Poster presentations

Topic E

E1 - Peptide delivery approaches
E2 - Peptide libraries and molecular diversity
E3 - Peptides in diagnostics, pharmacology and biotechnology
E4 - Role of peptides in genomics and proteomics

P E1 - Cell Penetration by primary amphipathic peptides: A) structural properties and interactions with lipids

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Two primary amphipeptides have been synthesized by SPPS using the FMOC strategy:

P_α: Ac G A L F L A F L A A A L S L M G L	WSQP	K K K R K V Cya
P_β: Ac G A L F L G F L G A A G S T M G A	WSQP	K K K R K V Cya
Hydrophobic domain	*Linker*	*Hydrophobic domain*

In both peptides the linker (WSQP) and the hydrophilic domain (NLS sequence) are identical while the hydrophobic domain is a sequence issued from the fusion peptide GP41 of HIV1; in this last part some residues were substituted according to the AGADIR software in order to obtain the P_α peptide which is predicted to adopt a helical conformation.
Structural properties, physicochemical characteristics in monolayers, and interactions with lipids of both peptides are compared.
FTIR and CD measurements reveal that P_α is in a helical conformation whatever its environment, whereas P_β is non structured in aqueous solution and adopts a β-sheet conformation in a lipidic environment.
Surface pressure and surface potential measurements carried out on peptide monolayers indicate that hydrophobic part of both peptides are orientated parallel to the air-water interface.
The penetration experiments into liquid expanded lipidic monolayers give identical results for P_α with neutral and negatively charged lipids, whereas P_β is more selective for negative polar headgroups
Analysis of mixed lipid-peptide surface pressure isotherms show that both peptides are miscible with neutral and negatively charged lipids in a liquid expanded state. Interactions are expansive between P_α and lipids, but they are contractive between P_β and lipids.
Atomic force microscopy observations are made on Langmuir-Blodgett monolayers transferred onto mica in order to compare the lateral organization of peptides when engaged into lipidic media.
Taking into account all these results we can assume that P_α has a stronger hydrophobic character than P_β and therefore it interacts more strongly with the hydrophobic chains of lipids, while P_β is less hydrophobic and interacts also with polar heads. This difference could be at the origin of the different cellular observed when comparing cell penetrations of these two peptides (see part B: targetting subcellular compartments).

P E2 - Cell penetration by primary amphipathic peptides: B) targeting subcellular compartments.

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A number of natural and synthetic peptides are able to translocate various cell membranes and have been used successfully to deliver hydrophilic molecules such as nucleic acids. These systems have been now extended to carry peptides and proteins through the plasma membrane. Most methods involve transport of genes of interest into cells via the endosomal pathway leading to the rapid degradation of the genes in these acidic compartments and therefore their poor release into the cytoplasm. Alternatively, our group has designed a peptide-based gene delivery system called MPG that bypasses the endosomal pathway. MPG associates a fusion sequence derived from the hydrophobic fusion peptide of HIV gp41 and the hydrophilic nuclear localization sequence (NLS) of SV40 large T antigen. This peptide can promote efficiently the delivery of short oligonucleotides and larger plasmids into a wide variety of cell lines independently of the endosomal pathway. However, the mechanism of the translocation process is poorly characterized despite several structural studies carried out on these amphipathic peptides. Moreover, little is known about the intrinsic characteristics from the peptide (sequence, conformation, aggregation) governing its final localization. In this study, the translocation process and the final localization of two versions of MPG called Pα and Pβ were analyzed in cell tissue culture. Both peptides have a similar sequence but adopt a distinct conformation in the presence of lipids (α helices for Pα and β-sheet for Pβ) *see part A of this study "Structural properties and interactions with lipids"*. The objective was to define whether or not the conformation of the peptides governs the mechanism of their cellular uptake. The internalization pathway of each fluorescently labeled peptide was monitored by fluorescence microscopy and the cellular compartments in which these peptides accumulate, were identified by immunofluorescence using specific markers of the endosome, lysosome, golgi or endoplasmic reticulum. As expected, the cellular uptake of Pα involved an energy-dependent endocytosis pathway and was dramatically reduced in presence of endocytosis inhibitors such as cytochalasin D or nocodazole. Furthermore, the release from the endosome was improved by adding the destabilizing reagent chloroquine confirming this mechanism. In contrast, Pβ showed a very different behavior by crossing the plasma membrane without the formation of subcellular vesicles or caveolae. Since Pα and Pβ share similar sequence and hydrophobicity of their N-terminal part (the C-terminal part being identical), these results strongly suggest that the conformation of the peptide at the initial step of the translocation process influences the mechanism of cell entry favoring either a direct transfer through the lipid bilayer or an endocytosis pathway. Time course analyses in cell tissue culture were performed with these two MPG conformers and Pα was held in the plasma membrane much longer than Pβ. This result was in agreement with the structural study showing an increase of hydrophobicity induced by the helical conformation. Furthermore, these results gave new insights into the mechanism by which MPG derivatives are taken up by cells and represent a new approach to target specifically a cellular compartment by modifying the hydrophobic sequence of the vector peptide.

P E3 - Development of a new derivative of vasoactive intestinal peptide and its novel administration system, dry powder inhalation

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Vasoactive intestinal peptide (VIP) was first isolated from porcine duodenum by Said S.I. et al. in 1970 and acts as the neurotransmitter in vasodilation, hypotension, the secretion of regulatory hormones, the inhibiotory effect of gastric-acid secretion and gastric-motility, pancreatic exocrine, bronchodilation and anti-inflammation. Upon these biological activities, VIP was believed to be a candidate for a number of potential therapeutic products. However, the VIP, as well as the other general peptide, is extremely sensitive to the endogenous enzymes, therefore it has to be digested immediately after administration. In this investigation, the chemical modification of VIP led to the development of a potent agonist of the VIP receptor (Table 1). In addition, further study clearly indicated that the developed VIP analogue has valuable properties, including resistance to the peptidases and the extended duration of its bronchodilation in vivo and in vitro.
It is well-established that VIP displays wide distribution especially in the central and the peripheral nervous system, and its specific receptors are expressed in many tissues including the aorta, brain, lung and gastrointestinal tract. Therefore, there is the probability that undesired side effects will appear if these VIP-related compounds are used as pharmaceutical agents for the treatment of asthma by injection. To avoid such serious side effects, an effective dosage form was investigated, which allows a topical application. Considering their physicochemical and biochemical properties, dry powder inhalation (DPI), a novel drug delivery system, is suitable for the bronchial administration of VIP-related materials. This study's medical form is shown in Figure 1. Fine particles (drug and vehicle particles) were processed with the use of a Jet Mill, and the pharmaceutics were micronized into ca. 3 mm to ensure arrival at the bronchi. Then, the fine particles were stabilized physicochemically with the use of the carrier molecule (Fig. 1; LEFT), because the fine particles of peptide/protein generally have strong cohesive and adhesive attraction resulting in association with each other. But the adhesion between the large carrier and the particles also tended to be somewhat strong. Therefore, an investigation was conducted on the optimal condition of the DPI formula, providing high emission from the capsule and a fine respirable fraction (RF) value that was investigated using the cascade impactor after aerosolization at 28.3 L/min via a Jet Haler®. Wide-ranged modification of the DPI formula resulted in the marked increase of the RF value, and this new DPI system could be applied to any types of peptide/protein that were tested. Especially, with the use of erythritol as the carrier molecule, the RF value was 5 times as large as that of the lactose carrier.
These findings suggest that the use of this developed medication system is a useful approach for the treatment of asthma using novel VIP-analogue.

VIP	H S D A V F T D N Y T R L R K Q M A V K K Y L N S I L N -NH_2
VIP analogue	H S D A V F T D N Y T R L R *R Q I A V R R* Y L N S I L N *G R R* -NH_2

Table 1 - Sequences of VIP and VIP analogue. The italic letters indicate the amino acid residues that are different between VIP.

