frequency markedly (23.26 vs 9.17 %, $P < 0.05$). As for the blood physiological and biochemical indexes, the serum urea was lower in UMP than the control group (3.90 vs 2.88 g/100 mL). In addition, the plasma essential amino acids (EAA) between the two treatments had no significant difference. While, about non-essential

amino acids (NEAA), Glu ($P = 0.075$) in UMP group had a decreased tendency and Orn ($P = 0.017$) was decreased significantly when compared to the control group. Interestingly, NEAA in the liver, there were no significant difference between the two treatments. However, Val ($P = 0.025$) and Met ($P = 0.023$) as EAA in the liver were largely lower than the control group. In conclusion, UMP supplementation during the immediate post-weaning may have a positive influence on amino acids metabolism and improve growth performance.

Keywords: Uridine monophosphate, Amino acids, Growth performance, Weanling piglets.

Dietary supplementation with yeast extract at different times affected growth performance and plasma amino acids of growing pigs

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CVI-221 (Peptides and Peptidases: Yeast extract (YE) is rich in amino acids and nucleotides, which offer important nutritional implications for both humans and livestock. Nucleotides in the liver metabolized rhythmicity. To evaluate the effects of feeding YE at different times on growth performance and plasma amino acids of pigs, 36 pigs (23 ± 1 kg of weight, 63 ± 1 day of age, Landrace × Large White × Duroc) were randomly assigned into three groups: the control group, YE-B group, and B-YE groups, with 6 replicates in each group, two pigs per replicate. All the pigs were fed at 6:00, 10:00, 14:00, and 18:00. The pigs from the control group were fed with basal diets +0.5 %YE throughout the day, YE-B group with basal diets +1 % YE at 6:00 and 10:00, and basal diets at 14:00 and 18:00, while B-YE group with basal diets at 6:00 and 10:00, and basal diets +1 %YE at 14:00 and 18:00. Meanwhile, the three group pigs got equal amount of feed. On day 28, six pigs from each treatment group were chose for plasma samples and then sacrificed. Plasma biochemical indexes, amino acids, and liver amino acids contents were measured. The results showed that F:G in the YE-B group decreased significantly compared with the control group ($P = 0.047$) and decreased by 5.9 % than the B-YE group ($P > 0.05$). As for the biochemical indexes, GLB in the B-YE group significantly decreased ($P < 0.05$) and TP in the B-YE group had a decreased tendency ($P = 0.06$) when compared with the YE-B group. Compared with the control group, Met ($P = 0.006$) and Ile ($P = 0.034$) contents in B-YE group were significantly increased, and Phe in the B-YE group had an increased tendency ($P = 0.078$). Meanwhile, Ile in the YE-B group was lower than the B-YE group ($P = 0.046$). Furthermore, in the liver EAA, Val ($P = 0.015$) and Met ($P = 0.014$) in the B-YE group and YE-B group were both lower than the control group. Compared with the B-YE group, Cit in the YE-B group decreased markedly ($P = 0.049$). The experiment can be summarized that feeding basal diets +1 %YE in the morning had a positive influence on growth performance for pigs, and feeding YE at different times may effect the metabolism of amino acids.

Keywords: Yeast extract, Amino acids, Nucleotides, Pigs.

Cystathionine affects cysteine/cystine levels in brain cancer cells

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The transsulfuration pathway allows for the interconversion of homocysteine and cysteine with the intermediary formation of cystathionine, which may be used in superoxide radicals scavenging, blocking the tissue injury by the nucleoside antibiotic tunicamycin inducing endoplasmic reticulum stress, and can inhibit the apoptosis of U937 monocytic cells and HepG2 hepatoma cells through preventing the excretion of glutathione. Both cystathionine and cysteine are also the substrates for H_2S synthesis by cystathionine β-synthase, the predominant H_2S-generating enzyme in the brain. We studied the effect of cystathionine on cysteine/cystine levels and proliferation of human astrocytoma (U373) cells. The cells were cultured in DMEM supplemented with 0.25, 0.5 and 1 mM cystathionine for 24, 48 and 72 h. The RP-HPLC method was used for the determination of cysteine, cystine and cystathionine levels in astrocytoma cells. As compared to cells cultured in DMEM without cystathionine, the addition of cystathionine resulted in the increase of the intracellular cystathionine levels in a concentration-dependent manner. The levels of cystathionine detected after 24 h were higher than after 48 and 72 h. During the first 24 h of the culture, cysteine and cystine levels in the U373 cells were significantly decreased in the presence of 0.5 and 1 mM cystathionine compared to untreated cells (about 20–40 % of reduction). However, in the U373 cells treated with 0.5 and 1 mM cystathionine for 48 and 72 h, a reduction of intracellular cystathionine level with culture duration was associated with increased cysteine and cystine levels (about 20–40 % of the control) and stimulation of cell proliferation (about 7 % for 48 h and about 14 % for 72 h). Our data indicate that an elevated level of cystathionine results in the increased cysteine/cystine levels and increased cell proliferation. Further investigations are required to elucidate the decrease of intracellular cysteine/cystine levels observed during the first 24 h of culture duration.

Changes in sulfur metabolism caused by lead, mercury, and cadmium ions in frog tissues

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Many heavy metal ions-related pathologies result from an oxidation of cellular components due to diminished levels of available antioxidant reserves in the body—mainly glutathione, and from changes of the activity of enzymes having antioxidant properties caused by competition of heavy metal ions with divalent cations, such as copper or zinc, in active sites of these enzymes. Cysteine, a substrate for glutathione synthesis, may undergo anaerobic desulfuration resulting in sulfane sulfur-containing compounds formation. Sulfane sulfur-containing compounds can play a role in the regulation of sulfur metabolism and can have antioxidant properties. The aim of the study was to investigate changes in cysteine desulfuration in selected tissues of *Pelophylax ridibundus*, *Xenopus laevis,* and *Xenopus tropicalis* exposed to lead (28 mg/l), mercury (1.353 mg/l), and cadmium (40 mg/l) during 10 days. In homogenates of liver, kidney, heart, testicles, brain, and skeletal muscle of the control animals and animals exposed to heavy metal ions, the level of sulfane sulfur, the expression, and the specific activity of rhodanese (TST),

3-mercaptopyruvate sulfurtransferase (MPST) and cystathionine γ-lyase (CTH) were determined. Simultaneously, the level of reduced (GSH) and oxidized (GSSG) glutathione, cysteine and cysteine, and expression of genes encoding selected antioxidative enzymes (cytoplasmic and mitochondrial superoxide dismutase, catalase, glutathione peroxidase, and thioredoxin reductase) were determined to verify whether exposition to heavy metal ions resulted in oxidative stress in animal tissues. In all the examined tissues, the level of the final product of lipid peroxidation—malondialdehyde (MDA)—was also determined. Based on the results, it should be noted that lead, mercury, and cadmium ions induce oxidative stress in all the examined tissues. Changes in the activity and/or expression of TST, MPST and CTH were also observed. Changes in their activity can result from modifications (oxidation, binding heavy metal ions) of sulphydryl groups located in the active centers of the enzymes or from changes in expression of genes encoding these enzymes. Changes found in gene expression, in response to oxidative stress, confirmed a role and significance of sulfurtransferases and/or sulfane sulfur in antioxidative processes.

