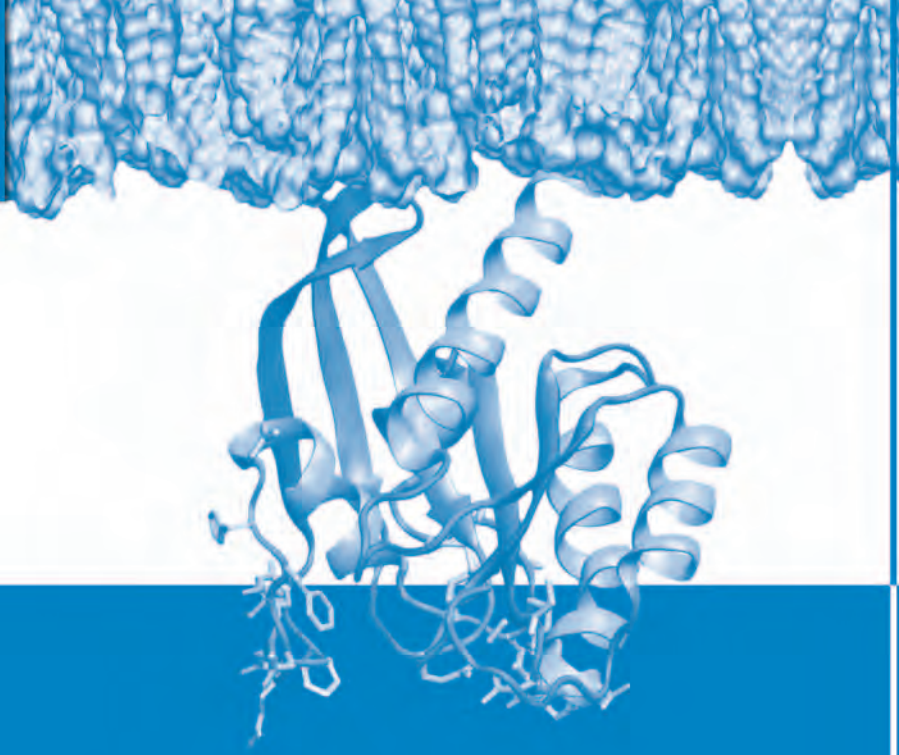

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12th Naples Workshop on Bioactive Peptides

2nd Italy-Korea Symposium on Antimicrobial Peptides

PEPTIDES AT WORK: FROM STRUCTURE TO APPLICATIONS

twelfth Naples Workshop on Bioactive Peptides

2nd Italy-Korea Symposium on ANTIMICROBIAL PEPTIDES

ABSTRACTS AND PROGRAM

Centro Congressi d'Ateneo "Federico II" – Via Partenope, Napoli
June 4-7, 2010

Twelfth Naples Workshop on Bioactive Peptides
Second Italy-Korea Symposium on Antimicrobial Peptides

PEPTIDES AT WORK: FROM STRUCTURE TO APPLICATION

Organized by:
University of Napoli "Federico II" – Dipartimento delle Scienze Biologiche
University of Roma "Tor Vergata"
Centro Interuniversitario di Ricerca sui Peptidi Bioattivi
Istituto di Biostrutture e Bioimmagini del Consiglio Nazionale delle Ricerche
DFM Scarl

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Acknowledgments

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The Organizing Committee gratefully acknowledges the contribution given
to the organization of the event by the following companies

Content

PROGRAM

Friday, June 4th

12:00 am **Registration**

2:30 pm - 2:55 pm **Welcome Addresses**

Antimicrobial Peptides — Session I

Chair: Stefania Galdiero (Naples, Italy) and Kyung-soo Hahm (Gwangju, Korea)

2:55 pm - 3:20 pm L1 Antimicrobial peptides from amphibian skin
Donatella Barra, Roma, Italy

3:20 pm - 3:45 pm L2 Molecular activation and regulation mechanisms for the induction of antimicrobial peptides after microbial pathogen challenges in insects
Bok Luel Lee, Pusan, Korea

3:45 pm - 4:10 pm L3 Lipopeptaibol trichogin GA IV: role of the three Aib residues on conformation and bioactivity
Fernando Formaggio, Padova, Italy

4:10 pm - 4:35 pm L4 Antimicrobial and anti-inflammatory activities of designed tryptophan-rich model antimicrobial peptides
Song Yub Shin, Gwangju, Korea

4:35 pm - 5:05 pm **Coffee Break** — sponsored by ICX Nomadics

Antimicrobial Peptides — Session II

Chair: Simona M. Monti (Naples, Italy) and Lorenzo Stella (Rome, Italy)

5:05 pm - 5:30 pm L5 Bacterial membrane lipids in the action of antimicrobial agents
Richard Epand, Hamilton, Canada

5:30 pm - 5:55 pm L6 Antimicrobial peptides: structure, topology and mechanisms of membrane permeabilization
Burkhard Bechinger, Strasbourg, France

5:55 pm - 6:20 pm L7 Folding versus aggregation: a critical evaluation of membrane-active peptides
Anne Ulrich, Karlsruhe, Germany

6:20 pm - 6:45 pm L8 Potential mechanisms of bacterial resistance to antimicrobial peptides and how to overcome them
Yechiel Shai, Rehovot, Israel

7:00 pm - 8:00 pm **Welcome Party**

Saturday, June 5th

Murray Goodman Young Investigators' Session

(Sponsored by Georg Thieme Verlag KG)

Chair: Alessandra Romanelli (Naples, Italy) and Luis Moroder (München, Germany)

8:30 am - 8:50 am	Y1	Ghrelin agonist and inverse agonist tracers for PET imaging Constance Chollet, Leipzig, Germany
8:50 am - 9:10 am	Y2	Synthesis, conformation, and bioactivity of integramide A, a peptide inhibitor of HIV-1 integrase, and selected analogs Marta De Zotti, Padova, Italy
9:10 am - 9:30 am	Y3	Non-cationic dipeptide mimetic oligomers as cell penetrating non peptides (CPNP) Lubomir Vezenkov, Naples, Italy
9:30 am - 9:50 am	Y4	Purification and characterization of peptides with antimicrobial activity isolated from fleshfly neobellieria bullata Tereza Neubauerova, Prague, Czech Rep.
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Murray Goodman Young Investigators' Session

(Sponsored by Georg Thieme Verlag KG)

Chair: Nina Dathan (Naples, Italy), Muriel Amblard (Montpellier, France)

10:20 am - 10:40 am	Y5	NMR structural characterization of a bioactive β-hairpin peptide and of its interaction with VEGFR-1D2 Donatella Diana, Naples, Italy
10.40 am - 11:00 am	Y6	A hierarchical approach to mimetics of the N-terminal 1-11 segment of PTH Andrea Caporale, München, Germany
11:00 am - 11:20 am	Y7	New methodology for the synthesis of peptides alcohol using O-N acyl shift Julien Tailhades, Montpellier, France
11:20 am - 11:40 am	Y8	The importance of being kinked: role of Pro residues in the selectivity of helical antimicrobial peptides Sara Bobone, Rome, Italy
12:20 am - 3:00 pm		**Lunch Break**

Antimicrobial Peptides — Session III

Chair: Maria Luisa Mangoni (Rome, Italy), Yoon-sik Lee (Seoul, Korea)

3:00 pm - 3:25 pm	L9	A novel tetrabranched antimicrobial peptide that neutralizes bacterial lipopolysaccharide and prevents septic shock in vivo Alessandro Pini, Siena, Italy
3:25 pm - 3:50 pm	L10	Development and application of novel antibiotic peptides from potato Yoonkyung Park, Chosun, Korea

Saturday, June 5th

3:50 pm - 4:15 pm	L11	Mechanisms of pore formation of antimicrobial peptides and viroporins inserted in a cell plasma membrane Giorgio Rispoli, Ferrara, Italy
4:15 pm - 4:40 pm	L12	Biochemical and biophysical characteristics of gaegurins and their derivatives Byeong Jae Lee, Seoul, Korea
4:40 pm - 5:05 pm	L13	Penetration of Bac7, a proline-rich antimicrobial peptide, into bacteria-characterizing the entry sequence Alessandro Tossi, Trieste, Italy
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Antimicrobial Peptides — Session IV

Chair: Daniela Marasco (Naples, Italy) and Renato Gennaro (Trieste, Italy)

5:35 pm - 6:00 pm	L14	Polyamines can increase resistance of Neisseria gonorrhoeae to mediators of innate human host defense Maira Goytia, Decatur, GA, USA
6:00 pm - 6:25 pm	L15	Oncocin, a novel designer peptide for the treatment of gram-negative infections Ralf Hoffmann, Leipzig, Germany
6:25 pm - 6:50 pm	L16	Effect of PEGylation on antimicrobial peptides Kesavakurup Santhoshkumar, Trivandrum, Kerala, India
6:50 pm - 7:15 pm	L17	Antimicrobial activity and cytotoxicity of new antimicrobial peptides (melectin, lasioglossins, halictines and macropin) from wild bee venom and their analogues Jirina Slaninova, Prague, Czech Rep.
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Bioactive Peptides — Session I

Chair: Rita Berisio (Naples, Italy) and Evaristo Peggion (Padova, Italy)

8:30 am - 9:00 am	L18	Regulation of intracellular proteolysis Robert Huber, München, Germany
9:00 am - 9:25 am	L19	Development of an orally active conotoxin for the treatment of pain David Craik, Brisbane, Australia
9:25 am - 9:50 am	L20	Design and synthesis of protease inhibitors, prodrug forms and click peptides Yoshiaki Kiso, Kyoto, Japan

9:50 am - 10:15 am	L21	Antiviral proteins from natural product extracts Barry R. O'Keefe, Frederick, MD, USA
10:15 am - 10:40 am	L22	Design and applications of protein epitope mimetics John A. Robinson, Zürich, Switzerland
10:40 am - 11:10 am		**Coffee Break**

Bioactive Peptides — Session II

Chair: Laura Zaccaro (Naples, Italy) and Chandralal Hewage (Dublin, Ireland)

11:10 am - 11:35 am	L23	Novel synthetic methodologies to prepare unnatural aminoacids and peptidomimetics Mauro F.A. Adamo, Dublin, Ireland
11:35 am - 12:00 am	L24	Amino acids and derivatives as attractive starting materials for the synthesis of active biomolecules Jean Martinez, Montpellier, France
12:00 am - 12:25 am	L25	Intramolecular macrocyclization reactions in 310-helical peptides: stereo- and regioselectivity properties Claudio Toniolo, Padova, Italy
12:25 am - 12:50 am	L26	Ureido-based scaffolds as peptide mimics : connecting structure and function Gilles Guichard, Bordeaux, France
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Chair: Giuseppina De Simone (Naples, Italy) and Inta Liepina (Riga, Latvia)

3:00 pm - 3:20 pm	O1	The microwave revolution: recent advances in microwave assisted peptide synthesis Grace Vanier, Matthews, NC, USA
3:20 pm - 3:40 pm	O2	Quantification of the cellular uptake of cell penetrating compounds by MALDI-TOF MS using HCCA tag Gilles Subra, Montpellier, France
3:40 pm - 4:00 pm	O3	Drug-armed branched peptides: turning non-specific cytotoxic drugs into tumor-selective agents Luisa Bracci, Siena, Italy
4:00 pm - 4:20 pm	O4	Probing the opening of lipase lid using site-directed spin-labelling and EPR spectroscopy Frederic Carriere, Marseille, France
4:20 pm - 4:40 pm	O5	Using SPOT synthesis for drug design: a peptide based approach to design CAL selective PDZ inhibitors Lars Vouilléme, Berlin, Germany
4:40 pm - 5:00 pm	O6	Clarification of signaling mechanisms for mitocryptides, novel neutrophil-activating peptides hidden in mitochondrial proteins Hidehito Mukai, Kyoto, Japan

Sunday, June 6th

5:00 pm - 5.30 pm		**Coffee Break**
5:30 pm - 7:30 pm		Poster Session Discussion of posters with EVEN numbers
		Free evening

Monday, June 7th

Bioactive Peptides — Session IV

Chair: Marilisa Leone (Naples, Italy) and Carlo Pedone (Naples, Italy)

8:30 am - 9:10am		Celebrating an anniversary Jean Martinez, Montpellier, France Claudio Toniolo, Padova, Italy
9:10 am - 9:35 am	L27	Side chain-to-side chain (Sc2Sc) clicked-cyclopeptides - Synthesis, conformation and biological activity Michael Chorev, Cambridge, MA, USA
9:35 am - 10:00 am	L28	In silico identified drug candidates and peptide-drug conjugates against M. tuberculosis infection Ferenc Hudecz, Budapest, Hungary
10:00 am - 10:25 am	L29	Solution structure and function of the folded and highly conserved C-terminal domain common to spider dragline silk proteins Horst Kessler, Garching, Germany
10:25 am - 10:50 am	L30	NrTP, a snake-derived CPP, targets very specifically the nucleoli of tumor cells David Andreu, Barcelona, Spain
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Bioactive Peptides — Session V

Chair: Emilia Pedone (Naples, Italy) and Reudiger Pipkorn (Heidelberg, Germany)

11:20 am - 11:45 am	L31	Structural disorder and induced folding within the measles virus nucleoprotein: experimental assessment and functional implications Sonia Longhi, Marseille, France
11:45 am - 12:10 am	L32	Discovery of potent, cyclic calcitonin gene related peptide (CGRP) receptor antagonists John P. Mayer, Indianapolis, IN, USA
12:10 am - 12:35 am	L33	Chemical challenges for the construction of protein mimics Rob M.J. Liskamp, Utrecht, The Netherlands
12:35 am - 1:00 pm	L34	Imide-click reaction. Application for peptide ligation and prodrug design Oleg Melnyk, Lille, France
1:00 pm - 3:00 pm		**Lunch Break**

Bioactive Peptides — Session VI

Chair: Luciana Esposito (Naples, Italy) and Adam Prahl (Gdansk, Poland)

3:00 pm - 3:25 pm	L35	Molecular interaction of PPARγ with its ligand Yuji Kobayashi, Osaka, Japan
3:25 pm - 3:50 pm	L36	Design and application of material forming peptides: from antibacterials to mammalian cell delivery Joel Schneider, Bethesda, MD, USA
3:50 pm - 4:10 pm	O7	Attenuation of ischemia/reperfusion injury in mouse hearts by BH4 peptide Prisca Boisguerin, Berlin, Germany
4:10 pm - 4:30 pm	O8	Self-assembled peptide biomaterials of de novo design Maxim G. Ryadnov, Teddington, UK
4:30 pm - 6:30 pm		Poster Session Discussion of posters with ODD numbers. Coffee break will be served during this session
7:30 pm		Bus transfer to “Hotel Gli Dei”
8:30 pm- 00:30 am		**Gala Dinner**

INVITED LECTURES

L1

Peptide effectors of innate immunity

D. Barra

Istituto Pasteur- Fondazione Cenci Bolognetti, Dipartimento di Scienze Biochimiche "A. Rossi Fanelli"
Sapienza Università di Roma, 00185 Roma

Peptides with diverse biological activities are particularly abundant in skin secretions of amphibians.[1] Many of these compounds are pivotal components of the innate immune response, while others are related to mammalian hormones or neurotransmitters. The skin peptides are usually synthesized and stored in cutaneous granular glands, and continuously released to provide protection from invading microorganisms. The location of the glands on the surface of the body makes frogs a useful model for in vivo studies on the induction of the innate immune response.[2]

We have focused the attention on the peptide components of amphibian skin secretions, discovering a number of molecules displaying antimicrobial activity,[3] as well as small proteins with different activities, among which the most interesting is Bv8.[4] Mammalian homologues of Bv8 have been described also in humans (prokineticin 1/EG-VEGF and prokineticin 2/human Bv8), and found to display chemokine-like activities.

These two classes of molecules can be considered as the effectors of the evolutionary ancient immune system, an essential arm of the vertebrate defence, having overlapping functions: antimicrobial peptides that inactivate invading bacterial, fungal, or viral pathogens; and Bv8/ rokineticins, that recruit and activate leukocytes.

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L2

Molecular activation and regulation mechanisms for the induction of antimicrobial peptides after microbial pathogen challenges in insects

B. L. Lee

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Pusan National University, Busan, 609-735, Korea

Innate immunity is a crucial host defense mechanism against microbial infection in all animals. The insect Toll signaling pathway is activated upon recognition of Gram-positive bacteria and fungi, resulting in the expression of antimicrobial peptides via NF-κB-like transcription factor. This activation is mediated by a serine protease cascade leading to the processing of Spätzle, which generates the functional ligand of the Toll receptor. Recently, we analyzed the serine protease cascade regulating the Toll signaling pathway using larvae of the beetle, Tenebrio molitor. This large insect enabled us to collect large amount of hemolymph (the insect blood) allowing purification of serine proteases. Our studies demonstrated that the recognition of bacterial Lys-type peptidoglycan (PG) by the PG recognition protein-SA (PGRP-SA)/Gram-negative binding protein 1 (GNBP1) complex and the recognition of fungal β-1,3-glucan by GNBP 3 activates pro-Spätzle via the sequential activation of three serine proteases: modular serine protease (MSP), Spätzle processing enzyme-activating enzyme (SAE) and Spätzle processing enzyme (SPE). Additionally, we provide biochemical evidences of how the Tenebrio PGRP-SA/GNBP 1 complex-mediated bacteria recognition signal or GNBP3-mediated fungi recognition signal is transferred to pro-Spätzle leading to the production of antimicrobial peptides. Injection of β-1,3-glucan into Tenebrio larvae induced production of two antimicrobial peptides, Tenecin 1 and Tenecin 2, which are also inducible by injection of the active form of Spätzle-processing enzyme-activating enzyme or processed Spätzle. Furthermore, we identified four different serpins that are involved in the regulation of Toll signaling cascade. We examined in vitro and in vivo biological functions of these serpins during Toll activation cascade.
In summary, our work supports a model in which bacterial Lys-type PG and fungal β-1,3-glucan recognition signals activate a common proteolytic cascade involving three different serine protease zymogens that are sequentially processed. This Toll activation processes were tightly regulated by specific serpins.

L3

Lipopeptaibol trichogin GA IV: role of the three Aib residues and cationicity on conformation and bioactivity

F. Formaggio[1], M. De Zotti[1], B. Biondi[1], L. Stella[2], N. Jeong[3], Y. Park[3], K.-S. Hahm[3,4] and C. Toniolo[1]

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3. Department of Bio-Materials, Research Center for Proteineous Materials, Chosun University, 501-759 Gwangju, Korea
4. Department of Cellular and Molecular Medicine, College of Medicine, Chosun University, 501-759 Gwangju, Korea

The lipopeptaibol trichogin GA IV is a linear peptide of fungal origin with antibiotic activity.[1,2] With the aim at investigating the role of the Aib residues on its conformation and biological activity, we synthesized by solid-phase methods four analogues in which one or two Aib residues were replaced by Leu. As basic residues are abundant in antibacterial peptides, we also prepared selected trichogin analogues with Lys replacing one or more Gly residues. This latter modification allowed us to assess, in addition, the impact of amphiphilicity (in particular cationicity) and hydrosolubility on bioactivity.

Trichogin-GA-IV	Oct-Aib-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Lol
Oct-[Leu1]-trichogin	Oct-**Leu**-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Lol
Oct-[Leu4]-trichogin	Oct-Aib-Gly-Leu-**Leu**-Gly-Gly-Leu-Aib-Gly-Ile-Lol
Oct-[Leu8]-trichogin	Oct-Aib-Gly-Leu-Aib-Gly-Gly-Leu-**Leu**-Gly-Ile-Lol
Oct-[Leu1,8]-trichogin	Oct-**Leu**-Gly-Leu-Aib-Gly-Gly-Leu-**Leu**-Gly-Ile-Lol
Oct-[Lys5,6]-trichogin	Oct-Aib-Gly-Leu-Aib-**Lys-Lys**-Leu-Aib-Gly-Ile-Lol
Oct-[Lys2,5,6,9]-trichogin	Oct-Aib-**Lys**-Leu-Aib-**Lys-Lys**-Leu-Aib-**Lys**-Ile-Lol
Oct-[Lys2,5,9,Aib6]-trichogin	Oct-Aib-**Lys**-Leu-Aib-**Lys-Aib**-Leu-Aib-**Lys**-Ile-Lol
Oct-[Lys2,Leu-OMe]-trichogin	Oct-Aib-**Lys**-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe
Oct-[Lys9,Leu-OMe]-trichogin	Oct-Aib-Gly-Leu-Aib-Gly-Gly-Leu-Aib-**Lys**-Ile-Leu-OMe

Table I. Amino acid sequences of trichogin GA IV and its newly synthesized analogues

The Aib4 residue appears to play a key role in both antibacterial activity and plasma stability. A single Leu substitution, at position 1 or 8, does not significantly alter the helical conformation of trichogin while, interestingly, it improves its antibacterial activity and decreases its hemolytic potency. The Lys-containing analogues display a significant antifungal action. In addition, they are the trichogin analogues, among those synthesized, with the highest antibacterial potency. We are currently trying to counterbalance their relevant hemolytic activity by inserting Leu residues at appropriate positions.

References

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L4

Antimicrobial and anti-inflammatory activities of designed tryptophan-rich model antimicrobial peptides

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To develop novel short Trp-rich antimicrobial peptides (AMPs) with potent cell specificity (targeting bacteria but not eukaryotic cells) and anti-inflammatory activity, a series of 11-meric Trp-rich model peptides with different ratios of Leu and Lys/Arg residues, XXWXXWXXWXX-NH_2 (X indicates Leu or Lys/Arg), was synthesized. $K_6L_2W_3$ displayed an approximately 40-fold increase in cell specificity, compared with the natural Trp-rich AMP indolicidin (IN). Lys-containing peptides (K_8W_3, K_7LW_3 and $K_6L_2W_3$) showed approximately 2- to 4-fold higher cell specificities than did their counterparts, the Arg-containing peptides (R_8W_3, R_7LW_3 and $R_6L_2W_3$), indicating that multiple Lys residues are more important than multiple Arg residues in the design of AMPs with good cell specificity. The excellent resistance of D-enantiomers ($K_6L_2W_3$-D and $R_6L_2W_3$-D) and Orn/Nle-containing peptides ($O_6L_2W_3$ and $O_6L_2W_3$) to trypsin digestion compared with the rapid breakdown of the L-enantiomers ($K_6L_2W_3$ and $R_6L_2W_3$), highlights the clinical potential of such peptides. $K_6L_2W_3$, $R_6L_2W_3$, $K_6L_2W_3$-D and $R_6L_2W_3$-D caused weak dye leakage from bacterial membrane-mimicking negatively charged EYPG/EYPE (7:3, v/v) liposomes. Confocal microscopy showed that these peptides penetrated the cell membrane of Escherichia coli and accumulated in the cytoplasm, as observed for buforin-2. Gel retardation studies revealed that the peptides bound more strongly to DNA than did IN. These results suggested that one possible peptide bactericidal mechanism may relate to the inhibition of intracellular functions via interference with DNA/RNA synthesis. Furthermore, some model peptides, containing $K_6L_2W_3$, $K_5L_3W_3$, $R_6L_2W_3$, $O_6L_2W_3$, $O_6L_2W_3$, and $K_6L_2W_3$-D inhibited LPS-induced inducible nitric oxide synthase (iNOS) mRNA expression, the release of nitric oxide (NO) following LPS stimulation in RAW264.7 cells and had powerful LPS binding activities at bactericidal concentrations. Collectively, our results indicated that these peptides have potential for future development as novel antimicrobial and anti-inflammatory agents.

L5

Cationic antimicrobial agents that cluster anionic lipids: relationship to microbial species specificity

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Bacterial membranes vary widely in the major phospholipids that comprise the cytoplasmic membrane. In particular, some bacterial species contain large fractions of zwitterionic lipids together with anionic lipids (generally Gram negative bacteria), while other species contain a large fraction of anionic lipids in their membranes. Only the former type of membrane will segregate anionic and zwitterionic lipids in the presence of cationic antimicrobial agents. This provides an objective criterion for predicting which bacterial species will be most susceptible to the action of certain antimicrobial agents. In order to be effective in clustering anionic lipids, antimicrobial agents must have a high density of positive charge, be capable of passing through the outer membrane of Gram negative bacteria, be conformationally flexible and contain sufficient hydrophobicity to partition into a membrane and stabilize a domain.

Several methods have been used to demonstrate the clustering of anionic lipids by cationic antimicrobial agents including DSC, FTIR and NMR as well as the imaging methods of freeze fracture electron microscopy and by AFM in combination with polarized fluorescence microscopy.

Bacteria that have lipid compositions making them susceptible to the clustering of anionic lipids in the presence of neutral or zwitterionic lipids exhibit lower minimal inhibitory concentrations for toxicity, demonstrating the importance of this mechanism for the action of a class of antimicrobial agents.

L6

Host defense peptides: structure, topology and mechanisms of membrane permeabilzation

B. Bechinger

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A number of cationic host defense peptides such as magainins and cecropins, and more recently phylloseptins (1), catestatin (2), DDK (3) or the heterodimeric distinctin (4), have been investigated by biophysical approaches including CD-, multidimensional solution and solid-state NMR spectroscopies. When interacting with membranes these peptides adopt amphipathic structures and in many of the above mentioned cases α-helical conformations. Furthermore, the cationic linear peptides preferentially align along the bilayer interface parallel to the membrane surface. Whereas at higher concentrations they disrupt the membranes the question remains how in such a configuration they can cause pore formation also at lower peptide-to-lipid ratios. The interactions within such complex peptide-lipid mixtures are best described by phase diagrams where the molecular geometry, electrostatics and hydrophobic interaction contributions provide first explanations about the resulting macroscopic phase properties (5).
By designing amphipathic peptides that carry several histidines along the polar face of these amphipathic helices we were able to design a system whose membrane alignment can be triggered merely by changing the pH of the surrounding environment (6). These peptides are transmembrane at neutral pH but in-plane at pH 5 (7). Interestingly, their antimicrobial action is more pronounced at low pH indicating that the in-plane oriented configuration indeed exhibits a high degree of biological activity and has allowed us to design new derivatives active against pathogens. Interestingly these novel peptides also exhibit potent nucleic acid transfection activities in agreement with recent ideas that antimicrobial and cell penetrating peptides exhibit a continuous spectrum of activities with overlapping functionalities (8, 9).

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L7

Folding versus aggregation: a critical evaluation of membrane-active peptides

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Structure analysis of membrane-bound peptides by solid-state NMR has yielded much insight into the conformations and preferred alignments of typical antimicrobial peptides. Besides these natural sequences, also many man-made peptides have been studied with similar membrane-perturbing features, such as designer-made antibiotics, cell-penetrating peptides, and fusogenic agents. We have thus compared and contrasted numerous amphiphilic peptides from natural sources as well as artificial ones. In contrast to the former, we observed that many man-made sequences have a tendency to aggregate once they bind to the lipid bilayer. However, this phenomenon appears to be largely unrecognized, as there are few other techniques available to reveal such aggregation of membrane-bound molecules. Here, we will demonstrate by a number of case studies how solid-state NMR of aligned membranes in combination with oriented circular dichroism is able to reveal peptide immobilization as well as the formation of β-conformers as a clear sign of concentration-dependent peptide aggregation. The functional consequences of a non-productive aggregation or a necessary self-assembly will be addressed.

L8

Potential mechanisms of bacterial resistance to antimicrobial peptides and how to overcome them

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Short peptides, lipopeptides, and protein toxins are produced by all forms of life and have various pathways to combat pathogen invasion. Gene-encoded cationic host defense antimicrobial peptides (AMPs) are the largest group within these toxins. They are believed to overcome bacterial resistance because they have preserved their biological function throughout evolution. In addition, their killing mechanism involves the perturbation of the cytoplasmic membrane, damage hard to fix. Lipopolysaccharide (LPS) the cell wall of Gram-negative bacteria, and lipotechoic actid (LTA) the cell was of Gram-positive bacteria, serve as the first protection layer against antibiotics, including AMPs, and the ability to traverse this barrier depends upon the composition of LPS and LTA, as well as the biophysical properties of the peptide. In line of this, studies have shown that some bacteria can resist AMPs via two possible mechanisms. The first mechanism is the physical barrier imposed by LPS or LTA which prevent AMPs to reach their target. We found that the alteration of the stability of this α-helical AMP by the incorporation of ~30% D-amino acids can overcome LPS barrier, by preventing peptide aggregation. The second possible resistance mechanism involves modification of the charge of head groups of LPS (or LTA). Studies with Salmonella have shown that some AMPs can trigger genes expression which is controlled by the two-component regulatory system, PhoP/PhoQ. The sensor protein, PhoQ, senses the presence of AMPs when they displace divalent cations from their metal binding sites, which lead to activation of PhoP. However, the suggestion that antimicrobial peptides would be natural ligands of the sensor PhoQ is not fully supported and our recent studies reveal that the biochemical and biophysical properties of the AMPs might control the induction of such resistance.