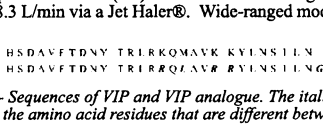

Fig. 1 - Schematic presentation of DPI. Carrier with fine particles are separated and dispersed by the aid of a Jet Haler®.

P E4 - Cellular internalisation of calcitonin derived carrier peptides-investigation of mechanism and applications

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The therapeutic potential of biopolymers, e.g., peptides, proteins or oligonucleotids, for the treatment of numerous human diseases is very promising. However, since most of these compounds show only poor bioavailability, it is difficult to establish effective therapeutic strategies. Examples of limiting factors are the hydrophobicity and charge selectivity of the lipid membrane, tight junctional and enzymatic barrier functions of the biomembranes as well as the large molecular size and high charge density or polarity of the biopolymers. Various attempts to overcome these problems have been made so far, e.g., chemical modifications or galenical formulations. Carrier peptides with membrane translocating ability are considered as very promising tools for drug delivery systems. Carrier peptides are also helpful tools for the investigation of signal transduction, intracellular trafficking or cell-cell communication. Currently used methods are either harmful to the cells (disrupting the cell membrane) or can only be applied to a limited number of cells (e.g. microcapillary injection). With little or no effect on cell viability carrier peptides can shuttle their cargoes (e.g., peptides, proteins or nucleotides) across cell membranes. Several sequences are currently used as carrier peptides, e.g. HIV Tat-protein derived peptides, penetratin or transportan. We study the suitability of human calcitonin (hCT) derived carrier peptides. Due to its physiological origin as a human peptide hormone, intrinsic toxicity or antigenicity are not expected. Since the N-terminal part of the hormone, that is responsible for the activation of the hCT-receptor, hormone side effects are unlikely with respect to a future therapeutic application.
The mechanism of uptake of carrier peptides is still not completely understood. However, different mechanisms seem to be involved for the different sequences, also the size of the cargo could influence the cellular uptake. Former studies revealed that hCT and its C-terminal fragment hCT (9-32) are internalised by the nasal epithelium and various cell lines via an endocytotic pathway [1]. In this study we further examined the uptake and intracellular fate of a hCT-derived carrier peptide by fluorescence microscopy and confocal laser scanning microscopy (CLSM), constructed an enzymatic cleavage site for rapid release of the cargo once internalised and added a nuclear translocation sequence (NLS). We also coupled the anti-tumor drug daunorubicin via two different linkers to the carrier peptide, one linker acid-labile, the other stable at pH 5. Daunorubicin coupled via the acid-labile linker was active, while in the other case no cytotoxic activity could be determined. This confirms the endocytotic uptake, release of the acid-labile conjugated drug in the endosomal compartments (pH ~ 5-6), while the stable conjugate is inactive.

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P E5 - Enhanced intracellular PNA concentration and antisense activity mediated by a cell-penetrating amphipathic model peptide

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The α-helical amphipathic model peptide KLALK LALKA LKAAL KLA-NH_2, previously shown to enter mammalian cells nonendocytically and to shuttle polar compounds into the cell interior, was covalently attached to a 12-mer peptide nucleic acid (5'-GGA GCA GGA AAG-3') directed against the m-RNA of the nociceptin/orphanin FQ receptor. The cellular uptake into CHO cells and neonatal rat cardiomyocytes of the fluorescein-labelled PNA and its peptide-conjugate was studied both by means of confocal laser scanning microscopy and of capillary electrophoresis combined with laser-induced fluorescence detection. For the PNA alone an internalization approaching the external concentration was found after 30 min exposure to the cells, which, however, was significantly lower at reduced temperature and after 2-deoxyglucose/sodium azide treatment, suggesting endocytosis to be involved. The conjugation of the PNA with the cell-penetrating model peptide resulted in an up to ten-fold enhanced intracellular PNA level as compared to that achieved with the PNA alone. Reduced temperature or energy depletion showed no significant influence in this case, suggesting a nonendocytic mechanism to be operative. Analogously, incubation of spontaneously beating neonatal rat cardiomyocytes with the conjugate lead to a significantly stronger downregulation of the nociceptin/orphanin FQ receptor than found after exposure to the PNA alone, as inferred from the reduction of the chronotropic effect of the neuropeptide nociceptin to lower than 10 percent and to about 50 percent, respectively. Treatment with respective scrambled PNA-sequences remained without influence, indicating specificity for the observed effects. Our results raise the possibility of exploiting simple synthetic peptides for improving the intracellular availability of PNA's, and so to overcome one of the most serious disadvantages compromising the utilization of these valuable compounds as scientific tools or potential therapeutics.

P E6 - A novel immunoadjuvant carrier system for synthetic vaccines

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The objective of this study was to explore the possibility of developing peptide vaccines for immunization without the use of any conventional adjuvant. A Lipidic-Polylysine Core-peptide (LCP) construct [1] was developed by combining Tam's Multiple Antigenic Peptide system [2] and Jung's multiple palmitoyl- containing system [3]. The LCP system (Figure 1) is constructed by incorporating multiple copies of one or two peptide epitopes on a lipophilic multiplying core. In this anchor, three copies of lipoamino acids (LAA) and two glycines were employed as spacers between the solid support and the lipoamino acids. The incorporated peptides are Group A Streptococci (GAS) epitopes from the bacterial surface M protein. The M protein is composed of a highly variable amino terminus region, which defines the GAS serotype. It is an important virulence factor during GAS infection and antibodies directed to the M protein type-specific and conserved regions are important in the development of protective immunity. The synthesized LCP constructs contain peptide epitopes either from the N-terminus type-specific region and/or the conserved C-terminus region. One of the constructs, which served as a control, contains the same non-specific peptide sequence in all positions, and one of the constructs has no LAA content; it was constructed only on the branching Lys-residues containing a Gly-spacer between the solid support and the peptide-construct. Serum IgG antibody responses to the constructs were measured in mice immunised with the LCP constructs alone or with adjuvant. Mice were also challenged with the GAS strains corresponding to the serotype of the peptides incorporated into the construct. The synthesis and immunological properties of the lipid-core compounds will be discussed.

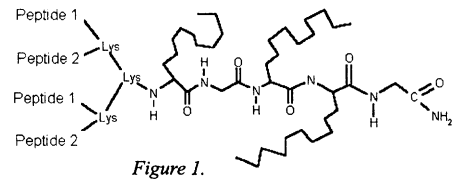

Figure 1.

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P E7 - Efficient intracellular delivery of proteins and low molecular weight substances via polyoma virus-like particles (PVLP)

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For most polypeptides and many low molecular weight substances (LMW) it is impossible to cross the cell membrane. Mimicking the natural ability of viruses to enter cells we developed a delivery system based on polyoma virus-like particles (PVLP). We found an efficient way to achieve selective PVLP-encapsidation of peptides and LMWs. The molecules of interest are bound to the inner side of the recombinantly expressed polyoma virus major capsoid protein VP1 by a molecular anchor which is derived from the minor coat protein VP2.
Polyoma capsoids loaded with EGFP-anchor fusion proteins (EGFP-PVLP) showed successfull intracellular delivery of EGFP into Swiss 3T3 mouse fibroblasts. The intracellular distribution was monitored by fluorescence microscopy using the autofluorescence of EGFP and indirect immunofluorescence for VP1. The EGFP-capsoids attached to the cell membrane and were subsequently internalized. EGFP was found in vesicular structures and could be colocalized with VP1.
To demonstrate the delivery of a biological active molecule Methotrexate (MTX), an inhibitor of folate-dependent enzymes, an MTX-anchor conjugate was encapsidated by PVLP. After assembly the MTX-PVLP capsoids were incubated on an acute lymphoblastic T-cell leukemia cell line (CCRF-CEM). VP1-encapsidated MTX induced cell death whereas free VP2-MTX-conjugate or empty PVLP had no effect. The PVLP drug delivery system offers high flexibility in application since the components are seperately available, modifiable and assembled in vitro to the operative entity.