14th International Congress on Amino Acids, Peptides and Proteins Redox regulation of a multifunctional enzyme, 3-mercaptopyruvate sulfurtransferase

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A multifunctional enzyme, 3-mercaptopyruvate sulfurtransferase (MST) catalyzes the transsulfuration reaction of 3-mercaptopyruvate or thiosulfate to thiol-containing compounds or cyanide as a cysteine-catabolizing enzyme. During the catalytic process, persulfide is formed at the catalytic site of the cysteine residue as a reaction intermediate and a sulfur-acceptor substrate donates the outer sulfur of the persulfide to form a new persulfide molecule. MST also serves as an antioxidative protein to maintain microenvironmental redox homeostasis. Recently, MST has been reported to form hydrogen sulfide and polysulfides. Finally, we advocate a possibility that MST also produces sulfur oxides in the redox cycle of the stable reaction intermediate. The enzyme is regulated by redox changes via two redox-sensing molecular switches consisting redox-active cysteine residues. The catalytic cysteine serves as a switch in itself. Under oxidizing conditions, a cysteine-sulfenate is formed resulting in inhibition of catalytic activity. Thioredoxin reduces the sulfenate to restore the activity, but glutathione cannot do. The other switch involves cysteine residues on the surface of the enzyme. Under oxidizing conditions, dimer, an inactive form, is formed via the intermolecular disulfide bond. On the other hand, under reducing conditions, monomer, an active form, is increased. Thioredoxin reduces the disulfide bond more effectively than dithiothreitol. We produced MST-knockout mice as a disease model of mercaptolactate-cysteine disulfiduria. They showed anxiety-like behavior, and 5HT type2A receptor density was increased in hippocampus. However, the relationship of MST defect to this pathogenic mechanism has not been identified yet.

Vascular regulatory roles of hydrogen sulfide: cooperative interactions between hydrogen sulfide and nitric oxide

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Vasodilatation is one of the earliest recognized biological actions of the gaseous biological mediator hydrogen sulfide (H_2S). Another important action of H_2S is the stimulation of angiogenesis (promotion of the growth of new blood vessels). These responses are apparent in various in vitro experimental systems, as well as in animal models in vivo. The responses show significant regional differences, with the vascular relaxant effects being most pronounced in microvessels. The underlying pathways are multiple and range from the activation (opening) of K_{ATP} channels to the stimulation of the guanylyl cyclase/cGMP pathway in the vascular smooth muscle cells. This talk will overview the vasodilatory and angiogenic roles of H_2S, and will present all of the known biochemical mechanisms involved in these responses. Special emphasis will be made to the role of H_2S produced by 3-mercaptopyruvate sulfurtransferase (3-MST), which (in addition to cystathionine-beta synthase [CBS] and cystathionine-gamma-lyase [CSE]) emerges as a third major biological source of H_2S. Importantly, a significant portion of the vasodilatory and angiogenic effects of H_2S occurs in concert with the similar roles of nitric oxide (NO). These cooperative interactions include (a) H_2S-induced release of NO from the various 'NO stores' such as nitrite; (b) H_2S-mediated activation of the endothelial isoform of NO synthase (eNOS) through modulation of the PI3 kinase pathway; (c) direct effects of H_2S on eNOS by sulfhydration and modulation of its dimeric/monomeric state and (d) H_2S-mediated inhibition of cGMP phosphodiesterase activity, thereby enhancing the biological half-life of NO. As a result of these interactions, the biological effects of H2S are often diminished in systems where eNOS activity is diminished; an observation that has important pathophysiological implications.

Trimethylglycine (betaine) accentuates cisplatin-induced hepatotoxicity by suppressing oxidative stress and inflammation and down-regulation of nuclear factor kappa and caspase-3 in experimental rats

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Oral presentation

Introduction: Cisplatin is widely used in the treatment of solid tumors but its full clinical utility is limited to its adverse effects as hepatotoxicity. The aim is to investigate the effect of trimethylglycine supplementation on cisplatin-induced hepatotoxicity.

Materials and methods: Animals were allocated into four groups; (1) saline control group; (2) cisplatin group in which rats were

injected with a single intraperitoneal injection of cisplatin (7 mg/kg); (3) trimethylglycine group, in which trimethylglycine was given at a dose of (250 mg/kg/day) orally via gavage for 21 days; (4) cisplatin + trimethylglycine group in which trimethylglycine was given orally via gavage for 21 days prior to cisplatin injection and daily for 5 days after cisplatin.

Results: Cisplatin injection deteriorated the liver functions as reflected by elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. Cisplatin increased hepatic lipid peroxides measured as thiobarbituric acid-reactive substances (TBARS), and reduced the concentrations of reduced glutathione, glutathione peroxidase, catalase and superoxide dismutase in hepatic tissues. Expressions of nuclear factor-kappa B (NF-κB) and the apoptotic marker, caspase-3 were up-regulated in hepatic tissues of cisplatin-treated rats. Cisplatin increased the hepatic concentrations of inflammatory mediators: nitrite and tumor necrosis factor-α (TNF- α). Hisopathological changes were observed in cisplatin group. Trimethylglycine pre-treatment protected against deterioration in liver functions abrogated the decline in antioxidants and suppressed the increase in TBARS, nitrite and TNF-α concentrations. Moreover, trimethylglycine inhibited NF-κB and caspase-3 activation and improved the histological changes induced by cisplatin.

Conclusions: The protective mechanisms of trimethylglycine in cisplatin-induced hepatic injury may be related to the attenuation of oxidative stress, along with the inhibition of inflammatory response and apoptosis.

Plasma methionine and S-adenosylmethionine are potential biomarkers to determine tolerable upper intake level of methionine

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Although many animal studies have reported that dietary excess of methionine causes toxic changes including growth suppression and hemolytic anemia, the biochemical mechanism and biomarkers for methionine toxicity have not been well elucidated. The present study identified "toxic metabolites" or biomarker metabolites of toxicity in rats fed excessive methionine. Since our previous study has shown relation of plasma homocysteine with toxic changes caused by excessive intake of methionine, effects of 2-week of homocysteine feeding were studied to compare to those of equimolar methionine. Feeding of supplemental methionine (1.2 or 2.4 %) significantly increased splenic non-heme iron content, an indicator for splenic hemolysis, while feeding of homocysteine (1.1 or 2.2 %) did not. However, 3.8 % betaine addition in combination with 2.2 % of homocysteine promoted remethylation of homocysteine to methionine and increased splenic non-hem iron significantly, indicating that homocysteine is not a direct causative of methionine toxicity. Elevations of hepatic methionine and S-adenosylmethionine (SAM) coincident with the toxic change, but not S-adenosylhomocysteine (SAH) and homocysteine, suggested that methionine and SAM are involved in methionine toxicity. Product-to-precursor ratios of methionine metabolites in the liver in rats fed graded doses of additional methionine (0, 0.3, 0.6, 0.9, 1.2, 2.4 %) for 2 weeks suggested metabolic process from SAM to SAH would be saturated at 0.9 % or more of additional methionine. Accumulations of methionine and SAM in the plasma were also evident at 0.9 % or more of additional methionine feeding. These results appeared to indicate that 0.9 % of additional methionine was excessive. Indeed, 4-week multi-dose (0, 0.1, 0.3, 0.9, 2.7 %) toxicity study of methionine documented that NOAEL and LOAEL of methionine were 0.3 and 0.9 %, respectively. These results suggested that plasma methionine and SAM are potential biomarkers for methionine toxicity and that they might be useful to determine safe intake level and tolerable upper intake level of methionine.