L9

A novel tetrabranched antimicrobial peptide that neutralizes bacterial lipopolysaccharide and prevents septic shock *in vivo*.

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We describe the novel antimicrobial peptide M33 (KKIRVRLSA)[1] that derived from the optimization of the previously reported peptide M6[2], whose sequence appeared not stable because of the natural modification of some residues. M33 was synthesized in a tetra-branched form that we previously demonstrated to induce general peptide resistance to proteolysis, obtaining molecules very suitable for in vivo use[3]. Branched M33 results markedly selective for Gram-negative bacteria showing low Minimal Inhibitory Concentrations for multi-drug resistant clinical isolates of Pseudomonas aeruginosa, Klebsiella pneumoniae, Acitenobacter baumannii and other bacteria. M33 shows a poor toxicity for eukaryotic cells, it binds LPS and DNA and it does not produce appreciable haemolysis even upon prolonged incubation with red blood cells. We also demonstrated that it is not immunogenic upon repeated injections in animals[4].
M33 is able to neutralize LPS derived by P. aeruginosa and K. pneumoniae preventing TNF-α release from LPS activated macrophages. This is of great interest because main sepsis symptoms are triggered by LPS release from lysated Gram-negative bacteria.
The peptide is able to avert septic shock symptoms, and consequently death, in animals infected with reference strains and multi-drug resistant clinical isolates of E. coli and P. aeruginosa, when administered in doses comparable to traditional antibiotics and compatible for a clinical use.
M33 peptide is currently under investigation for the cure of severest symptoms due to Pseudomonas lung infections in Cystic Fibrosis.

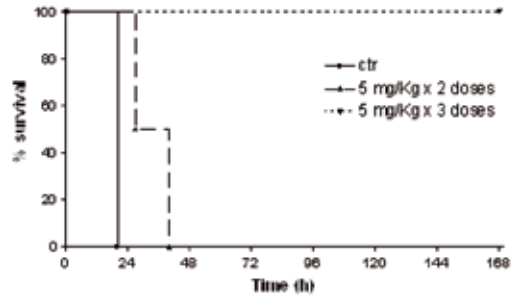

Figure 1. In vivo activity of tetrabranched peptide M33. Mice were ip infected with a lethal amount of P. aeruginosa and then treated with the indicated doses of M33. Two doses delayed animal death. Three doses prevented symptom onset and death.

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L10

Development and application of novel antibiotic peptides from potato

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A 5.6 kDa trypsin-chymotrypsin protease inhibitor was isolated from the tubers of the potato (Solanum tuberosum L cv. Gogu, Gogu valley) by extraction of the water-soluble fraction, dialysis, ultrafiltration, and C18 reversed-phase high performance liquid chromatography. This inhibitor, which we named potamin-1 (PT-1), was thermostable and possessed antimicrobial activity but lacked hemolytic activity. PT-1 strongly inhibited pathogenic microbial strains, including Candida albicans, Rhizoctonia solani, and Clavibacter michiganense subsp. michiganinse. Automated Edman degradation showed that the N-terminal sequence of PT-1 was NH_2-DICTCCAGTKGCNTTSANGAFICEGQSDPKKPKACPLNCDP HIAYA-. Next study, we investigates the use of refined potato protein (RPP) obtained from the potato for their antimicrobial properties and its effects on growth performance, apparent nutrient digestibility, small intestinal morphology and microbial populations in feces and large intestine of weanling pigs. Pigs (n = 280; Landrace × Yorkshire × Duroc; average initial body weight of 5.9 ± 0.66 kg; 23 ± 3 d of age) were allotted on the basis of their body weight and sex to five dietary treatments (each treatment comprised of 4 replicate pens with 14 pigs in each) in a randomized complete block design. The dietary treatments were: PC (positive control; basal diet + 150 ppm apramycin sulfate and 10 ppm colistin sulfate), and RPP (basal diet added with 0, 200, 400 and 600 ppm RPP). The RPP showed in vitro antimicrobial activity and at the concentration of 150 ppm inhibited the growth of tested microbes (Staphylococcus aureus, Salmonella Choleraesuis, Salmonella Gallinarum and E. coli). Pigs fed with antibiotic diet showed better growth performance and had lower populations of total bacteria, coliforms and Staphylococcus spp. in the feces and large intestine when compared with pigs fed RPP diets. Increasing the levels of RPP in diets linearly improved performance and reduced the populations of total bacteria, coliforms and Staphylococcus spp. in feces and contents of colon and rectum. These results suggest that antibiotics were more effective in improving the performance of pigs, while feeding of RPP also improved the performance and reduced the harmful microbes being more effective at higher levels. Thus at higher levels RPP obtained from the potato tubers of Gogu valley can be a potential replacement of antibiotics in the feed of weanling pigs.

L11

Mechanisms of pore formation of antimicrobial peptides and viroporins inserted in a cell plasma membrane

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The biophysical characteristics of synthetic, naturally occurring peptides forming membrane pores, were investigated by inserting them in the plasma membrane of isolated photoreceptor rod outer segments (OS) of frog and their red blood cells (RBC). The OS membrane resistance recorded in whole-cell configuration was ≥1 GΩ once the OS main endogenous conductance was blocked with light; the RBC had lower resistances but high enough to allow the detection of the exogenous current produced by the peptides up to single channel resolution. The peptides were applied to (and removed from) the recorded cells in ≤50 ms; cells were held at -20 mV in symmetric K+ (120 mM).

1μM of synthetic alamethicin F50/5 applied to the OS produced a reversible, inward rectifying current that activated exponentially with a time constant of ~300 ms to a steady state amplitude of 0.7 nA within ~200 ms from peptide application; at low concentration the F50/5 produces single channel activity of conductance ranging between 50-100 pS. The hydrophilic Gln residues at positions 7, 18 and 19 of alamethicin F50/5 were not a key factor for pore formation, but this analogue produced larger pores (~500 pS), with a lower probability of formation than alamethicin F50/5; both peptides produced pores according to a barrel-stave model. Surprisingly, these alamethicines produced smaller current amplitudes once inserted in the RBC in respect the OS: therefore, the lack of hemolysis does not ensure the lack of side-effects of an antimicrobial peptide.

The cecropin-melittin hybrid peptide (Acetyl-KWKLFKKIGAVLKVL-CONH2), produced partially reversible permeabilization at concentrations up to 10 μM. At difference with alamethicines, membrane permeabilization was voltage-independent, repetitive peptide application caused the progressive increase of the steady-state current amplitude, and no discernible single-channel events were detected at low peptide concentrations. Collectively, these results indicate that cecropin-melittin peptide permeabilize the membrane according to a toroidal model.

A peptide spanning residues 35-55 of picornavirus 2B, a non-structural protein required for effective viral replication, produced voltage independent currents at 100 nM concentration. Current activated with a delay of 5 s to a steady state amplitude of ~1 nA within 25 s (10-90%) and was irreversible, indicating a carpet model of permeabilization.

L12

Antimicrobial peptides from frogs: activities, physicochemical properties and applications

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Frogs produce and secrete many kinds of antimicrobial peptides. In this presentation, we will discuss on antimicrobial peptides from two species of Korean frogs: Six antimicrobial peptides from R. rugosa whose amino acids ranging from 22 to 37 residues, and six from R. dybowskii whose amino acids ranging from 15 to 37 residues. All these peptides contain the heptapeptide motif (Rana box, CXXXXXC) at their C-terminus like most of antimicrobial peptides isolated from Ranidae. All the peptides are predicted to form alpha-helical structure. NMR study showed that long peptide (37 amino acids) formed two helical structures and a flexible loop region between the helices. Amphipathicity, length of alpha helix and hydrophobic momentum affected the antimicrobial activity of peptides significantly. Each peptide showed different potency on Gram positive, Gram negative, fungi and cancer cells. GGN5 and GGN6 which are potent on fungi also have high anticancer activity. They and their derivatives showed cytotoxicity against even multi-drug resistant cancer cells by causing apoptosis. Some antimicrobial peptides and dental drugs such as xylithol and chlorohexidine showed synergistic effects on the growth of oral Streptococci.

L13

Penetration of Bac7, a proline-rich antimicrobial peptide, into bacteria - characterizing the entry sequence

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The proline-rich AMPs, isolated from both mammals and invertebrates, are active mainly against Gram-negative bacteria, and act intracellularly being internalized without an apparent damage to bacterial membranes. We have elucidated a putative entry mechanism for Bac7, a proline-rich peptide from cow, and have attempted to identify the region responsible for the peptide's activity. A set of progressively shorter fragments of Bac7 were labelled with a fluorescent probe and the antibacterial activity correlated with the internalization efficiency into E. coli, by using data from antimicrobial activity assays, flow cytometry and confocal microscopy. Results showed that an N-terminal 16-residue fragment is still fully active and efficiently internalized into the cells. Further shortening had dramatic effects on both these functions. In addition, to evaluate the role of N-terminal arginine residues, which appear to be required for activity, these were systematically substituted with lysine, D-Arg, nitro-Arg [4], dimethyl-Arg [4], citrulline or omitted, and the activity of the resulting analogues investigated. The data show that the basicity and propensity to hydrogen bonding of the two Arg residues are important elements for antimicrobial activity. Bacterial variants with mutated transport machinery or altered outer membrane characteristics were used to dissect membrane and transport effects. Results reveal some important requirements for the activity or these proline-rich peptides and suggest a minimal length necessary for both the antimicrobial activity and internalization.

L14

Polyamines can increase resistance of *Neisseria gonorrhoeae* to mediators of innate human host defense

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Antimicrobial peptides are a strategic microbicidal alternative to antibiotics, especially in this era of multi-drug resistant pathogens. *Neisseria gonorrhoeae*, a strict human pathogen of the urogenital tract, has developed resistance to many antibiotics used to treat this infection. Hence, it is imperative to find new compounds to fight this pathogen, responsible for 62 million infections per year worldwide. Understanding the resistance mechanisms developed by the bacteria in its natural environment should help us design more efficient microbicidal compounds. In this respect, we hypothesized that polyamines, biogenic cationic compounds of the human urogenital tract, could alter the resistance levels of N. gonorrhoeae to cationic antimicrobial peptides (AMPs). Our experiments demonstrate that N. gonorrhoeae is particularly adapted to resist high concentrations of polyamines that bathe urogenital mucosal surfaces, and that polyamines can increase resistance to AMPs, such as LL-37 and polymyxin B, and to complement-mediated killing by normal human serum. We observed that the polyamine effect was independent of bacterial polyamine transporters and that antibiotics that exert their activity intracellularly (i.e., ciprofloxacin, nalidixic acid or spectinomycin) are not affected by polyamines. We propose that polyamines could enhance gonococcal survival during infection by reducing bacterial susceptibility to host-derived antimicrobials that function in innate host defense.

L15

Oncocin, a novel designer peptide for the treatment of Gram-negative infections

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Multi-resistant bacterial strains continue to increase in number, triggering extensive research to identify novel classes of antimicrobial compounds with new modes of action and to optimize these for therapeutic applications. Whereas the Gram-positive pathogen methicillin-resistant *Staphylococcus aureus* (MRSA) attracts most interest in the public sphere, highly resistant Gram-negative pathogens are emerging, causing serious health care problems. Currently, three species of Enterobacteriaceae (*Escherichia coli, Klebsiella pneumoniae* and *Enterobacter cloacae*) and two non-fermenting species (*Acinetobacter baumannii* and *Pseudomonas aeruginosa*) are causing much concern due to the rapid spread of multi or extremely resistant strains. One promising line of research focuses on antimicrobial peptides that could represent valid drug leads. Here, we describe the design of novel peptide derivatives with (i) superior antimicrobial activities against all five aforementioned Gram-negative bacteria, (ii) high serum stabilities, (iii) no hemolytic activity, (iv) no eukaryotic cell toxicity, and (v) bactericidal activity to overcome potential resistance mechanisms. The minimal inhibitory concentrations (MIC) in 1% TSB medium for the most promising oncocin derivatives ranged from 0.25 to 4µg/mL for 32 different strains and clinical isolates. Most interestingly, the lead compounds were equally active against resistant bacteria. Another important feature for systemic treatments was their high serum stability with half-lives of 4 to 8 hours in full serum. The non-lytic intracellular mode of action explains nicely the complete absence of any hemolytic or toxic effects on mammalian cells studied in vitro at peptide concentrations of up to 600 µg/mL. In conclusion, the optimized oncocin derivative represents a very promising candidate for subsequent in vivo models and may serve as a novel lead compound for an antibacterial drug class against multiresistant Gram-negative pathogens.

L16

Effect of PEGylation on antimicrobial peptides

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Antimicrobial peptides (AMPs) are diverse group of molecules that can attach to and insert into membrane bilayers to form pores. [1,2] AMPs of various structural classes like tachyplesin-I, indolicidin, and bactenecin were synthesised to investigate the influence of amino acid composition, amphipathicity, cationic charge and size on their antimicrobial activity. These peptides showed very high activity against *E. coli* and *P. Aureginosa*. We also investigated the effect of PEGylation of these peptides on antimicrobial activity. The Peptidyl resins were PEGylated using carboxyl mPEG ranging from 500-5000 Da. Synthetic yield these PEGylated peptides using commercial resin is very low (30-50%) as revealed from HPLC analysis. Presence of high quantity of deletion and truncated PEGylated peptide in the cleavage products obtained from the commercial resin further complicated its purification. We designed new polymer tetra functional PEG crosslinked polystyrene (Baily's Beads) to overcome various problems associated with the synthesis. In this polymer the reactive functional sites are segregated into a reactive shell and to a chemically robust inert core. Confocal microscopic studies of fluorescently labelled polymers showed that the reactive functional sites are confined to its surface of the new polymer resulting in a core-shell polymer compare to commercial polymers where the functional sites are distributed uniformly throughout the bead volume. Confinement of the resin bound peptides only to the polymer surface facilitated the easy accessibility of bulky PEGs on to their N-terminals resulting very high yield and purity of the target molecules. Native peptides and their bio-conjugates were subjected to antibacterial and RBC haemolysis assays. Bioassay showed that PEGylation could enhance the therapeutic indexes AMPs and it may alter the supramolecular assemblies and contribute positively to improve the therapeutic indexes.

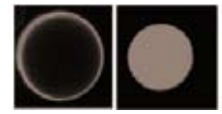

Figure 1. Confocal laser scanning microscopic image of Baily's bead and Merrifield resin

	Tach	PEG Tach	Indo	PEG- Indo	Bac	PEG-Bact
E. coli	> 2	> 2	b/w 50-100	b/w 100-120	>100	>100
P. Aureginosa	b/w 12.5-25	b/w 12.5-25	>120	>120	>100	>100
RBC lysis at 37 µM	4.4 %	1.2 %	5.7 %	0.89 %	1.93%	1.0 %

Table 1 Antibacterial and haemolysis assay of AMPs and PEG- AMP conjugates

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L17

Antimicrobial activity and cytotoxicity of new antimicrobial peptides (melectin, lasioglossins, halictines and macropin) from wild bee venom and their analogues

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Recently, we have isolated, characterized and synthesized interesting antimicrobial peptides (AMPs) from the venom reservoirs of wild bees. These peptides (melectin, lasioglossins, halictines and macropin) and their analogues display high antimicrobial activity against Gram-positive and –negative bacteria, antifungal activity and low or moderate hemolytic activity towards rat and human red blood cells [1-4].
Here we describe cytotoxicity of the above mentioned AMPs and some of their analogs towards 4 types of sessile cells – two of them normal ones (human umbilical vein endothelial cells, HUVEC, and rat endothelial kidney cells, IEC) and two cancer cells (HeLa S3 and colorectal carcinoma cells).
The results showed that the cell lines tested are variously sensitive to the AMPs and their analogues. Hela S3 cells are the most sensitive ones, the concentration causing 50% death of the cells is in the case of the most toxic analogues derived from halictines 2.5-10 μM. Five to 20 times higher concentrations are necessary to kill the other tested cell lines to the same degree. Their antimicrobial activity, the MIC values, determined previously in [3] range between 1-10 μM. These results bring hopeful outlooks to find out medically applicable drugs on the basis of antimicrobial peptides.
Experiments using fluorescently labeled peptide lasioglossin III (Fl-VNWKKILGKIIKVVK-NH_2, Fl, 5,6-carboxyfluorescein) as tracer showed that the peptides enter the animal cells in higher quantities only after they reach the toxic concentration, their concentration is the highest in the vicinity of the nucleus (no overlap with DAPI staining), in the nucleolus and in granules which are situated at very similar places as mitochondria.

Acknowledgements

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L18

Regulation of intracellular proteolysis

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Within cells or subcellular compartments misfolded and/or short-lived regulatory proteins are degraded by protease machines, cage-forming multi-subunit assemblages. Their proteolytic active sites are sequestered within the particles and located on the inner walls. Access of protein substrates is regulated by protein subcomplexes or protein domains which may assist in substrate unfolding dependent of ATP. Five protease machines will be described displaying different subunit structures, oligomeric states, enzymatic mechanisms, and regulatory properties.

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L19

Development of an orally active conotoxin for the treatment of pain

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Peptides derived from the venoms of marine cone snails have attracted recent attention as potential therapeutic agents for the treatment of chronic pain. Although conotoxins share the attractive features of peptides in general of having exquisite selectivity and potency for particular ion channels, membrane receptors or transporters, they also share the general disadvantages of peptides of short biological half-lives and poor oral bioavailability. Cyclisation has been used in the past as a strategy in the pharmaceutical industry for stabilising and locking the conformation of small peptides of 5-12 amino acids. This cyclisation strategy can also be applied to conotoxins to produce additional stabilisation with the potential to dramatically increase the therapeutic potential of these molecules. In this study, we describe the development of a conotoxin analogue that has potent analgesic activity in the chronic constriction injury model of neuropathic pain in rats when administered orally. This result demonstrates the effectiveness of the cyclisation approach for the stabilisation of peptide therapeutics. Neuropathic pain is caused by damage to the nervous system and is among the most severe forms of chronic pain. Although there are medications available for the treatment of neuropathic pain, these are effective in only 40-60% of patients and there is a great need for new effective treatments for this condition. This approach has been successfully applied to other conotoxins highlighting the potential in drug design.

L20

Peptidomimetic chemistry: design of protease inhibitors, prodrug forms and click peptides

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Based on the substrate transition-state mimetic concept, we designed and synthesized novel classes of inhibitors of aspartic proteases such as renin, HIV protease, malarial plasmepsin II, containing the hydroxymethylcarbonyl (HMC) isostere[1-3]. Among them, tripeptide KNI-272 was a highly selective and superpotent HIV protease inhibitor. Physicochemical studies suggested that the HMC isostere is an ideal transition-state mimic[4]. We applied the substrate transition state concept to develop inhibitors against β-secretase (BACE1) targeting Alzheimer's disease (AD)[5,6], and designed a novel BACE1 inhibitor, KMI-429 that reduced amyloid β peptide (Aβ) production in transgenic and wild-type mice[7].
We developed the O-acyl isopeptide method[8] for the synthesis of difficult peptide sequences including Aβ. The native Aβ1-42 tends to aggregate due to uncontrolled polymerization complicating AD research. On the basis of our study with the "O-acyl isopeptide method"[8-10], we developed novel photo- and pH-triggered "click" peptides that readily convert to the native Aβ1-42 upon activation. Click peptide Aβ1-42 analogs migrated to generate Aβ1-42 with a 'click' reaction via an O-N intramolecular acyl migration[8-12]. The BACE1 inhibitors and amyloid β 'click' peptide that we developed will pave the way to defy Alzheimer's disease.

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L21

Antiviral proteins from natural product extracts: potent antiviral activity of griffithsin

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Our laboratory has discovered several novel antiviral proteins and peptides from nature. Here we will discuss the unique structural basis and potent antiviral activity of a recent discovery from a marine organism. The antiviral protein griffithsin (GRFT), isolated from the red alga Griffithsia sp., is a 12.7 kDa protein originally identified based on its picomolar activity against HIV in cell-based in vitro assays. GRFT was found to have a unique three-dimensional structure with three carbohydrate binding sites per monomer and to block viral fusion and entry. More recent studies have shown that GRFT can be manufactured on a large-scale by recombinant production in Nicotiana sp. This has allowed for a wider range of studies on this marine natural product. Since its discovery, GRFT has been tested for activity against a broad spectrum of viruses. Here we report recent progress on the development of GRFT for use as a systemic, topical and intranasal antiviral agent. Results from recent *ex vivo* and *in vivo* studies showing GRFT's activity against HIV, ebola Zaire and the SARS coronavirus will be presented.

L22

Design and applications of protein epitope mimetics

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The folded 3D structures of peptides and proteins provide excellent starting points for the design of synthetic molecules that mimic key epitopes (or surface patches) required for biological activity. Protein epitope mimetics (PEMs) should capture both the structural and conformational properties of the target epitope, as well as the biological activity. By transferring the epitope from a recombinant to a synthetic scaffold that can be produced by parallel combinatorial methods, it is then possible to optimize properties through iterative cycles of library synthesis and screening.

One very interesting naturally occurring scaffold is based on the β-hairpin motif, which is used by many proteins to mediate molecular recognition events. This motif is readily amenable to PEM design, for example, by transplanting hairpin loop sequences from folded proteins onto hairpin-stabilizing templates, such as the dipeptide D-Pro-L-Pro. However, β-hairpin peptidomimetics can also be exploited to mimic other types of epitope, such as those based on α-helical secondary structures. Some examples taken from earlier work will be presented in this lecture[1,2].

In one case, some naturally occurring cationic host-defence antimicrobial peptides were taken as a starting point for PEM design. After several rounds of optimization, a lead compound was produced that showed potent antimicrobial activity against the Gram-negative bacterium Pseudomonas aeruginosa. The mechanism of action of this compound has been studied intensively, and appears to target an outer membrane protein required for the biogenesis of the outer cell membrane. The peptidomimetic also shows potent antimicrobial activity in a mouse septicemia model. Drug-resistant strains of Pseudomonas are a serious health problem, so this family of antibiotics may have important therapeutic applications.

In another case, the focus of interest has been in the use of epitope mimetics as vaccine candidates. In order to elicit strong immune responses using synthetic epitope mimetics, we have developed a novel delivery system, based on synthetic virus-like particles (SVLPs)[3]. SVLPs have a size and shape similar to those of some recombinant VLPs, but are completely of synthetic origin. In recent work, we have shown how the properties of these SVLPs can be tailored by synthesis, to include T helper epitopes and Toll-like receptor ligands, as well as allowing multivalent display of B cell epitope mimetics. The SVLP technology combined with the use of synthetic antigen mimetics may represent a powerful chemistry-driven approach to synthetic vaccine design.

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L23

Novel synthetic methodologies to prepare unnatural aminoacids and peptidomimetics

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Preparation of unnatural α, β and γ aminoacids in enantiopure form is an important and challenging task in organic synthesis. In particular, methodologies which are modular in nature and make use of organocatalysts, e. g. chinchona phase transfer catalysts, are of great interest due to their low environmental impact compared to metal catalysed processes. We have developed reagent **1** as a highly reactive cinnamate equivalent.[1] In this paper we will describe the use of reagents **1** in chiral phase transfer catalyses to obtain α and γ aminoacids in high yields and in enantiomeric excesses up to 98% ee.[2]

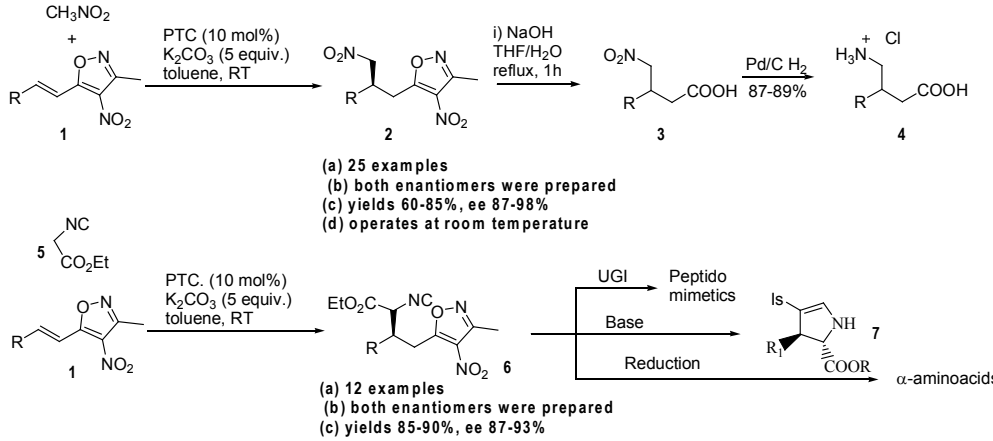

Figure 1. preparation of α and γ aminoacids in high enntiomeric excess.

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L24

Amino acids and derivatives as attractive starting materials for the synthesis of active biomolecules

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Amino Acid Derivatives are important starting materials for the synthesis of biomolecules. Among these derivatives, N-Carboxyanhydrides (NCA) as well as diketopiperazines (DKP) present interesting reactivity.

We have used NCA for the synthesis of:

- Peptides in «solid» media, without using solvent (V. Declerck, F. Lamaty, & J. Martinez, Patent N° 0753970 Filed March 2007; Angew. Chem. Int. Ed., 2009);
- Supported reagents for the formation of disulfide bonds in peptides (M. Cristau, S. Cantel, G. Subra, & J. Martinez, US Patent Filed June 2006).

On the other hand, N-protected DKP were used for the stereoselective synthesis of Pyrrolidine-2,4-Diones through the Transannular Rearrangement of Activated Lactams (TRAL reaction) that was discovered in our laboratory (G. Dewynter, D. Farran, J. Martinez, Patent n°0753973, March 2007, PCT Int. Appl. 2008, WO 2008125421; Farran et al., Angew. Chem. Int. Ed. 2007; Farran et al. Org. Letters, 2007; Org. Biomol. Chem., 2008).

Acknowledgements

These syntheses as well as some of their applications will be described and discussed.

L25

Intramolecular macrocyclization reactions in 3_{10}-helical peptides: stereo- and regioselective properties

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In our ongoing efforts to bridge peptide chemistry and organic chemistry, we are currently investigating two types of intramolecular macrocyclization reactions in 3_{10}-helical peptides:
(I) The 3-(4-benzoylphenyl)alanine (Bpa) residue is widely used as a photoaffinity label for the study of intramolecular (peptide) ligand-receptor (protein) interactions, where it is thought to primarily function by H-abstraction from a Met residue followed by covalent C-C bond formation of the resulting radical pair. So far, we have carried out a detailed analysis of the chemical and 3D-structures of the diastereomeric, intramolecular i,i+3 photoreaction products, arising from the terminally-protected, 3_{10}-helical hexapeptide Boc-Aib-L-Bpa-$(Aib)_2$-L-Met-Aib-OMe substrate using chromatographic and spectroscopic techniques in addition to X-ray diffraction. The stereo- and regioselective aspects of this macrocyclization reaction have been examined in detail. Moreover, in collaboration with dr. K. Wright (University of Versailles, France) we are currently working on the synthesis, characterization and applications of two conformationally restricted Bpa congeners.
(II) The intramolecular ring-closing metathesis (RCM) is a useful and emerging method for reinforcing the conformational and metabolic stabilities of helical peptides. In this connection, we have recently shown that an RCM-derived, 18-membered macrocycle is the result of cross-linking the side chains of i,i+3 amino acids in the terminally-protected, 3_{10}-helical, 8-mer Boc-$(Aib)_3$-L-Ser(Al)-$(Aib)_2$-L-Ser(Al)-Aib-OMe. Remarkably, we have characterized by X-ray diffraction not only the starting linear peptide, but the major, olefin E-diastereomeric, stapled product and its hydrogenated derivative as well. Interestingly, in this peptide substrate the all-hydrocarbon tethers do not disturb significantly the original 3_{10}-helicity. In this connection, we are currently preparing a set of ladder peptides by taking advantage of different types of metathesis reactions including RCM.