P E8 - Specificity of PDZ interaction investigated by NMR spectroscopy and synthetic peptide libraries

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PDZ domains are modular protein interaction domains that bind to short C-terminal peptides [1] as well as to internal peptides folded as a β-finger in a sequence-specific fashion. The protein AF6 contains a single PDZ domain and has been originally identified as a fusion to ALL-1 in acute myeloid leukaemia [2]. It has been demonstrated that the PDZ domain of AF6 interacts with a subset of members of the ephrine (Eph) subfamily of receptor tyrosine kinases (RTKs) via their C-termini [3].
We investigated the interaction between the PDZ domain of AF6 and short synthetic peptides [e.g. AQMNQIQSVEV] of the C-terminal region of EphB2-RTK using NMR spectroscopy and synthetic peptide libraries generated by spot synthesis [4].
Spot synthesis allows the parallel screening of thousands of cellulose-bound peptides to study protein-protein interactions. Conventionally the peptides are synthesized from C- to N-terminus and are thus C-terminally fixed to the solid support. Here we developed a new strategy for cellulose-bound peptides with free C-termini, using Fmoc-amino acid 3-bromopropyl esters, mercapto functionalized cellulose membranes and a chemoselective cyclization step [5]. Using this technique, the binding specificity of the AF6 PDZ domain was characterized. Substitutional analysis, in which each position was exchanged by all 20 natural amino acids, of the wildtyp peptide AQMNQIQSVEV show that the four C-terminal positions (SVEV) are key residues for binding. The NMR measurements (e.g. ^{15}N-HSQC) enable the exposure of the PDZ domain binding pocket. The successive titration of a ligand to the PDZ domain showed chemical shifts of the PDZ domain amino acid, that are involved in the interaction with the ligand. Based on these results we have designed new peptide libraries using L-amino acids, D-amino acids and other non-natural amino-modules to obtain analogs with enhanced affinity for the PDZ domain. In the future the generated analogs will be also analysed by NMR spectroscopy to investigate adaptability of the AF6 PDZ binding pocket. These derivates should give new insights into the biological function (specificity) and the cellular localisation of the PDZ domain, which might be utilized for further pharmacological studies.

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P E9 - Peptide chemistry: the source of combinatorial methods

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It is a commonplace today that introduction of combinatorial synthetic methods brought about a revolution in pharmaceutical research and also enriched other branches of science. The roots of combinatorial chemistry grew out from peptide chemistry. There were good reasons for that. First of all, the number of possible peptide sequences is limited. It was easy to speculate about all tetrapeptides and about their potential preparation, for example, because their number, 160 thousand, could easily be calculated. The second reason is that introduction by Merrifield of the solid phase synthesis made preparation of the majority of peptides very simple: just repetition of the few operations of a coupling cycle. Furthermore, the peptides could be produced without the need of complicated and tme consuming purification steps. The presentation will show that the majority of the combinatorial synthetic and deconvolution methods were exemplified by using peptide chemistry.
The examples will include split-mix synthesis and the deconvolution methods: iteration, positional scanning, omission and amino acid tester libraries, binding tests with tethered libraries and the use of encoding methods. Further examples are string synthesis, light directed spatially addressable synthesis, biological methods of preparations of peptide libraries and procedures of parallel synthesis. Some earlier non-peptidic methods that did not received wide acceptance will also be mentioned.

P E10 - Automated, parallel synthesis of a guanidinio-carbonyl pyrrole receptor library for stereo-selective complexation of small peptides and amino acid carboxylates in aqueous solutions.

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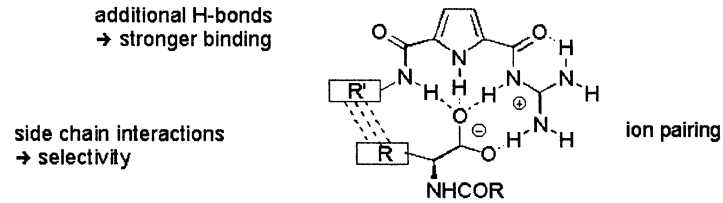
Getting a deeper insight into molecular recognition is necessary for a better understanding of diseases like Altzheimer, BSE and its human versions, where self-aggregation of peptide fragments plays an important role. 2-(guanidiniocarbonyl)-1*H*-pyrroles bind carboxylates by ion pairing in combination with multiple hydrogen bonds even in highly polar solvents. Hence, they can be used as receptors for the stereoselective complexation of amino acid and small peptide carboxylates in aqueous solvents. To explore the binding properties of such systems in terms of binding strength and steresoelectivity of the recognition event, a systematic variation of the receptor structure is necessary. In this context, we used solution phase automated synthesis to prepare 24 different receptors by coupling the parent guanidiniocarbonyl-pyrrole-carboxylate zwitterion with various amines using PYBOB as the coupling reagent in DMF. Reaction and workup (incl. evaporation of the solvent and precipitation of the products as picrates) has been carried out unattended on a CHEMSPEED ASW-2000 workstation. This research project is part of a collaboration between Chemspeed and Dr. Carsten Schmuck of the University of Cologne, Germany.



P E11 - The library of p-nitrophenyl esters immobilized on celluloze membrane. Synthesis and degradation by tissue homogenates of Lewis lung carcinoma bearing mice

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It is expected that a conjugate of pro-drug with some oligopeptides could be selectively splitted inside a specific tissue introducing active agents directly into the target organs. In case of alkylating cytostatic agents, this could allow to introduce an alkylating molecule directly into the cancer tissue.
In order to select the tissue-specific amino-acid sequence of oligopeptide chain, a spatially addressed library of *p*-nitrophenyl esters of oligopeptides immobilized on cellulose membrane has been obtained.
To enhance an exposition of C-terminal fragment to degrading enzymes, oligopeptides were anchored to the cellulose support with N-terminal amino-acid via triazine linker. The library of peptides has been synthesized in the so-called "inversed approach"[1], starting from the N-terminal amino-acid. All peptide and ester bonds were obtained by means of CDMT in the presence of NMM under standard conditions.
After digestion of the library with appropriate tissue homogenate, susceptibility of the sequences towards the treatment were detemined by colorimetric test. The amount of *p*-nitrophenyl esters remaining undegraded was measured after developing coloration of the matrix in reaction with appropriate dying reagent[2].
Peptides degraded the most intensive in the presence of homogenate of LL metastatic colonies and relatively resistant towards blood serum were selected for further studies.

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P E12 - Evolution of Puumala virus neutralization site mimic

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We have previously selected from a random peptide library expressed on the pIII protein of the filamentous phage fd-tet a peptide insert FPCDRLSGYWERGIPSPCVR, which recognizes the Puumala virus glycoprotein 2-specific neutralizing monoclonal antibody (MAb) 1C9 with Kd of 2.85×10^{-8}. We have now created a second-generation phage-displayed peptide library in which each amino acid was mutated randomly to another with a certain probability. Peptides binding tightest to MAb 1C9 and peptides, which bind to a partially cross-reactive MAb 4G2 were selected. The resulting peptides were synthesized as spots on cellulose membrane and allowed to bind to the MAb 1C9. Binding-improving changes were combined in the peptide ATCDKLFGYYERGIPLPCAL with Kd of 1.49×10^{-9}. The change of tryptophan to tyrosine in a second-generation MAb 1C9-selected peptide improved the binding affinity. In selections with MAb 4G2 loss of proline in the second position correlated with improved binding of phage-displayed peptides to MAb 4G2 as compared to binding to MAb 1C9. The alanine scanning and determination of minimal binding site with peptides synthesized on membrane were compared with the second-generation library repanning with MAb 1C9. The comparison shows that semi-random second-generation phage libraries may be used to determine the importance of each amino acid in the reacting peptide. An additional benefit is the finding of variants with new properties.