Antimicrobial activity and protease stability of short cationic peptides containing arginine mimetics

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The emergence of multidrug-resistant (MDR) pathogens underscores the need for new antimicrobial agents to overcome the resistance mechanisms of these organisms. Antimicrobial peptides (AMPs) have been intensely investigated in the last 3 decades as a potential source of complementary antimicrobial agents with a lower propensity to select for drug resistance phenotypes than that of current antimicrobials. Most AMPs are cationic peptides that assume a specific amphipathic conformation, either in the presence of bacterial membranes (amphipathic α-helical peptides, e.g., cathelicidins and magainins or constitutively (cyclic and β-sheet AMPs, e.g., the defensins, stabilized by disulfide bridges. Cationic charge and hydrophobicity are two basic determinants of cell selectivity, which modulate the peptides' electrostatic interactions with the lipid head group and partitioning into the lipid bilayer, respectively. The bovine cationic dodecapeptide bactenecin (also called dodecapeptide) is one of the smallest naturally occurring cationic antimicrobial peptide. A linear variant, **Bac2A** (RLARIVVIRVAR-NH_2), in which the cysteine residues were replaced with alanines, showed similar activity against Gram-negative bacteria but improved activity against Gram-positive bacteria. The features of **Bac2A**, namely small size, linearity, and activity against both Gram-positive and Gram-negative bacteria, make this peptide an ideal lead structure to develop new antimicrobial drugs. On the basis of these data, we designed and prepared new **Bac2A** derivatives. In order to identify compounds with a good antimicrobial profile, we synthesized two series of short fragments of **Bac2A** containing arginine analogs and several amino acids (such as halogenated L/D-tryptophan, L/D-tyrosine, and L/D-phenylalanine), respectively, in order to confer both lipophilic properties and positive-charged portions. Their purity characterized by RP-HPLC and capillary electrophoresis was 87–94 %. The peptides also showed modest increases in protease resistance, relative to the parent peptide. The antibacterial activity of synthesized peptides was studied by a disc diffusion assay and by minimum inhibitory concentration (MIC) against selected Gram-negative and Gram-positive bacteria.

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Synthesis, analysis, and cytotoxic effects of novel SMAC-based peptides

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Apoptotic cell death is an essential mechanism for maintaining homeostasis and normal development of multicellular organisms. Inappropriate regulation of apoptotic pathways can lead to severe pathological consequences, including cancer, inflammation, and neurological conditions. One hallmark of cancer cells is a compromised ability to undergo apoptosis. Targeting critical apoptosis regulators in order to promote apoptosis in cancer cells is thus a promising strategy for the development of new classes of anticancer drugs. Smac protein is a pro-apoptotic protein that functions as an endogenous inhibitor of IAP proteins (XIAP, cIAPs) and thus reactivates caspases and apoptosis. Smac exerts its activity through direct interaction that involves the AVPI tetrapeptide of Smac and a well-defined groove on the surface of these IAP proteins. The structural information as well as inverse correlated levels of Smac and IAPs proteins in cancer cells prompted the design of AVPI-mimetics and Smac-mimetics as agents evading apoptosis block. Regarding the data and experience with other cytotoxic compounds, our group devoted efforts in design AVPI-mimetics. We synthesized AVPI-tetrapeptide analogs as we included D-Pro and L-Hyp instead of proline itself. Recently, we have focused on the synthesis, structure, and properties of AVPI-RGD hybrids, which are derivatives of AVPI with RGD analogs. RGD is a peptide that is known to interact with specific overexpressed proteins on the membrane of cancer cells. Since AVPI derivatives and RGD peptides possess useful characteristics, the combination of the two functionalities will result in the generation of compounds with structural and biological properties of great interest. Currently, we are running cell viability assay (MTT test) and apoptosis assay (Comet assay) in order to determine the biological effects of the synthesized mimetics.

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Synthesis, analysis and cytotoxic effects of new RGD-mimetics

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Among the peptide family, short peptides are very appealing for drug discovery and development because of their cost-effectiveness, possibility of oral administration, and simplicity to perform molecular structural and quantitative structure–activity studies. Small peptides are under development as possible anti-tumour agents as well. Arginine–Glycine–Aspartic acid (RGD) is the specific recognition site of integrins with theirs ligands, and regulates cell–cell and cell–extracellular matrix interactions. The RGD motif can be combined with integrins overexpressed on the tumour neovasculature and tumour cells with a certain affinity, becoming the new target for imaging agents, and drugs, and gene delivery for tumour treatment. RGD peptides conjugated with different cytostatic agents are likely to exhibit an anti-tumour and anti-angiogenic synergetic effect. During the last few years, a number of RGD-cytotoxic drugs were developed and showed promising activities in vitro and in vivo. Despite the few RGD analogues being approved for clinical use, development of orally active RGD peptidomimetics have been significantly hindered because of low bioavailabilities. This is largely due to the metabolic lability of this class of compounds in the presence of proteases and peptidases and because of their high polarity and charge. Following our long-term program for the design of biologically active peptides based on the non-protein amino acids, some short RGD-mimetics have been synthesized and their cytotoxic potentials were studied [1]. This communication reports synthesis and biological activity of new RGD mimetics, containing the sequence **Xaa-GD**, where **Xaa** is Arg-mimetic (Agb or Agp) with a view to improve the stability and cytotoxic activity. Synthesis was performed by the conventional and manual stepwise Fmoc/tBu solid-phase methods. Their purity characterized by RP-HPLC and capillary electrophoresis was 87–94 %. All the compounds were evaluated in vitro for their cytotoxic activities on non-tumour 3T3 cells and tumour cell lines HepG2.

1. Balacheva A, Iliev I, Detcheva R, Dzimbova T, Pajpanova T, Golovinsky E (2012) In vitro assessment of the cytotoxic effects of novel RGD analogues. BioDiscovery 4:1–6.

Chemical stability of new analogues of octreotide

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Somatostatin (SST-14 H2N-Ala-Gly-c(Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys)-COOH) is a hormonal cyclopeptide produced by neuroendocrine neurons of the hypothalamus, gastrointestinal, immune cells, as well as by certain tumours. It has a broad range of biological action that includes inhibition of growth hormone (GH), insulin, and glucagon and other hormone secretions, suppression of gastric and pancreatic exocrine secretion, gut motility, cell proliferation and also plays a role as a neurotransmitter.

Some modified octapeptide analogues of somatostatin with the following structure D-Phe-c(Cys-Phe-D-Trp-Xxx-Yyy-Cys)-Thr-NH_2, where Xxx is Lys or Orn and Yyy is Aib (α-aminoisobutyric acid), Ac5c (1-aminocyclopentanecarboxylic acid) or Ac6c (1-amino cyclohexane carboxylic acid) have been synthesized [1].

We evaluate cytotoxic potential of the parent compound and new analogues in cultured non-tumour Lep-3 cells and panel of tumour HepG2; HT-29; MDA-MB-23 and HeLa cell lines by the MTT assay also. The new synthesized analogues have shown improved growth-inhibitory effects on HT-29 cells.

The purpose of this study was to evaluate the stability of the new synthesized analogues in different values of pH, available physiologically. Both the analogues have shown significant stability and kipped 90 % of their outcome concentration in different pH values.

Reference

Svetlana Staykova, Diana Wesselinova, Lyubomir Vezenkov, Emilia Naydenova. (2015) Synthesis and in vitro antitumor activity of new octapeptide analogs of somatostatin containing unnatural amino acids *Amino Acids*, **47**(5):1007–1013.

Docking studies of novel neurotensin(8–13) analogues

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Neurotensin (NT) is a neurohormone and/or neuromodulator containing 13 amino acid residues. The neurotensin receptors (NTR) are transmembrane receptors that bind neurotensin. The active fragment of neurotensin is 8–13 (RRPYIL). The main drawback in the use of NT or any other endogenous peptide as a drug is extremely short half-life as a result of their rapid degradation by the action of peptidases. To overcome this problem, various neurotensin analogues were synthesized, which include linear peptides, cyclic peptides and non-peptide molecules. Chemical modification of the native peptide, however, may result in a radical change in receptor affinity and specificity. The purpose of this work is using computational methods to identify interactions between neurotensin and neurotensin receptor analogues (NTR1) and to establish the relationship between structure and action. Analysing the results of docking is apparent that the tested analogues of neurotensin bind in the active site of the receptor, but significantly weaker than NT. Replacement of arginine residue in the second position with canavanine strongly increases the total energy of the ligand–receptor complex; therefore, it is more unstable than the complex NT/NTR1.