L26

Ureido-based scaffolds as peptide mimics : connecting structure and function

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Selecting the appropriate building units and deciphering the associated "folding codes", are determining steps towards the elaboration of discrete oligomers with defined folding patterns. Though non natural amino acids remain unmatched for structural diversity and versatility in foldamer chemistry, foldamers based on amide bond surrogates have emerged.[1] Strategies developed for oligoamides to impose conformational restriction and to promote folding – e.g. local conformational control, solvophobic interactions or long range H-bond interactions – largely apply to aromatic and aliphatic urea oligomers. Secondary structural motifs adopted by urea strands include duplexes, helices, sheets and turn segments.[2] Our groups has investigated enantiopure oligomers consisting of urea bridging units and bearing proteinogenic side-chains. These oligomers show a remarkably strong propensity to fold into stable helical secondary structures reminiscent of the α-helix.[3-5] Because of their diversity in side chain appendages, and also their resistance to enzymatic degradation, urea-based helical foldamers are promising scaffolds for use in a range of biological and biomedical applications. Noteworthy, short sequences designed to mimic globally amphiphilic α−helical host-defense peptides display broad antibacterial activity with selectivity for prokaryotic versus mammalian red blood cell membranes.[6, 7]

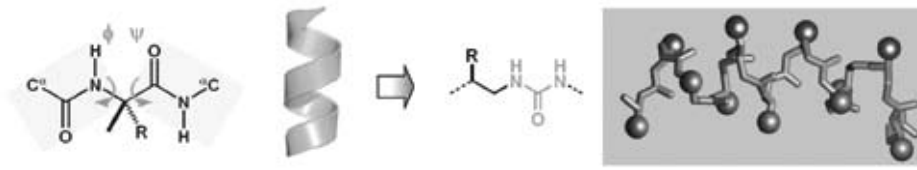

Figure 1. α-peptide versus urea-based helical backbones.

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L27

Side chain-to-Side chain (Sc2Sc) clicked-cyclo peptides. Synthesis, conformation and biological activity

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The prospective of peptide-base drugs and the abundance of novel attractive targets for therapeutic intervention that require disruption of protein-protein interactions (PPIs) present challenging drugability issues. Of special interest are excised helical domains that are frequently found to interact with hot spots at the critical interfaces of the PPI sites. Overcoming inherent metabolic susceptibility, bioavailability and cell permeability are some of the problems associated with the use of peptides as potential therapeutics. To this end, different modes of cyclization that introduce structural rigidification are practiced to improve selectivity and overcome the above mentioned deficiencies. Our recent introduction of Cu^{I}-catalyzed azide–alkyne 1,3-dipolar Huisgen cycloaddition to form a clicked side chain-to-side chain (Sc2Sc) cyclization provides a bioorthogonal approach to generate an intramolecular bridge containing the peptidomimetic [1,2,3]triazolyl moiety. We developed the synthesis of building blocks and the heterodetic cyclopeptides and studied the impact of the size of this bridge, the orientation and the position of the triazolyl moiety within this bridge on the conformational propensities and biological activities.

L28

New, *in silico* identified drug candidates and their peptide conjugates with antimycobacterial activity

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One third of the world's population is believed to be latently infected with Mycobacterium tuberculosis, the causative agent of tuberculosis. The pathogen can remain in infected macrophages in dormant phase for over years. Therapy of the disease takes at least six months and could have serious side effects. The development of novel antimicrobials to counter the emergence of bacteria resistant to current therapies is urgently needed. In silico docking methods and structure-based drug design are useful tools for identifying potential agents. Novel docking algorithms could be used to (i) analyze the structure of crucial bacterial enzymes for possible binding sites involved, (ii) generate drug candidates with binding potential to the target protein and (iii) rank them according to their binding affinities.

The clinically used antibiotics usually have low effect against intracellular bacteria. The cellular uptake of the antitubercular agents can be enhanced by peptide conjugates containing T-cell specific oligopeptide carrier[1]. Based on in silico docking calculations on their binding to enzymes here we report on the identification new drug candidates. To confirm the antibacterial effect, the minimal inhibitory concentration (MIC) and the colony forming unit (CFU) data of these compounds as well as some of their peptide conjugates were determined on M. tuberculosis H_{37}Rv culture. The cytotoxicity and the cytostatic effect of these compounds were also measured by MTT assay on human cells and cell lines. Twenty-eight out of fifty-seven in silico determined compounds showed relevant antitubercular activity under these conditions. The MIC values of the most effective molecules were lower than that of isoniazid (0.05μg/ml, 1.47×10^{-7} M). The IC_{50} value on MonoMac6 monocytic cell line was four orders of magnitude lower than the MIC value of the most active compounds. We also report on the synthesis and specific delivery of the compounds to the infected macrophages using tuftsin [2,3] and/or scavenger receptor specific polypeptides.[4]

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L29

Solution structure and function of the folded and highly conserved C-terminal domain common to spider dragline silk proteins

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Spider silk is one of the most impressive protein fibers known, with a toughness of the dragline silk greater than that of man-made synthetic fibers such as Kevlar [1]. The proteins are stored as a very highly concentrated (up to 50%) solution without any aggregation but form stable silk on demand in less than a second. The key of this surprising result is in the folded domain structure of the C-terminal end [2].
Spider silk mainly consists of large protein components with long repetitive core sequences and non-repetitive amino- and carboxyterminal (NR) domains, which covalently link individual silk monomers. These NR domains are highly conserved among different spider silk proteins and across all known species of spider. We present the nuclear magnetic resonance (NMR) solution structure of the carboxyterminal NR domain from the garden spider Araneus diadematus dragline silk protein ADF3 [2]. The structure is a novel dimeric α-helical fold with a large dimerization surface and interleaved helical segments. We additionally provide evidence for the functional properties of the NR domains. These domains are quite sensitive to the presence of an inter-monomer disulfide bridge and an intra-monomer salt bridge. Additionally, this domain tends to unfold at high protein concentrations. This can be prevented by the addition of sodium chloride, which is also present during silk protein storage in the spider. Furthermore, this domain is responsible for the correct alignment of the repetitive sequence elements required for stable fiber formation. These data provide a structural basis for a better understanding of the complex mechanism of the structural transformation, taking place during fiber formation.

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L30

NrTP, a snake-derived CPP, targets very specifically the nucleoli of tumor cells

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We have recently reported[1] the preferential nucleolar localization in live HeLa cells of designed peptides resulting from the structural dissection of a South American rattlesnake toxin. Named NrTPs (nucleolar targeting peptides), these are strongly cationic 12-13 amino acid sequences with two repetitive KKG triads. Here we report that NrTP1 (YKQCHKKGGKKGSG) and its retro version (rNrTP1, GSGKKGGKKHCQKY) are both able to translocate the cell membrane and quite specifically home in the nucleoli of tumor cell lines such as human pancreatic adenocarcinoma (BxPC-3), human ductal mammary gland carcinoma (BT-474 or human colorectal adenocarcinoma (Caco2). Mouse neuroblastoma cells (N2a) also uptake NrTP1 rather efficiently at concentrations in the 6-50 μM range, as measured by flow cytometry. The preliminary identification of putative targets of NrTP1 in the nucleolus by proteomic methods will be discussed. The findings are noteworthy for at least two reasons: (1) among scores of cell penetrating peptides, NrTPs are unique for their singular ability to target a specific subnuclear compartment, and (2) the nucleolus being the site of ribosome biogenesis and other vital cell functions, NrTPs may be useful for delivering drugs aimed at nucleolar proteins involved in cell cycle regulation, thus controlling the fate of tumor cells.

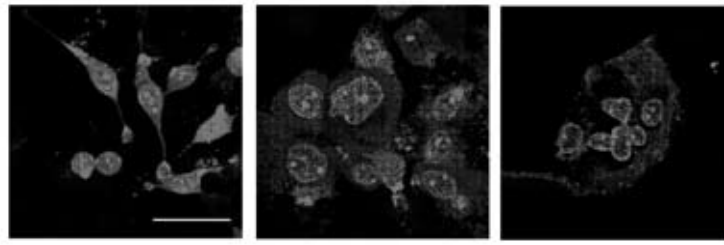

Figure 1. Internalization of rhodamine B-labelled NrTP1 with (left) HeLa, (center) BT-474 mammary adenocarcinoma and (right) BxPC-3 pancreatic adenocarcinoma cells.

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L31

Structural disorder and induced folding within the measles virus nucleoprotein: experimental assessment and functional implications

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In the last decade there has been an increasing amount of experimental and computational evidence pointing out that eukaryotic genomes code for a high proportion of intrinsically disordered proteins (IDPs). IDPs are functional proteins that lack stable II and III structures under physiological conditions in the absence of a partner and that rather exist as conformational ensembles. IDPs are often involved in biological processes implying manifold protein-protein interactions, such as cellular regulation, transcription and signal transduction. Indeed, intrinsic disorder is a distinctive and common feature of "hub" proteins, with disorder serving as a determinant of protein interactivity.

In the course of the structural and functional characterization of the measles virus (MeV) replicative complex, we discovered that the nucleoprotein (N) and the phosphoprotein (P) contain long (up to 230 residues) disordered regions possessing sequence and biochemical features that typify IDPs. We then extended these findings to the N and P proteins of Paramyxoviridae, and we showed that the presence of intrinsically disordered regions is a conserved feature within the replicative complex of these viruses.

We thoroughly characterized the intrinsically disordered C-terminal domain of MeV N (N_{TAIL}), as well as the molecular mechanisms governing its disorder-to-order transition upon binding to the C-terminal X domain of P (XD). The presence of the disordered N_{TAIL} domain exposed at the surface of the viral nucleocapsid, allows the establishment of interactions with various viral and cellular partners, thereby leading to multiple biological effects, including tethering of the polymerase complex, stimulation of viral transcription and replication, immunosuppression, stimulation of cytokine expression, and virus assembly. Furthermore, the persistence of a disordered N_{TAIL} appendage even after complex formation may enable the establishment of interactions with additional partners, thereby providing a scaffold for the intervention of viral and/or cellular co-factors that can modulate the strength of the N_{TAIL}-XD interaction.

Taking into account the fact that the protein flexibility of disordered regions is known to confer an increased plasticity that enables promiscuity and allows protein interactions to occur with both high specificity and low affinity, we propose that the main advantage of the abundance of disorder within viruses would reside in pleiotropy and genetic compaction, where a single gene would encode a single (regulatory) protein product able to establish multiple interactions via its disordered regions, thereby exerting multiple concomitant biological effects.

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L32

Discovery of potent, cyclic Calcitonin Gene Related peptide (CGRP) receptor antagonists

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The triptan class of 5-$HT_{1B/1D}$ agonists including sumatriptan, rizatriptan and others are the established clinical standard for the treatment of episodic migraine headache. The therapeutic value of these agents can be compromised particularly in cases of patients who are susceptible to their cardiovascular side-effects. Calcitonin Gene Related Peptide (CGRP), a potent dilator of cerebral and dural vasculature is known to be elevated in plasma and cerebral spinal fluid during migraine attacks. Selective blockade of the CGRP1 receptor offers the promise of controlling migraine headache more effectively without the side-effects commonly associated with the use of triptans. Recent disclosure of clinical studies with small molecule CGRP receptor antagonists has confirmed the potential of this therapeutic strategy. Our efforts to develop a novel, peptide based CGRP1 antagonist focused on the C-terminal portion of the peptide which is known to bind to the CGRP receptor but lack the agonist properties of the native ligand. Extensive SAR studies of the C-terminal (CGRP 27-37) region identified a novel cyclic structure: Bz-Val-Tyr-cyclo[Cys-Thr-Asp-Val-Gly-Pro-Phe-Cys]-Phe-NH_2 with a K_b value of 0.13nM against the cloned human CGRP1 receptor. Additional SAR studies directed at enhancement of potency and improvement of physicochemical properties yielded a series of analogs with K_b values of 0.02 -0.05 nM range. Several of these were studied in two in vivo models: dural plasma protein extravasation in the rat and inhibition of capsaicin induced forearm blood flow in the cynomolgus monkey.

L33

Chemical challenges for the construction of protein mimics

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We wish to develop chemical approaches for the construction of protein mimics using scaffolds and dendrimers. These should lead to obtaining smaller equivalents of entire proteins, which are (fully) functional such as enzyme inhibitors, synthetic vaccines and perhaps ultimately synthetic antibodies.
In addition, we wish to develop approaches for the synthesis of peptide-based polymers using click chemistry. This may be particularly attractive for the preparation of protein mimics with repetitive sequences and provide an avenue towards new biomaterials. Another source of inspiration for new bio(nano)materials are the properties of peptides and proteins involved in protein aggregation diseases. Insights in the principles and rules of the molecular basis of self-assembly of peptides may lead to the construction of peptide nanotubes and nanospheres.

L34

Imide-click reaction. Application for peptide ligation and prodrug design

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This presentation will describe the interest of imide-click ligation, i.e. the reaction between peptide thioacids and azidocarbonyl derivatives, for access to complex peptide scaffolds.
The reaction of peptide thioacids with sulfonyl azides is well known and allows the formation of a stable N-acyl sulfonamide bond.[1,2] One of the significant differences between N-acyl sulfonamide and imide groups is the ability of latter to react with nucleophiles. We present two different applications that exploit the usefulness of imide ligation and electrophilicity of imide group. First, the reaction was exploited for the design of a novel prodrug strategy allowing the facile assembly/disassembly of drug conjugates. The first step involves an imide bond formation between a peptide carrier and a drug by reaction of a peptide thioacid with an azidoformate derivative of the drug. This imide bond plays a key role in the disassembly process leading to drug release. Indeed, enzyme-catalyzed cleavage of a peptide bond unmasks an amine group, which cyclizes on the imide to liberate the drug. Typically, the disassembly process can be triggered by an enzyme (prostate specific antigen, PSA) specific for the target tissue (prostate cancer). In the second application, imide ligation was exploited for diblock peptide synthesis. In a first step, reaction of a C-terminal peptide azide (fragment 1) with an N-terminal thioaspartyl peptide (fragment 2) leads to a construct in which the side-chain of Asp and the last residue of fragment 1 are connected by an imide bond. Unmasking of the alpha-amino group of fragment 2 triggers acyl migration of fragment 1 from the side-chain to the amino group through a 6-membered intermediate, and thus to the formation of a native peptide with an Asn residue at the ligation site.
Overall, the work presented shows that imide ligation complements advantageously the existing chemical peptide ligation toolbox.

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L35

Molecular interaction of PPARγ with its ligand

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PPARγ is one of the metabolic nuclear receptors with a role in maintaing glucose and lipid homeostasis. Thus it has been the target of antidiabetic insulin sensitizing drugs which bind the receptor as ligands to modulate receptor activity and target gene transcription by recruiting cofactors.

Although two derivatives of thiazolidinedione (TZD), pioglitazone (Pio) and rosiglitazone (Rosi), which are potent and selective PPARγ full agonists, are commercially available antidiabetic drugs, unfortunately they are associated with weight gain and several adverse effects such as edema. It is especially significant that pulmonary and macular edemas are linked to congestive heart failure. Although structures of the complexes of PPARγ with some ligands have been investigated to explain how ligands differentiate the functional activities, this mechanism remains unclear. Here we carried out thermodynamic analysis in addition to X-ray crystallographic analysis on the series of complexes to gain deeper insight into complex formation.

The ligands used here are Pio and Rosi, FK614 and nTZDpa which are classified by the in vivo efficacy into full, intermediate and partial agonists, respectively. We determined the structure of the binary complex of the ligand-binding domain (LBD) of human PPARγ for FK614 with 1.97Å resolution and obtained the refined structures by re-investigation those for Pio and Rosi with 1.60Å and 1.48Å, respectively. (The previously reported structure with 2.05Å was used for nTZDpa. Structure 15, 1258, 2007) The results showed that FK, an intermediate agonist, binds to Helix 3 which is located in-between Helix 12 and the β-strand in the cavity of LBD where the former is the binding site of the full agonists and the latter is that of the partial agonists.

The isothermal titration experiments first provided the precise thermodynamic parameters of the association-dissociation systems of PPARγ and its ligand. Interesting information was elicited from the set of data for these ligands at various temperatures. For example, the *K*d values of the full agonists are smaller than those of the intermediate and partial agonists. The Δ*C*p values were almost the same for Pio, FK and nTZDpa which were larger than that of Rosi. This indicated that the contribution of hydrophobic interaction to the stability of Rosi's complex is less than those of the others.

The structural information of the complexes with good resolution obtained above allowed us to explain further the binding modes of each ligand by these thermodynamic parameters. The activity-binding mode relation-ship should give a clue to the rational drug design for diabetes.

L36

Design and application of material forming peptides: from antibacterials to mammalian cell delivery.

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Hydrogels are an important class of biomaterial used in drug and cell delivery, three-dimensional cell culture and tissue engineering, to name a few applications. We have been developing a peptide-based hydrogel system that allows the direct encapsulation of therapeutics during gel formation. Loaded gels can be directly shear thin delivered via syringe to a target site where the therapeutic can be released at controlled rates. In addition to delivering drugs, mammalian cells can also be encapsulated and delivered without effecting cell viability. Among several important considerations for implantation of hydrogel materials, a main concern is the introduction of infection. The surface of many of our gels exhibit inherent antibacterial activity. They demonstrate broad spectrum antibacterial activity against gram positive (Staphylococcus epidermidis, Staphylococcus aureus, and Streptococcus pyogenes) and gram negative (Klebsiella pneumoniae and Escherichia coli) bacteria, all prevalent in hospital settings. By employing peptide design principles, a new generation gel has been prepared that can kill Methicillin-resistant Staphylococcus aureus (MRSA) on contact. This gel displays shear-thin/recovery rheological behavior and therefore can be easily syringe-delivered to either treat contaminated surfaces such as wound sites or applied as coatings to inhibit MRSA infection.

"MURRAY GOODMAN" YOUNG INVESTIGATORS' SESSION

Y1

Ghrelin agonist and inverse agonist tracers for PET imaging

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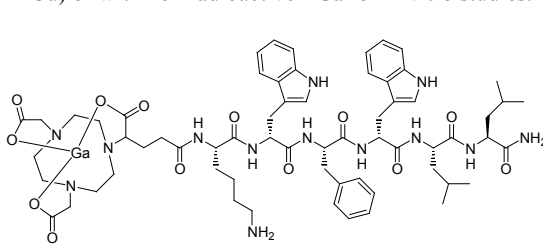
Ghrelin, a gastro-intestinal peptide hormones is the only known endogenous orexigenic signal. It plays a central role in the short and long term regulation of hunger and energy homeostasis.[1]a Therefore, ghrelin antagonists and inverse agonists have emerged as potential anti-obesity drugs.[2]
Positron Emission Tomography (PET) is a non-invasive imaging method allowing the localisation and quantification of a radiotracer in vivo.[3] Thus, development of microPET for small animals provides to biochemists a unique tool for pharmacokinetic studies of bioactive molecules. In this context, ghrelin agonist and inverse agonist tracers were developed in the aim to provide a better understanding of ghrelin mode of action and for further design of ghrelin inverse agonists.
Human ghrelin$_{1-28}$ and short ghrelin inverse agonists were synthesised on solid-phase and NODAGA, a bifunctional chelator,[4] was coupled to the resin-bound peptides at different free amine positions. The NODAGA-peptides were then labeled in solution with radioactive isotopes suitable for PET imaging (^{68}Ga or ^{64}Cu) or with non radioactive ^{69}Ga for in vitro studies.



Ghrelin Inverse Agonist: **Ga-NODAGA-KwFwLL-NH$_2$**

[Lys^{16}(Ga-NODAGA)]-ghrelin$_{1-28}$

Figure 1. Ghrelin and ghrelin inverse agonist tracers

The activity of ^{69}Ga-NODAGA-peptides was evaluated by inositol phosphate turnover assay. [Lys^{16}(^{69}Ga-NODAGA)]-ghrelin$_{1-28}$ was equipotent to the native ghrelin. Introduction of the ^{69}Ga-NODAGA complex at the N-terminus of ghrelin inverse agonists decreased their potency from 1.5 to 6 fold. Nevertheless, pharmacokinetic profiles of the radiolabeled peptides were investigated by microPET on rodent.

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Y2

Synthesis, conformation, and bioactivity of integramide A, a peptide inhibitor of HIV-1 integrase, and selected analogs

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AIDS is produced by HIV-induced infections. HIV integrase is an important enzyme as it is critical for the integration of the HIV genome into that of the host cell. This complex process is exclusively carried out by a viral enzyme not found in the host cell. Therefore, this protein represents a safe target for the development of single or combined anti-HIV therapy. Integramide A is a 16-mer long, effective peptaib inhibitor of HIV-1 integrase (Figure 1). Here, we describe a versatile synthetic strategy in solution to afford this natural compound, its diastereomer at positions 14 and 15,[1] and the analogs of the two above mentioned peptides in which all of the three (2S,4R)-Hyp residues at positions 2, 9, and 13 are replaced by L-Pro.[2]

Integramide A: Ac-D-Iva-L-Hyp-L-Ile-L-Iva-L-Leu-Aib-Aib-Aib-L-Hyp-L-Leu-L-Iva-Aib-L-Hyp-L-Iva-D-Iva-Gly-OH

Figure 1.Chemical structures of Aib and Iva, and amino acid sequence of integramide A.

We also present our data on in-depth conformational analysis and biological activity against HIV-integrase of these peptides. In particular, the study performed on the Pro-containing analogs definitely confirms that the mixed α-/3_{10}- helical conformation of natural integramide A plays a key role in its mechanism of inhibition. Moreover, our data provide evidence that the amphipathic character of this helical structure is not strictly required for the activity of integramide A. These observations will hopefully help us to further clarify the precise mechanism of inhibition of this interesting peptaib and to identify shorter bioactive peptide sequences.

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Y3

Non-cationic dipeptide mimetic oligomers as cell penetrating non peptides (CPNP)

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The delivery of drugs into cells remains a major limitation in several therapies. Peptide vectors referred to as cell-penetrating peptides (CPPs) have emerged as promising tools for the intracellular delivery of bioactive cargoes (drugs, peptides, siRNA).[1] Several classes of CPP have been reported, most of them are basic or amphipathic peptides[2] and often display high α-helical propensity.[3]

In our study we have focused on the possible application of non-charged oligomers constructed by assembly of constrained dipeptide mimics[4] as a new class of cell penetrating carriers. These oligomers are characterized by their well defined secondary structures, stability to protease degradation and small size. After in depth biological studies, we have proven that these compounds can compete for their cellular uptake with the polyarginine - a well established CPP.

The cellular uptake (fig.1) of the compounds was evaluated by total fluorescence uptake and confocale laser scanning microscopy. The values obtained by the mentioned techniques were equivalent or better than those of the L-Arginine octamer. The cellular penetration of the described short oligomers offers a novel class of vectors with the particularity to be non-cationic. These features should open new avenues in the field of compounds able to cross the cell membrane and give the possibility for some interesting medicinal applications

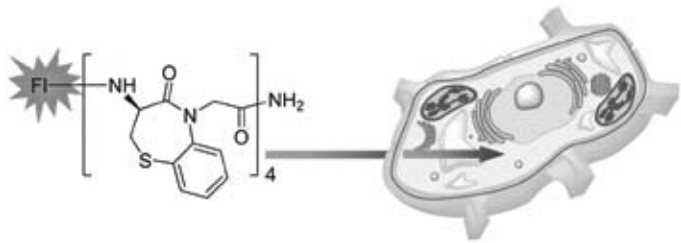

Fig. 1 Small oligomers were synthesized by solid phase synthesis and marked by fluorescence tag. Their cellular uptake and sub cellular localization were determined.

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Y4

Purification and characterization of peptides with antimicrobial activity isolated from fleshfly *Neobellieria bullata*

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Microbiological research is focused on solution of pathogen resistance problem. The number of resistant pathogenic microorganisms has increased in last years. In the future there will be fewer possibilities to cure multiresistance infections by existing antibiotics. One of the potential solutions seems to be isolation or design of the short cationic peptides naturally synthetised as a part of innate immunity[1]. In our project larvae of the fleshly *Neobellieria bullata* were chosen as a source of these peptides.

The haemolymph was picked up from larvae, gradually centrifuged and precipitated by acidified methanol. Subsequently these fractions were separated by chromatographic methods (SPE column, FPLC, RP-HPLC) to obtain fractions of short peptides. Identification and characterization of these fractions were performed by tricine electrophoresis, mass spectrometry MALDI-TOF analysis and N-terminal sequencing.

Antimicrobial activity of fractions was screened by diffusion method. Testing model organisms were gram-negative bacteria: Escherichia coli, Pseudomonas aeruginosa, gram-positive bacteria: Staphylococcus aureus, Bacillus megaterium, Listeria innocua, and fungi: Candida scotii, Aspergillus ochraceus and Mucor species.

Several fractions showed antimicrobial activity against bacteria Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus megaterium and fungi Candida scotii. Tricine electrophoresis proved presence of low molecular peptide. MS analysis of antimicrobial active fractions determined molecular masses less than 10 kDa and the N-terminal sequencing was successful only on the part of the sequence. The sequences do not have any similarity match with the databases of peptide with antimicrobial properties.

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Y5

NMR structural characterization of a bioactive β-hairpin peptide and of its interaction with VEGFR-1_{D2}

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Angiogenesis, the process of new blood vessel formation, is a complex process that involves a number of different growth factors and it is essential for a variety of physiologically important events such as embryogenesis, wound healing and tissue repair but is also critical in a number of diseases such as tumor progression, psoriasis, rheumatoid arthritis and diabetic retinopathy.[1,2] Consequently, the molecules that induce or mediate angiogenic events are potentially important targets for the treatment of these diseases. VEGF-A (or VEGF), the most important inducer of angiogenesis, is a homodimeric protein belonging to the family of cysteine knot growth factors, and it binds with high affinity two tyrosine kinase receptors VEGFR-1 (also called Flt-1) and VEGFR-2 (or KDR).[1]

Recent studies have been reported the VEGF homolog, Placenta Growth Factor (PlGF), which plays an important role in pathological angiogenic events, exerting its biological activities through binding to VEGFR-1. Solid state structure of the PlGF bound to the second immunoglobulin-like domain of VEGFR-1[3] revealed the interacting regions of the growth factor. In order to find new active biomolecules which can modulate the interaction of PlGF with its own receptors, we designed and synthesized peptides mimicking the interacting region 87-100 of PlGF, which in the natural protein adopt a β-hairpin conformation. The designed peptides were characterized by NMR in water solution and tested to assess their biological properties. Moreover, in this work, we use NMR methodologies to elucidate the structural requirements for the interaction of these bioactive peptides with VEGFR-1_{D2}.

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Y6

Hierarchical approach on the N-terminal 1-11 segment of PTH

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Recent advances in understanding the chemistry of life have shown that peptide-macromolecular interactions constitutes the main physico-chemical mechanisms by which living processes are controlled and modulated. The prevalence and diversity of peptides, along with their participation in crucial physiological functions, have renewed the interest in using these compounds as therapeutics. The development of peptide or peptido-mimetic ligands, which can target the receptors or modulate biological activities, is currently a top priority in biology and medicine. Therefore, establishing systematic structure-based approaches for the design of such ligands is a very important issue[1]. Starting from the hierarchical approach proposed by Hruby[1], analogues of the N-terminal 1-11 segment of the paratyroid hormone (PTH) with conformational constraints were synthesized and analyzed by CD and 2D-NMR. Recently, PTH(1-11) analogues[2] with helicity-enhancing substitutions yield potent agonists of PTH(1-34), which is fully active in vitro and in vivo and reproduces all biological responses characteristic of native PTH. In the present work we describe our results on the 3D-structural preferences of PTH(1-11) and their effect on bioactivity. Starting from the most active, modified PTH(1-11), H-Aib-Val-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-NH_2 (Har, Homoarginine)[2], we synthesized a set of "D-scans" analogues[3] and two series of analogues containing the C^{α}-tetrasubstituted (αMe)Val and (αMe)Nle residues in crucial positions by SPPS, employing the Fmoc-protocol and combining standard coupling methods with specific methodologies for the C^{α}-tetrasubstituted, hindered amino acids[3]. All peptides were 3D-structurally analyzed by CD studies to compare their conformation with the results of the biological tests. The conformational properties of the new analogues were investigated in a mixed solution of 80% water and 20% TFE[3]. Then, we performed 2D-NMR measurements on all analogues. A negative difference in the values of chemical shifts of the αCH protons ($\Delta\delta < -0.1$ppm) between those of helical segments and randomly coiled segments were taken to identify the peptide 3D-structural features. Finally, NOESY and ROESY experiments, combined with molecular dynamics calculations, allowed us to correlate the data of the biological tests with the 3D-structural results.