P E13 - Binding specificity of 42 synthetic WW domains

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WW domains are small protein modules consisting of about 35 amino acids with two highly conserved tryptophan residues. They are the smallest natural antiparallel beta-sheet structures known and stable without disulfide bridges. WW domains are found in a wide range of signaling and regulatory proteins mediating protein-protein interactions through binding to short proline-rich sequences. They have been divided into subgroups according to their binding to different consensus motifs, which may include phosphorylated amino acids [1]. Detailed knowledge of WW specificity patterns should lead to a better understanding of intracellular interaction networks.
42 WW domains (29 human, 2 mouse and 11 yeast) were synthesized and used in soluble form for ^{1}H-NMR-studies and printed on a cellulose membrane for solid phase binding assays (the "WW array"). The binding partners comprised peptide ligands known from the literature as well as designed prolin-rich model peptides, including tyrosine or serine/threonine phosphorylated peptides. 28 of the 42 synthetic WW domains were found to be folded without ligand and for 32 domains binding to peptide ligands was detected. For selected domains further binding studies with sets of substitution analogs of different ligands were performed to identify key interacting residues. Some domains were found to bind stretches of prolines without the need for any other specificity determining residues.
The experiments revealed that distinct WW sequence patterns correlate with the binding to specific ligand motifs. These sequence-activity relationship is being used for the prediction of potential activities of other members of the WW family, particularly for domains with unknown function.
As a matter of particular interest in WW domain recognition processes we investigated ligand phosphorylation which has been proposed to play a regulatory role in vivo, acting as a "negative" or "positive switch" [1]. A cellulose-bound peptide library consisiting of 600 potential target sequences for kinases was synthesized by spot-synthesis [2] in phosphorylated and unphosphorylated form and screened with different WW domains. Selective binding to phosphorylated peptides but not to the unphosphorylated counterparts was found for several domains.

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P E14 - Modification of secondary structures by combinatorial chemistry: a new solid phase based screening test for protein folding detection

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Modification of proteins to obtain new structures with improved stability to physiologic media has become of great interest in peptide chemistry. However, the size of this kind of systems has limited the number of methodologies that can be applied to biological approaches (phage display libraries, mutation of proteins...) or de novo design strategies, restraining either the number of amino acids (natural amino acids only) or structures that can be analyzed.
With the aim of improving both the rate of analysis of structures and the number of amino acids that can be used, we present a new methodology for the synthesis, screening and deconvolution of one-bead-one-compound combinatorial libraries directed to the modification of secondary structures in protein natural domains. This methodology has beed developed using the B domain of the protein A as model. The screening test (see figure below) is based on the detection of well folded domains through their activity, such is the interaction with mammalian immunoglobulins G in our case. Folding process is directed by non-covalent interactions between the secondary structure to be modified, which remains reversibly attached to solid phase, and the rest of the domain in solution. Deconvolution can be accomplished by MALDI-TOF PSD (Post-Source Decay) with the picomolar amount of peptide contained on a single bead, directly cleaved on MALDI-TOF sample plates.

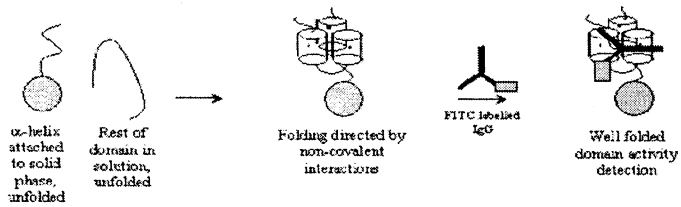

Fig. 1 - Scheme of the solid phase based screening test developed for protein folding detection

P E15 - Investigation of heterospecific coiled coil interactions by means of synthetic peptide libraries

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The coiled coil is an ubiquitous protein motif that is often used to control oligomerisation. It is found in many types of proteins, including transcription factors (e.g. GCN4), viral fusion peptides, SNARE complexes and certain tRNA synthetases. Very long coiled coils are found in proteins such as tropomyosin, intermediate filaments and spindle-pole-body components [1].
Coiled coils are ideal candidates for protein folding and design studies, as they represent probably the simplest tertiary structure. They involve a number of alpha-helices wound around each other in a highly organized manner. There may be between two and five helices in the structure, although dimers and trimers are the most common [2]. The helices may be from the same or from different proteins [3].
Our goal is to understand the driving forces of heterospecific coiled coil interactions using synthetic solid phase bound peptide libraries of GCN4 leucine zipper variants. Ultimately, we want to select for metabolically stabel dimeric coiled coils with a high degree of heterospecificity.
We synthesized two libraries of GCN4 leucine zipper variants by means of SPOT synthesis [4,5] which were tested for heterospecific binding to wild type GCN4 leucine zipper: 1) a substitutional analysis where each residue of the GCN4 leucine zipper was individually substituted with all 20 natural amino acids and 2) a library where double substitutions were introduced on the constitutive zipper residues.
These approaches allowed the finding of novel GCN4 leucine zipper variants that bind heterospecifically to wild type GCN4 leucine zipper.

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P E16 - Selection of peptides homing to angiogenic vessels and the application of the novel peptides to the anti-neovascular therapy

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In vivo selection of peptides having affinity to a target tissue, phage peptide library is a useful technology. On the other hand, cancer chemotherapy targeted to angiogenic vessels is expected to cause indirect tumor regression through the damage of the neovasculature without the induction of drug resistance. To develop a neovasculature-specific drug delivery system, we isolated novel peptides homing to angiogenic vessels formed by a dorsal air sac method from a phage-displayed 15mer peptide library. Three distinct phage clones selected by the biopanning prensented following amino acid sequences, PRPGAPLAGSWPGTS, DRWRPALPVVLFPLH, or ASSSYPLIHWRPWAR and they showed the marked accumulation in murine tumor xenografts, respectively. The truncated peptides were synthesized from these peptides and we found that the pentamer sequences containing WRP or PRP showed similar inhibitory activity against the accumulation of the selected phage clones to the tumor tissue. Then we synthesized C18-acylated pentamer peptides and prepared cholesterol-lecithin liposomes containing the C18-pentamer peptides. Liposome modified with C18-APRPG showed high accumulation in murine tumor xenografts, and APRPG-modified liposome encapsulating Adriamycin effectively suppressed experimental tumor growth. Next, we investigated whether the peptides selected in murine angiogenic model have affinity for angiogenic endothelium derived from human tissues. Confocal observation demonstrated that the APRPG-modified liposome specifically bind to the human umbilical vein endothelial cells (HUVEC) only when the cells are stimulated with VEGF. Furthermore, histochemical analysis demonstrated that biotinylated PRP-containing peptide specifically bound to angiogenic endothelium in human tumor tissues. These data indicate that PRP-containing peptides may be useful for human cancer treatment.

P E17 - A constrained α-helical library

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Peptide libraries are useful in the discovery of new compounds that can be used as vaccines, diagnostics or leads in the development of new drugs. Successful peptide libraries often incorporate structural elements that restrict the conformation of the peptide chain(s). We have previously described the synthesis of a 27-residue disulfide-bridged homo-two-stranded coiled-coil template with two lactam bridges per strand which displays 5 library positions on the surface of each helix for screening. With this design, we were able to show that specific residues in the 5 library positions of the coiled-coil template could mimic the binding of a lipopolysaccharide to its antibody [1]. Subsequently, we improved upon this design by changing the template to a hetero-two-stranded molecule with a "backing strand" and a single library display strand. The advantages of this heterostranded design (compared to the homostarnded design) was the dramatic improvement in stability of destabilizing sequences, improved solubility of hydrophobic sequences and a single display which eliminated the complications associated with dual display [2]. In the present study, we have now further modified the template to incorporate a DNP-group on a Lys side chain in the display and backing strands for colorimetric and antibody detection (DNP-specific antibody) in direct binding assays. As a test case, we show the screening of the library against the target proteins, troponin c and calmodulin known to bind to a variety of ligands (helical and non-helical). We show the identification of several unique hits based on the new detection strategy which has allowed us to derive troponin c specific inhibitors compared to calmodulin.