Analgesic activity of new RGD analogues

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Peptides containing unnatural amino acids are used in a multitude of applications including structure–activity studies, diagnostics, and new drug discovery, improvements in bioavailability, molecular markers, and biologically active pharmaceuticals. The RGD sequence is present in many extracellular matrix proteins and intracellular proteins, including caspases. Synthetic RGD peptides may affect adhesion, migration and tumour metastasis, or directly induce apoptosis. In an effort to improve the antitumor activity of the RGD molecule, several RGD peptides were synthesized, and their cytotoxic properties were analysed in vitro [1]. Next, we assumed that such oligopeptides containing arginine residue are able to induce analgesia, given that arginine exert modulating effects on pain transduction. The purpose of this study was to investigate the possible analgesic effects of several synthesized RGD analogues (RGD methyl ester, AgbGD, AgpGD) and their interactions with the μ-opioid receptor. The analgesic activity was evaluated and compared with the parent peptide in male Wistar rats (120–150 g). All peptides were intraperitoneally (i.p.) administrated at a dose 1 mg/kg. The involvement of the opioid receptors was demonstrated using a non-selective opioid receptor antagonist naloxone (at a dose 1 mg/kg, i.p.). Nociception was measured by paw-pressure test. According to the obtained results, the new analogues elicited antinociceptive effects. The incorporation of arginine mimetics in RGD molecule leads to alteration in analgesic activity of the parent molecule. The involvement of the opioid receptors in such effect was proved.

1. Balacheva A, Iliev I, Detcheva R, Dzimbova T, Pajpanova T, Golovinsky E (2012) In vitro assessment of the cytotoxic effects of novel RGD analogues. BioDiscovery 4:1–6.

Hyperammonemia decreases branched-chain amino acid concentration in blood plasma and enhances their catabolism in skeletal muscle

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It has been proposed that enhanced ammonia detoxification to glutamine (GLN) is the main cause of decreased concentrations of branched-chain amino acids (BCAA; valine, leucine and isoleucine) in blood plasma and that the decreased BCAA levels are important pathogenic factor in encephalopathy and protein-energy malnutrition development in liver disease. Two separate experiments were performed using Wistar rats in which the effects of hyperammonemia on GLN, BCAA and protein metabolism were evaluated. In the first, in vivo study, the hyperammonemia was induced by infusion of ammonium acetate/bicarbonate mixture and parameters of protein and leucine metabolism were measured using L-[1-^{14}C] leucine infusion. In the second, in vitro study, soleus muscle (SOL, slow-twitch, red muscle) and extensor digitorum longus (EDL, fast-twitch, white muscle) were incubated in a medium with or without 0.5 mM ammonia. We measured the exchange of amino acids between the muscle and the medium, amino acid concentrations in muscle, leucine oxidation, total and myofibrillar proteolysis and protein synthesis. Ammonia infusion increased ammonia and GLN and decreased BCAA and alanine levels in blood plasma, increased leucine oxidation and decreased protein synthesis in skeletal muscle. Under in vitro conditions, hyperammonemia increased GLN concentration and leucine oxidation in muscles and enhanced GLN and decreased BCAA release from muscles to incubation medium. The effects were more pronounced in EDL. The results indicate that hyperammonemia directly activates the BCAA metabolism in skeletal muscle and that the cause of decreased plasma BCAA levels observed in liver cirrhosis is hyperammonemia.

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Tissue distribution of cystinyl aminopeptidase (CysAP/IRAP) activity in control and captopril-treated WKY and SHR. Its relationship with blood pressure, water balance and plasma glucose

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Abstract: Cystinyl aminopeptidase activity (CysAP) is involved in a diversity of functions such as blood pressure (BP) control, water balance (WB), glucose homeostasis and cognitive processes. In addition, CysAP has a close functional relationship with the renin–angiotensin aldosterone system (RAAS). It was characterized as the high-affinity Angiotensin IV binding site i.e. AT_4 receptor. Marked differences in the pattern of CysAP activities depending on the origin of the sample analyzed, such as plasma or the soluble (Sol) or membrane-bound (MB) fraction from tissue homogenates, were observed. There are studies providing the local regional distribution of CysAP in selected tissues such as the central nervous system or kidney. However, so far, no comprehensive analysis has been performed evaluating the inter-tissue distribution and differentiating the subcellular origin of the sample. To examine this subject, we analyzed plasma activity and tissue distribution of Sol and MB CysAP in SHR and WKY. We also searched for a possible association of CysAP with WB, BP and plasma glucose (PGLU) under basal conditions. Furthermore, the effect of ACE inhibition by captopril treatment on the CysAP levels was investigated. Sol or MB activities were generally higher in SHR compared to WKY notably in hypothalamus and kidney (particularly renal medulla) than in the other tissues with exception of adrenals. The effect of captopril on CysAP was variable mainly decreasing CysAP in SHR, whereas it increased in WKY. Except for Sol CysAP in WKY, captopril reduced CysAP in renal medulla. The distribution of Sol CysAP was more homogeneous among tissues of WKY than in SHR. Furthermore, the distribution of MB CysAP was more heterogeneous than Sol CysAP in both WKY and SHR. The majority of the significant correlations between the activities detected in the different tissues and the measured clinically relevant parameters (BP, WB, PGLU) were observed mostly in hypothalamus and renal medulla. These correlations were mainly positive in control groups and essentially negative in the captopril-treated groups. These results suggest significant functional differences among plasma, Sol or MB activities and support a particular role of hypothalamus, renal medulla and renal cortex CysAP in glucose metabolism, aqueous balance and/or blood pressure control. Since important differences between WKY and SHR and after captopril treatment were observed in the present study, a direct relationship between the activity of CysAP and the functional status of RAAS may also be hypothesized.
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Interaction of Leptin and GABAB-agonist and antagonist on thermoregulation

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Effects of the obese gene peptide leptin have been widely recognized and studied currently, as well as leptin interaction with other neuromodulators or neurotransmitters. The present study was set to determine the effects of leptin, GABAB-agonist baclofen and GABAB-antagonist CGP35348, applied separately or in combinations, on thermoregulation of male Wistar rats. In vivo experiments were made by intraperitoneal (i.p.) administration of substances. The core body temperature of rats was measured by thermistor probes (TX8) and monitored on multichannel recorder Iso-Thermex 16. In vitro experiments were made on neurons from the preoptic area/anterior hypothalamus (PO/AH) by conventional electrophysiological equipment (set up), using rat brain slice preparations. In vivo separate injection of leptin as well as of GABAB-antagonist CGP35348 produced significant hyperthermia in rats while the GABAB-agonist baclofen caused a decrease in the core body temperature. The probable synergy between the hyperthermic effects of leptin and GABAB-antagonist CGP35348 did not occurr. On the contrary, effect of this combination was significantly lower compared to the result of separate administration of substances. When leptin was applied just prior GABAB-agonist baclofen neither one of their separate effects appeared. In vivo effects determined were in correlation with in vitro changes of firing rate observed in PO/AH neurons. Leptin increased dose-dependently firing rate of PO/AH neurons. The effect of leptin and GABAB-antagonist combination on firing rate was significantly lower compared to the result of their separate administration. Neither effect of leptin nor of GABAB-agonist baclofen appeared when applied in combination on PO/AH neurons. The data from this study provide a new point of view concerning the interactions of neuromodulatory acting peptides and GABAergic neurotransmission on the level of central temperature controller—the neurons of the PO/AH.

A Protective Role of L-Arginine on the Intestine of Weaning Piglets Ingesting Deoxynivalenol-Contaminated Feed

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Abstract: This study was conducted to investigate the effects of supplementary L-arginine (Arg) on piglets fed Deoxynivalenol (DON)-contaminated feed. Eighteen weaning pigs (28-day-old Landrace × Yorkshire) were divided into 3 groups randomly. Arginine (Arg) groups fed 1.0 % Arg on a 7-day period for immune-fortification while control and toxic group fed basal diet. Then the toxic group and Arg group (continually fed with Arg for fortification as before) fed 6 mg/kg DON-contaminated diet 7 days later. The control group

fed with DON-free diet at same time. Voluntary feed intake for 21 days and consequently, DON caused obvious damages to piglets of toxic group. However, clinic parameters, like intestinal morphology, the amino acids concentrations in the serum, jejunum and ileum were notably ($P < 0.05$) improved by the supplementary Arg. Furthermore, the mRNA levels of y + L amino acid transporter 1, cationic amino acid transporter, excitatory amino acid carrier type 1 that down-regulated in the jejunum of toxic group increased ($P < 0.05$) in the Arg group. In conclusion, Arg plays a protective role in the small intestine to alleviate the adverse effects of DON.