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Y7

New methodology for the synthesis of peptides alcohol using O-N acyl shift

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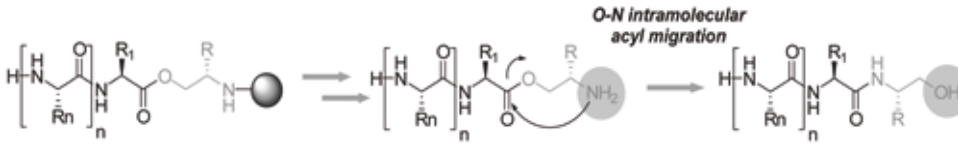
Peptides containing a C-terminal alcohol function (C-terminal peptide alcohols) constitute an important class of compounds. They exhibit important biological activities like antimicrobial (peptaibols) or anticancer (Octreotide). The absence of the carboxylic acid function in C-terminal position limits their synthesis on solid support. We developed a new strategy based on an O-N acyl intramolecular transfer reaction. In this strategy, the C-terminal β-amino alcohol was introduced on a 2-chlorotrityle resin by its free amine function. The free supported alcohol function offers a new starting point for the solid phase peptide synthesis (SPPS) of the targeted peptides alcohol.



The end of the SPPS, cleavage of the resin and elimination of the side chain protection with TFA released an unprotected isopeptide as TFA salt. The native peptide alcohol was generated in phosphate buffer or in basic organic media via the O-N intramolecular acyl migration on the corresponding deprotonated isopeptide. This methodology will be illustrated by the synthesis of Octreotide[1, 2].

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Y8

The importance of being kinked: role of Pro residues in the selectivity of helical antimicrobial peptides.

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Antimicrobial peptides (AMPs) are small molecules (usually less than 40 residues long) with strong bactericidal activity, linked to their ability to perturb the permeability of bacterial cells. For this reason, these peptides are investigated as lead compounds for the development of a new class of antibiotic drugs, to fight the insurgence of drug-resistant bacteria. AMPs often show an amphiphilic composition and a cationic character. From the structural viewpoint the most frequent conformation (after membrane-association) is α-helical. Moreover, many helical AMPs have a kink or a hinge in the middle of their structure, caused by Pro or Gly residues. In order to understand the role of this kink, such as its relevance to peptide activity and selectivity, we designed a series of analogues of the amphipathic, helical and cationic AMP P5 (KWKKLLKKPLLKKLLKKL), in which the central Pro residue was moved from its central position, or removed altogether. The displacement of the Pro residue had its most relevant effect in a dramatic increase in the toxicity against erythrocytes, with the most toxic peptide being the analogue P5F, lacking the Pro residue. Circular dichroism experiments and molecular dynamics simulations indicate that both P5 and P5F are helical when associated to lipid bilayers. By contrast, in water the fraction of helical structure is significantly reduced for P5, while P5F maintains its helical conformation. Fluorescence experiments showed that the kinked P5 peptide, which exhibits the highest selectivity for bacterial cells, has a dramatically higher affinity for negatively charged vesicles (mimicking the composition of bacterial membranes) than for neutral liposomes (which are similar to mammalian cells). On the other hand, analogue P5F exhibits comparable affinities for anionic and neutral membranes. HPLC retention times and theoretical calculations indicate that in water the helix-breaking Pro residue allows P5 to attain a closed conformation, in which its hydrophobic residues are partially shielded from the solvent. This property might explain its low affinity towards neutral bilayers, since in this case the hydrophobic effect is the main driving force of peptide-membrane association. The observed differences in the biological and biophysical properties of the two analogues highlight the role of the central Pro-induced kink in the selectivity of AMPs, and provide hints for the design of new, highly selective compounds.

ORAL PRESENTATIONS

O1

The microwave revolution: recent advances in microwave assisted peptide synthesis

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One of the greatest breakthroughs in solid phase peptide synthesis (SPPS) in the past decade is the use of microwave irradiation to overcome incomplete and slow reactions typical of conventional SPPS. Microwave energy has been applied successfully in a manual and automated approach for enhancing synthesis of peptides and peptidomimetics. We have recently demonstrated common side reactions such as racemization and aspartimide formation are easily controllable with optimized methods that can be applied routinely[1]. Our latest research has focused on the microwave assisted synthesis of modified peptides. Such modifications include N-and C- terminal modifications, cyclizations, and the incorporation of unnatural amino acids. These peptides were synthesized in a fraction of the time compared to conventional peptide synthesis without the need for unusual or expensive reagents and in a fully automated fashion to give peptides in high yield and purity.

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O2

Quantification of the cellular uptake of cell penetrating compounds by MALDI-TOF MS using HCCA tag

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Estimation of cell penetrating peptides (CPPs)[1] cellular uptake relies often on radioactivity or fluorescence measurement. Results strongly differ from one study to another depending of the protocol used and in particular inaccurate distinction between membrane trapped and internalised CPP. The pioneer work of Burlina et al. set a great improvement in proposing a highly reproducible quantification method based on MALDI-TOF MS to measure the concentration of the internalised peptides.[2] Here we describe and validate a new method to absolutely quantify CPP internalised by MDA-MB-231 breast cancer cells. Contrary to the existing protocols, this sensitive strategy does not require any purification or separation steps thanks to matrix discrimination effect induced by HCCA/HCCE Matrix/tag combination.[3, 4] At least, we will describe the first series of non peptidic, non cationic, cell penetrating compounds based of short oligomeric sequences of benzothiazepine scaffold.

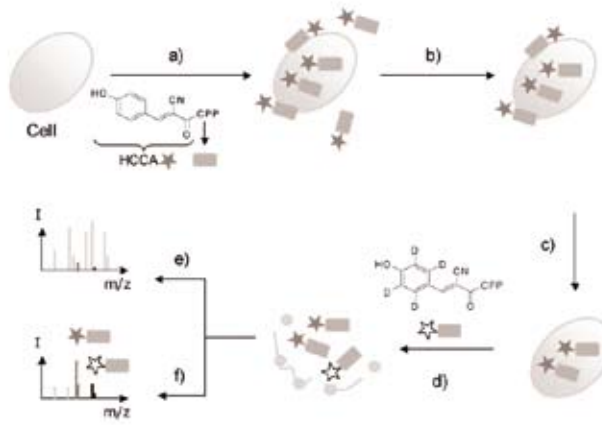

Figure 1. Principle of quantification using HCCA tagged penetrating compounds

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O3

Drug-armed branched peptides: turning non-specific cytotoxic drugs into tumor-selective agents

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Selective tumor targeting agents, able to ensure both a diagnostic and a therapeutic application (theranostics), would greatly advance the development of personalized cancer therapy. We have studied the use of protease-resistant tetra-branched peptides containing the sequence of the human regulatory peptide neurotensin (NT), as specific tumor targeting agents[1], demonstrating that peptides synthesized in a oligo-branched form can function as very effective theranostic molecules in oncology[2]. We set up a general branched scaffold[3], which allows conjugating different functional units to tetra-branched peptides, making them efficient target-selective carriers, either for in vitro or in vivo cell tracing, or for cell therapy. Tetra-branched NT peptides (NT4) are able to selectively and specifically deliver functional units for cell imaging or killing, to many different human cancer cells. NT4: 1-bind to cell membrane receptors more efficiently than monomeric homologous sequences[1,2]; 2- can efficiently discriminate between tumor and healthy tissue in human surgical samples from colon or pancreas adenocarcinoma in a high number of patients, with very good statistical significance[4]; 3- when conjugated to chemotherapy drugs, can induce selective killing of different human cancer cells from colon, pancreas or prostate carcinoma, in vitro[2,3] and in xenografted mice[2,4].

Different drug-armed NT4 have been synthesized and tested in vitro and in vivo and we demonstrated that they allow killing of tumor cells through a mechanism mediated by peptide receptors, which greatly increase drug selectivity toward receptor-positive cells. The switch to a receptor-selective drug internalization produces three consequences: i) it dramatically reduces drug non-specific cytoxicity; ii) it greatly increases in vivo activity of the drug; iii) it may induce reverse of innate cell resistances, when these are produced by mechanism of cell internalization or export of the drugs. Drug-armed oligo-branched peptides can combine the high selectivity produced by multimeric binding to membrane receptors over-expressed by cancer cells with the high efficiency of chemotherapy drugs which interfere with different cellular pathways. By increasing selectivity of small molecules towards tumor cells, NT4 act as Trojan horses, which selectively transport chemotherapy drugs into tumor cells.

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O4

Probing the opening of lipase lid using site-directed spin-labelling and EPR spectroscopy

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Lipases are model enzymes which have been studied since many years for setting up the basis of "Enzymology at Interfaces"[1]. These enzymes are highly soluble in water but they are acting on an unsoluble substrate, the triglycerides from oils and fats. Lipases have first to bind the lipid-water interface before hydrolyzing their substrate. This adsorption step results in a drastic change in the environment of the enzyme. Most proteins would be largely unfolded under such conditions but lipases adapt to this new environment by changing part of their 3D structure. A specific structural feature of several lipases is the presence of a molecular "lid" covering the active site. Under its "closed" conformation, the lid mainly exposes a hydrophilic surface. In the presence of lipids or amphiphiles, the lid becomes "open" and a large hydrophobic surface becomes exposed around the active site. This hydrophobic surface is involved in the interaction of the lipase with the lipid-water interface and the lid becomes part of the active site once it is open.

EPR spectroscopy is well adapted for studying the conformational changes in the lid in human pancreatic lipase (HPL) in solution and in the presence of amphiphiles/lipids. Thanks to a spin label grafted onto the lid of HPL, specific EPR spectra components corresponding to the closed and open conformation of HPL lid were identified and were used today to monitor the lid opening under various conditions[2-3]. Double spin labelling was used for measuring the amplitude of the lid opening by pulse EPR and the double electron-electron resonance (DEER) technique [4].

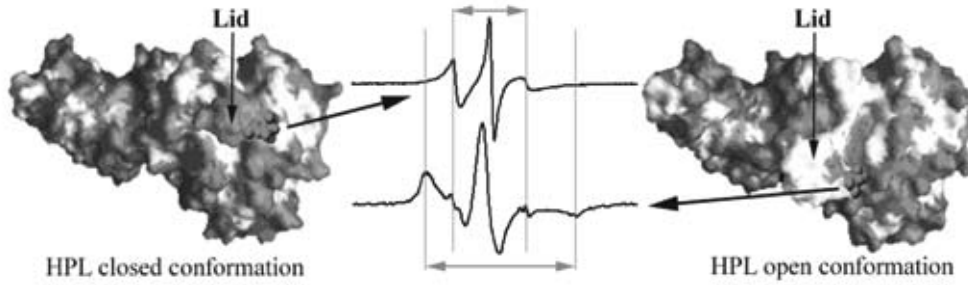

Figure 1. Closed (left) and open (right) 3D structures of human pancreatic lipase, with a spin label grafted to the lid and the distinct EPR spectra corresponding to the closed and open conformations.

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O5

Using SPOT synthesis for drug design: a peptide based approach to design CAL selective PDZ inhibitors

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The cystic fibrosis transmembrane conductance regulator (CFTR) is an epithelial chloride channel mutated in patients with cystic fibrosis. Its expression and functional interactions in the apical membrane are regulated by several PDZ domain containing (PSD-95, disc large, zonula occludens1) proteins. In particular, the CFTR-associated ligand (CAL) limits cell-surface expression of the most common disease-associated mutant ΔF508-CFTR by lysosome targeting [1, 2] whereas the Na^+/H^+ Exchanger-3 Regulatory Factors 1 and 2 (NHERF1, 2) increase CFTR-mediated chloride efflux [3]. We are looking for a molecular inhibitor of the ΔF508-CFTR/CAL interaction, which would significantly increase the plasma membrane lifetime of ΔF508-CFTR and therefore reduce the pathogenicity of Cystic Fibrosis. To be most effective, such a bioactive peptide should competitively bind the CAL-PDZ domain binding pocket without interfering with the favourable interactions between the ΔF508-CFTR and NHERF1 and NHERF2, respectively. Starting from a library of 6223 peptides with free C-termini synthesized by our modified SPOT synthesis, we found new CAL ligands which indeed have higher binding affinities as the wt-CFTR. By combining different peptide libraries (e.g. combinatorial libraries, substitutional analyses, profile libraries), we could further optimize the CAL ligands to CAL specific inhibitors, which are able to increase Cl^--efflux on the plasma membrane. In vitro pull-down assays and mass spectrometry analysis indicate this peptide inhibitors are potent and selective for endogenous CAL. Finally, the validations of our experiments were performed by means of fluorescence polarisation assays (K_d-/K_i-measurements). The received results lead to the assumption that we are heading in the right direction towards reducing the pathogenicity of Cystic Fibrosis by a peptide based pharmacological approach.

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O6

Clarification of signaling mechanisms for mitocryptides, novel neutrophil-activating peptides hidden in mitochondrial proteins

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Cryptides are bioactive peptides hidden in protein structures that have recently been designated by us.[1] Cryptides are produced from various proteins by proteolysis during their maturation and degradation processes and involve in the endogenous signaling. Previously, we have purified and identified two neutrophil-activating cryptides, mitocryptide-1 and mitocryptide-2 that are derived from mitochondrial cytochrome c oxidase subunit VIII and cytochrome b, respectively.[2,3] We have also found the existence of many mitochondrial protein-derived peptides that activate neutrophils utilizing the bioonformatic approach.[1-3] Most of these cryptides had features, in common, in their distributions of positively-charged and hydrophobic amino acid residues, but homologies in their primary structures were not apparent[1]. According to these results, we proposed a regulatory mechanism of acute inflammation involving mitocryptides. Here, we report cellular signaling mechanisms induced by mitocryptides in neutrophilic/granulocytic cells.

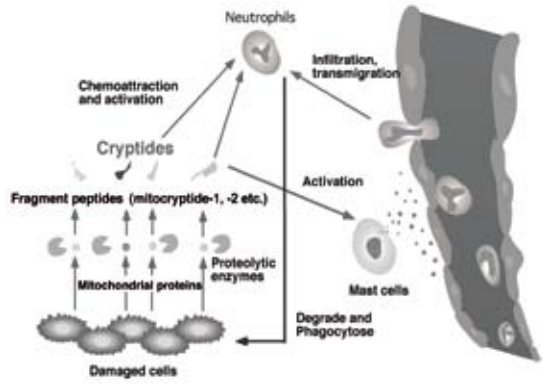

Figure 1. Proposed mechanism of acute inflammation involving mitocryptides

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O7

Attenuation of ischemia/reperfusion injury in mouse hearts by BH4 peptide

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Acute myocardial infarction (AMI) is a frequent and disabling disease, and infarct size is a major determinant of myocardial functional recovery and mortality after AMI. Lethal reperfusion injury is caused by restoration of coronary blood flow after an ischemic episode. This phenomenon culminates in apoptotic death of cardiac cells that were viable immediately before myocardial reperfusion. The involvement of a highly regulated form of cell death during myocardial ischemia/reperfusion (I/R) may lead to novel therapeutic interventions in the reperfusion phase.

Recently, Ono and co-worker[1] showed that a BH4-peptide (derived from the BH4 domain of the Bclx-L protein) prevents apoptosis when coupled to the Tat[2] cell penetrating peptide (CPP). CPP are short peptides, able to penetrate cell membranes and to translocate different cargoes into cells[3]. Beside conventional CPPs such as Tat, a new generation of synthetic CPPs such as (RXR)4[4], Bpep[5] or Pip2b[6] were developed together with our co-workers to improve the internalisation properties of the CPP-cargo conjugates.

Here, we present our new results on the anti-apoptotic effect of BH4-peptide coupled to these CPPs and provide insights into their intracellular trafficking. Transduction efficiency was assessed by flow cytometry and by laser scanning confocal microscopy. Biological activity was tested in vitro as the ability to protect primary mouse cardiomyocytes from death induced by staurosporine and in vivo using wild-type mice submitted to an ischemia/reperfusion protocol (40 minutes of left coronary artery occlusion and 60 minutes of reperfusion). Furthermore, we determine the route of CPP-BH4 internalisation via endocytosis and the interaction with endosomal membranes by leakage assay and by structural analyses using CD spectroscopy.

Taking together, our findings demonstrate that CPP-BH4 peptide conjugates promote an anti-apoptotic activity both in vitro and in vivo, and imply that they may become useful therapeutic agents for clinical application.

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O8

Self-assembled peptide biomaterials of *de novo* design

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Peptide self-assembly is attracting growing attention. The main drive for its advancement is the current demand for new materials programmed at the nanoscale. Elucidating first principles allowing such level of control may provide better understanding of macromolecular organization and its relation to function in a variety of biomedical contexts. This in turn requires a clear path to the construction of functional nanostructures, with mimicking Nature's designs providing perhaps the most straightforward strategy for success.[1]

Basic protein folding motifs such as α-helices and β-sheets are appealing elements for prescriptive supramolecular engineering. In particular, these prove to be instrumental in designing constructions necessarily functional in cellular environments. In this report, recent advances in de novo design of peptide self-assembling systems possessing antimicrobial,[2] cell-supporting[3] and encapsulating[4] properties will be discussed.

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POSTER PRESENTATIONS

P1

Molecular cloning of novel peptides belonging to brevinin-1 and brevinin-2 families from the skin of Indian bronzed frog *Hylarana temporalis*

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The present work is designed to elucidate the natural peptides present in the skin secretion of Indian bronzed frog *Hylarana temporalis*, currently considered endemic to India and Sri Lanka. Polyadenylated mRNA directly isolated from the lyophilised skin secretion of H.temporalis was used to construct the cDNA library. The library was subjected to 3'-and 5'– rapid amplification of cDNA ends (RACE) procedures and a full length cDNA was obtained successfully. After ligation and transformation, clones were picked randomly to perform plasmid PCR for confirming the size of inserted fragments within plasmids. Three different cDNA sequences, one encoding brevinin-1 and two encoding brevinin-2 precursor proteins were identified From the homology search, it was found that the three peptides are novel and they were named brevinin-1TEa, brevinin-2TEa, brevinin-2TEb respectively (TE=temporalis) in accordance with nomenclature rules recently suggested for frog skin peptides [1]. The deduced open reading frames encoding the biosynthetic precursors of brevinin-1TEa consisted of 70 amino acid residues, while that of each brevinin-2TEa and brevinin-2TEb consisted of 71 and 72 amino acids respectively. The conserved prepro regions of each precursor open reading frame includes a putative 22 amino acid residue signal peptide followed by an N-terminal acidic spacer domain which terminated in a dibasic cutting site Lys- Arg (K-R) for trypsin like proteases cleavage to release different mature C-terminal antimicrobial peptides[2]. Predicted secondary structural parameters of the mature peptides reveal the α-helical nature of peptides (70-75%). The grand average hydropathicity of the mature peptide was found to be positive indicating hydrophobic nature of the deduced peptides which may enable them to interact easily and efficiently with microbial membrane. Net charge, hydropathicity, number of amino acid residues etc. clearly shows possible biological activity and selectivity of the deduced peptides in the present study.

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Isolation and characterisation of antimicrobial peptides from plants

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Decreasing efficiency of antibiotics developed during last century is responsible for efforts of the microbiological research to find new antibiotics and compounds with antimicrobial effect, for example peptide based antibiotics. Misusing of antibiotics in human and veterinary medicine led to increased amount of resistant and multiresistant pathogens. In our project tissues of three plants – purple coneflower (Echinacea purpurea), tomato (Solanum lycopersicum) and flax (Linum usitatissimum) were chosen as sources for isolation of such peptides. Purple coneflower is known for its antimicrobial and immunostimulating effects while tomato for comparison due to its wide availability. Flax is commonly used in textile industry and its alcoholic extract is used as folk remedy.

Extraction of peptides was performed using extraction buffer containing protease inhibitors. Peptides were then precipitated with ammonium sulphate and fractionated using chromatographic methods (SPE column, RP-HPLC) and membrane filtration. Fractions were characterised by electrophoresis (Tricine-PAGE) and by mass spectrometry MALDI-TOF. The antimicrobial activity of the fractions was screened by agar diffusion method. Several fractions exhibited inhibitory effects to model microorganisms – bacteria (Bacillus megaterium, Enterococcus faecalis, Escherichia coli, Micrococcus luteus, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus equi) and fungi (Candida scotii, Fusarium sp., Mucor sp., Trichoderma virens).

As for echinacea (Echinacea purpurea) no antimicrobial peptides are known meanwhile. Crude extract fractions possessed antibacterial and antifungal activities. After RP-HPLC fractions were tested against three bacteria: Pseudomonas aeruginosa, Enterococcus faecalis and Bacillus megaterium.

In tomato (Solanum lycopersicum) several antimicrobial proteins and peptides have been described. By tricine gel electrophoresis we have detected bands of molecular masses from 20 to 50kDa in crude extract and its hydrophilic fraction. These bands were cut off the gel and characterized by MS.

From flax (Linum usitatissimum) no antimicrobial peptides and proteins were described yet. We have obtained several fractions after RP-HPLC that possessed antimicrobial activity against tested bacteria: Pseudomonas aeruginosa, Enterococcus faecalis and Bacillus megaterium.

Acknowledgement

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P3

Faststep™ : a new approach to increase throughput for screening and kinetics

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Achieving high analyte throughput while maintaining high quality kinetic data is often difficult. Increasing the number of sensing channels, or probes, is an effective means of overcoming this problem but these high channel, or probe count, systems are often confined to core facilities and often lack the sensitivity of lower channel count systems. Pilot experiments to identify the appropriate analyte concentration range for each analyte is usually required and is both time consuming and expensive. This requirement for pilot experiments may be considerably reduced by using a single analyte gradient injection (**Faststep™**) that covers a wide analyte concentration range in a single injection. In contrast, conventional fixed concentration injections require a series of analyte dilutions to be prepared in order to cover an equivalent concentration range. More importantly, the overhead associated with preparing, loading and injecting each analyte dilution in such a series is eliminated. We show that **Faststep™** , as implemented on SensíQ Pioneer, eliminates the need for sample dilutions, and the associated multiple injections per cycle, and produces high quality kinetics data that is equivalent to the conventional kinetics format. **Faststep™** also reduces regeneration (if required) to a single injection step for a given analyte. Most importantly, **Faststep™** greatly improves analyte throughput and reduces the human error associated with preparing and loading multiple analyte dilutions while reducing the complexity of the instrument routines and protocol writing.

P4

A new Gd(III)-complex based system for the MR molecular imaging of MMPs in multiple sclerosis

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Matrix Metalloproteinases (MMPs) constitute a family of endoproteases that exert their function in the extracellular matrix and are differentially expressed in tissues or pathological states, such as tumors and neurodegenerative disease. In particular, multiple sclerosis (MS) is characterized by over-expression of MMP-9, whose activity is detected in the CSF and blood serum of MS patients.[1] The assessment of the activity of selected members of MMPs in a given tissue or anatomical district would be of great value for the typization and staging of the pathological process. If such an assessment could be done in vivo by imaging techniques, its diagnostic content would be even greater. MRI is one of the most clinically relevant imaging techniques, because of its great spatial resolution (<100 μm with modern high field equipment), lack of invasiveness (no ionizing radiation) and possibility to image soft tissues at any depth within the body. Contrast agents (CAs) based on paramagnetic materials are usually employed to enhance image contrast.

Recently, a way to the MR molecular imaging of MMPs has been presented based upon a MMP-2 cleavable peptide functionalized at the N-terminus with a Gd(III)-DOTA chelate as the reporter unit, in which the MMP activity can be detected on the basis of the differences in wash-out kinetics between the Gd(III) probe cleaved by MMPs and the intact probe.[2] We are developing a new methodology for the simultaneous targeted delivery of therapeutics and visualisation of drug release by MR molecular imaging. The concept underlying this methodology relies on nanosized systems, based on β-cyclodextrin/β-poly-ciclodextrin assemblies, carrying i) a suitably designed, MMP responsive Gd(III)-based MRI contrast agent, ii) a MMP-inhibitor (as the drug); and iii) a vector targeting the region of interest. In this communication, we report about the design, synthesis and characterization of Gd(III)-based MMP cleavable probes. These probes are based upon a peptide sequence cleavable by MMPs, conjugated to a Gd-DOTA unit at one peptide terminus and at the other terminus to a hydrophobic alkyl chain that enables the formation of host/guest complexes with β-CD.

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P5

New bradykinin analogues modified in position 7 with 2-aminomethyl phenylacetic acid and 3-aminophenylacetic acid

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In the present work, achiral non-coded amino acids, 2-aminomethyl phenylacetic acid (X_1) or 3-aminophenylacetic acid (X_2), were substituted in the position 7 of the model B_2 receptor antagonist [D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]BK. The primary amino group of the analogues was either free or acylated with 1-adamantaneacetic acid (Aaa). Previously, we reported that acylation of the N-terminus of B_2 receptor antagonists with various bulky groups (e.g. 1-adamantaneacetyl, 1-adamantanecarbonyl, 4-tert-butylbenzoyl, palmitoyl, etc.) is regularly improving their antagonistic potency in the rat uterotonic test (up to 33 times). Biological activity of the compounds was assessed in the in vitro rat uterus test[1, 2] and the in vivo rat blood pressure test[3]. X_1^7 substitution resulted in almost complete loss of activity in uterus assays and decreased antagonistic pressor activity of analogues. X_2^7 substituted analogues showed residual agonistic or low antagonistic activity in the rat uterus test and low activity in the pressor test. Acylation of the N-terminus increased antagonistic properties of the resulting peptides in the pressor test. Our studies provide new information about structure-activity relationship of the BK antagonists which may help designing more potent BK receptor blockers.

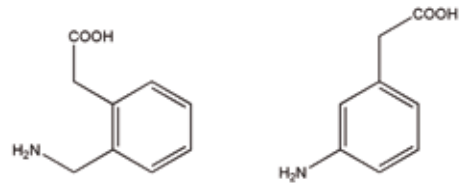

Figure 1. Structures of non-coded amino acids used (2-aminomethyl phenylacetic acid (X_1) and 3-aminophenylacetic acid (X_2), respectively)

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P6

Neuroglobin-Prion protein interaction: what's the function?

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Neuroglobin (Ngb) is a heme-protein discovered in the nervous system of vertebrates. Ngb plays a role in neuroprotection, with a still unclear mechanism. It might have a role in O_2 supply and/or reactive oxygen species detoxification in neurons and/or activating protective pathways [1,2]. Recently, it was demonstrated retinal co-localization of Ngb [3] and cellular Prion Protein (PrP^C), also a protein of unknown function expressed in the nervous system [4]. In the same paper it has been shown that PrP^C aggregates rapidly in the presence of Ngb, whereas it does not aggregate in the presence of myoglobin suggesting electrostatic complementarity between the unstructured PrP^C N-terminus and Ngb, with no influence on PrP structure or Ngb ligand binding [3]. Considering the above results, we have evaluated Ngb-PrP^C association in vitro by Surface Plasmon Resonance (SPR) technique. Our experimental data indicate a sub-micromolar K_D value for the Ngb-PrP^C interaction and no detectable complex formation with horse heart myoglobin. A combined approach of automated docking and molecular dynamics studies carried out on short stretches of PrP N-terminus have identified some potential electrostatically-interacting regions with Ngb. To verify this hypothesis we have performed the synthesis of these peptides by solid phase methods and we tested their interaction with Ngb by SPR. Preliminary results confirm the specific interaction between synthetic PrP peptides and Ngb suggesting a crucial role of PrP^C positively-charged regions in protein-protein association.

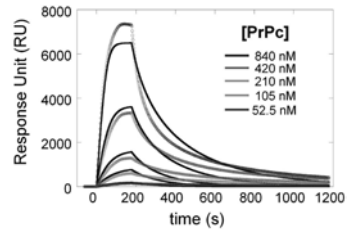

Figure 1. Sensorgrams of PrP^C binding to Neuroglobin using Biacore X100 SPR-assay

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P7

NMR structural studies of frog skin peptide alyteserin-1C and its interactions with membranes

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High resolution NMR Spectroscopy is an efficient method for structure determination of peptides. In this study we used the solution NMR spectroscopy methods along with molecular modelling to investigate the solution structure of antimicrobial peptide alyteserin-1C. alyteserin-1C is a short antimicrobial peptide, isolated from the skin of midwife toad Alytes obstetricans (Alytidae), composed of 23 amino acids (GLKEIFKAGL GSLVKGIAAH VAS). It showed broad spectrum of antibacterial activity against *Escherichia coli* (MIC-25 μM) and Staphylococcus aureus (MIC-100 μM) and LC_{50} value against human erythrocytes was 145 μM [1]. Due to its antibacterial activity and low haemolytic properties, alyteserin-1C is being considered as a potential target of antibacterial drug development.