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P E18 - Solid-phase tryptophan modification: use of a rational combinatorial chemistry approach to develop new analgesic drugs

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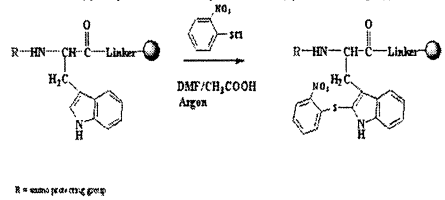
Increasingly, indoles are considered to be promising molecules as lead structures for the design of new drugs. Compounds containing the indole ring are reported to exhibit numerous biological activities, and include different G-protein coupled receptor ligands and peptido-leukotriene antagonists[1]. Small peptide molecules containing the 2-[(o-nitrophenyl)sulfenyl] tryptophan moiety are also reported to show antinociceptive activity [2].
Starting from these lead compounds we have designed a small library by solid phase strategy in order to obtain new analgesics with good affinity and selectivity for the VR1 nociceptive receptor [3]. This promising therapeutic target of the neuropatic pain and other pathological conditions involving C-fibers neurons acts through the desensibilization of the receptor caused by specific ligands. The choice of the appropriate conditions for the derivatization of the tryptophan ring in solid phase is the key factor in establishing an efficient strategy to obtain the library and so to explore the ability of the 2-substituted indole moiety as a pharmacophore group for the antinociceptive activity. In the present comunication the establishment of the conditions to obtain the compounds, as well as the biological results, will be discussed.

Tryptophan Modification Synthetic Scheme



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P E19 - Comparison of fragment condensation and stepwise approaches to the synthesis of synaptobrevin peptides

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It is known, that various strains of the bacterium *Clostridium botulinum* produce seven antigenically different protein neurotoxins (BoNT/A - BoNT/G) which cause the syndrome botulism [1]. BoNT/B cleaves vesicle-associated membrane protein (VAMP) or synaptobrevin at a single peptide bond between Gln-76 and Phe-77. One of the most robust and specific assay system for the detection of BoNT/B is based on the cleavage of a synthetic peptide substrate representing amino acid sequence 60-94 of VAMP [2]. In order to develop simplified variant of test-system we have synthesised VAMP(60-94) extended by C-terminal cysteine residue (VAMP(60-94)*) for the subsequent modifications and its fragments VAMP(60-76) and VAMP(77-94)*. A priori, fragment condensation approach seems to be the most promising using single N-terminal 14-mer in schemes 14+3 and 14+22. It should be considered also that all above-mentioned peptides comprise "difficult sequence". Synthesis of VAMP(77-94)* and VAMP(74-94)* have been performed using the combination of BOC/Bzl and Fmoc/But strategy and Merrifield resin. N-terminal 14 aa fragment, prepared on 2-Cl-Trt resin have had high purity and poor solubility in DMA. Both 14+3 and 14+22 fragment condensation reactions was fulfilled by DIC/HOBt method in DMA. Cleavage and deprotection of all three peptides by TFMSA produce a complex mixture of products, except to VAMP(77-94)*. The AAA analysis of VAMP(60-94)* after an attempts of ion exchange and HPLC purification indicate an increased amount of C-terminal peptide fragment despite its absence in crude product. In the second synthetic protocol we have applied stepwise elongation, DIC/HOBt couplings, HF cleavage/deprotection for VAMP(60-94)* and TFMSA treatment for VAMP(60-76) and VAMP(77-94)*. Finally, we repeat synthesis of VAMP(60-94)* using DIC/HOAt coupling protocol. The comparison of all three methods have shown similar product purity in the case of DIC/HOBt and DIC/HOAt schedule and complex mixture formation for the fragment condensation technique. These results confirms the literature data regarding strong influence of reaction volume on the yield and purity of final product in the last case [3]. For the purification of VAMP(60-94)* we have found very useful to combine ion-exchange membrane chromatography (IEMC) [4] and HPLC technique. IEMC provide rapid and convenient post-cleavage purification of crude peptide and permits to exclude the stage of size-exclusion chromatography. In the course of assay system development VAMP(60-94)* substrate was cleaved by BoNT/B. Resulting fragments have been detected by RP-HPLC and Tricine-SDS-PAGE. The developed assay was specific to BoNT/B, showing no cross-reactivity with other clostridial neurotoxins, and had a sensitivity for BoNT/B 1-10 ng/ml in HPLC and 0.1-1 ng/ml in Tricine electrophoresis method. Trypsin treatment of BoNT/B samples, which converts the single-chain toxin to the active di-chain form, was found to increase the sensitivity of assay from 5 to 10 fold. Thus, stepwise approach in conjunction with IEMC/HPLC purification of final product seems to be the method of choice for the preparative synthesis of VAMP(60-94)* substrate in the course of simplified BoNT/B assay system development.

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P E20 - Angiotensin I-converting enzyme inhibitory properties of an equine casein tryptic digest and characterisation of some active peptides.

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Peptides with biological activity have been found in enzymatic hydrolysates from various food proteins. Caseins and whey proteins from human and bovine milks have been particularly studied and caseins are a source of numerous biologically active peptides. Peptides with an angiotensin I-converting enzyme (ACE) inhibitory activity have been identified in hydrolysates of bovine αs1-, αs2-, β- and κ-casein. Mare's milk has been poorly studied, but its therapeutic use in some countries seems to suggest that it could be a potential source of bioactive peptides. Sodium caseinate was obtained from Haflinger mare's milk by precipitation at pH 4.2. Different casein fractions were prepared from sodium caseinate by batch fractionation on diethylaminoethyl-cellulose DE23 with increasing concentrations of $CaCl_2$. A fraction, eluted with 15 mM of $CaCl_2$, contained a great proportion of αs1-casein, as shown by electrophoretic analysis. This fraction, subsequently named F1, was hydrolysed by trypsin during 2 h at 37°C. The F1 tryptic hydrolysate inhibited totally the ACE when tested at 50 µM primary amines. In order to identify the active peptide(s), 36 peptidic fractions of F1 tryptic hydrolysate were separated and purified by reversed-phase HPLC on C18 column. Their inhibitory activity of ACE was assayed in vitro, at 50 µM primary amines, by HPLC measurement of the release of hippuric acid from an ACE synthetic substrat (hippuryl-His-Leu). Among them, 11 peptidic fractions had ACE inhibitory activity higher than 50%, but only 5 were in sufficient amount in the hydrolysate to allow characterisation. It can be noticed that the tryptic carboxy-terminal peptide of equine αs1-casein had no inhibitory activity on ACE whereas the bovine carboxy-terminal hexapeptide is one of the more potent ACE peptidic inhibitor found in milk protein hydrolysates. This might be due to the resistance of the 186K-T187 bond to tryptic hydrolysis and to the insertion of one more residue in the equine sequence. The 5 peptidic fractions were repurified in isocratic conditions on C18 column and the amino acid sequence of the peptides contained in these fractions was determined by microsequencing. Three peptides coming from the tryptic hydrolysis of equine αs1-casein and two peptides from tryptic hydrolysis of equine β-casein were identified. F1 fraction contained principally αs1-casein, but also significant amount of β-casein. The identified peptides corresponded to αs1-CN-(f28-40), αs1-CN-(f155-177), β-CN-(f32-40), β-CN-(f196-218). The third tryptic peptide from αs1-casein corresponded to αs1-CN-(f130-153) or αs1-CN-(f130-154). Presence of an arginyl residue at the carboxy-terminal extremity of peptides can potentiate their ACE inhibitory activity. Consequently, the β-CN-(f32-40) was chemically synthesised and its ACE IC50 was precisely determined to be 285 ± 15 µM. This IC50 was included in a scale frequently found for peptidic ACE inhibitor from bovine caseins, but it was about 30 fold higher than that of the best of them. Nevertheless, many ACE peptidic inhibitors from food proteins with IC50 between 100 and 500 µM have effective antihypertensive activity in vivo after per os administration. Mare's milk might be a potential source of peptidic ACE inhibitors. However, other peptides from F1 fraction tryptic hydrolysate should be precisely tested for their ACE inhibitory activity as the total inhibitory activity of F1 fraction was not recovered in any characterized peptide.