Keywords: Deoxynivalenol, Arginine, Intestinal function, Amino Acids transporter.

Biology-oriented diverted synthesis of glutamate analogs

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Ionotropic glutamate receptors (iGluRs) play a pivotal role in higher brain functions such as learning and memory by mediating the majority of fast excitatory neurotransmission in the mammalian central nervous system. iGluR is also thought to be fully or partly involved in nociception and closely related to brain disorders such as Alzheimer and Parkinson diseases. Structurally, iGluR is composed of four subunits assembling as homomers or heteromers to form the ionchannel. The neurotransmitter, such as glutamic acid, generally binds to the ligand-binding domain (LBD) of iGluR and causes structural change of the transmembrane domain. It has been of particular interest to develop selective and specific ligands for iGluR as a drug candidate to treat neuronal diseases mentioned above, and many modulators have been reported and investigated for clinical trials. In 2008, we synthetically constructed compound collection of artificial glutamate analogs which are structurally inspired by excitatory amino acids such as dysiherbaine and kainic acid. In vitro and in vivo evaluations have identified some biologically interesting compounds including IKM-159. IKM-159 weakly inhibits AMPA-type iGluR with some selectivity to GluA2 and GluA4 subunit proteins. Structural study of the complex indicated the unique interactions with GluA2 LBD. To develop more potent and/or selective modulators for iGluRs, we further studied diverted synthesis of IKM-159 analogs. Here we report our recent progress on these synthetic studies.

Synthesis of C-terminal octapeptide $B_{23–30}$ of B-chain human insulin by classical peptide method to be used in semisynthesis of human insulin iodinated at tyrosine B_{16}

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C-Terminal protected octapeptide $B_{23–30}$ of the B-chain human insulin (benzyloxycarbonyl-glycyl-phenylalanyl–phenylalanyl-*O*-t-butyltyrosyl-*O*-t-butyl-threonyl-prolyl-*N*-methylsulphonylethyloxy-carbonyllyzyl-*O*-t-butylthreonyl-t-butyl ester) was synthesized by the stepwise peptide synthesis, using DCC and 1-hydroxybenztriazole as a condensing agent for fragment condensation of B_{29}–B_{30} with B_{27}–B_{28} dipeptides and B_{23}–B_{26} with B_{27}–B_{30}. Cleavage of benzyloxy-carbonyl groups from N-terminal and coupling with DOP B_1–B_{22}, iodinated at Tyrosine B_{16} and then combined to natural A-chain giving Human insulin iodinated at Tyrosine B_{16} selectively.

Keywords: Peptide synthesis, Human insulin, Iodinated tyrosine, N-Methyl sulphonyl ethloxycarbonyl, DOP B-chain.

Icosahedral chart of the 20 commonly occurring amino acids

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The 20 commonly occurring amino acids are Alanine, Arginine, Asparagine, Aspartic Acid, Cysteine, Glutamine, Glutamic Acid, Glycine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Proline, Serine, Threonine, Tryptophan, Tyrosine, and Valine. The corresponding 3-letter symbols of the amino acids are Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val. The corresponding 1-letter symbols of the amino acids are: A, R, N, D, C, Q, E, G, H, I, L, K, M, F, P, S, T, W, Y, and V. A Platonic polyhedron is a polyhedron with congruent faces and the same number of faces meeting at each vertex. The 5 Platonic polyhedrons are the Tetrahedron with 4 faces, the Cube with 6 faces, the Octahedron with 8 faces, the Dodecahedron with 12 faces, and the Icosahedron with 20 faces. Since there are 20 commonly occurring amino acids and there are 20 faces to an icosahedron, a useful heuristic device for learning and remembering the chemical structures, the names, the 3-letter symbols, and the 1-letter symbols of the amino acids can be constructed by placing the chemical structure, the 3-letter symbol, and the 1-letter symbol for each amino acid on a single face of an icosahedron. The United States Design Patent Number US D721,005 S, by the author, entitled ICOSHEDRAL CHART OF THE 20 COMMONLY OCCURRING AMINO ACIDS shows what such an icosahedron with the chemical structure, 3-letter symbol, and 1-letter symbol, for each amino acid on each face of such an icosahedron would look like from front, right, left, top, and bottom views. Design Patent US D721,005 S also contains two cut-and-assemble half-patterns which can be cut out and assembled to make the icosahedron shown in the design patent. Once the icosahedron is assembled, it can be tossed at random like a 20-sided dice to generate random amino acid sequences. For example, one such random amino acid sequence that is 100 amino acids long, which was generated by randomly tossing the icosahedron is KWLLTEKSTPSEKWIEDGRTVCLFMPHAMNHKGRESYCGYCY NIMTSKETRECGGKNPQARDKGKKNNMCHFSLCRWYQSMNH HWNGIRFREERGVGED.

Limitation of proteolysis in soils of forests and other types of ecosystems by diffusion of substrate

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Proteinaceous materials are hypothesised to represent approximately 40 % of total nitrogen in soils. Proteolytic enzymes depolymerize proteins into amino acids that serve as a source of carbon and nitrogen

for soil-dwelling microorganisms. Proteolysis is generally considered to be the rate-limiting step in mineralization of nitrogen in soils. Methods developed for measurement of proteolysis in soils may differ in the type of substrate, incubation conditions and analytical procedures. Measurements of proteolysis are usually performed in soil slurries to reduce substrate diffusion limitation. Knowledge on limitation of proteolysis in soils of forests and other types of ecosystems by diffusion of substrate will be presented.

Amino acids in root exudates of *Ambrosia artemisiifolia*

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Amino acids are generally second or third most abundant class of low-molecular-weight organic compounds released from plant roots by passive diffusion into the rhizosphere; intense amino acid exudation was proposed at root tips and legume nodules. Amino acids released from plant roots serve as a source of carbon and nitrogen for soil-dwelling microorganisms and may play different roles in the rhizosphere including chemotaxis of rhizosphere-colonizing bacteria, alteration of pseudomonad biology, modulation of genes involved in sporulation in *Bacillus subtilis*, synthesis and regulation of the activity of auxin phytohormones, protection from pathogens, formation of biofilm by rhizosphere-dwelling bacteria and its disassembly or allelopathy. Knowledge on exudation of amino acids by roots of invasive *Ambrosia artemisiifolia* (Asteraceae) will be presented.
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Limitation of activity of acid *phosphomonoesterase* in soils

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Phosphomonoesterases are a group of relatively non-specific phosphohydrolases that catalyse degradation of a wide variety of phosphomonoesters. Extracellular acid phosphomonoesterase in soils is produced by bacteria, fungi and plant roots and its potential and not real activity is measured; assay conditions such as concentration of substrate, temperature, pH etc., can affect the measured activities. A study of limitation of activity of extracellular acid phosphomonoesterase in soils by diffusion of substrate was conducted. Soil samples were collected under Norway spruce stands located at different altitudes and under beech stand. Knowledge on limitation of activity of extracellular acid phosphomonoesterase in organic and mineral soils of forest ecosystems by diffusion of substrate will be presented.