Therefore, to understand the basic structural requirements for the biological activity of Alyteserin-1C peptide, the solution structure was investigated by proton NMR spectroscopy and molecular modelling in various solvent systems including SDS and DHPC micellular media to modulate anionic and zwitterionic membranous media. The structure of Alyteserin-1C is characterized by full-length alpha-helix between residues Leu^2-Ala^{22}. Furthermore, NMR studies have been carried out to identify the interaction of this particular peptide with biological membranes using various paramagnetic probes.

Frog skin peptides have been considered as potential therapeutic agents [2]. Valuable information obtained from this project will be used to examine the structural and functional properties of this peptide and its analogues and also understand the structure activity relationship of alyteserin-1C. Therefore, this application has a great potential in the design of novel therapeutics that could be targeted towards several different pathogens.

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P8

Biomedical nanotechnology: preparation and characterization of new functionalized gold nanoparticles

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Over the past decade gold nanoparticles have attracted much interest since they are versatile agents with a variety of biomedical applications including use in highly sensitive diagnostic assays, thermal ablation and radiotherapy enhancement, as well as drug and gene delivery[1]. Several stabilizing agents, that interact with nanoparticle surface, are usually added to improve their stability. In particular, recently we have studied the use of peptide sequences based on the GGC motif as capping agents in the preparation and characterisation of monolayer-protected gold nanoparticles[2]. These capped systems can be considered as a good scaffold for a functionalization with molecules for the targeting of receptor tumor marker such as integrin receptors. The aim of this research is the wet chemistry preparation and characterization by ATR-FTIR, XRD and TEM of new capped gold nanoparticles functionalized with molecules encompassing the RGD motif critical for the integrin binding.

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P9

Anti-pseudomonas activity of frog skin antimicrobial peptides in *Caenorhabditis elegans* infection model

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The emergence of multidrug-resistant (MDR) microorganisms makes it increasingly difficult to treat infections. These infections include those associated with *Pseudomonas aeruginosa*, which is hard to eradicate, especially in patients with a compromised immune system[1]. Naturally occurring membrane-active cationic antimicrobial peptides (CAMPs) serve as attractive candidates for the development of new therapeutic agents[2]. Amphibian skin is one of the richest sources for such peptides, but only a few studies on their in vivo activity and mode of action were reported. Here we investigated: (i) the activity and mechanism underlying the killing of short CAMPs from frog skin (e.g., temporins and esculentin fragments[3]) on a MDR clinical isolate of *P. aeruginosa*; (ii) their in vivo antimicrobial activity and mode of action, using the mini-host model of *Caenorhabditis elegans*[4]. Our data revealed that in vivo, both temporin-1Tb and esculentin(1-18) were highly active in promoting the survival of pseudomonas-infected nematodes, although temporin-1Tb did not show significant activity in vitro, under the experimental conditions used. Importantly, esculentin(1-18) permeated the membrane of Pseudomonas cells inside the gut of the infected nematode. To the best of our knowledge, this is the first report showing the ability of a CAMP to permeate the microbial membrane within a living organism. Besides shedding light on a plausible mode of action in vivo of frog skin CAMPs, our data suggest that temporins and esculentins would be attractive molecules as templates for the development of new therapeutics against life-threatening infections.

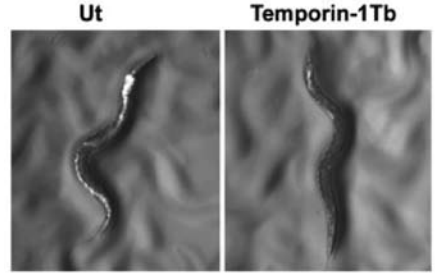

Figure 1. Effect of temporin-1Tb on the colonization of GFP-expressing *P. aeruginosa* within the nematode gut, with respect to the untreated animal (Ut)

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P10

Milk proteins as precursors of antimicrobial peptides. A computational study based on the BIOPEP database

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Bioinformatics tools and methods are today widely used to investigate the structure of biomacromolecules, to classify proteins in view of the similarities between sequential motifs, and to predict the functions of proteins based on their primary structure. The present study relied on the BIOPEP database of proteins and bioactive peptides (*http://www.uwm.edu.pl/biochemia*), developed at the Department of Food Biochemistry as part of an original research project. The BIOPEP database of protein and bioactive peptide sequences facilitates the search for bioactive fragments in polypeptide chains, the hierarchical classification of proteins according to the developed algorithms and the design of proteolytic processes for the purpose of acquiring or removing bioactive peptides. The main data sheet of the BIOPEP application comprises four interlinked databases of protein sequences, biologically active peptides, allergenic proteins and proteolytic enzymes. All four databases are regularly updated and presently comprise 707 and 2457 protein and peptide sequences, 88 allergenic proteins with its (their) epitops, 224 sensory peptides and 27 proteolytic enzymes.

The analysis of profile of biological activity of milk proteins and the frequency of occurrence of fragments exhibiting antimicrobial activity were determined in the research work. Based on achieved results the milk proteins were evaluated (classified) as precursors of antimicrobial peptides. Moreover it was determined which proteolytic enzymes of gastrointestinal track and extracellular microbial enzymes of starters and fermented dairy products can release bioactive peptides.

P11

Immunoadsorption in patients with IgG-subclass specific peptides

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Heart diseases are the most frequently cause of death in industrial countries. Among heart diseases dilated cardiomyopathy (DCM) is the most common form of non-ischemic cardiomyopathy. It occurs more frequently in men than in women and show a prevalence of 500.000 people e. g. in Germany. DCM affected the muscle cells (myocardium) of the heart with the consequence that the heart becomes weakened as well as enlarged and the heart function is dramatically decreased. Among other factors, disorder of the humoral immune system has been found as a pathogenic factor in DCM, whereby autoantibodies affecting cardiac structures, especially antibodies of the IgG-3-subclass.[1, 2] Recently, clinical studies demonstrated the positive effects of immunoglobulin adsorption in patients with DCM.[3, 4]. Protein A and protein G are both potent immunoglobulin adsorber molecules, however, immunoglobulin subclass selectivity of these proteins is poor (PrA: IgG-1, IgG-2, IgG-4; PrG: all IgGs). An effective immunoadsorption in DCM patients should only eliminate the IgG-3 subclass population without affecting other IgG subclasses. Recently, peptides have been developed as specific IgG-3 antibody binders.[5] Unfortunately, those peptides are directed against the variable part of the antibodies diminishing its general use for IgG-3 immunoadsorption.

Here we present a novel and tricky strategy to develop highly selective human IgG-3 antibody binding peptides. Firstly, the constant Fc-region of the human IgG-3 antibody (IgG-3-Fc) was generated in E. coli. Secondly, synthetic peptide arrays[6] were tested for IgG-3-Fc binding peptides. Thirdly, hits were validated for IgG-3 selectivity by probing the candidates with all other IgG-subclasses. Fourthly, specificity of the IgG-3-Fc binding was checked by substitution analyses and additionally the specificity and affinity of the hits were improved by replacement analyses. Finally, the optimized hits were coupled to sepharose materials and the IgG-3 antibody adsorber-capacity of such mini-columns was probed with sera and mixtures of immunoglobulins.

Briefly, from a peptide array of 5520 randomly generated sequences 86 candidates were found as potential IgG-3-Fc binders. Further optimization and validation ends up with ten hits which are now attached on sepharose columns. Probing the adsorber-capacity is on the way.

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P12

Interaction of grape and peach defensins with lipid membranes

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Plant defensins are small (2-10 kDa) basic cysteine-rich antimicrobial peptides occuring in various plant species. They share a common three-dimensional structure, stabilized by eight disulphide-linked cysteines and composed of three antiparallel β–strands and one α–helix. Most plant defensins posseses antifungal or antibacterial activity but are non-cytotoxic to mammalian and plant cells. In some plant tissues, the expression of defensins is induced in response to fungal infection, whereas in other tissues they are expressed constitutively.

Plant defensins induce membrane permeabilization through specific interaction with high affinity binding sites on fungal cells, resulting in Ca^{2+} uptake, K^{+} efflux, alkalinization of the medium and membrane potential changes (1).

The genes encoding for a peach (P. persica) and a grape (V. vinifera) defensin, Pp-Dfn1 and Vv-AMP1 respectively, were expressed in E. coli and purified to homogeneity. Defensins were tested for antimicrobial activity against B. cinerea, M. laxa and P. expansum and showed to have a significant inhibitory effect on their spore germination.

Biophysical analysis demonstrated that these recombinant proteins were able to interact with both model and natural membranes. Binding of defensins to pure lipid monolayers was dependent on lipid composition, increasing with the sphingolipids content. Moreover there is an increase in lipid-protein interaction when whole lipid extracts from B. cinerea, M. laxa or P. expansum spores were used. Interaction with sphingolipids and other fungal membrane lipid components might lead to insertion of defensins into the membrane resulting in membrane destabilization.

Acknowledgement

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P13

NPY analogues containing *cis*-pentacin residues as potential Y1 receptor ligands

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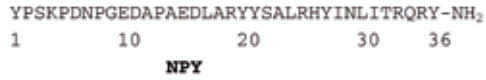
Neuropeptide Y (NPY) is a member of pancreatic polypeptide family of hormones, which activates a class of receptors named Y receptors that belong to large superfamily of the G-protein coupled receptors.[1] NPY is a 36-residue peptide amide and was first isolated from extracts of porcine brain in 1982.[2] This hormone



is one of the most abundant neuropeptide found in the mammalian central nervous system.
Applications already studied are treating feeding disturbances and anxiety.[3] Recently, novel, possibilities of human breast cancer treatment emerged: It was proven that Y_1 receptors are expressed predominantly in human breast carcinomas, while Y_2 receptors were found to be preferentially in healthy human breast.[4] Thus, Y_1 receptor selective ligands provide a handle to selectively recognize breast cancer cells and could be effective anti-cancer drug carriers.
The C-terminal part of NPY is crucial for its ability to bind to Y_1 receptor, however, C-terminal fragments of NPY — truncated peptides are very weak ligands for the Y_1 receptor. This phenomenon was explained by its high conformational liability. Only peptides that are conformationally constrained by incorporation of unnatural amino acid residues (e.g. β-aminocyclopropanecarboxylic acid), were evidenced to be highly active and selective Y_1 receptor ligands.[5]
This paper presents application of cis-pentacin for constraining the structure of C-terminal part of NPY. The synthesis and biological evaluation of novel NPY analogues will be presented.

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P14

Structural behaviour of selective anti-Cryprococcus peptides in membrane mimicking environments

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Many drugs are available for the treatment of systemic or superficial mycoses, but only a limited number of them are effective antifungal drugs, devoid of toxic and undesirable side effects. In particular there remains an urgent need of antifungal drugs active against *Cryptococcus Neoformans* since criptococcossis. is the main cause of fatal meningoencephalitis in AIDS patients and in patients who have undergone organ transplants.

According to a polypharmacological approach, we recently reported the synthesis and antifungal activity of a set of peptides designed to simultaneously target the fungal cell surface and lanosterol demethylase, a key enzyme involved in ergosterol synthesis. Our peptides include amino acid sequences characteristic of membrane-active antimicrobial peptides, and due to the presence of His residues, they carry the imidazole ring characteristic of azole compounds. The peptides were tested against various fungal species, and showed a therapeutic promising antifungal specificity against *C. Neoformans*.

Here we present a structural characterization of the mentioned peptides performed by means of CD, NMR and fluorescence spectroscopy in membrane mimicking environments. Our investigation was intended to evaluate a potential correlation between the peptides membrane destabilizing property and their antifungal activity.

P15

^{18}Fluoro-active-peptides: optimization of radiolabelling synthesis

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The application of biologically active peptides labelled with positron-emitting nuclides has emerged as a useful and interesting field in nuclear medicine. Small synthetic receptor-binding peptides are currently the preferred agents over proteins and antibodies for diagnostic imaging of various tumours. Due to the smaller size of peptides, both higher target-to-background ratios and rapid blood clearance can often be achieved. Among a number of positron-emitting nuclides, ^{18}F appears to be the best candidate for labelling bioactive peptides by virtue of its favourable physical and nuclear characteristics. In recent years, various techniques have been developed which allow efficient labelling of peptides with ^{18}F without affecting their receptor-binding properties . Moreover, the development of a variety of prosthetic groups has facilitated the efficient and site-specific labelling of peptides with ^{18}F[1]. Recently, we have developed a new and selective $\alpha_v\beta_3$ antagonist, RGDechi, that has been labelled with Fluoro-18 and tested in vivo by microPET/CT imaging studies[2].

Starting from the results obtained the aim of this research has been the optimization of synthesis and purification conditions to improve the radiolabelling yield of the final product. In particular, HPLC purification conditions has been modified to allow the direct injection of the radiotracer to the animal avoiding the solvent evaporation step. In vivo studies by microPET/CT have been perfomed to evaluate the properties of the final compound.

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P16

Structural characterization and interaction studies of human lipocalin-type prostaglandin D synthase (L-PGDS)

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Lipocalin-type prostaglandin D synthase (L-PGDS) catalyzes the isomerisation of the 9,11-endoperoxide group of PGH2 (Prostaglandin H2) to produce PGD2 (Prostaglandin D2) with 9-hydroxy and 11-keto groups in the presence of sulphydryl compounds. PGH2 is a common precursor of all prostanoids, which include thromboxanes, prostacyclins and prostaglandins. PGD2 is synthesized in both the central and peripheral nervous system and it is involved in many regulatory events. L-PGDS, the first member of the important lipocalin family to be recognized as an enzyme, is also able to bind and transport small hydrophobic molecules and was formerly known as β-trace protein, the second most abundant protein in human cerebro-spinal fluid. L-PGDS is also detected in brain, testis and prostate, endothelial cells, placenta and heart tissue and even in macrophages infiltrated in atherosclerotic plaques. In these tissues it participates in many physiological activities as well as in the response to diseases. Currently the main structural and biochemical studies, present in the literature, concern recombinant rat and mouse L-PGDS. In this work we use recombinant human L-PGDS in order to solve its three-dimensional structure by X-ray diffraction and test its affinity for several ligands using Surface Plasmon Resonance (SPR). Wild type human L-PGDS and three mutants (C65A; C65A-K59A; C89/186A) were expressed using E. coli cell strains and subsequently purified by a chitin affinity column, size exclusion and hydrophobic interaction chromatography. Large and highly ordered crystals were used to collect X-ray diffraction data using either a rotating-anode generator or a synchrotron source. The multiple isomorphous replacement method was used to solve the phase problem. In the electron density maps an unidentified density was observed apparently interacting with lysine 59 inside the L-PGDS-C65A cavity; the foreign molecule is probably PEG, an additive present in the crystallization liquors. This hypothesis is supported by the fact that the L-PGDS-C65A/K59A crystals, which grow without PEG, show a completely free protein cavity. A seeding experiment of L-PGDS-C65A/K59A crystal, grown in L-PGDS-C65A crystallization conditions, partially confirmed this hypothesis since the foreign molecule was present in the L-PGDS-C65A/K59A cavity. Another crystal form was obtained by mixing L-PGDS-C65A/K59A with the amyloid β peptide (1-40). Although the amyloid β peptide is not visible in the maps, the packing of the protein molecules has changed in the presence of the peptide suggesting interaction of the two molecules. Wild type L-PGDS small crystals were recently obtained and will be tested as soon beam time at a synchrotron source becomes available.

SPR experiments are also in progress and will be used to verify interaction of L-PGDS with PEG, the amyloid β peptide and other ligands and to determine their binding constants.

P17

Unfolding study of cold shock protein from *Mycobacterium tuberculosis* by molecular dynamics simulations

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Bacterial cold shock proteins (Csps) are small systems of about 67 residues over-expressed as response to cold stress and having a role in transcriptional and translational events due to their ability to bind single stranded nucleic acids.[1] Csps so far characterized show similar structures with a closed five stranded antiparallel β-barrel. They represent a good model for β-protein folding studies since they are well-behaved systems. Indeed, Csps have no disulfide bonds, cis peptide bonds, co-factors, or post-translational modifications that may complicate folding[2]. The protein from mesophile Bacillus subtilys (Bs-CspB) and from thermophile Bacillus caldoliticus (Bc-Csp), were both found to undergo reversible two-state folding, with an energetically polarized transition state.[3] Although sharing a high identity rate (82%) and a very similar 3D structure, those two proteins significantly differ in their folding stability. Mutational studies and molecular dynamics simulations were employed to rationalize the stability difference.[4] We have recently undertaken a study of CspA from *Mycobacterium tuberculosis* (MTB). Using electrophoretic mobility shift assays (EMSA), we show that MTB-CspA is able to bind ssDNA, similar to its homologous proteins. However, structural investigations by in-solution techniques reveal that the protein structure is less ordered than expected. This apparent difference in structural stability among MTB-CspA and the homologous proteins was investigated by molecular dynamics simulations at high temperature. The starting structure for MTB-CspA simulations was obtained by homology modeling by using Ec-CspA (1mjc) and Bs-CspB (1csp) as templates. In parallel, unfolding simulations of Bs-CspB and Bc-Csp were undertaken for comparison. The parallel study of homologous proteins provides insight on the effects of multiple substitutions on the structure stability.

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P18

Synthesis and characterization of magnetic nanoparticle assemblies based on PNA-DNA duplexes

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Due to their promising properties, nano-scaled magnetic particles with biocompatible coating, covalently linked to drugs, proteins, enzymes, antibodies, or nucleic acids, have been widely investigated for their high potential in therapeutic and diagnostic applications,[1] ranging from the magnetic resonance imaging (MRI)[2] to the treatment of cancer by hyperthermia,[3] the magneto trasfection or the drug-delivery.[4]
Here we report the synthesis of magnetic conjugates consisting of modified dextran-magnetite particles and PNA/DNA strands. Due to the polyvalent nature of the nanoparticles, mixing the complementary PNA and DNA nanoconjugates should lead to particle assemblies based on duplex structures. The formation of these supramolecular systems was investigated by optical spectroscopies, scanning and transmission electron microscopies and by the response of the nanoparticle-conjugates to an external magnetic field. Furthermore, serum stability assays on the duplex-based magnetic assemblies were performed in order to evaluate their resistance to enzymatic degradation.

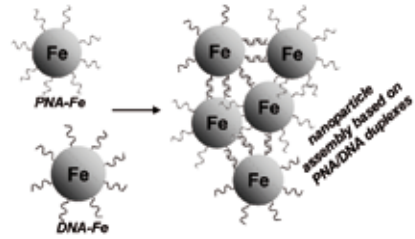

Figure 1. Nanoparticle assembly formed by the complementary PNA and DNA nanoconjugates

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P19

From nature, through chemical synthesis, towards the use of nucleobase-containing amino acids in biotechnology

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Nucleobase-containing amino acids, also referred to as nucleoamino acids, represent an interesting class of molecules of biotechnological importance presenting an amino acid residue conjugated to a DNA or RNA nucleobase through different linker moieties.

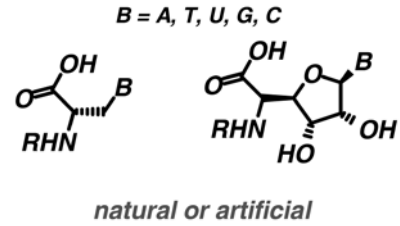

Figure 1. Schematic representation of some nucleobase-containing amino acids

Interestingly, some of them are natural, such as willardiine derivatives[1] and amino nucleosides (such as puromycin and cystocin), which are well-known for their antimicrobial activity,[2] whose biological role was investigated by us in this study. Nevertheless, it is possible to obtain by chemical synthesis both natural and artificial nucleobase-containing amino acids to be employed in biotechnology and medicine as also reported in this work.

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P20

Novel insights on cationic peptides

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Cationic peptides which are known to act as nucleic acid compacting devices and cell-penetrating carriers are also interesting in biotechnology due to other useful characteristics such as their significant antimicrobial activity[1] and inhibitory action towards plant proteinases[2] and also for the possibility to realize cationic hydrogels based on positively-charged poly amino acids.[3]

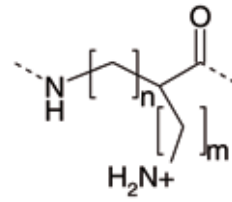

Figure 1. Repeating unit of a cationic peptide

In this work we describe a study of a novel positively-charged peptide based on modified amino acids suitable for the Fmoc solid phase synthesis. Furthermore, some structural and binding properties of this polycationic peptide were investigated in order to explore its possible application in biotechnology and medicine.

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P21

Synthesis, preferred conformation, and membrane activity of heptaibin, a medium-length peptaibiotic

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The medium-length peptaibiotics are characterized by a primary structure of 13-15 amino acid residues and include inter alia some samarosporins, stilbellins, bergofungins, and emerimicins. Despite the interesting antibiotic and antifungal properties exhibited by these membrane-active peptides, their exact mechanism of action is still unknown.

Here, we present our results on heptaibin, extracted from the culture of Emericellopsis sp. BAUA8289 and chemically characterized by Ishiyama et al. ten years ago (1). This peptaibiotic shows growth inhibition against fungi and Gram-positive bacteria (1).

The heptaibin primary structure is as follows:

Ac-Phe-(Aib)$_3$-Val-Gly-Leu-(Aib)$_2$-Hyp-Gln-Aib-Hyp-Aib-Phol

where Phol is the 1,2-aminoalcohol (S)-phenylalaninol.

Heptaibin was prepared using SPPS and the Fmoc-protection/HATU C-activation methodology. Cleavage from the 2-chlorotrityl resin was achieved by treatment with 30% HFIP in dichloromethane. Special attention was devoted to prevent 2,5-dioxopiperazine formation, particularly when the N-terminal sequence of the growing chain is the H-Aib-Hyp- dipeptide, and to avoid the acid hydrolysis of the labile -Aib-Hyp- tertiary amide bond. The final product was purified and fully characterized. A detailed conformational analysis was performed by use of FT-IR absorption, CD, and 2D-NMR combined with MD calculations, revealed that the peptide backbone is folded in a mixed 3_{10}-/α- helical structure. Fluorescence leakage experiments showed that heptaibin is a membrane-permeabilizing compound.

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P22

Membrane insertion of para-cyanophenylalanine-labeled alamethicin analogues. Correlation of fluorescence and infrared absorbtion data.

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Different classes of peptides, such as antimicrobial, cell penetrating and fusogenic peptides, exert their bioactivities by interacting with cellular membranes. Therefore, the determination of their location and orientation inside a lipid bilayer is a fundamental step in the characterization of their mechanism of action. In this respect, the α-amino acid analogue para-cyanophenylalanine (pCNPhe) is a very promising probe, since it can be employed both in fluorescence and in IR absorption experiments. Its fluorescence quantum yield is 0.11 (as compared to 0.025 for Phe). The C≡N stretching vibrational transition is located at around 2230 cm^{-1} (i.e., far from the water background absorption) and is sensitive to the medium polarity. In this study, we exploited the peculiar properties of pCNPhe to investigate the membrane interaction of the [Glu(OMe)^{7,18,19}] alamethicin analogue by synthesizing three peptides in which Ala4, Val9 or Val15 were substituted by pCNPhe. Liposome leakage kinetics data indicate that the label does not perturb significantly the peptide activity. Furthermore, pCNPhe fluorescence is sensitive to the fluorophore environment, allowing a characterization of peptide aggregation and water-membrane partition. The position of the fluorophore in the membrane was determined by fluorescence, depth-dependent quenching experiments, performed as a function of the peptide to lipid ratio. Correlation of these data with the IR absorption spectrum of the C≡N group allowed a determination of its dependence on the depth of insertion in the bilayer, while polarized ATR-FTIR experiments provided indications on peptide orientation in the membrane. Overall, these data offer a picture of alamethicin insertion in the membrane and confirm that pCNPhe is an extremely useful probe in fluorescence and IR absorption studies of peptide-membrane interactions.

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P23

In vivo efficacy of the antimicrobial peptide Bac7(1-35) against *S. typhimurium* infection in mice

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Bac7 is a proline-rich peptide with a potent in vitro antimicrobial activity against Gram-negative bacteria[1]. Here we investigated its activity in biological fluids and in vivo using a mouse model of *S. typhimurium* infection. The efficacy of the active 1-35 fragment of Bac7 was assayed in the presence of serum and plasma, and its stability in these biological fluids analyzed by mass spectrometry. The ability of the peptide to protect mice against Salmonella was assayed in a typhoid fever model of infection by determination of survival rates and bacterial load in liver and spleen of infected animals. Bac7(1-35) retained a substantial activity in biological fluids and showed a very low toxicity in vivo. In the animal model of infection, it significantly increased the number of survivors and the mean survival time of treated mice, reducing the bacterial load in their organs. In addition, the peptide's biodistribution was evaluated by using time-domain optical imaging. The Alexa-labelled peptide reaches the kidney and the bladder respectively 1 and 3 hours after injection. The in vivo and ex vivo analyses performed after 24 h confirm that the compound was totally excreted.

To improve the Bac7(1-35) half-life and biodistribution, we conjugated Bac7, via ester formation, to polyethylene glycol (PEG 20 kDa), a non-toxic, non-immunogenic and FDA-approved polymer that could enhance its halftime and biodistribution.[2].

The efficacy of the conjugated was assayed in vitro via viable colony count assay and its stability analyzed by mass spectrometry. Preliminary results show an increased persistence of the pegylated peptide in biological fluids compared to unpegylated Bac7(1-35).

Our results provide a first indication for a potential development of Bac7-based compounds in the treatment of salmonellosis and, eventually, a valuable strategy for improve their bioavailability.

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P24

Oxyfold : effective and simple solid supported reagent for disulfide bond formation

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The importance of disulfide bridges in peptide conformation and activity make their formation a fundamental step of peptide synthesis.

Formation of disulfide bond is probably one of the most challenging steps to achieve regarding the formation of unwanted by-products and oligomerization. In order to minimize the latter phenomenon, disulfide bridge cyclizations are performed under high diluted conditions which require time consuming removal of solvent at the end of the reaction. Dimethyl sulfoxide (DMSO) is one of the oxidizing reactants most commonly used for this. One of its major drawbacks is its elimination from the reaction medium, which requires evaporation under strong vacuum or repeated lyophilizations. Furthermore, the dimethyl sulfide generated during the reaction is volatile and toxic.

On the other hand, supported reactants are particularly advantageous for promoting intramolecular reactions. In fact, they are known to cause a phenomenon of "pseudodilution" which makes it possible to minimize oligomerization and to use much smaller amounts of solvents[2].

Here we present the synthesis and the use of novel oxidation reactants on solid support for disulfide bond formation. This family of supported reagents consists in a series of oxidized methionines grafted onto a solid support. We demonstrate the efficiency and easiness of these supported reagents for the formation of disulfide bridges in peptide.

Their characteristics make the use of our supported reagents a fast, cheap and green procedure for disulfide bridge formation.

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P25

Searching for a minimal entry sequence in proline-rich antimicrobial peptides

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Antimicrobial peptides (AMPs) constitute an effective component of natural immunity for host defense against microbial agents [1]. The proline-rich are a group of linear AMPs, isolated from both mammals and invertebrates, active mainly against Gram-negative bacteria, which act intracellularly being internalized without an apparent damage to bacterial membranes. Previous studies had indicated that the N-terminal portion of Bac7, a proline-rich peptide from cow, is the region responsible for the activity [2, 3]. To search for the minimal entry sequence and to investigate whether this sequence overlaps with that of the minimal antimicrobial fragment, a set of progressively shorter fragments of Bac7 were synthesized and labelled with the fluorescent probe BODIPY. In this manner their antibacterial activity and internalization into E. coli have been analyzed by using antimicrobial activity assays, flow cytometry and confocal microscopy. Results showed that the N-terminal 16-residue fragment is still fully active and is efficiently internalized into the cells. Shortening of its length has dramatic effects on both these functions. The presence of BODIPY on Bac7 fragments of 13-15 residues also has an influence on antimicrobial activity.
In addition, to evaluate the role of the first two N-terminal arginine residues in the sequence, these were systematically substituted with lysine, D-Arg, nitro-arg [4], dimethyl-arg [4], citrulline or omitted, and the activity of the resulting analogues investigated. The data show that the basicity and propensity to hydrogen bonding of the two Arg residues are essential elements for antimicrobial activity. These results reveal some important requirements for the activity or these proline-rich peptides and suggest a minimal length necessary for both the antimicrobial activity and internalization. The identification of the minimal cell penetrating fragment might also be used to carry drug-molecules into Gram-negative bacteria that are unable to cross their cell membranes.