P E21 - Preparation of a radiolabeled peptide-PNA conjugate for imaging oncogene expression

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Non-Hodgkin's lymphoma (NHL) is among the most common hematopoietic malignancies and is at present incurable. Overexpression of the *B-cell lymphoma/leukemia-2* (*bcl-2*) gene in intermediate- to high-grade NHL correlates strongly to development of resistance to radiation and chemotherapy, high relapse rate, and poor survival. Our working hypothesis is that a radiometal-labeled antisense peptide nucleic acid (PNA) against *bcl-2* mRNA, coupled to a cell-permeating peptide, might be used for diagnostic imaging in NHL patients. The prognostic information provided by non-invasive detection of *bcl-2* may lead to identification of patient risk groups, who might respond better to alternative treatments like radioimmunotherapy, antisense therapy, or anti-angiogenic metronomic chemotherapy.
We describe here the synthetic preparation of an 18-residue anti-*bcl-2* PNA covalently attached to the membrane-permeating peptide PTD-4 and modified with the macrocyclic chelating agent DOTA (DOTA = 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid). We synthesized a fully protected derivative of DOTA that can be incorporated into any sequence position of a peptide or PNA by standard solid-phase synthesis techniques. A combination of manual and automated stepwise elongation was used to synthesize the peptide-PNA conjugate. An innovative process involving purification of partially protected intermediates and subsequent deprotection has been accomplished to obtain the desired compound. The structure of the final product was confirmed by HPLC/MS.
The peptide-PNA conjugate was labeled with the diagnostic imaging radiometal ^{111}In ($T_{1/2}$ = 2.81 d; EC 849 keV (100%); γ 173 keV (89%), 247 keV (94%)) in 0.2 M ammonium acetate, pH 5.0, for 1 h at 90 °C. Normal phase thin-layer chromatography and size exclusion HPLC indicated that ^{111}In incorporation was 97.0%. Proof-of-principle studies with the ^{111}In-labeled peptide-PNA conjugate will lead to the evaluation of this agent for non-invasive detection of *bcl-2* expression in a cell-free system, in NHL cells in culture, and in a tumor-bearing animal model.

P E22 - Synthetic peptides based biosensors in the immunodiagnosis of hepatitis g infection

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GB virus C (GBV-C) and hepatitis G virus (HGV) are strain variants of a recently discovered enveloped RNA virus belonging to the Flaviviridae family.
HGV is spread worldwide and infections with the virus have been found both among healthy individuals and in different patient groups. Many reports have shown that the virus may be transmitted via blood and/or blood products, indicating surprisingly high prevalence rates. Controversial data exist concerning the potential of HGV to cause hepatitis in humans. Most infections appear to be innocent and assymptomatic [1,2]. The first method described to diagnose HGV infection was a reverse transcription polymerase chain reaction that is still the only available method for diagnosing an ongoing infection. Later, an assay that detects antibodies to the HGV envelope E2 protein was developed and is now commercially available. In recent years, synthetic peptides that mimic specific epitopes of infectious viral proteins have been used in diagnostic systems for various diseases [3,4]. Peptide sequences that mimic the immunodominant epitopes of viral proteins have been used to enhance the sensitivity and specificity of diagnostic systems. In the present study, new putative epitopes, located in structural (E2) and non-structural (NS3) proteins of HGV, were identified by computer-aided prediction of antigenicity and synthesized in solid-phase, following an Fmoc/tBut strategy, for its use in immunoassays.
The corresponding synthetic peptides were used as antigens in enzyme-linked immunosorbent assays (ELISAs). To verify the performance of these new assays in comparison to the recombinant E2 protein existing commercial test, antibodies to these peptides were searched for in different panels of serum samples (hemodialyzed and chronic hepatitis C patients and in assymptomatic control subjects). Furthermore, the course of HGV markers was studied prospectively in patients exposed to HGV by blood transfusion. Our results could be useful for the development of new diagnostic peptide based assays.

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P E23 - A point-of-care test using a heptapeptide IgG epitope for diagnosis human parvovirus B19

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Human parvovirus B19 is a small nonenveloped virus, the icosahedral capsid of which consists of virus proteins (VPs) 1 and 2. It has been shown that VP2 in denatured form binds antibodies only from patients with acute or recent infection, in contrast to its conformational epitopes, to which antibodies are synthetised even many years after B19 infection.
The aminoacid sequences of the VP1 and VP2 were studied with overlapping synthetic peptides attached to cellulose membranes, A heptapeptide epitope showed the strongest reactivity with acute-phase IgG but not with IgG from samples representing past immunity to B19. A new diagnostic EIA was set up using a synthetic peptide containing this VP2 linear epitope together with VP2 capsids exhibiting conformational epitopes [Kaikkonen et al. J Clin Microbiol 1999;37:3952-6].
Using this epitope we set up a rapid test for human parvovirus B19 infections. In the test the antigens are bound to a nitrocellulose membrane. A serum sample is added together with buffer and a gold-conjugated antigen. A positive result is obtained when the antibodies bind to the antigen band together with the gold conjugate, forming a specific colored line.
Serum samples were collected from patients with acute B19 infection, and control samples were drawn many years after infection; additional control sera came from subjects devoid of B19 antibodies. We evaluated 3 different peptide antigens all containing the heptapeptide epitope and empty parvovirus capsids expressed in the baculovirus system. A single-chain peptide alone did not bind to the membrane, and as conjucated with bovine serum albumin it gave a weaker signal than the branched peptides. An 8-branched peptide bound well to nitrocellulose, but it had lower sensitivity and specificity than a branched peptide of four 24-amino-acid sequences, with the heptapeptide sequence in the middle. The 4-branched peptide was evaluated further for use in an immunochromatographic point-of-care test.
Using as antigen the empty capsids consisting of VP2, a B19-virus IgM assay was similarly set up. The capsids were immobilized in the nitrocellulose, and the samples were applied together with gold-conjugated anti-human-IgM. The assay was shown to be as sensitive and specific as conventional parvovirus EIAs.

P E24 - Development and preliminary evaluation of immunoglobulins Y against thymosin peptides

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α and β thymosins are bioactive peptides, which are widely distributed in mammalian tissues and highly conserved among mammalian species (~5 to ~11 kDa). Their main biological role has been related to cell proliferation and differentiation. According to accumulated data of ours as well as to literature information, thymosins are poorly immunogenic and no anti-thymosin antibodies are yet commercially available. In the present study we immunized hens and rabbits against either intact thymosins (prothymosin α, thymosin β 4, thymosin β 10, thymosin β 15), or thymosin epitopic fragments (prothymosin α [101-109], thymosin β 10 [1-16], thymosin β 15 [31-43], etc.), conjugated to keyhole limpet hemocyanin. The immunization schedule depended on the animal species, the main difference being the immunogen dose. The rabbit antisera were obtained at a quantity of ~15 mL for each antigen, while their titres averaged 1:5,000 [which corresponds to ~300 ng of rabbit immunoglobulins G (IgGs) per mL], when tested by a standard ELISA system. On the other hand, egg yolk immunoglobulins Y (IgYs) were obtained at ~90 % purity using a suitable isolation protocol (combination of the polyethylene glycol method and the acidic water method), at quantities of several grams (>100 eggs / hen, 90-120 mg IgY per egg) for each antigen. The working IgY solution concentrations averaged 150 ng IgY per mL, when tested by the above ELISA system, which indicates that, in addition to the production of higher quantities, the immunization of hens led to antibodies of a better titre. The IgY antibodies are currently under evaluation in competitive ELISA immunoassays, with encouraging preliminary results. Thus, the use of anti-prothymosin α IgY antibodies enabled the development of an ELISA standard curve of better sensitivity and a broader working range compared to the curve obtained by the corresponding rabbit antiserum. This curve was successfully applied to the measurement of prothymosin á in total protein extracts from MCF-7 and MDA breast cancer cell lines. Since we have long experience in antibody development, we consider the "IgY approach" as a very promising way of obtaining high quantities of specific antibodies, with improved immunoreactivity characteristics compared to mammalian IgGs, by using animals that are easy to handle and to house. This is especially important when poorly immunogenic peptides, such as thymosins, are used as antigens.