Effect of methionine amino acid over the performance process and bacterial community structure of a partial bench-scale nitritation biofilter

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Abstract: Biotechnological applications of autotrophic nitrogen removal technologies for the treatment of digested sludge liquor have proliferated during the last decade. Four partial bench-scale nitritation biofilters were fed continuously with synthetic anaerobic digester supernatant containing four different concentrations of methionine (0, 100, 300 and 500 mg/L) in order to evaluate the effect of this amino acid on the performance and bacterial community structure of this biological system. Synthetic anaerobic digester supernatant containing 100 mg/L methionine reduced (60 %) the biotransformation of ammonia to nitrite, while concentrations of 300 and 500 mg/L completely inhibited this biological process. Moreover, with respect to the no-methionine scenario, addition of the amino acid promoted an increase of 30, 65 and 230 % of total bacterial biomass within the system when the digester sludge liquor was amended with 100, 300 and 500 mg/L methionine, respectively. Also, *tag*-pyrosequencing studies showed that the presence of methionine affected the bacterial community in the bioreactors, causing a virtual disappearance of the ammonium oxidizing bacteria such as *Nitrosomonas.* However, significant increases in the heterotrophic microbiota were observed including as more representative genera to *Lysobacter* and *Micavibrio.* In conclusion, the presence of high concentrations of methionine in the anaerobic digester supernatant, which can be treated in autotrophic nitrogen removal technologies, may represent a negative factor in the performance of these systems and therefore this amino acid must be removed from these influents before its treatment.

Quantitative proteomics reveals protein kinases and phosphatases in the individual phases of contextual fear conditioning in the C57BL/6 J mouse

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A series of protein kinases and phosphatases have been linked to contextual fear conditioning (cFC) but information is mainly derived from immunochemical studies. It was, therefore, decided to use a high-throughput quantitative proteomics approach to concomitantly determine protein kinases and phosphatases in hippocampi of mice in the individual phases of cFC. The C57BL/6J mice were divided into four groups in the cFC paradigm: mice with foot shock only (controls), acquisition, consolidation, and retrieval phase. Hippocampal proteins were in-solution digested and used for label-free quantitative proteomics studies using LC-MSMS. Several members of kinase/phosphatase signaling modules of mitogen-activated protein kinase (MAP3K10, RAF1, KSR2), Ca^2+/calmodulin-dependent protein kinase (CaMKIIα, DAPK1), protein kinase C (PRKCD), and subunits of protein phosphatases 1, 2A, 2B(3), 2C were identified in our study. Next, tyrosine protein kinases ABL1, SgK223, LYN; receptor tyrosine kinases IGF1R and NTRK3; serine/threonine kinases STK3, STK25, CDK4, VRK1, WNK1, and CDKL3; and kinase ADCK1 were significantly different from foot shock controls as well as receptor-type tyrosine protein phosphatase F (PTPRF), tyrosine protein phosphatases DNAJC6, UBASH3B, and ACP1, and protein phosphatase SSH2. The finding that levels of 32 out of 323 identified protein kinases or -phosphatases/subunits were modulated in cFC significantly extends knowledge on hippocampal signaling and represents a major step forward in the analysis of signaling molecule networks.

Quantitative, high-resolution localization of ionotropic glutamate receptors in the hippocampal CA1 area

Ryuichi Shigemoto, IST Austria

AMPA-type and NMDA-type glutamate receptors are major excitatory ionotropic receptors in hippocampal pyramidal cell synapses. Using quantitative SDS-digested freeze-fracture replica labeling, we investigated synaptic and extrasynaptic localization of GluA1-4 (panAMPA), GluA1, GluA2 subunits of AMPA receptors, and NR1, NR2A, and NR2B subunits of NMDA receptors in mouse CA1 pyramidal cells. Immunogold labeling for both GluA1 and GluA2 subunits were extensively observed not only in spine synapses of CA1 pyramidal cells but also in extrasynaptic sites throughout the dendritic membrane with density of up to 20 % of that in synaptic sites. The number of synaptic AMPA receptors was positively correlated with the size of IMP clusters, which represents postsynaptic density area. The synaptic as well as extrasynaptic receptors often made clusters with similar local density. In GluA1 knockout mice, the extrasynaptic AMPA receptors were mostly gone indicating that GluA1-containing AMPA receptors constitute the extrasynaptic receptors in pyramidal cells. In contrast with AMPA receptor subunits, NMDA receptor subunits were mostly observed in synaptic sites with much lower density in extrasynaptic sites. Although the number of NR1 and NR2A subunits positively correlated with IMP cluster size, number of NR2B had no correlation with the size of synapse. This difference causes distinct NR2A/NR2B ratios between small synapses on thin spines and large synapses on mushroom spines, which may result in preferential induction of long-term potentiation in small synapses.

Analysis of pre- and postsynaptic brain receptors, transporters and channels by mass spectrometry

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Receptors, transporters and channels (RTCs) are the key elements in the synaptic membranes. Nonetheless, mass spectrometry-based proteomics studies on the analyses, quantitation and the presynaptic or postsynaptic localisation of the RTCs are limited. Determining the site and level of RTCs in synaptic membranes is essential as RTCs may have different functions when differentially segregated. Synaptosomes from rat cortical tissue were isolated, pre- and postsynaptic membranes were enriched, enzymatically digested, modified by stable-isotope peptide labelling and analysed by liquid chromatography mass spectrometry. A total of 4784 synaptic proteins were identified including 274 receptors, 394 transporters/channels and 1377 transmembrane proteins. Of these, 1,781 are potential drug targets and 834 are linked to brain disorders/diseases. These findings not only provide an unprecedented resource and analytical tool for neurological RTCs, but also enable a deeper interpretation of previous work and provide a solid foundation for future design of studies in neurochemistry and neuropharmacology.

Involvement of intra-coerulear galanin in the pathophysiology and pharmacotherapy of depression

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Our knowledge about central changes underlying depressive disorders is still incomplete. While disturbances in monoaminergic neurotransmission are clearly involved, increasing evidence also proposes a role of the neuropeptide system galanin in the pathophysiology and

treatment of depression. Here, we investigated whether transcriptional processes of galanin are affected by depression-like behaviour. The abundance of galanin mRNA was increased in the paraventricular hypothalamus, the central amygdala and the locus coeruleus (LC), but not in the dorsal raphe of HAB rats characterised by high trait anxiety- and depression-related behaviour as compared with their low anxiety/depression LAB counterparts. Conversely, long-term (42 days, p.o.) treatment with desipramine, paroxetine or tranylcypromine caused a general reduction in galanin mRNA expression in the LC of unselected rats indicating a common response to antidepressant pharmacotherapy. This collective modulation of galanin mRNA in the LC by the antidepressants is opposite of the finding in the HAB model suggesting that altered coerulear galanin may be associated with depression-related behaviour. To provide functional evidence of a role of coerulear galanin in trait anxiety/depression, we demonstrate that local microinfusion of galanin, but not of the galanin receptor 2 agonist M1145, caused a pronounced increase in the immobility of LAB rats displayed in the forced swim test indicating enhanced depression-like behaviour. In contrast, the enhanced immobility of HAB rats was reduced by the unselective galanin receptor antagonist M35 applied either intra-LC or icv. Collectively, the present data suggest that HAB subjects represent a valuable model organism for studying the role of galanin in depression-related behaviour.