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P26

Studies on the modes of action of the human cathelicidin LL-37

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The human cathelicidin LL-37 is an important multifunctional component of the innate immune system with direct activity against Gram-positive and -negative bacteria, fungi, and viruses, and with the capacity to modulate host-cell activities. The antimicrobial activity is due to its ability to permeabilize and to disrupt bacterial membranes. However, its mechanism of action is not yet clear. Biophysical studies on model membranes and biological assays were conducted with human LL-37 and its primate orthologues[1] to compare how different structural features influence the interaction with bacterial cell wall components, and, as a consequence, modulate the antimicrobial activity. The data showed that human LL-37 and some of its primate orthologues that are in a structured/aggregated form in bulk solution, act on biological membranes in a different manner from other primate orthologues, such as rhesus RL-37, which are unstructured and monomeric[2]. Given the importance of the peptide's conformation and oligomerization to the biological activity, we synthesized three different disulfide-linked LL-37 dimers by adding a Cys residue to either the C- or N-termini, (forming a C-terminal parallel dimer, an N-terminal parallel dimer and an antiparallel dimer). CD studies indicate that the dimeric forms have an increased propensity to form stacked helices and that the behaviour of the dimers is somewhat different from one another in bulk solution. Biological studies indicate that disulfide-linked LL-37 dimers are less efficient against E. coli cells compared to unlinked LL-37, possibly because of stronger interactions with the lipopolysaccharide (LPS) layer, or increased segregation due to binding to medium components. By selection of an E. coli library of transposon-insertion mutants, we previously isolated an LPS mutant showing decreased sensitivity to LL-37 in comparison to the wild-type strain, a phenotype due to the inactivation of waaY, a gene responsible of the heptose II phosphorylation in the inner core of LPS[3]. Work is in progress to understand the mode of action of LL-37 and the different dimers by using this and other mutants of LPS synthesis, and to verify whether variations in the number of negatively charged phosphate groups on the outer membrane may modulate the capacity of the peptide to bind to and permeabilize the membrane of target cells.

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P27

In vivo biological activity of a ApoA-I mimetic peptide

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Apolipoprotein A-I (ApoA-I), the major protein component of HDL, plays a key role in reverse cholesterol transport, mainly by stimulating the efflux of cholesterol and activating the enzyme LCAT. LCAT converts cholesterol into cholesteryl esters and addresses them to HDL for transport into the circulation. The binding of haptoglobin (Hpt) to ApoA-I is associated with inhibition of LCAT activity[1]. On the basis of the above information, high levels of Hpt, as occurring during the acute phase of inflammation[2], were suggested to be a major cause of both poor cholesterol removal from peripheral cells and low level of HDL cholesterol in the circulation. Previously, we showed that an ApoA-I mimetic peptide (P2a), with amino acid sequence overlapping the stimulatory site for LCAT, was effective in displacing Hpt from ApoA-I, and able to rescue in vitro the stimulatory function of ApoA-I in the presence of high Hpt levels[3].
In this work we characterized the biological activity of peptide P2a in an experimental model of inflammation. In particular, we verified the ability of peptide P2a to rescue LCAT dependent cholesterol esterification *in vivo* in presence of high level of Hpt.

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P28

Synthesis of novel L-diamino acid-based cationic peptide for biomedical applications.

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Cationic poly amino acids, known to act as nucleic acid compacting devices, are also interesting in biotechnology applications, due to several useful characteristics such as their inhibitory action towards plant proteinases[1], significant antimicrobial activity,[2] and also for the possibility to realize cationic hydrogels.[3]

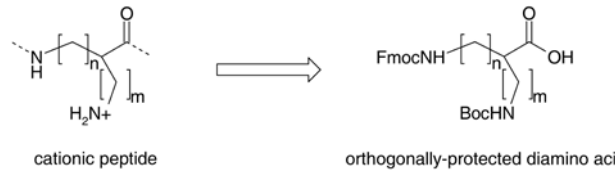

Figure 1. Positively charged peptide can be easily synthesized by using suitably protected diamino acids

In this work we describe the synthesis and characterization of a novel positively-charged peptide based on modified amino acids suitable for the Fmoc solid phase synthesis. The novel cationic peptide, which resulted well-soluble in water, was purified by RP-HPLC and characterized by LC-ESIMS which confirmed the identity of the product. The structural characteristic, as well as the biological properties of this positively-charged molecular device are currently under investigation.

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P29

Non-covalent strategy to deliver pro-apoptotic KLA-peptide

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Cell-penetrating peptides (CPPs) have been the topic of many research articles for twenty years.[1] They show high potential for a non-invasive and specific delivery of otherwise membrane-impermeable pharmacological agents, e.g. bioactive peptides. Therefore we studied the delivery of a bioactive peptide by mixing it with a CPP in a non-covalent approach. The otherwise membrane impermeable peptide KLA was chosen, which is a 14-amino-acid pro-apoptotic peptide, non-toxic outside the cell, but toxic when internalized due to disruption of mitochondrial membranes.[2] Using the three common cell penetrating peptides FP-NLS (MPG), Integrin, and Penetratin, which have been intensively studied by us before [3], we analysed the cellular KLA-delivery in the often utilised epithelial cell line Cos-7, in the breast adenocarcinoma cell line MCF-7, and in the immune cells RAW 264.7. First, we evaluated the optimal mixing ratio of KLA and CPP while keeping the CPP concentration constant. Then we monitored the quantity of the cellular KLA and CPP uptake using a microplate reader and the quality via confocal microscopy. We also studied the role of the CPPs Cterminus regarding its uptake itself as well as its influence on KLA-delivery. Therefore the three CPPs were synthesised either with a carboxylated or carboxamided C-terminus. Since KLA is able to induce apoptosis, its ability to reduce cell viability after cellular delivery by a CPP was examined using a MTT test. Fortunately, we were able to deliver the KLA peptide with our non-covalent strategy simply by mixing it with a CPP in all cell lines studied. The KLA-delivery-efficacy depends on the CPP used and on the CPPs C-terminal conformation. Surprisingly, a high cellular CPP-uptake does not indicate an efficient KLA-delivery. The KLA-peptide was also able to reduce cell viability when delivered by a CPP, e.g. when delivered by carboxylated Integrin, it reduced cell viability by about 30%. Our study reveals the possibility to deliver a membrane impermeable peptide in a non-covalent fashion whilst remaining its bioactive property.

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P30

The crystal structure of hCA I/Topiramate complex provides useful insights into rational drug design of anti-obesity drugs

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Carbonic Anhydrases (CAs, EC 4.2.1.1) constitute an ubiquitous family of metalloenzymes that catalyze the reversible hydration of carbon dioxide to bicarbonate ion.[1] These enzymes represent an interesting target for the design of specific inhibitors for the treatment of a variety of diseases such as glaucoma, acid-base disequilibria, cancer, epilepsy and some other neurological disorders.[1,2] Recently, CA inhibitors have also been explored for treatment of obesity, a growing widespread medical problem, which has been recognized as a critical global health issue, since it presently encroaches on over 300 million individuals worldwide.[3,4]

Topiramate (TPM) is a widely used antiepileptic drug, which has been demonstrated to act as an efficient weight loss agent.[5,6] Since several studies have pointed out that TPM is a potent *in vitro* inhibitor of several CA isozymes, it has been hypothesized that its anti-obesity properties could be ascribed to the inhibition of CAs involved in *de novo* lipogenesis, namely CA VA or/and CA VB within the mitochondria as well as CA II within the cytosol.[3,7,8] Consequently, the study of the interactions of TPM with all human CA isoforms represents an important step for the rational drug design of selective CA inhibitors to be used as anti-obesity drugs.

Here we report the crystal structure of the adduct which TPM forms with hCA I, showing for the first time a profound reorganization of the CA active site upon binding of the inhibitor. Moreover, a structural comparison with hCA II-TPM and hCA VA-TPM adducts, previously investigated, has been performed showing that a different H-bond network together with the movement of some amino acid residues in the active site may account for the different inhibition constants of TPM toward these three CA isozymes.

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P31

Structural characterization of human carbonic anhydrase VII

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Members of the carbonic anhydrase (CA) family are ubiquitos metallo-enzymes present in prokaryotes and eukaryotes that are encoded by five different gene families (α, β, γ, δ, ε). All human CAs belong to the α class and 15 isoforms have been presently identified in different tissues and organs.[1] CAs catalyse the conversion of CO_2 to the bicarbonate ion and protons and play a crucial role in various physiological processes, that include the respiration and gas exchange, pH homeostasis, renal excretion of anions and urine formation, production of electrolytes in many tissues/organs, biosynthetic reactions, etc. Therefore, their malfunctioning can lead to several pathological effects. [1] In this scenario, CAs have recently became interesting targets for pharmaceutical research. In fact, developing isozyme-specific inhibitors should be highly beneficial in obtaining novel classes of drugs.

CA VII is a recently identified cytosolic member of the CA family, overexpressed in brain, whose malfunctioning has recently been reported in the development of epilepsy.[2] In this work, recombinant human CA VII has been functionally and structurally characterized. Biochemical studies revealed that this isozyme acts like a very efficient catalyst, while the analysis of its crystallographic structure gives additional insights into the catalytic mechanism of the CA enzyme family. Furthermore, these results have provided important implications for the rational drug design of selective CA inhibitors with clinical applications.

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P32

Fluctuations and the rate-limiting step of peptide-induced membrane leakage

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Peptide-induced vesicle leakage is a common experimental test for the membrane-perturbing activity of antimicrobial peptides. The leakage kinetics is usually very slow, requiring minutes to hours for complete release of vesicle contents, and exhibits a biphasic behaviour. We report here that, in the case of the peptaibol trichogin GA IV, all processes involved in peptide-membrane interaction, such as peptide-membrane association, peptide aggregation, and peptide translocation, take place in a time-scale much shorter than the leakage kinetics. On these bases, we propose a stochastic model in which the leakage kinetics is determined by the discrete nature of a vesicle suspension: peptides are continuously exchanging among vesicles, producing significant fluctuations over time in the number of peptide molecules bound to each vesicle, and in the formation of pores. According to this model, the fast initial leakage is caused by those vesicles which, after the random distribution of peptides among liposomes, already contain at least one pore, while the slower release is associated to the time needed in an intact vesicle to occasionally reach the critical number of bound peptides necessary for pore formation. Fluctuations due to peptide exchange among vesicles represent therefore the rate-limiting step of such a slow mechanism.

P33

Structural characterization of carbonic anhydrase inhibitors incorporating new metal binding functionalities

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Carbonic anhydrases (CAs) are ubiquitous metalloenzymes, which catalyze the reversible hydration of carbon dioxide to bicarbonate ion. CAs are wide-spread enzymes, present in mammals in at least 16 isoforms, differently distributed in many tissues and organs.[1] Since at these sites CAs play a crucial role in various physiological and patho-physiological processes, they have recently become interesting targets for pharmaceutical research. Indeed, several CA inhibitors (CAIs) incorporating a sulfonamide/sulfamate/sulfamide moieties are currently clinically used for the treatment or prevention of a multitude of diseases such as glaucoma, solid tumors, and epilepsy.[2] Unfortunately, these molecules are still far from being optimal drugs. They show several non-desired side-effects, mainly because of their lack of selectivity for the different CA isoforms. As a consequence, many CAIs are continuously synthesized and tested for their CA inhibition action. A very promising alternative strategy for CAI drug design consists of the development of different zinc binding groups. Besides sulphonamides and their bioisosteres such as sulfamates and sulfamides, five other different zinc binding groups have been reported to efficiently bind to the zinc ion, i.e. the ureate/hydroxymates, the mercaptophenols, metal-complexing anions, N-hydroxy-sulfamides and N-hydroxy-sulfonamides. Few information are at the moment available on this latter CAI class.[2]

Here, we report the inhibition study on two new CAIs containing the N-hydroxy- and the N-methoxy-sulfonamide moiety, respectively, and their crystal structure in complex with the ubiquitous cytosolic human carbonic anhydrase II. The identification of the molecular interactions driving the inhibitor/CA binding could be very useful for the rational drug design of selective CAI with clinical applications.

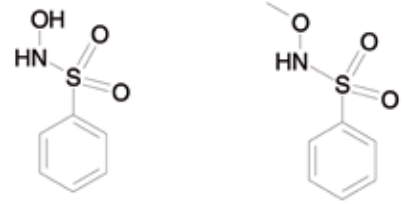

Figure 1. Schematic representation of the N-hydroxy- and N-methoxy-benzenesulfonamide inhibitors.

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P34

Host defense mechanisms elicited in *Salmonella Typhimurium* colitis

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The host antimicrobial defense system in the gut is well characterized. However, it remains unclear how it shapes the gut flora under normal steady state conditions and influences it during an inflammatory response. Antimicrobial peptides (AMPs) as well as reactive oxygen or nitrogen species might be involved.
To study the interactions of the mucosal innate immune system with the gut flora we use *Salmonella enterica* subspecies I serovar Typhimurium (*S. Typhimurium*) in a mouse enterocolitis model. *S.* Typhimurium is able to trigger intestinal inflammation as a strategy to outcompete the microbial flora which in the non-inflamed gut prevents *S. Typhimurium* from growing to high density [1]. Strikingly the host innate immune system strongly upregulates AMPs upon infection with a virulent *S. Typhimurium* strain.
We hypothesize that AMPs differentially kill *S. Typhimurium* and members of the commensal gut flora. This differential killing could be exploited by *S. Typhimurium* to outcompete the gut flora under inflammatory conditions. Further work is required to understand the mechanisms of differential killing by AMPs as well as its effect on *S. Typhimurium* competition with the gut flora.

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P35

Preliminary characterization of *Brucella suis* hystidinol dehydrogenase

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Brucella is a causative agent of brucellosis, which is the most widespread zoonosis worldwide. In humans there are three pathogenic *Brucella* species, *Brucella Suis, B. Abortus* and *B. Melitensis.* [1] They are intracellular pathogens that can survey and multiply within the phagocytic cells of the mammalian host. The pathogen is capable of establishing persistent infections in humans which are difficult to eradicate, even with antibiotic therapy.

The enzyme hystidinol dehydrogenase (HDH) is essential for intramacrophagic replication.[2] In particular, this enzyme converts L-histidinol to L-histidine through a L-histidinaldehyde intermediate, with the concomitant reduction of two molecules of nicotinamide adenine dinucleotide (NAD). During evolution the HDH sequence has been well conserved and since it is absent in mammals, has become a novel target for the development of anti-*Brucella* agents. To date, HDH enzymes have only been cloned and characterized from *Salmonella typhimurium* [3] and *Escherichia coli*.[4]

In the present study we have focused our attention on HDH from *B. suis*. We have cloned, expressed and purified the full length enzyme and a preliminary characterization has been carried out. By combining biochemical methodologies with Light Scattering analysis we have found that the enzyme is a non covalent homodimer, which shows a very compact and globular shape, as also reported for *E.coli* enzyme. However, the enzyme was not stable in solution due to the presence of a reactive cysteine residue (Cys 371), which led to the formation of covalent dimers after few days. In order to handle a more stable enzyme form, this cysteine was mutated in serine and the corresponding mutant overexpressed in *E.coli*.

Crystallization experiments have been performed both on native and mutant enzyme, with the better results obtained on the mutant form. Crystal structure resolution is currently underway.

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P36

SPLC: solid phase cross-linking, a new tool to approach protein structure in solution

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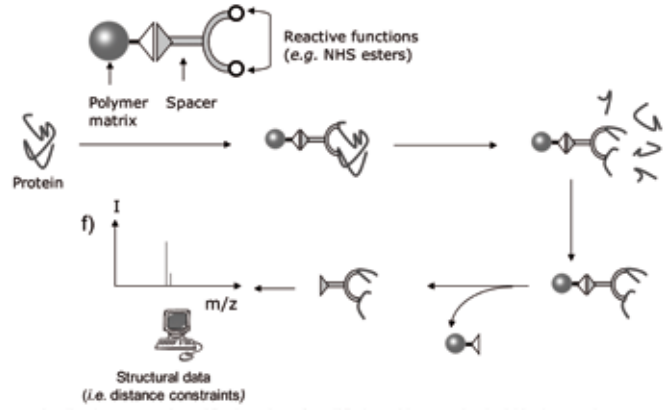
The technique of chemical cross-linking followed by mass spectrometry analysis has proven to bring valuable information about protein structure when NMR or X-ray crystallography data are lacking.[1] However, the detection of a significantly large number of cross-linked peptides in complex biological mixtures represents the real bottleneck of this method.[2] To address this issue, we developed mono and bifunctional supported chemical reagents able to selectively react with accessible amino groups of a protein allowing easy and specific detection of modified peptides contained within the protein.
Interestingly, the whole process is realized on solid support including synthesis of the cross-linkers, reaction with the protein and enzymatic digestion. All undesired soluble materials including unreacted protein and non-covalently linked peptides were easily removed by simple washings avoiding chromatography or purification steps. [3] Software treatment gave structural results in accordance with NMR and X ray data of the two model proteins.[4]



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P37

Studies on temporin B analogues

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Temporins are antimicrobial peptides (AMP) produced and secreted by the granular glands of the European red frog (*Rana temporaria*)[1]. They are amphipathic α helical peptides, 10–14 amino acids long, containing only 1 or 2 positively charged amino acids (R or K). Temporins are active prevalently against Gram positive bacteria, including methicilline and vancomycine resistant staphylococci and enterococchi, and are non toxic to mammalian cells. In a recent work it was shown that a synthetic temporin B (TB) analogue acquires the capacity to act in synergism with temporin A (TA) and to exert antimicrobial and anti-inflammatory activity *in vivo* against Gram positive and Gram negative bacteria[2]. In order to understand which amino acids are necessary for the TB biological activity and whether there is a relation between the activity and the structure of the peptide, we have studied 13 analogues of TB, obtained after substitution of each amino acid by an alanine (Table).

LLPIVGNLLKSLL	TB	LLPIVGALLKSLL	TB N7A
ALPIVGNLLKSLL	TB L1A	LLPIVGNALKSLL	TB L8A
LAPIVGNLLKSLL	TB L2A	LLPIVGNLAKSLL	TB L9A
LLAIVGNLLKSLL	TB P3A	LLPIVGNLLASLL	TB K10A
LLPAVGNLLKSLL	TB I4A	LLPIVGNLLKALL	TB S11A
LLPIAGNLLKSLL	TB V5A	LLPIVGNLLKSAL	TB L12A
LLPIVANLLKSLL	TB G6A	LLPIVGNLLKSLA	TB L13A

Table: Sequences of the TB analogues

Peptides were obtained by solid phase synthesis as C-terminal amides, purified by RP-HPLC and characterized by mass spectrometry. Molecules were analyzed by Circular Dichroism to determine the peptide secondary structure. The antimicrobial activity of the peptides was tested on both Gram positive and Gram negative bacteria, including *S. enterica*, *E. coli*, *S. aureus* and *Listeria*. As demonstrated by the CD spectra, all peptides show the tendency to adopt an α helical structure in SDS, while they are random coils in phosphate buffer. Although no clear relationship between the secondary structure and the antimicrobial activity of the peptides comes out, it is clear that some substitution, are detrimental to solubility, as the replacement of serine with alanine. Substitution of glycine and valine in position 5 and 6 results in peptides which show good antimicrobial activity when administered together.

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P38

A SPR strategy for high-throughput ligand screenings based on synthetic peptides mimicking a selected subdomain of the target protein: a proof of concept on HER2 receptor

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The discovery of pharmaceutical agents is a complex, lengthy and costly process, critically depending on the availability of rapid and efficient screening methods. In particular, when targets are large, multidomain proteins, their complexity may affect unfavorably technical feasibility, costs and unambiguity of binding test interpretation.

A possible strategy to overcome these problems relies on molecular design of receptor fragments that are: sensible targets for ligand screenings, conformationally stable also as standalone domains, easily synthesized and immobilized on chip for Biacore experiments.

To test the feasibility of such approach on a case with potential applicative interest, we developed a surface plasmon resonance (SPR)-based screening method for drug candidates toward HER2, a Tyr-kinase receptor targeted in anticancer therapies.[1-3] HER2 was mimicked by HER2-DIVMP, a modified fragment of it immobilized onto the sensor surface specifically modeling HER2 domain IV in its bounded form, designed by structural comparison of HER2 alone and in complex with Herceptin, a monoclonal therapeutic anti-HER2 antibody.[4]

This design and its implementation in SPR devices was validated by investigating Herceptin- HER2-DIVMP affinity, measuring its dissociation constant (KD = 19.2 nM). An efficient synthetic procedure to prepare the HER2-DIVMP peptide was also developed. The HER2-DIVMP conformational stability suggested by experimental and computational results, makes it also a valuable candidate as a mold to design new molecules selectively targeting domain IV of HER2.

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P39

Synthesis and characterization of new γ sulphate PNA

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Peptide Nucleic Acid (PNA) is nucleic acid mimic in which the sugar phosphodiester backbone was replaced by the achiral N-(2-aminoethyl) glicine unit[1]. The neutral backbone allows PNA to hybridize to DNA and PNA with high affinity and sequence selectivity through Watson-Crick base-pairing, while the unnatural polyamide linkage allows PNA to withstand enzymatic degradation by protease and nucleases[2]. These properties, along with the ease of synthesis, made PNA an attractive reagent for many applications in chemistry, biology and medicine[3]. However, the full potential of PNAs as tools for regulating gene expression has not yet been fully realized due their poor cellular uptake, limited solubility and reduced affinity toward proteins. Several modifications have been made to the PNA backbone in the attempt to improve its properties[4]. Since binding of DNA to proteins is mostly mediated by hydrogen bond and charge-charge interaction between phosphates and charged amino acids, it is desiderable to introduce hydrogen bond donors/acceptors and charged units into the PNA backbone.
In this communication we present the synthesis of new PNA monomers with a sulphate group on the hydroxymethyl side chain located in the γ position of the backbone.

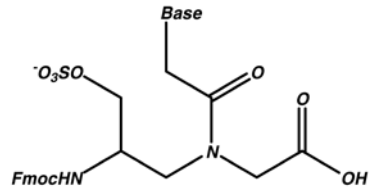

Figure 1. γ sulphate PNA monomer

Coupling conditions on solid phase for each modified monomer were optimised. One homopirimidine PNA oligomer containing T γ sulphate monomers (Ts) (CTsCCTsCCTsC), whose sequence was chosen to perform specific biological assays, was obtained in high purity after HPLC purification and characterized by LC-MS and CD. The stoichiometry of the complex formed with a complementary DNA, its secondary structure and its thermal stability were investigated. Furthermore the ability to work as an antigene towards ErbB2 gene was evaluated on SK-BR-3 cell line over-expressing the HER2/c-erb-2 gene product by Elisa assays and FACS analyses.

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P40

A single amino acid addition enhances the fusion promotion activity of a membranotropic region of HSV-1 glycoprotein H

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Herpes simplex virus type 1 (HSV-1) induced membrane fusion remains one of the most elusive mechanisms to be deciphered in viral entry. The structure resolution of glycoprotein gB has revealed the presence of fusogenic domains in this protein and pointed out the key role of gB in the entry mechanism of HSV-1. A second putative fusogenic glycoprotein is represented by the heterodimer comprising the membrane anchored glycoprotein H (gH) and the small secreted glycoprotein L (gL), which remains on the viral envelope in virtue of its non-covalent interaction with gH. Different domains scattered on the ectodomain of HSV-1 gH have been demonstrated to display membranotropic characteristics.[1] The segment from amino acid 626 to 644 represent the most fusogenic region identified by studies with synthetic peptides and model membranes[2]. We have identified the minimal fusogenic sequence present on gH. An enlongation at the N-terminus of a single histidine (His) has proved to profoundly increase the fusogenic activity of the original sequence. Nuclear magnetic resonance (NMR) studies have shown that the addition of the N-terminal His contributes to the formation and stabilization of an α-helical domain with high fusion propensity.

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P41

Thermodynamics of melittin binding to lipid bilayers. Aggregation and pore formation

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Lipid membranes act as catalyst for protein folding. Both α-helical and β-sheet structures can be induced by the interaction of peptides or proteins with lipid surfaces. Melittin, the main component of bee venom, is a particularly well-studied example for the membrane-induced random coil-to-α-helix transition. Melittin in water adopts essentially a random coil conformation. The cationic amphiphatic molecule has a high affinity for neutral and anionic lipid membranes and exhibits ~50 – 65% α-helix conformation in the membrane-bound state. At higher melittin concentration, the peptide forms aggregates or pores in the membrane. In spite of the long-standing interest in melittin – lipid interactions, no systematic thermodynamic study is available. This is probably caused by the complexity of the binding process. Melittin binding to lipid vesicles is fast and occurs within milliseconds, but the binding process involves at least four steps, namely, (i) the electrostatic attraction of the cationic peptide to an anionic membrane surface, (ii) the hydrophobic insertion into the lipid membrane, (iii) the conformational change from random coil to α-helix, and (iv) peptide aggregation in the lipid phase. We have combined microelectrophoresis (measurement of the ζ-potential), isothermal titration calorimetry, and circular dichroism spectroscopy to provide a thermodynamic analysis of the individual binding steps. We have compared melittin with a synthetic analogue, [D]-$V^{5,8}$,I^{17},K^{21}-melittin, for which α-helix formation is suppressed and replaced by β-structure formation. The comparison reveals that the thermodynamic parameters for the membrane-induced α-helix formation of melittin are identical to those observed earlier for other peptides with an enthalpy h_{helix} of -0.7 kcal/mol and a free energy of g_{helix} of-0.2 kcal/mol per peptide residue. These thermodynamic parameters hence appear to be of general validity for lipid-induced membrane folding. As g_{helix} is negative, it further follows that helix formation leads to an enhanced membrane binding for the peptides or proteins involved. In this study, melittin binds by ~2 orders of magnitude better to the lipid membrane than [D]-$V^{5,8}$,I^{17},K^{21}-melittin which cannot from an α-helix. We also found conditions under which the isothermal titration experiment reports only the aggregation process. Melittin aggregation is an entropy-driven process with an endothermic heat of reaction (ΔH_{agg}) of ~2 kcal/mol and aggregation constant of 20 – 40 M^{-1}.

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P42

Comparison of distance information in [TOAC1,Glu(OMe)7,18,19] Alm F50/5 from PRE measurements with data obtained from an X-ray diffraction based model

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Peptaibol antibiotics are membrane-active linear peptides of fungal origin that are characterized by a high population of α-aminoisobutyric acid (Aib), an N-terminal acetyl group, and a C-terminal 1,2-amino alcohol.[1] Alamethicins (Alms) are a group of closely sequence-related peptides composed of 19 amino acid residues.[2] In this work, distance information from Paramagnetic Relaxation Enhancement (PRE) studies on [TOAC^{1},Glu(OMe)^{7,18,19}]Alm is compared with distances observed in a model derived from the X-ray structure of the closely related peptide [TOAC^{16},Glu(OMe)^{7,18,19}]Alm[3,4] (Figure 1). The methodology for PRE determination is discussed as well as the generation of the X-ray based model structures. The distances obtained from PRE measurements are in close agreement with those derived from the X-ray diffraction based model. This finding suggests that this information could be implemented as long-range distance restraints in NMR based structure determination.

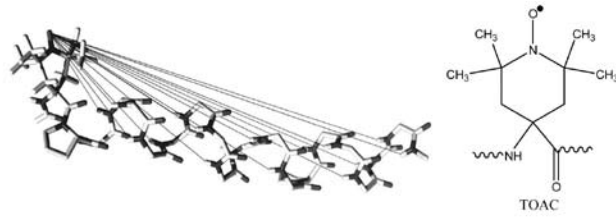

Figure 1. Distance determination in [TOAC^{1},Glu(OMe)^{7,18,19}] Alm based on PRE and an X-ray diffraction based model.