P E25 - Introduction of lanthanide(III) chelates to oligopeptides on solid phase

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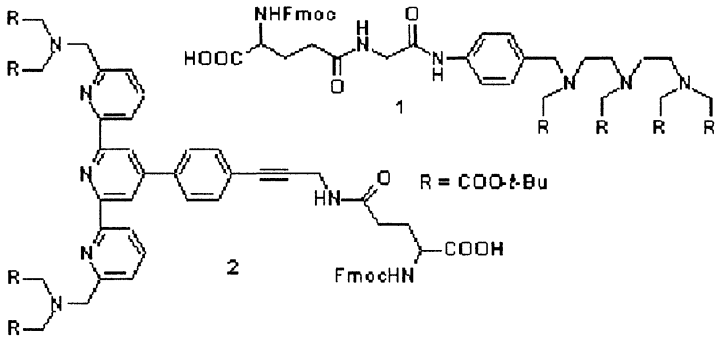
Due to their unique luminescence properties lanthanide(III) chelates are often used as non-radioactive markers in a wide variety of routine and research applications. Since lanthanide(III) chelates give strong, long decay-time luminescence, they are ideal labels for assays where high sensitivity is required. Time-resolved fluorometric assays based on lanthanide chelates have found increasing applications in diagnostics, research and high throughput screening. The heterogeneous DELFIA® technique is applied in assays requiring exceptional sensitivity, robustness and multi-label approach [1]. Development of highly luminescent stable chelates [2], extends the use of time resolution to homogeneous assays based on energy transfer (TR-FRET) quenching, or changes in the chelate's luminescence properties during a binding reaction.
We describe here synthesis of oligopeptide building blocks (1, 2) that allow introduction of non-luminescent and luminescent lanthanide(III) chelates to synthetic oligopeptides using standard machine assisted chemistry.



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P E26 - Long acting derivatives of the GLP-1 agonist exhibit high potency and extended pharmacokinetics when bioconjugated to albumin *in vivo*

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Glucagon-Like Peptide 1 (GLP-1) is a potent 30 A.A. that inhibits glucagon secretion, stimulates insulin secretion in a glucose dependant manner, restores beta cell function and promotes beta cell proliferation. Although GLP-1 exhibits considerable potential for treating Type II diabetes, the native peptide faces both a rapid degradation ($T_{1/2}$: 1-2 min.) by the serum protein DPP IV and a rapid renal clearance thereby precluding effective use as a therapeutic agent. To circumvent these limitations, the Drug Affinity Complex (DAC) technology was applied to generate novel, stable and long-acting GLP-1 receptor agonists. The DAC:GLP-1 constructs bioconjugate *in vivo* selectively and covalently to the Cys^{34} free thiol of circulating serum albumin. Once anchored, DAC:GLP-1 retains potency, prolongs duration of action and displays extended pharmacokinetic profile. In addition, the technology prevents the activation of the immune system, an important element in drug safety. 11 analogues of DAC:GLP-1 along with 2 analogues of DAC:Exendin-4 were prepared using a manufacturing compatible solid phase synthetic approach. *In vitro*, the DAC constructs bind rapidly to purified human serum albumin (HSA) with efficiencies >98%. The native GLP-1 sequence was rapidly hydrolysed to its inactive form GLP-1 (9-36) in human plasma, while no metabolite was detected for the lead DAC:GLP-1 construct. Moreover, the lead DAC:GLP-1:HSA bioconjugate binds to human receptor hGLP-1R with nanomolar affinities and stimulates cAMP production. Rodent pharmacokinetic studies combined with western blot analyses show the lead DAC:GLP-1 compound binding highly selectively to rat albumin (RSA) *in vivo* while radioimmunoassay indicated terminal half lives of ~20 hrs for the lead DAC:GLP-1:RSA bioconjugate (RSA $T_{1/2}$: 48 h). An excellent pharmacodynamic dose response is observed *in vivo* (db/db mice). These findings show a highly potent stable DAC:GLP-1 construct exhibiting a promising biological and pharmacokinetic profile thereby introducing the DAC™ technology as a powerful alternative in peptide drug delivery.

P E27 - Proteomics scale protein interaction studies facilitated by high throughput synthesis of phosphopeptides

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A comprehensive understanding of intracellular signaling requires knowledge of protein-protein interactions. One important subset of these interactions is mediated by the SH2 family of binding domains and ligands. Whereas all SH2 domains bind a phosphotyrosine (pY) containing ligand, specificity within the homologous SH2 family is determined by the amino acids flanking pY. As a part of AxCell Bioscience's commitment to mapping the entire human proteome, we have recently completed a study of the interactions between 11 SH2 domains and 36 potential ligand peptides through a high throughput in vitro binding assay. This is a quantitative assay that measures binding of synthetic peptide ligands containing pY to fusion proteins containing SH2 binding domains. Because the domain proteins are relatively large (about 250 AA), they are generally produced as fusion proteins with glutathione S-transferase (GST) via standard molecular biology techniques, including PCR from human tissue cDNA libraries. The synthetic peptides, on the other hand, are prepared in high throughput using Mimotopes SynPhase™ solid phase synthesis support with standard Fmoc chemistry [1]. Detection of binding is facilitated by biotin included at the N-terminus of the ligand peptides. Results of this preliminary set of 396 interaction data (11 domains x 36 ligands) confirm that specificity of binding is afforded by amino acids on either side of pY in the ligand. This suggests that the assay is robust and appropriate for high throughput quantitative analysis of protein interactions. We are now studying the specificity of over 100 SH2 domains for over 1000 potential pY containing ligands.

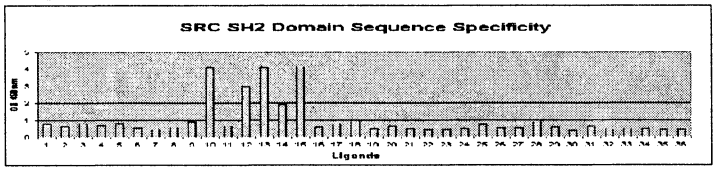

Fig. 1 - Binding specificity of src SH2 domain.

Reference
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P E28 - High throughput synthesis of small protein domains

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Using high throughput technologies, we currently generate about 1000 pure, fully characterized synthetic peptide ligands per month in support of AxCell Biosciences' proteomics business [1]. In an attempt to accelerate the production of protein domains used to assay interaction (binding) with those peptide ligands, we recently investigated the possibility of chemical synthesis of small protein domains on Mimotopes' SynPhase(tm) acrylic-grafted polypropylene solid phase support. Synthesis of 35-41mer peptides was carried out as described [2] in a 96 well microtiter plate with Fmoc chemistry. Parallel synthesis of 60 WW domains was facilitated by a PinPal(tm) Amino Acid Indexer [3]. Dinitrophenol was attached at the N-termini to facilitate immobilization on microplate wells (via an anti-DNP antibody) in subsequent high throughput binding assays in which the synthetic protein domains were screened against synthetic peptide ligands. The resulting affinity data are comparable to data obtained from the assays performed with protein domains produced through expression as fusion proteins. This study demonstrates that small protein domains may be generated in high throughput as an alternative to genetic technology, in which expression of many protein domains is challenging.