Intestinal depletion of NaPi-IIb/Slc34a2 in mice: renal and hormonal adaptation

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Secondary active transport of phosphate (Pi) across epithelial cells is mediated by two families of Na^+-coupled Pi cotransporters, namely Slc20 and Slc34. The Slc34 family consists of three members: NaPi-IIa, NaPi-IIb, and NaPi-IIc. NaPi-IIa and NaPi-IIc are particularly abundant in kidney, whereas NaPi-IIb is widely expressed, with intestine, lung, and testis among the organs with highest mRNA levels. In humans, mutations in NaPi-IIb associate with pulmonary alveolar microlithiasis and testicular microlithiasis. The intestinal expression of NaPi-IIb in mice is restricted to the ileum, where the cotransporter localizes specifically at brush border membrane (BBM). Its abundance is regulated by vitamin D3 and dietary Pi. Constitutive full ablation of NaPi-IIb is embryonically lethal, whereas the global but inducible removal of the transporter leads to intestinal loss of Pi and lung calcifications. We have generated a constitutive but intestinal-specific NaPi-IIb-deficient mouse model, by crossing floxed-NaPi-IIb pups with villin-Cre mice. The offspring retains expression of NaPi-IIb in lungs and testis, whereas the cotransporter is not detected in the intestinal epithelia. This constitutive intestinal ablation of NaPi-IIb results in viable and fertile pups, with normal growth. Homozygous mice are characterized by fecal wasting of Pi and complete absence of Na/Pi cotransport activity in BBM vesicles (BBMV) isolated from ileum. In contrast, the urinary excretion of Pi is reduced in these animals. The plasma levels of Pi are similar in wild-type and NaPi-IIb-deficient mice, suggesting that the renal retention of Pi fully compensates for the intestinal loss. In females, the reduced phosphaturia associates with higher expression of NaPi-IIa and higher Na/Pi cotransport activity in renal BBMV, as well as with reduced plasma levels of intact FGF-23. A similar trend is found in males. Thus, NaPi-IIb is the only luminal Na^+-dependent Pi transporter in the murine ileum and its absence is fully compensated in adult females by a mechanism involving the bone–kidney axis.

Cortico-limbic neuropeptidases after acute restraint stress

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Abstract: Oxytocin, vasopressin as well as enkephalins are neuropeptides involved in the stress response and in memory processes. These neuropeptides are partially regulated by several neuropeptidase activities such as oxytocinase/vasopressinase and enkephalinase activities. The medial prefrontal cortex, amygdala, and hippocampus are brain areas involved in the stress response. These areas constitute a circuit in which they interact with each other for such a response. Therefore, in order to investigate the role of these neuropeptidases in the response to stress, we analyzed the profile of regional distribution of these enzymatic activities as well as their patterns of interaction into this circuit after causing an acute restraint stress in adult male rats. With regard to the regional study, while most activities showed a preponderance of the amygdala on hippocampus and medial prefrontal cortex, in both groups of control and stressed animals, enkephalinase activity demonstrated a change after stress increasing in hippocampus and decreasing in amygdala. The analysis of the correlation study suggested that neuropeptidase activities are connected with the functional status of this cortico-limbic circuit, changing significantly the pattern observed in controls after acute restraint stress. In controls, the suggested connection implied essentially a positive interaction between medial prefrontal cortex and amygdala, without a clear relationship between the other regions. In marked contrast, after the applied stress, there was a highly significant change in the pattern of interactions, activating a positive correlation between medial prefrontal cortex and hippocampus and between amygdala and hippocampus and diminishing the interaction medial prefrontal cortex versus amygdala observed in controls. The global analysis of interactions between neuropeptidases and areas suggests a differential role for oxytocinase/vasopressinase activity in the medial prefrontal cortex in comparison with the rest of activities and areas. The present results support a possible role for neuropeptidases and consequently for their corresponding neuropeptidergic substrates vasopressin, oxytocin, and enkephalin in response to stress of the regions analyzed.

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Expression and Characterization of Alanine Racemase from the Green Alga *Chlamydomonas reinhardtii*

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The unicellular green alga *Chlamydomonas reinhardtii* has served as a model system to study many fundamental biological processes. We demonstrated that D-Ala has no inhibitory effect on cell growth, and alanine racemase activity was exhibited by *Chlamydomonas reinhardtii*[1)]. The amino acid sequence deduced from a hypothetical alanine racemase gene of *C. reinhardtii* showed few similarities to those for reported pyridoxal 5′-phosphate depending enzymes, suggesting that the gene product has a novel protein structure. We here report expression and enzymatic characterization of the product of putative alanine racemase gene in order to crystalize it for conformational analysis. A modified alanine racemase gene (*alr'*) whose codons were optimized for *E. coli* was synthesized and used to construct pET41b/*alr'* and to transform BL21 (DE3). SDS-PAGE of the crude extract revealed that the gene product was overexpressed. The N-terminal sequence of the protein with 45-kDa-molecular mass was MRALVSKARLAH and corresponded to the amino acid sequence deduced from the hypothetical alanine racemase gene. The gene product was purified to electrophoretic homogeneity from the recombinant cells. Amino acid racemase activity was assayed with D- and L-Ala, L-Thr, L-Pro and L-Asp as substrates. The result indicated that the enzyme was highly specific for D- and L-Ala. Alanine racemase activity for L-Ala was 38.1 nmol min^{-1} mg^{-1}, and K_m value was 12.0 mM. Activity of threonine aldolase was also assayed using alcohol dehydrogenase. The activity of D-threonine aldolase was detected, and the enzyme exhibited activity of 296 nmol min^{-1} mg^{-1}, whereas no activity of L-threonine aldolase was detected.

1. Nishimura et al. (2007) Amino Acids, 32:59–62

Structure-based computational design of HMGR/HDAC dual-action drugs for prevention and treatment of cancers and their metastasis

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Statins are HMG-CoA reductase (HMGR) inhibitors and are broadly used for the control of hypercholesterolemia. In 2007, we discovered that statins are also inhibitors of histone deacetylase (HDAC)[1], which provided the epigenetic basis for the beneficiary effects of statins for cancer inhibition and prevention. Recent evidences further indicated that the HDAC inhibitors and statins exhibited significant synergistic efficacy against cancer cell line. We proceeded to design single agents that concurrently modulate the activities of HDAC and HMGR, also known as "designed multiple ligand (DML)" in drug discovery. With the structural information from crystal complexes of HDAC and HMGR, three generations of DML using the "conjugation", "merging" and "fusion" strategies were designed and evaluated by molecular docking. The first generation, "conjugation", was designed by simply linking HDACi and HMGRi with different lengths of carbon linker. The optimal length of linker was determined by molecular docking followed by consensus scoring. To reduce the molecular weights, the second and third generations of compounds were designed by increasing the degree of overlapping among the pharmacophores of HMGRi and HDACi. All generations of compounds exhibited potent inhibitory activities against HDAC and HMGR with IC_{50} values in the nanomolar range. These compounds also effectively reduced the HMGR activity as well as promoted the acetylation of histone and tubulin in cancer cells, and were not toxic to normal cells. Our new compounds achieve several hundred folds increase of activity in the protein assays compared to the original statin compounds.[2]

1. Yi-Chu Lin, Jung-Hsin Lin, Chia-Wei Chou, Yu-Fan Chang, Shu-Hao Yeh, and Ching-Chow Chen (2008) Statins increase p21 through inhibition of histone deacetylase activity and release of promoter-associated HDAC1/2. Cancer Res 68:2375–2383

2. Jhih-Bin Chen, Ting-Rong Chern, Tzu-Tang Wei, Ching-Chow Chen, Jung-Hsin Lin*, and Jim-Min Fang* (2013) Design and synthesis of dual-action inhibitors targeting histone deacetylases and HMG-CoA reductase for cancer treatment. J Med Chem 56:3645–3655

The effect of charge state of arginine on heat-induced protein aggregation

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Protein aggregation is a common problem during purification, refolding, heat-treatment and storage in biotechnological and pharmaceutical fields. Arginine (Arg) is one of the most effective additives in suppressing protein aggregation. In order to understand the molecular mechanism of Arg as an aggregation suppressor, we investigated the effects of charged state of Arg on protein aggregation. Lysozyme at 1.0 mg/ml was heated at 90 °C in 50 mM Na-phosphate and glycine buffer (pH 7.0–10.0), resulting in denaturation followed by aggregation of the protein. The aggregation process was followed by turbidity measurement. At neutral pH, Arg dramatically suppressed protein aggregation, whereas at and above pH 9.0, the suppression effect disappeared. This pH corresponds to the pK of Arg's α-amino group, suggesting its involvement in suppressive effect of Arg. This was confirmed by testing aggregation suppression effects of acetylarginine, which showed weaker effects on protein aggregation than Arg at neutral pH. These results suggest that the positive charge of the α-amino group on Arg plays an important role in suppression of heat-induced protein aggregation. These findings provide a new insight into the mechanism of aggregation suppression effects of Arg.