[Glu(OMe)^{7,18,19}] Alm : Ac-U-P-U-A-U-A-Q*-U-V-U-G-L-U-P-V-U-U-Q*-Q*-Phol
[TOAC^{1},Glu(OMe)^{7,18,19}] Alm : Ac-TOAC^{1}-P-U-A-U-A-Q*-U-V-U-G-L-U-P-V-U-U-Q*-Q*-Phol
[TOAC^{16},Glu(OMe)^{7,18,19}] Alm : Ac-U-P-U-A-U-A-Q*-U-V-U-G-L-U-P-V-TOAC^{16}-U-Q*-Q*-Phol
Q* = Glu(OMe), Phol = phenylalaninol, U = Aib

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P43

Addition of pyridine in PNA couplings improves the reaction yields: development of an efficient and low cost protocol for the PNA synthesis by manual Fmoc-chemistry

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Peptide Nucleic Acid are DNA analogues with a peptide like backbone, having the nucleobases attached to the backbone through a methylen carbonyl linker[1]. The ability to bind complementary DNA and RNA with high affinity, and the resistance to degradation by nucleases and proteases stimulated the research on the therapeutic applications of PNAs, as antisense, antigene and decoy[2,3].
Synthesis of PNA oligomers has been carried out using a variety of monomers and coupling conditions.
Nowdays PNA syntheses are usually carried out on automated synthesizers, in virtue of the higher yields of the oligomers, as compared to those obtained by manual synthesis and protocols for the manual synthesis are often an adaptation of protocols employed in automated synthesis.
With the aim to develop an efficient and low cost protocol for the manual synthesis of PNA oligomers, we have explored a new combination of activators and bases. We tested several coupling conditions, using HOBT/HBTU as activators, in place of HATU and bases as NMM and pyridine. The protocol we developed, relies on coupling reactions carried out with of 2.5 equivalents of PNA monomers activated with HOBt/HBTU, in the presence of pyridine/NMM and it has been tested on four PNA oligomers with a length ranging from 9 to 12 bases and a purine content up to 70%.
We report the results obtained using the standard and all the new tested protocols The protocol developed will allow the obtainement with good yields of difficult sequences, avoiding expensive activators and large excesses of PNA monomers.

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P44

Design and biological characterization of a proangiogenic peptide reproducing the β-hairpin region 87-100 of PlGF

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Vascular endothelial growth factor (VEGF) is a highly-conserved, homodimeric signaling glycoprotein exhibiting multiple functions, including modulation of angiogenesis, vasculogenesis, vascular leakage, neurogenesis, inflammation, hematopoiesis and lymphangiogenesis. VEGF is characterized by a Cys-knot structural motif and is encoded by a single gene that can splice differentially to give at least eight isoforms of the protein that differ in the number of amino acids. However, VEGF possesses several homologs, including the Placental Growth Factor (PlGF) that was originally discovered in placenta. As well as others growth factors, VEGF and its homologs elicit diverse biological activity through the interaction with membrane receptors (as VEGFR1, VEGFR2 and VEGFR3) which then transfer the signal to cell interior inducing specific biochemical pathways. The impairment of signaling pathways activated by VEGF and its homologs may contribute to the onset, development and progression of several common and lethal human diseases, including cancer, cardiovascular disorders, diabetic complications, retinal degeneration, and chronic inflammation.[1,2]

The development of molecules able to function as modulators interfering with the molecular recognition between VEGF or its homologs with their receptors is gaining a big pharmacological interest for therapeutic and diagnostic applications. In this regard, bioactive peptides targeting VEGF receptors are considered lead compounds due to the great number of advantages that they present, such as modest size, easy and well established synthetic procedures, possibility to modulate physico-chemical properties and conformation. [3] In order to identify a peptide with pharmacological applications in angiogenesis related diseases, we designed, synthesized and characterized the biological activity of a peptide mimicking the region 87-100 of PlGF, which in the natural protein assume a β-hairpin structure and is part of the interaction interface with the domain 2 of its receptor VEGFR1. In vitro and in vivo biological assays demonstrated its ability to interact with VEGF-R1 and interfere with VEGF-related angiogenesis.

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P45

Role of membranotropic and antiviral sequences from *Herpes Simplex* virus type I glycoproteins B and H in the fusion process

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The entry of enveloped viruses involve attachment followed by close apposition of the viral and plasma membranes. Then, either on the cell surface or in an endocytotic vesicle, the two membranes fuse by an energetically unfavourable process involving the destabilization of membrane microenvironment in order to release the viral nucleocapsid into the cytoplasm. The mechanism by which herpesviruses fuse with cellular membranes to permit virus entry is still relatively poorly understood. This process is proving difficult to unravel, largely due to the fact that multiple viral envelope proteins appear to function in concert to mediate the fusion event. The core fusion machinery, conserved throughout the herpesvirus family, involves glycoprotein B (gB) and the non-covalently associated complex of glycoproteins H and L (gH/gL). Both gB and gH possess several hydrophobic domains necessary for efficient induction of fusion, and synthetic peptides corresponding to these regions are able to associate to membranes and induce fusion of artificial liposomes.[1,2] We selected peptides from gB and gH and also analysed the behaviour of HIV gp41 fusion peptide and the cationic antimicrobial peptide melittin to increase our molecular understanding of the mechanism of membrane fusion. Several biophysical methodology have been used, such as surface plasmon reasonance and electron spin resonance spectroscopy. The results suggest that membrane interacting peptides all act via a similar mechanism, which is substantially different from that of the non-cell selective lytic peptide melittin. The results suggest that membrane interacting peptides all act via a similar mechanism, which is substantially different from that of the non-cell selective lytic peptide melittin. These results might shed further light on HSV-induced membrane fusion.[3]

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P46

Novel analogs of human beta defensins 1 and 3 showed enhanced antimicrobial activity

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Human beta-defensins (hBDs) are antimicrobial peptides of human innate immunity. Epithelial cells secrete human beta defensins (hBDs). They kill bacteria and fungi in a quite aspecific way and/or by pore formation in the target membrane; they also exert chemotactic properties towards immature dendritic cells and T-cells, coupling the human innate immune response to the cellular response. It is well known that, the antibacterial activity of beta-defensin 1, 2 and 4 is modulated by the salt concentrations; the high levels of NaCl observed, for example in the respiratory layer of cystic fibrosis patients, can inhibit the activity of beta-defensins, contributing to chronic infections and bacterial colonisation. On the other hand, the antibacterial activity of human beta defensin 3 is not modulated by the salt concentration of the medium. To increase our insight into the pathophysiology of beta defensins and to clarify the potential role of these peptides as therapeutic targets, we performed in vitro tests to evaluate the antimicrobial (against *Pseudomonas aeruginosa*), pro-chemotactic (toward neutrophils) and the antiviral activity (against *herpes simplex virus*) of beta-defensin variants obtained by chemical synthesis. We have shown that the hBD1 internal region and the hBD3 C-terminal region are critical for antibacterial activity also at high salt concentrations, whereas the deletion of the N-terminal region of hBD3 results in an increase of antibacterial activity. All analogs inhibited Herpes simplex virus; antiviral activity was enhanced by the hBD1 internal region and the hBD3 C-terminal region. These new peptides may have therapeutic potential.

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P47

Structural investigation of CCK8 peptide amphiphiles in self-assembled supramolecular aggregates

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Peptide amphiphiles (PAs), consisting of a hydrophilic peptide sequence and a hydrophobic moiety, self-assemble in highly ordered nanostructures.[1] In these structures peptides are displayed on the nanostructure surface, with orientation order resulting from β-sheet formation during the self-assembling process. The peptide moiety, remaining on periphery of the nanostructure, enables different chemical and biological functionalities. Previously we reported the synthesis and the structural characterization of $(C18)_2$L5CCK8 peptide amphiphile.[2] This molecule contains two alkyl chains as hydrophobic segment, a spacer consisting of five ethoxylic units, and CCK8 peptide, as hydrophilic moiety. $(C18)_2$L5CCK8 self-assembles in well-ordered nanostructures in aqueous solution and could be employed for target selective drug delivery system on a biological target. In fact, CCK8 peptide on aggregate surface is able to interact with high affinity towards to cholecystokinin receptors overexpressed by cancer cells. According to literature CCK8 assumes a random-coil conformation in aqueous solution, whereas shows a β-sheet structural motif in $(C18)_2$L5CCK8 PA aggregates. NMR studies, between CCK8 and the N-terminus of CCK receptor in DPC micelles, suggest that CCK8 assumes a pseudo-helical structure in the presence of receptor fragments.[3] These findings imply CCK8 in PA aggregates could not be able to interact with the receptor. Now we report the synthesis, the structural characterization and in vitro binding properties of five novel supramolecular aggregates obtained by starting from cationic PAs. All monomers are synthesized according to SPPS, based on Fmoc strategy, and aggregates were formulated by dissolving PAs in aqueous solution. Each monomer contains the same hydrophilic and hyrophobic moiety of $(C18)_2$L5CCK8, while the spacer is represented by three lysine residues and an ethoxylic linker. The presence of several positive charge on lysine residues should avoid β-sheet conformation. Supramolecular aggregates of PAs were structurally characterized by DLS and fluorescence spectroscopy. CD studies, at different pH and ionic strength, confirm that peptide sequence in cationic PAs is not able to assume any secondary structure. Cellular uptake of PA aggregates, studied by flow cytometry on A431 cells overexpressing the CCK_2-R by stable transfection, confirmed the behavior expected on the basis of conformation results.

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P48

NMR analysis of the interaction between VEGF mimicking peptides and VEGFR-1_{D2}

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Angiogenesis is the process characterized by the formation of new blood capillaries from preexisting vessels. It is the result of a complex balance of positive and negative regulators, and vascular endothelial growth factor (VEGF) is one of the most important pro-angiogenic factors, essential for growth, mitogenesis, and tube formation of endothelial cells. The biological effects of VEGF are mediated by its binding to two tyrosine kinase receptors, VEGFR-1 and VEGFR-2, resulting in activation of signal transduction and regulation of physiological and pathological angiogenesis[1]. The extracellular portion of these receptors is comprised of 7 immunoglobulin domains; domains deletion studies have shown that, for VEGFR-1, the ligand binding function resides within the first three domains, while for VEGFR-2 only domains 2 and 3 are critical for ligand binding[2,3]. In the last years, the study of molecular mechanisms of angiogenesis has stirred renewed interest due to the recognition of the role played by this process in several pathologies of large social impact and to the pharmacological interest rising from the possibility of modulating this phenomenon. Many approaches have been pursued to modulate VEGF-receptors interaction, and new molecular entities as peptides have been reported to bind to the extracellular region of the VEGF receptors [4]. Recently, we have developed peptides able to modulate the VEGF-dependent angiogenesis. These peptides, designed on VEGF N-terminal helix 17-25, bind to VEGF receptors and are biological active *in vitro* and *in vivo* experiments[5-7]. Here, we report the NMR characterization of the interaction of the bioactive peptides with the recombinant VEGFR-1_{D2}[8]. This analysis allowed to determine the peptide binding sites on the receptor and the peptide residues involved in the interaction.

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P49

Antimicrobial properties of human hepcidin 20 and 25

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Hepcidin 25 (hep-25) is a peptide primarily produced by human liver [1] with a central role in iron homeostasis[2, 3]. Its isoform, hepcidin 20 (hep-20) lacking the first five aminoacids of the amino-terminal portion, has an unknown function. This sequence is crucial for iron regulation by hep-25 and contains a molecular motif able to bind metals [4]. In this study, both peptides were studied in vitro for their antibacterial properties. Although both hepcidins showed a wide spectrum of bactericidal activity against both Gram-positive and Gram-negative clinical isolates with different antibiotic resistance profiles, hep-20 was usually found to be active at lower concentrations than hep-25. Among the bacterial species tested, Staphylococcus aureus was the less susceptible to both peptides. Bactericidal activity of hep-25 was slightly enhanced in the presence of copper, while the same metal did not affect the activity of hep-20. The presence of human serum partially inhibited the bactericidal effects of both peptides while a synergistic effect on antibacterial activity was observed in buffer at pH 7.4, when hep-20 and hep-25 were used in combination against Enterococcus faecium. Killing kinetics, carried on in sodium-phosphate buffer at pH 7.4, demonstrated that bactericidal activity occurred not earlier than 30-90 minutes of incubation. With the aim to comprehend the mechanism of action of hep-20 and hep-25, a study on the potential membrane permeabilization effect was carried out by means of enzymatic assays and artificial phospholipids vesicles. As a result the two peptides did not affect bacterial membrane integrity thus suggesting a mechanism of action other than membrane poration. The verification of the hypothesis of a potential intracellular molecular target, as suggested by the observation of the long-time bactericidal effects, is object of our forthcoming studies.

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P50

Antimicrobial and mammary cell-activating properties of bovine cathelicidin peptides in bovine mastitis – A comparative analysis

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Bovine mastitis is an inflammatory condition of the mammary gland that decreases milk yield and alters milk composition. It is a most costly and widespread infectious disease of dairy cattle and is usually caused by bacterial invasion of the udder [1].

Cathelicidins are cationic and amphipathic antimicrobial peptide components of the innate immune system of mammals[2]. Quite a few distinct cathelicidin members have been identified in cattle, including α-helical, Pro-rich, Trp-rich and disulphide-bonded peptides[3]. In this study we examined the biological properties of selected bovine cathelicidins, i.e., BMAP-27 and –28 (α-helical), Bac5 (Pro-rich) and indolicidin (Trp-rich). Their promise as novel therapeutics for prevention and treatment of bovine mastitis was investigated with respect to i) cathelicidin gene expression in healthy and infected bovine mammary tissue and in lipopolysaccharide (LPS)-treated cells; ii) antimicrobial activities against microbial pathogens isolated from bovine mastitis; iii) ability to trigger defense responses in bovine mammary cells.

All cathelicidin genes were found to be upregulated in LPS-stimulated bovine neutrophils but not in infected bovine quarters or epithelial cells. The corresponding peptides showed a varied spectrum activity in vitro against 28 bacterial isolates from bovine mastitis also including antibiotic resistant strains (MIC values 0.5 to 32 μM). They were also effective against clinical isolates of the yeast-like alga *Prototheca* spp. (MIC values 1 to 16 μM), an emerging bovine pathogen which does not respond to routine mastitis therapy [4]. The antimicrobial activity was significantly enhanced when each peptide was tested in pairwise combinations, reaching the synergy threshold when indolicidin was present. Whereas Bac5 and indolicidin were active under a limited range of experimental conditions, the α-helical peptides BMAP-27 and -28 were inhibited in milk from healthy cows but were highly effective in mastitic milk, in whey and in blood serum. Moreover, both BMAPs but not Bac5 or indolicidin, triggered the expression of tumor necrosis factor alpha (TNF-α) in bovine mammary epithelial cells.

Overall our results encourage studies aimed to develop novel therapeutics based on BMAP peptide sequences for topical treatment of mastitis.

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P51

A novel and highly selective IgE-binding peptide with anti- allergic properties: an SPR and cell-based study

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Binding of IgE to the high affinity receptor FcεRI on the surface of mast cells and basophils is the key step of allergic reactions. The receptor contains a β-chain, a dimer of γ-chains and an extracellular α-chain that binds with high affinity to the Fc of IgE ($K_D \approx 10^{-9}$ M). Receptor cross-linking through allergen/antibody interactions activates an intracellular signalling in mast cells that leads to degranulation and to release of histamine or other mediators of the allergic response [1] and it has been shown that preventing the IgE-FcεRI binding is an effective way to block the early events of the allergic response [2,3]. The crystallographic structure of the complex FcεRI(αchain)-IgE(Fc) has cleared the molecular details of this interaction and has opened the way to the design of novel modulators of allergic responses [4,5]. We have recently reported a set of peptide-based receptor mimetics containing key residues from the D2 domain (interaction site 2) and from the D1-D2 junction of FcεRIα chain (interaction site 2) joined by a linker. Peptides have a μM affinity for IgE and show a distinct 2-site mechanism of recognition for the immunoglobulin, as it occurs in the native receptor. We report here a new IgE-binding peptide – named *Pep-E* - where the linker has been optimized in terms of length. *Pep-E* shows a largely improved affinity (> 30-fold) compared to the first generation peptides [5], the same specificity and a 2-site mechanism of binding for IgE Binding to IgE has been performed using the SPR technique on a BIAcore instrument (Pharmacia Biosensor) and on a SensiQ (Nomadics). For this purpose, biochips were prepared by coupling IgE, IgG and IgA to a CM5 sensor (Biacore), whereas only IgE were bound on a COOH1 SensiQ chip. Notably, assays with the two instruments provided a comparable 600 nM affinity even when the peptide was conjugated to a fluorescent dyes. IgE-binding and the potential anti-allergic properties of *Pep-E* were further assessed on RBL2H3 cells, confirming disruption of IgE interaction with the cell-bound native receptor and blocking of histamine release following IgE-antigen stimulation. The IC_{50} for the histamine release assay was about 100 μM.

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P52

Fast automated synthesis of an antimicrobial peptide analog using UV-monitoring on the Tribute

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Protaetiamycine is a naturally occurring 43-mer antimicrobial peptide obtained from the larvae of the *Protaetia brevitarsis* beetle. A 9-mer analog, 9Pbw0 (RLWLAIGRG-NH2), was synthesized by Shin et al. which showed good antifungal activity against *Candida albicans*[1]. 9Pbw0 was synthesized on the Tribute peptide synthesizer using the IntelliSynth UV-Monitoring and Feedback Control System to control and monitor the deprotection reactions, and fast coupling times using the activator HCTU.

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P53

Small peptides as antagonists of PED-PEA15/PLD1 protein complex

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In the field of drug discovery through the combinatorial preparation and HTS of arrays of compounds, peptide libraries play a pivotal role for the rapid identification of high- or medium-affinity target ligands that can be subsequently optimized in terms of potency, selectivity and stability [1]. Here, we describe a simplified approach [2] by which rationally designed small synthetic peptide libraries were screened to identify inhibitors of the complex between PED-PEA15 and PLD1, recognized as involved in molecular mechanisms of insulin resistance occurring in type 2 diabetes. Firstly we confirmed that the main PLD1 region involved in PED-PEA15 recognition is its N-terminal region (D4α)[3] through SPR technique and, further, applying a fully automated screening of two simplified peptide libraries in positional scanning format, we identified small peptides able to inhibit PED-PEA15/D4α interaction. The selection of inhibitors was carried out employing combined competitive and direct ELISA and SPR experiments providing IC50 values in the micromolar range. Our results showed that the protein complex appears to be susceptible to peptides with relatively high aromaticity and H-donor capacity, these small sequences can be used as good 'growing' molecules into higher-affinity compounds for SAR studies to aid the design of more efficient inhibitors through medicinal-chemistry approaches.

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P54

Design and characterization of peptides involved in IkappaB-alpha/tat interaction

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Knowledge of protein-protein interactions is a major goal for the modern structural biology, as it represents the starting point for understanding the molecular basis of diseases and for identifying molecules to inhibit or promote such interactions[1-2]. These mechanisms may involve large protein surface areas including several contact sites, or can be mediated by "hot spots". IkappaB-alpha is an inhibitor of NF-kB transcription factors, and harbors six ankyrin repeat domains that show differential flexibility with diverse protein partners[3]. The HIV-1 trans-activator of transcription (Tat) is a protein of 86-101 amino acids that is essential for viral replication[4]. We recently showed that IkappaB-alpha blocks the HIV-1 expression and replication in an NF-kappaB-independent manner by directly binding to Tat through its C-terminal region spanning from amino acids 72 to 287[5]. Here, we have further investigated the IkappaB-alpha amino acid sequence required for the binding to Tat by structural mapping the physical and functional interaction domains of both proteins. On the basis of available structural and composition data, several peptides covering the amino acid sequence of IkappaB-alpha from 262 to 317 amino acids as well as the Cys-rich and Arg-rich motifs of Tat were designed and synthesized. The conformational properties of peptides were investigated by CD spectroscopy, while their binding capacities to Tat protein were analyzed through SPR technique and co-immunoprecipitation assays. The results point out to a singular peptide that shows a significant binding affinity to Tat. This peptide may represent a structural model for developing new and specific inhibitors of the Tat transcriptional activity.

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NMR studies of the heterotypic interaction between Ship2-Sam and EphA2-Sam

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Sterile alpha motif (Sam) domains are protein binding modules that play a significant role in many biological processes mainly *via* homo- and hetero-dimerization.[1] It has been recently reported that the lipid phosphatase Ship2 is able to regulate the endocytosis of the EphA2 receptor, a process that has been investigated as a potential way to decrease tumor malignancy.[2] A heterotypic Sam-Sam domain association is needed to engage Ship2 at the receptor site.[2] We have performed NMR and ITC (Isothermal Titration Calorimetry) studies on the Sam domain of Ship2 and determined its three-dimensional structure and its possible mode of interaction with the Sam domain from the EphA2 receptor.[3] These studies have also resulted in the identification of a minimal peptide region of Ship2 that retains binding affinity for the Sam domain of EphA2 receptor.[3] This peptide and the detection of key structural elements important for EphA2 receptor endocytosis provide possible routes for the development of novel small molecule antagonists with potential anti-cancer activity.

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P56

An automated High Throughput (HT) assay for the identification of new protease inhibitors

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Selective expression of proteases and their controlled proteolityc activity is a hallmark of developmental as well as pathological processes in biology. Abnormal proteases activity is one of the critical factors leading to the development of pathological conditions including acute pancreatitis, neurodegeneration, etc (1, 2). Thus, administration of protease inhibitors has been considered of potential interest for the treatment of diseases associated to protease damage. Protease activity can be conveniently measured by using Fluorescence Resonance Energy Transfer (FRET) and substrate peptides that contain an N-terminal fluorescence acceptor group, like 4-(4-dimethyllaminophenylazo)benzoic acid (DABCYL), and a matched C-terminal fluorescence donor group, such as 5-(2-aminoethylamino)-naphthalene-1-sulfonic acid (EDANS). DABCYL acts to quench the fluorescence of the EDANS fluorophore, since the emission band of EDANS (490 nm) displays excellent overlap with the broad visible absorption band of DABCYL. As this is a distance-dependent effect, when a substrate peptide chain holding the two groups together is cleaved by an enzyme, it enables the donor to move away from the quenching group thus inducing an increase in the emitted fluorescence. We have used the DABCYL-EDANS couple for the development of a homogeneous and highly sensitive High Throughput (HT) assay for the screening of protease inhibitors, using trypsin as the model protease. A simple synthetic trypsin substrate is readily cleaved by the enzyme, allowing the fluorescence signal of EDANS to increase in dependence of enzyme activity and reaction time. The automated assay proceeds through single step in low volume white 384-well plates and has been developed using a complete Hamilton platform endowed of a 8-channel liquid handler with volume dispensing control, a robotic arm and a multiwell fluorescence reader. To develop and optimize the assay, we have investigated the parameters that can affect assay performances. Reaction times, enzyme/substrate ratios, and the influence of different DMSO amounts have been changed in order to establish the conditions for achieving optimal signal-to-noise (S/N) ratios and Z′ factor values. To assess the method reliability, we have used the automatic procedure to determine the IC_{50} of a known trypsin inhibitor and next we screened a library of 400 single compounds identifying a novel trypsin inhibitor with IC_{50} in the low micromolar range. In conclusion, using a novel fluorescent peptide substrate, we have developed an automated assay for the screening of trypsin inhibitors. Extension of the method to other protease is rapid and straightforward, involving only the synthesis and characterization of suitable substrates.

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P57

Biochemical characterization of HP0862, a new Pantothenate Kinase isoform from *Helicobacter pylori*

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Helicobacter pylori is a gram negative bacterium that colonises the human stomach. It is present in more than half of the world's population and causes major diseases such as gastritis, mucosa-associated lymphoid tissue, peptic ulcers and stomach cancer[1]. *H.pylori* produces a new Pantothenate Kinase isoform (HP PanK) encoded by the CoaX gene referred as to HP0862 involved in a critical pathway for pathogen survival: that of coenzyme A (CoA) biosynthesis. In particular, pantothenate kinase catalyzes the first step of the universal five-step CoA biosynthetic pathway. HP PanK enzyme, belonging to the type III class, differs in sequence, structure and enzymatic properties from the previously characterized type I, found essentially in bacteria, and type II forms found in eukarya as well as in some bacteria; its activity is not regulated by CoA and thio-esters[2]. In our study we have cloned the HP CoaX gene in pRoEX-HTc and expressed the recombinant protein in *E.coli* BL21(DE3) cells. We have optimized the experimental conditions for purification and stability of the expressed protein. The purified HP0862 was analyzed by gel filtration chromatography and dynamic light scattering to investigate its state of oligomerization. Results show that HP0862 exists as a homodimer, as predicted by its homology with the other PanK III bacterial proteins. The purified protein was characterized by CD spectra analysis and mass spectrometry assay; crystallization trials using a nanodispensator were also carried out in order to define proteins 3D structure. *H.pylori* infections remain a significant global public health problem. Vaccine and antagonist compound development against this infection appears to be a preferable strategy. Actually there is a substantial requirement for new drug targets as alternative strategies for the treatment of *H.pylori* infections, since there is often resistance against traditional antibiotic therapy. Comparison of substrate binding and catalytic sites of PanK-III with those of PanK-II and PanK-I, reveals drastic differences in the binding modes for both ATP and pantothenate substrates, suggesting that these differences may be exploited in the development of new inhibitors specifically targeting PanK-III isoforms[3]. For this reason HP0862 is considered to be a good target for new therapeutic strategies.

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P58

New SOCS1-KIR mimetic peptides through focused symplified combinatorial libraries

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Suppressor Of Cytokine Signalling (SOCS) proteins are negative feedback regulators of several pathways involved in immune response, particularly the JAK/STAT (Janus kinase/Signal Transducer and Activator of Transcription)[1]. Usually their basal levels are low, but they can be selectively induced by cytokines, such as IFNγ. SOCS1 inhibits IFNγ signalling for its capacity to bind and inactivate JAK2 protein and consequently to block the IFNγ-induced tyrosine phosphorylation of IFNγ receptor (IFNγR) and STAT1 activation. It has been demonstrated that keratinocytes avoid the detrimental consequences of an excessive stimulation by IFN-γ over-expressing SOCS1 thus hindering the expression of many pro-inflammatory genes, including those involved in skin diseases, such as psoriasis and allergic contact dermatitis (ACD)[2]. A three-dimensional model of the complex between SOCS-1 and JAK2[3] shows that the Kinase Inhibitory Region (KIR) of SOCS-1 protrudes towards the catalytic region of JAK2 and occupies the ATP binding site. Here we present new peptides mimicking KIR-SOCS-1 binding activity identified through an ELISA-based screening of a focused simplified combinatorial peptide library[4]. On the basis of an Ala-scanning investigation we have firstly restricted KIR domain (52-67) to a shorter region (52-61) improving binding affinity, then several positions within this sequence have been randomized leading to the selection of new and more potent ligands as antagonists of SOCS-1. These new mimetics bind to JAK2 catalytic site (both in phosphorylated and non-phosphorylated form) in a dose dependent manner providing KD values in the high nanomolar range that are 15-fold lower respect to w-t KIR. Cellular experiments on STAT1 activation signaling suggest their potential application as modulators of disorders involving SOCSs overexpression.

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P59

Design and synthesis of new class of phosphonopeptides potent inhibitors of cathepsin C

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Cathepsins form quite large family of lysosomal proteases involved in many physiological functions in human body. Elevated activity of these enzymes in serum or the extracellular matrix often signifies a number of gross pathological conditions. Cathepsin-mediated diseases include: Alzheimer's disease, numerous types of cancer, autoimmune related diseases like arthritis and the accelerated breakdown of bone structure seen with osteoporosis. Cathepsin C (dipeptidyl dipeptidase I; EC 3.4.14.1) belongs to papain family of proteases[1] and sequentially removes dipeptides from the free N-termini of proteins and peptides. It has a broad substrate specificity being able to hydrolyse out nearly every possible dipeptide unit, with exception of those containing basic amino acids (Arg or Lys) at N-terminal position or Pro on either side of the scissile bond. It is also quite unusual in that it requires the presence of halide ions for its activity. The main function of cathepsin C is protein degradation in lysosymes, but it is also found to participate in the activation in cytotoxic T lymphocytes and natural killer cells (granzymes A and B), mast cells (tryptase and chymase), and neutrophils (cathepsin G and elastase) by removing their N-terminal activation dipeptides[2,3]. Loss of function mutations in the cathepsin C gene result in periodontal disease and palmoplantar keratosis[4]. The application of phosphonic analogues of peptides as inhibitors of proteases is based on the concept of the resemblance of the phosphonic moiety to the high-energy tetrahedral transition state of the amide bond hydrolysis. This, however, seems not to apply to cathepsin C, although introduction of phosphonic acid moiety to short peptides afforded from weak to strong inhibitors of this enzyme[5-7].
On poster the results of design, synthesis and activity towards cathepsin C of new group of phosphonodipeptides will be presented.