References
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[2] L. Chen, B. Korenstein, M. James, L. Kvecher, and J.M. Carter (2001) in *Peptides: Wave of the Future* - Proceedings of the 17th American Peptide Symposium, M. Lebl and R. A. Houghten, ed., Mayflower Scientific Ltd, Kingswinford, UK, pp 206-207.
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P E29 - Combining combinatorial chemistry and affinity chromatography protocols for systematically probing protein-ligand interactions: application to the development of highly selective phosphinic inhibitors of human betaine: homocysteine S-methyltransferase

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The challenge of functional genomics and proteomics is to translate sequencing data into a precise understanding of how proteins function in cells, tissues or whole organism. Small ligands, able to specifically interact with proteins, can be very effective tools in the search for the function of proteome. The classical approaches for identification of new protein ligands, as are structure-activity studies, molecular computer modeling or combinatorial techniques, ask for a single and purified protein target. However, the human organism contains thousands of proteins which existence we can only anticipate.
That is why we envisaged to develop a simple approach to considerably enhance the chance to discover new interactions between proteins and ligands. The principle of our new approach is based on the affinity chromatography coupled to combinatorial chemistry and consists in the interaction of thousands of proteins (crude biological samples) with a mixtures of immobilized potential ligands (combinatorial library of affinity supports). We chose phosphinic pseudopeptides as potential ligands in our approach.
Using this method we discovered phosphinic pseudotetrapeptides which are highly selective and potent inhibitors of Zn-metalloenzyme betaine: homocysteine S-methyltransferase (BHMT, EC 2.1.1.5). BHMT catalyses the transfer of methyl group from betaine to L-homocysteine giving dimethylglycine and L-methionine. The development of selective inhibitors of BHMT should help to better understand the physiological role of BHMT by pharmacology *in vivo*.
Our results validate the concept that combining combinatorial chemistry to affinity capture protocols makes possible to identify, without any *a priori* hypothesis, novel protein targets of phosphinic peptides. And *vice versa*, selective inhibitors for BHMT were developed despite the rather limited diversity of the library used to fish out the proteins.

P E30 - Mechanism-based detection and activity-profiling of protein kinases

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Proteomics, the study of the so called proteome which is the entirety of proteins expressed by a cell at a given time, basically relies on the two-dimensional separation of the proteins and their subsequent identification. However, since the proteome may consist of some 10.000 different proteins, resolution often remains incomplete. Furthermore, sensitive detection and quantification which are conventionally done using different staining methods (silver, Coomassie blue, fluorescent dyes) represent further problems since they are non-selective and often difficult to compare and to reproduce.
As a matter of principle it is possible to tag certain proteins covalently with a specific ligand (peptide or organic molecule) bearing a reporter group (biotin, radioactive tag, or fluorescent label) prior to two-dimensional separation. Subsequently the protein in question can easily be identified, e.g. fluorimetrically, and quantified. Especially tagging of enzymes has been achieved simply using irreversible enzyme inhibitors (suicide inhibitors).
Recently we extended the applicability of this strategy towards the use of reversible enzyme inhibitors. This was accomplished by a covalent linkage of a reversible inhibitor to an enzyme by means of photoaffinity labelling in order to avoid dissociation under the conditions of 2D electrophoresis.
The feasibility of this approach has been proven for protein kinases of plants (*Chlamydomonas reinhardtii*). A reversibly binding isoquinolinesulfonamide type inhibitor (H-9) was equipped with 4-benzoylphenylalanine as photoreactive group and carboxyfluorescein as fluorescent reporter group. Incubation of a thylakoid preparation containing several putative serine/threonine kinases with the engineered inhibitor followed by irradiation at a wavelength of 350 nm gave covalently linked enzyme-inhibitor-complexes.
The specificity of the photoaffinity labelling of a particular kinase is highlighted by the fact that in several cases only the catalytic subunits are tagged, whereas the regulatory units remain unlabelled.

P E31 - Peptides as tools for the discovery and activity-profiling of matrix metalloproteinases

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The term proteomics describes the analysis of the protein entirety expressed by a cell under certain conditions. The separation of such complex protein mixtures relies on two-dimensional gel electrophoresis. Due to the expression of more than 10000 different proteins at the same time the resolution often remains incomplete. Therefore more sensitive detection and quantification procedures have to be developed.
A new concept for mechanism-based detection and activity-profiling of proteins and protein families utilizes novel engineered probes consisting of a ligand, which is specific for the particular protein family, and a linker molecule for further derivatisation. Such derivatisations include attachment of reporter groups or immobilisation on a solid surface e.g. for affinity chromatography.
Derivatives of the matrix metalloproteinase inhibitor marimastat, a pseudo-peptidic hydroxamate inhibitor, have been rationally designed and synthesized. The synthesis can be done either on solid support or in solution. The marimastat derivative allows for immobilisation on solid support, e.g. on a chromatography matrix for affinity purification or on a surface plasmon resonance sensorchip for activity-profiling.
The marimastat derivative has been further modified with a reporter group (fluorescent label) and a photoreactive group (4-benzoylphenylalanine). After incubation with a cell extract the inhibitor is covalently bound to active MMP by irradiation. This procedure makes it possible to easily detect MMP, especially undiscovered members of that protein family after gel electrophoresis.

P E32 - Towards the delivery of double stranded nucleic acids: synthesis and biological studies

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Cellular uptake of various biological entities (peptides, drugs, proteins, antisense oligonucleotides) has been achieved by their coupling to cell penetrating peptides (CPP) such as peptides derived from Antennapedia or Tat proteins. The mechanisms by which these peptides and these chimeric constructs enter the cells remain unclear. However no active process of transport appears to be involved since the uptake occurs at 4°C or in the presence of inhibitors known to prevent energy-dependant transport. Further studies to elucidate the internalisation pathway of these structures are investigated in our group. Despite the lack of full knowledge of this translocating process, the peptide-mediated delivery of double-stranded nucleic acids entities should become an interesting tool for the cell uptake of small double-stranded deoxyribonucleic acids (DNAs) or ribonucleic acids (RNAs) since it avoids transit through the endosomial vesicles.
New strategies allowing to regulate specifically and efficiently gene expression have been described. Double-stranded DNA fragments (decoy DNA) corresponding to the binding site of transcription factor have been used to titrate these proteins. More recently small double-stranded RNA fragment (siRNA or interfering RNA) lead to gene silencing. In both cases delivery is a limiting factor and should be improved by conjugation to CPP. Moreover the results of the cellular delivery of such double-stranded nucleic acids molecules should highlight by which mechanisms these constructs are internalised.
Methodology of the chemical synthesis of these double-stranded nucleic acids bound to the Tat derived CPP and preliminary studies of their cellular uptake will be presented.

P E33 - Design of inhibitors of plasmepsins based on combinatorial specificity studies

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We have utilised a combinatorial approach to study the subsite preferences of enzymes of the aspartic endopeptidase class. We have constructed two libraries: 1) Lys-Pro-(Xaa)-Glu-P1#Nph-(Xaa)-Leu, and 2) Lys-Pro-Ile-(Xaa)-Nph#P1'-Gln-(Xaa). In each library one residue, P1 in library (1) and P1' in library (2) is varied among 19 different "pools" and the residue given as (Xaa) is a mixture of the same 19 amino acids. Each pool contains 361 peptides. Following incubation with different enzymes, product analysis is preformed by LC-MS with the products showing up in greatest amounts indicating the preferences in the positions shown by (Xaa). Spectrophotometric analysis of cleavage for each pool gives an evaluation of the preferences in subsite P1 or P1' for libraries (1) and (2), respectively. We have used the results of this work to design peptidomimetics targeted to each of five different enzymes from the malaria species that infect man: *Plasmodium falciparum, P. vivax, P. ovalae,* and *P. malariae*. These inhibitors are low nanomolar to picomolar inhibitors of the malarial enzymes.

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