New syntheses of γ-glycoamino acids and neoglycopeptides

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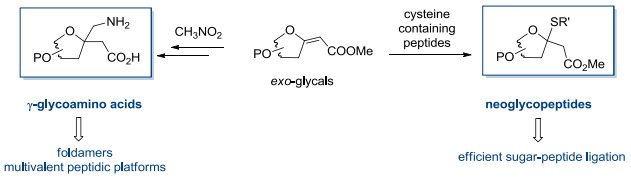
Synthetic glycopeptides with unnatural linkages can be used as probes for biochemical studies. Efficient accesses to these new glycopeptidic constructions of interest in the peptide and carbohydrate fields were recently developed in our group. Our approach was based on anomeric sugar olefins (commonly named *exo*-glycals)[1] which proved valuable substrates in the well-known Michael addition reaction. Nucleophilic addition of nitronate anion on these sugar olefins opened the way to new anomeric γ-glycoamino acids used as building blocks for glycofoldamers and multivalent platforms synthesis.[2] An efficient sugar-peptide ligation method was also developed by hydro-thiolation of *exo*-glycals with cysteine and cysteine-containing peptides [3].



Both approaches will be presented and their scopes and limitations discussed.

1. Pellegrini-Moïse N, Richard M, Chapleur Y (2014) Carbohydr. Chem. In: Rauter P, Lindhorst TK, Queneau Y (eds) 40:99–117.
2. Richard M, Felten A-S, Didierjean C, Ruiz-Lopez M, Chapleur Y, Pellegrini-Moïse N (2014) Eur. J. Org. Chem. 7364–7376.
3. Richard M, Didierjean C, Chapleur Y, Pellegrini-Moïse N (2015) Eur. J. Org. Chem. 2632–2645.

Post-translational modifications of clock proteins bolster circadian rhythms

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Circadian rhythms with a period of ~24 h are generated by an internal timekeeping mechanism referred to as circadian clock. The mammalian clocks are driven by a transcription–translation-based negative feedback loop, in which CRY1/2 repressor proteins are finely regulated by posttranslational modifications for temporally coordinated transcription rhythms of clock genes. We previously reported a posttranslational mechanism regulating the stability of CRY2 protein. The priming phosphorylation of CRY2 at Ser557 allows subsequent phosphorylation at Ser553 and the two-step phosphorylation of CRY2 leads to its proteasomal degradation (Kurabayashi et al., *MCB*, 2010; Hirano et al., *MCB*, 2014). On the other hand, FBXL3, an F-box-type E3 ligase, ubiquitinates CRYs and mediates their degradation. We found that its closely related paralog, FBXL21, also ubiquitinates CRYs. *Fbxl21* null mice exhibited normal periodicity of behavioral rhythms (tau: 23.9 h), while an extremely long period phenotype of *Fbxl3* null mice (tau: 27.7 h) was largely attenuated in *Fbxl3*/*Fbxl21* double-knockout mice (tau: 25.6 h). Intriguingly, the double knockout destabilized the behavioral rhythms in DD and sometimes elicited arrhythmic behaviors. Surprisingly, FBXL21 stabilized CRY proteins and antagonized the destabilizing action of FBXL3 by conjugating a polyubiquitin chain(s) different from those formed by FBXL3 (Hirano et al. & Yoo et al., *Cell*, 2013). These observations emphasize the physiological importance of antagonizing actions between FBXL21 and FBXL3 on CRYs providing stable oscillation of the circadian clock. Collectively posttranslational modifications of clock proteins play essential roles in timekeeping of the circadian circuits.

Therapeutic proteins and peptides: recent advances in the research and development for hereditary angioedema

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Human plasma proteome is an exceptional source of native proteins that are essential for understanding molecular basis of many diseases. Collected human plasma remains the major source of a wide range of therapeutic products for treatment and/or prevention of various clinical conditions, many of which are caused by a protein deficiency. In addition to albumin products, fractionation of human plasma yields immunoglobulins, alpha-1-antitrypsin, antithrombin, factors VIII and IX, C1-esterase inhibitor, and several other therapeutic proteins. During the last two decades, recombinant technologies and transgenic animal platform greatly contributed to the field and dramatically extended the therapeutic options now available for many serious diseases. This presentation will focus on recent advances in the research and development for hereditary angioedema (HAE), a rare potentially life-threatening disorder associated with a deficiency of functional C1-esterase inhibitor. Until recently, in the United States only fresh-frozen plasma, attenuated androgens or plasmin inhibitors were used to provide relief during acute attacks, but no HAE-targeted therapy was available. Since 2008, when first plasma-derived C1-esterase inhibitor concentrate was approved by the US Food and Drug Administration, several HAE-specific therapies have been developed. Current understanding of HAE mechanism(s) and recent therapeutic interventions will be discussed in this presentation, as well as some research approaches to enhance currently available HAE therapies.

Regulation and physiological function of cationic amino acid transporters

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Cationic amino acids (CAA), such as arginine, lysine, and ornithine, share the same transport proteins. Most mammalian cells express two types of CAA transport proteins, mediating activities of the so-called systems y^+ (specific for CAA) and y^+ L (CAA and neutral AA), respectively. Although only distantly related, the two transporter types belong to the same gene family: SLC7. The family members SLC7A1-A3 correspond to the CAT proteins (CAT for CAA transporter) mediating system y^+ activity. They seem to be the major entry path for CAA in most cells. SLC7A4 and A14 have also been attributed to the CAT-subfamily. Their function remains, however, elusive to date. Besides providing cells with CAA for protein synthesis and energy supply, CATs seem to be involved in important signal pathways such as nitric oxide, mTor, and neurotransmission. CAT-1 and CAT-2 are extensively regulated on the level of both transcription and translation. In addition, protein kinase C and small G proteins regulate the location of these transporters in the plasma membrane. SLC7A6 and 7 encode for system y^+ L transporter y^+ LAT2 and 1, respectively. They catalyze the exchange of CAA against NAA plus Na^+ and thus seem to be rather CAA exporters under physiological conditions. One of their functions in endothelial cells may be the export of CAA derivatives such as the nitric oxide synthase (NOS) inhibitor asymmetrical dimethyl arginine (ADMA). ADMA is an independent risk factor for cardiovascular disease. There is increasing evidence that accumulation of ADMA leads to endothelial dysfunction through inhibition of endothelial NOS. Reduced activity of y^+ LATs may thus contribute to endothelial dysfunction.

Proline methanologues: Design, Synthesis, Oligomeric Structures, and Potential Applications in Medicinal Chemistry

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Proline plays a prominent role as a component of mammalian proteins. It is a unique "cyclic" amino acid which forms tertiary amide bonds resulting in *cis–trans* isomerism in proline-containing peptides. It is also an important constituent of β-turns and reversed β-turns. Introducing substituents on the proline ring results in conformational preferences that have relevance in its susceptibility to enzymatic degradation. Attaching rings of different sizes to the proline backbone leads to bicyclic variants as peptidomimetics and to azabicyclic carboxylic acids and related derivatives as components of drugs for cardiovascular diseases among others. 4,5-Methanoprolines are particularly interesting *proline methanologues* because the fusion of the cyclopropane unit results in the flattening of the proline ring depending on the stereochemistry of the ring junctions. We shall discuss the synthesis, conformational properties, and oligomeric structures of *cis*- and *trans*-4,5-methano-L-prolines and applications in the design of potential drug prototypes. The first synthesis of *cis*- and *trans*-4,5-methanoprolines and their ring flattening effect were reported from our laboratory (*Angew. Chem. Int. Ed.* **1997**, *36*, 1881). Since then, these conformationally constrained methanologues have found extensive applications in medicinal chemistry and as components of marketed drugs.

Collaborators: Gilles Berger, Miguel Angel Vilchis Reyes, Ingrid Chab Majdalani