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P60

Acyl peptide hydrolase as new target protease of the PEBP inhibitor proteins: functional analysis and modeling study

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The PEBP (phosphatidylethanolamine-binding proteins) family consists of over 500 multifunctional proteins, which are involved in signalling mechanisms during cell growth and/or differentiation *via* modulation of kinase or serine protease activities.[1] Recently, a new PEBP protein named SsCEI (*Sulfolobus solfataricus* chymotrypsin-elastase inhibitor) has been purified from the archaeon *S. solfataricus*.[2] In order to further study the biological function of SsCEI, we have identified and characterized its endogenous target enzyme, a serine protease belonging to the acylpeptide hydrolase (APEH) family. APEH is a member of the prolyl oligopeptidase (POP) family of serine proteases which removes acylated amino acid residues from the N terminus of oligopeptides. It has been recently suggested that deficiencies or changes at the genetic and protein levels within members of APEH family are associated with a number of disorders including Alzheimer's disease and cancer.[3]

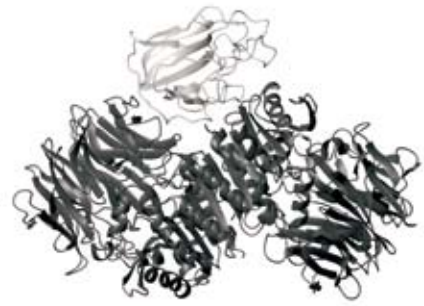

Figure 1. Model complex between $APEH_{Ss}$ dimer (black) and SsCEI (light grey)

To get insights into the enzyme structure, as well as into its interaction with the SsCEI inhibitor, a modelling study has been carried out. The 3D model of $APEH_{Ss}$ shows that it possesses the typical structural features of the POP family including an N-terminal β-propeller and a C-terminal α/β hydrolase domain. The predicted structural model of the $APEH_{Ss}$-SsCEI inhibition complex suggests a mechanism of steric blockage on substrate access to the active site or on product release. Like other POP enzymes, APEH may constitute a new therapeutic target for the treatment of a number of pathologies, so this study may represent a valuable starting point for further medical research.

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P61

Assessment of protein interaction network using synthetic peptides

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Synthetic peptides are a very useful tool for the elucidation of protein-protein interactions[1]. Using this approach, we have previously defined interactions between several proteins, including that between Phosphoprotein Enriched in Diabetes/ Phosphoprotein Enriched in Astrocytes, PED/PEA15) and PLD1[1,2]. PED/PEA15 is a 130 amino acids protein expressed in the cytosol of different cell types. It is involved in several important processes, such as apoptosis and insulin resistance. Among the others, PED/PEA15 interacts with Extracellular signal-Regulated Kinase (ERK) and with Phospholipase D1 (PLD1). Binding of PED/PEA15 to ERK in the cytoplasm prevents the nuclear translocation of the kinase, thus inhibiting ERK-dependent transcription and the consequent cellular proliferation[3]. Instead, PED/PEA15 binding to PLD1 impairs insulin sensitivity in skeletal muscle cells by a PLD1 downstream mechanisms involving activation of PKCs[2].

In this study we have designed new PED/PEA15-derived peptides which should reproduce the binding interface with selected targets. The corresponding synthetic peptides have been used in biochemical assays to study the molecular interfaces that feature the recognition with the binding partners.

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P62

Internally quenched β-amyloid peptides. New probes for the high throughput screening (HTS) of anti-aggregating compounds

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Many neurodegenerative diseases are associated with the β-sheet aggregation of misfolded proteins and their pathological deposition in specific brain areas. Small molecules that can interfere with this aggregation are therefore of interest as potential therapeutic candidates (1). We have developed a simple, one-pot and homogeneous assay amenable to automation by HTS systems, based on the use of an internally quenched fluorescent probe derived from the sequence of the Aβ peptide (residues 10-25) which exhibits a strong tendency to aggregate in absence of inhibitors. The probe contains several cleavage sites for proteolytic enzymes which, in absence of aggregation, can readily and efficiently split the molecule and activate the fluorescent signal thus allowing an accurate quantitation of the released fluorofore. Conversely, when aggregation occurs, cleavage sites are poorly or not accessible at all, thus no fluorescence is released.
The donor-acceptor pair is made by an EDANS group on the side chain of a glutamic acid at the peptide C-terminus (fluorescence donor), while the acceptor is a DABCYL group placed at the N-terminus. The presence of a lysine in the middle of the sequence allows processing by trypsin, a very efficient, cheap and specific enzyme, and shedding of the DABCYL quencher. Conditions for the automated execution of screening of β-sheet aggregation inhibitors have been optimized. We have investigated probe and enzyme concentrations (10 μM and 0.05 μM respectively) and time of reaction (30 min). Finally we have investigated the influence of DMSO concentration on S/N as most compounds are stored as stock solutions in neat DMSO at high concentrations (≥ 5 mM). The assay proceeds through simple steps, on 384-well plates, with reduced volume (20 μL total volume), for miniaturization and high throughput analysis on a Hamilton screening station. To rule out the possibility that inhibitors block the enzyme activity, a parallel screening with a non fibrillogenic, internally quenched peptide has been performed.

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Synthesis and antimicrobiological evaluation temporin a analogues substituted with L-alanine

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Temporins are antimicrobial peptides (AMPs) isolated from the skin of the red european frog *Rana temporaria*[1]. They are active particularly against Gram-positive bacteria, Candida species and fungi. They have the ability to bind and permeate both artificial and biological membranes.We have recently investigated two members of this AMPs family, temporin-1Tl (Tl) and temporin-1Ta (Ta).[2,3] At the same time, we developed new analogues of these peptides, among which Pro3TL (FVPWFSKFLGRILNH$_2$) exhibiting a higher antimicrobial activity and a lower hemolytic activity than the native peptide Tl. The strong activity of Ta against gram-positive cocci and Candida, as well as its small molecular weight 13 aa residues), make this peptide TA an interesting antimicrobial compound. To elucidate the molecular basis of the interaction of the native Ta with bacterial membrane and to develop new potent analogues with improved activity, but without hemolytic activity, we performed a structure-activity study replacing *L*-Ala in each position of this peptide. Here we report the preliminarly results of this study.

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P64

Tubomicella of 1,4-dihydropyridine lipid and its binding with DNA

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In our previous work[1] using molecular dynamics (MD) we showed that the gene transfection agent, 1,1'-{[3,5-bis(dodecyloxycarbonyl)-4-phenyl-1,4-dihydropyridin-2,6-diyl]dimethylene} bispyridinium dibromide (1,4-DHP lipid) formed a tubular micellae, if started from a lipid bilayer, and the existence of worm-like structures was confirmed by electron microscopy. In the present work using MD is shown that the 1,4-DHP lipid tubular micellae is stable in a bigger amount of water, as well as the systems of four or five tubular micellaes are stable. The rod of 15-mer DNA was put aside of the five tubular micellaes system in a periodic water box, and by MD, Amber 9.0 force field, was simulated how the 1,4-DHP lipid tubular micellaes 'swallow' the DNA rod. The 1,4-DHP rings tend to take tangential position towards the tubular micellae's surface.

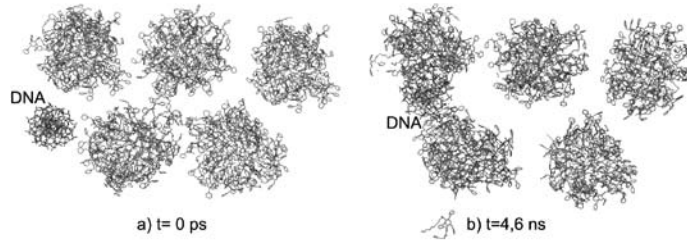

Figure 1. Five tubular micellaes of 1,4-DHP-lipid and DNA a) at the start of MD, b) after 4,6 ns of MD simulation

Acknowledgements

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P65

A Cul3-derived peptide binding different members of the KCTD family

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Cul3 ubiquitin ligase has been shown to require proteins containing BTB domain to target substrates. REN^{KCTD11}, a human BTB protein defined as a medulloblastoma suppressor, has revealed to bind Cul3 by immunoprecipitation analyses[1]. By homology modelling, a REN^{KCTD11} POZ/BTB-Cul3 complex with a 4:4 stoichiometry was generated. A peptide empassing the 49-68 Cul3 sequence was identified as responsible of interaction with the POZ/BTB domain of REN^{KCTD11}. The peptide was synthesised by Fmoc strategy and characterized by circular dichroism. Different peptides were designed, synthesised and characterised to define either the precise role of some residues or to increase the solubility. The binding affinities to the POZ/BTB of REN^{KCTD11} of the peptides were evaluated by ELISA assays. Other members belonging to KCTD family were analysed to verify the binding to the Cul3 derived peptides. The results obtained help to validate the involvement of these KCTD family members in the ubiquitination pathway. In addition they also demonstrate that the Cul3 peptide could be useful as a suitable scaffold to characterize at the molecular level the interaction between Cul3 and KCTD members as well as to develop molecules of therapeutic interest to be used as agonists or antagonists according to the peculiar role played by the KCTD family members investigated.

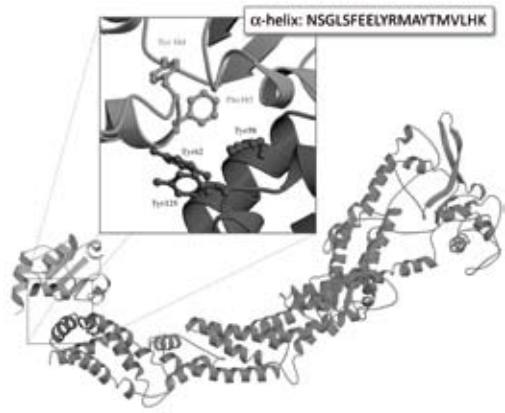

Figure 1: View of the interaction of a single subunit of the POZ/BTB domain (in grey) with Cul3 (in black) modelled on the SKP1-Cul1 structure used as a template (PDB code 1LDK).

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P66

^{111}In and ^{68}Ga labelled GluDTPA and DOTA-gastrin analogs: a biological and functional analysis.

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The development of suitable radioligands for targeting CCK-2 receptor expressing tumours, such as medullary thyroid carcinoma, is of clinical interest [1]. In the search for the best CCK-2R binding peptides, we have synthesized and evaluated two gastrin analogs. DGlu(1)-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2 (DGlu(1)-minigastrin) and DGlu-Glu(5)-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2 (DGlu-Glu(5)-minigastrin) have been synthesized on solid phase utilizing the Fmoc strategy. The N-terminal portion of both conjugates was derivatized by introducing the DTPAGlu or DOTA chelators to allow radiolobeling with ^{111}In(III) and ^{68}Ga(III) respectively. Saturation binding experiments were performed on A431-CCK2R overexpressing cells. All compounds showed K_d values in the nM range. Biodistribution experiments showed higher specific uptake of ^{111}In-GluDTPA-DGlu-Glu(5)-minigastrin on CCK2-R overexpressing xenografts compared to ^{111}In-GluDTPA-DGlu(1)-minigastrin and to previously described ^{111}In-GluDTPA-CCK8. The higher retention levels were associated with markedly elevated and undesired kidney uptake for ^{111}In-GluDTPA-DGlu-Glu(5)-minigastrin compared to the other compounds. Similarly, ^{68}Ga-DOTA-DGlu(1)-minigastrin showed slightly lower specific uptake in receptor positive xenografts (see table) but much lower kidney retention compared to ^{68}Ga-DOTA-DGlu(5)-minigastrin. Current indications suggest that the 5 Glu N-terminal residues while improving receptor targeting cause unacceptably high kidney retention. Future work will focus on improving the receptor targeting of the shorter DGlu(1)-minigastrin or CCK8 peptide sequences.

Biodistribution at 1h post-injection	**^{111}InGluDTPA-CCK8**	**^{111}In-GluDTPA-DGlu-Glu(5)-minigastrin**	**^{111}In-GluDTPA-DGlu-Glu(1)-minigastrin**	**^{68}GaDOTA-CCK8**	**^{68}Ga-DOTA-DGlu(5)-minigastrin**	**^{68}Ga-DOTA-DGlu(1)-minigastrin**
Kidneys %ID/g	4.1	48.1	2.6	1.2	36.6	2.4
Receptor positive xenograft %ID/g	1.6	2.9	1.2	1.5	3.1	2.2
Control Xenograft %ID/g	0.4	1.3	0.7	0.3	0.5	0.4
Positive xenograft/kidney ratio	**0.4**	**0.1**	**0.4**	**1.3**	**0.1**	**0.9**
Positive to control xenograft ratio	**3.7**	**2.2**	**1.7**	**4.6**	**6.7**	**5.2**
Binding Affinity (Kd,nM)	**23**	**25**	**30**	**12**	**22**	**28**

Table 1. Comparison of biodistribution and binding properties of the CCK2R binding radiolabeled peptides

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P67

Crystal structure of the ribosome inactivating protein PD-L3 from *Phytolacca dioica*

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Many plants accumulate proteins that are commonly referred to as ribosome-inactivating proteins (RIPs). These are N-glycosidases which depurinate a universally conserved sequence of the ribosome, named as alpha-sarcin loop. Removal of the specific adenine hinders the elongation factor dependent binding of aminoacyl-tRNA and, therefore, arrests protein synthesis at the elongation step. RIPs are also able to depurinate nonribosomal nucleic acid substrates, thus they have been renamed as adenine polynucleotide glycosylases (APG), on analogy with the EC nomenclature on nucleic acid glycosylases.
We have previously determined the crystal structure of various RIP forms which exist in leaves of *Phytolacca dioica*[1-5]. The crystal structure of the glycosylated PD-L3, which has been recently determined, will be discussed in relation to the importance of glycosylation for the activity of this class of RIPs.

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P68

Molecular dynamics of amylin amyloid single β-sheets

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The formation of amylin aggregates is strongly associated with β-cell degeneration in type II diabetes. Here we analyze the conformation of a 20-residue domain of amylin (Gln-Arg-Leu-Ala-Asn-Phe-Leu-Val-His-Ser-Ser-Asn-Asn-Phe-Gly-Ala-Ile-Leu-Ser-Ser, Amyl 10-29) as well as a backward and a scrambled version of this peptide to decipher the role of amino acid sequence and composition in amyloid formation. Six stranded parallel beta sheets of amylin 10-29, reverse amylin 10-29 and scrambled amylin were constructed, surrounded by chloride counterions to provide zero charge of the system, and by 5 Å layer of explicit water molecules and submitted to MD simulations in a periodic water box. The system temperature was raised stepwise from 10 K till 309 K during 45 ns of MD run. Afterwards an MD run was performed for 140 ns at 309 K temperature. Preliminary results for Amyl 10-29 show the stable ß- sheet across the ß-sheet regions Arg11-Val17 which time to time expand till Ser 19, and two strands having stable ß-sheet over the region Asp22-Leu27. In the region Ser19-Ser20-Asn21-Asn22 ß- sheet has W-shaped bend with the deeper vertex on Ser20 and smaller vertex on Asn22, suggesting that also the bent ß- sheet could be possible.

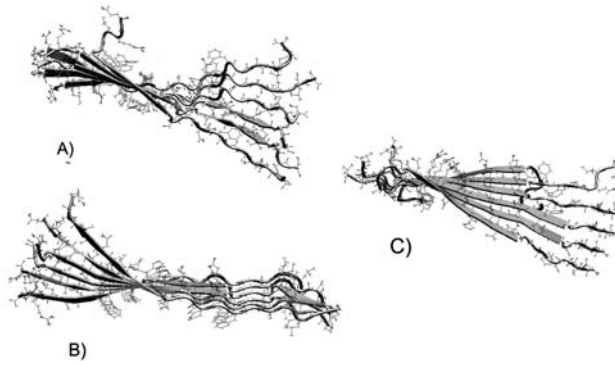

Figure 1. β-sheets of a) Amylin 10-29 b) reverse amylin 10-29, c) scrambled amylin 10-29, after ~60 ns of MD simulation.

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P69

Membrane-induced conformations of the neuropeptide substance P

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Ligand binding is an essential step for the functioning of the protein family G-protein coupled receptors (GPCRs). Substance P (SP) is the most potent ligand of Neurokinin 1 (NK1) GPCR receptor and is believed to be implicated in the generation of several neurodegerative disorders and diseases. Though it biological and pharmaceutical importance, the factors controlling the binding and the formation of active peptide conformation are not fully understood yet. To address these questions we studied the conformation of SP in membrane mimetic environments: micelles and liposomes, by using CD spectroscopy. Complementarily, we carried out fluorescence spectroscopy experiments to assess the insertion and the binding of the peptide to the membrane.

Our data show that the interaction SP with the membrane is rather complex, involving hydrophobic and electrostatic interactions and depends on membrane surface charge density. In an effort to understand the impact of each of these components in the stages of membrane binding and for the promotion of stable peptide conformations we performed experiments in solvents and in two membranes-mimic environments, varying the head charge and an acyl chain length of the lipid and the concentration of the detergent. The binding experiments demonstrate that the Trp side chain of SPW (SP with a Trp in place of Phe^8) is inserted into hydrophobic core when SPW is incorporated into negatively charged micelles and liposomes, but faces hydrophilic aqueous region in the zwitterionic DMPC liposomes. The strong contribution of the electrostatic interactions for the SP binding was further strengthened by DMPG/DMPC experiments.

The analysis of CD data revealed that the SP forms a partially α helical structure in the presence of the negatively charged micelles and vesicles. On the contrary, CD spectral features of SP in aqueous solutions, submicellar concentrations of SDS and in DMPC liposomes showed the presence of extended polyproline (PPII) formation, as confirmed by the CD temperature - induced spectra.

These findings imply that membrane matrix and the head group lipid charge are crucial for α helical conformation of the peptide. From the other side, the formation of PPII in the absence of membrane suggests that this formation most likely is dictated by sequence specific properties of the SP.

P70

A detailed picture of stereoelectronic effects associated with peptide group distortions in peptides and proteins

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The structure adopted by a protein is the result of a complex and subtle balance of a number of different stabilization interactions, both intrinsic and environmental.
By combining quantum-mechanical analysis and statistical surveys of protein/peptide structure database we here report a thorough investigation[1] of the conformational dependence of the geometry of peptide bond, the basic element of protein structures. Different peptide model systems have been studied by an integrated quantum mechanical approach, employing DFT, MP2 and CCSD(T) calculations, both in aqueous solution and in the gas phase. The backbone ψ dihedral angle turns out to play a major role in modulating the peptide bond geometry, including the deviation from planarity and other geometrical parameters, such as bond lengths, whose dependence on ψ has never been hitherto fully disclosed. These indications are fully corroborated by statistical surveys of very accurate protein and peptide structure databases.[1-5] Orbital analysis provides a reliable interpretation in terms of local stereo-electronic effects of the observed correlation. In particular we show orbital interactions between the σ system of C^{α} substituents and the π system of the amide bond are crucial for modulation peptide bond distortions. In contrast with the scheme of the classical resonance model, present data suggest that the deviations from planarity are not necessarily associated with a destabilization of the amide bond and, thus, with specific trends of the CO and CN bond distances. Although proteins assume extremely complicated structures with a hierarchical juxtaposition of basic elements, the variability of peptide bond geometry is thus remarkably reproduced by extremely simplified systems. This indicates that local factors are the main driving force of these distortions. The implications of the present findings for protein structure determination, validation and prediction are also discussed.

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P71

The role of cholesterol and calcium in substance P-model membrane interactions

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Substance P (SP), the natural ligand of Neurokinin 1 (NK1) GPCR receptor is a short positively charged neuropeptide. In the past years, SP and NK1 receptor interactions have been extensively studied. However, the role of cholesterol and calcium in this mechanism is still elusive. Cholesterol is amongst the compositions of the lipids found in the postsynaptic membrane, where NK1 receptor and SP ligand are expressed and functioning (O'Brien & Sampson, 1965). On the other hand, it has been reported that the calcium-bound conformation of tachykinins is required for the receptor activation (Ananthanarayanan & Orlicky, 1992) and the biological action of tachykinins depends on extracellular calcium concentration (O'Riordan *et al*., 2001). In the present work, we used circular dichroism (CD) spectroscopy to study the secondary structure of the peptide in the presence and absence of cholesterol and calcium in membrane mimicking environments. Fluorescence spectroscopy was explored to monitor the binding of the peptide to membranes. In order to measure intrinsic Trp fluorescence, [Trp^8]SP was used as an analogue of SP. The fluorescence binding experiments show that the binding of the [Trp^8]SP to DMPG vesicles decreases as the molar ratio of cholesterol increases, as it was revealed by the red-shift of Trp emission at around 342 nm, compared to that at 339 nm in pure DMPG vesicles. A decreases of the helical content of SP was found in the presence of 1:1 mol:mol cholesterol/DMPG ratio, as was judged by MRE at 222 nm of CD spectra . The difference CD spectral analysis revealed a presence of polyproline II structure at the expense of the helical conformation of the peptide with an addition of calcium.

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P72

Comparative drugability evaluation of MOR opioid peptides in functional brain research

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Eight MOR (μ-opioid receptor) peptides, six agonists and two antagonists, are comparatively evaluated in the mouse model for brain influx (multiple time regression and capillary depletion), brain efflux and metabolic stability in brain tissue as well as in plasma[1].

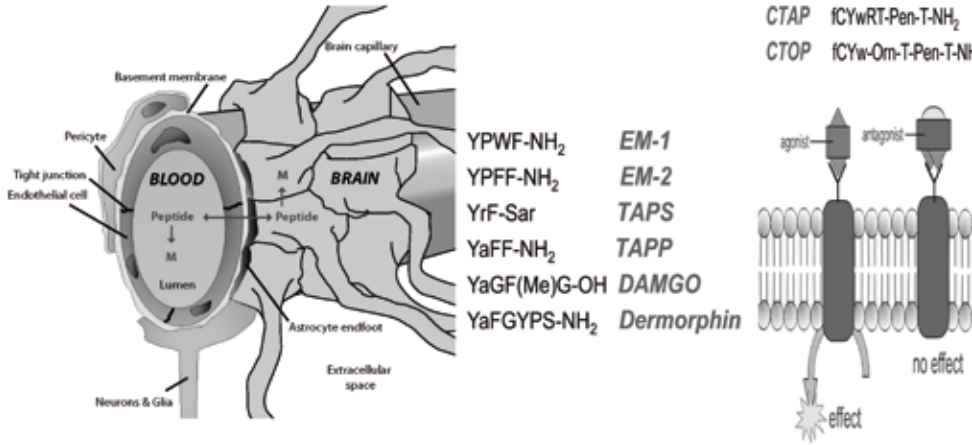

Figure 1. Blood Brain Barrier (left) and investigated MOR peptides (right)

Using these four individual responses d_i, a global desirability function D was created as follows:

$$D = \sqrt[n]{\prod_{i=1}^{n} {d_i}^{p_i}}$$

where p_i is a weighing factor attributed to the individual responses. Dermorphin yielded the highest D-value, indicating this peptide possessed the highest drugability characteristics compared to the other investigated peptides. In-vivo medical imaging confirmed the above D-based conclusions, and allowed a refinement of the weighing factors p_i.

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P73

Small peptidic APEH inhibitors selected by screening combinatorial libraries made of D-aminoacids

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The acylpeptide hydrolases (APEH), also referred to as acylaminoacyl peptidases or acylaminoacyl peptide hydrolases, represent one of the four members of the prolyl oligopeptidase (POP, clan SC, family S9) family[1]. All POP enzymes, differently from the classical serine protease families (trypsin and subtilisin) are able to hydrolyze only relatively short peptide substrates. Specifically, APEH catalyzes the removal of an N-acyl amino acid from blocked peptides, producing an acyl-amino acid and a peptide with a free N terminus shortened by one amino acid residue [2]. In human, APEH is expressed in a variety of cells and tissues, including erythrocytes, liver and brain. Although the physiological role of this enzyme in humans has not yet been clarified completely, its overexpression is associated with cardiovascular diseases, cancer, inflammation, haematological disease, neurological and urological disease [3]. Like other POP family enzymes, APEH is thus recognised as a new pharmacological target for the discovery of drugs for several diseases. In the attempt to obtain inhibitors or inhibitor precursors of APEH, we have set up a photometric screening assay that makes use of the recombinant enzyme and its synthetic specific substrate N-acetyl-L-alanine p-nitroanilide (AANA) and we have screened a combinatorial peptide library. The assay has been set up in 384-wells, low volume plates and using a multiwell reader able to perform kinetic measurements. The assay has been optimized in terms of enzyme-substrate ratio, volumes, time and temperature and has been used to screen a simplified synthetic peptide library [4] made of 4 residues in a Positional Scanning (PS) format, therefore contained 12x4 = 48 sub-libraries. The library was built with only 12 amino acids (12^4 = 20736 compounds) in the D configuration in order to avoid structural redundancies [4] and to select protease resistant inhibitors. The screening provided clear consensus sequences for both the inhibition and activation of APEH and the corresponding synthetic tetrapeptides were prepared and tested in the same assay confirming the compound activity. Although IC50s were in the high micromolar range, the tetrapeptide structure has provided a good starting scaffold for the design of novel, more potent inhibitors and activators for modulating the APEH enzymatic activity.

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P74

Activation of monocytic cells by immunostimulatory lipids conjugated to peptide antigens

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A major problem associated with the use of subunit vaccines including peptide epitope-based vaccine is the weak immunogenicity that they display in the absence of adjuvant. Although many experimental adjuvants are available, only squalene oil water emulsion and aluminium based salt adjuvants are currently licensed for use in humans. An effective way to overcome this predicament is to incorporate lipid groups that have self-adjuvating properties into the peptide. The lipidic polylisine core peptide (LCP) technology represents a potentially safe option for vaccine delivery in humans and gives the possibility to incorporate multiple copies of different peptide antigens. We have synthesised a LCP system that combines multiple antigen peptides derived from essential glycoproteins of herpes simplex virus type 1 with the 2-amino-dodecanoic lipoamino acids C_{12} (H_2N-$CH(CH_2)_9CH_3$-COOH).

The technology has been proven effective in a small number of experimental models. In fact, LCP-based vaccine candidates incorporating domains derived from *Chlamydia trachomatis,* group A streptococci, foot-and-mouth disease virus and papillomavirus were immunogenic and resulted in the induction of peptide antibodies in the absence of any additional adjuvant.

The focus of the present study is to elucidate the signalling mechanisms of LCP-induced activation of macrophages. We have tested the capacity of the synthesised LCP to activate the mitogen-activated protein kinase signalling cascades and the underlying cytokine production from macrophages.

P75

Structural determinats of *M. tuberculosis* agglutination guided by heparin binding hemagglutinin A

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Heparin-binding hemagglutinin (HBHA) is a virulence factor of tuberculosis which is responsible for extrapulmonary dissemination of this disease. We previously reported a thorough biochemical study of the protein in which we showed that HBHA is characterised by an elongated dimeric structure and provided experimental evidence of the presence of coiled coil regions in HBHA[1]. These data also suggested, consistent with previous findings, that bacterial agglutination is to be attributed to interactions between coiled coil motifs of HBHA molecules on the bacterial surfaces[1]. Indeed, coiled-coil motifs represent a natural mechanism for guiding and cementing protein–protein interactions. In this scenario, we undertook a study of HBHA oligomerisation process. Using various experimental techniques we derived a mechanism of bacterial cell-cell interaction via trans-dimerisation of HBHA[2], a process which involves the formation of trans-cellular HBHA dimers[3]. Furthermore, by combining computational approaches, site directed mutagenesis and peptide synthesis, we identified the region of HBHA which is responsible for dimerisation and plays a key role for HBHA structural integrity. Results will be presented in the poster.

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P76

Structural bases of *M. tuberculosis* daughter cell separation

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Mechanisms which regulate bacterial exit from dormancy are the same which are responsible for cellular division. Indeed, proteins which have been identified as responsible for resuscitation, like the Resuscitation Promoting Factor Interacting Protein (RipA) play a key role in bacterial division, as they and are likely responsible for septal peptidoglycan degradation[1,2,3]. RipA localizes at bacterial septa and has remarkable effect on bacterial phenotype. Indeed, the deletion of the gene encoding for RipA induces decreasing growth and an abnormal *M. tuberculosis* phenotype, consisting in branching and chaining bacteria[1]. This makes RipA an excellent candidate as a drug target against Tuberculosis.
RipA was cloned, expressed and purified in native conditions. Crystals were obtained using vapour diffusion techniques. The structure of RipA was solved by Multiwavelength Anomalous Dispersion using the anomalous signal from the Se atoms of selenomethionine-labeled enzymes. For functional characterization of the enzyme, lyophilized cells of *M. lysodeikticus* labeled with FITC were used as substrate.
The crystal structure of RipA allowed us to definitely characterize the enzyme function and its regulation mechanism. Cell wall degradation experiments revealed that this enzyme is active as cell wall hydrolase, by cleaving cell-wall peptide crosslinks. In parallel, mass spectrometry studies of the processed cell wall material provided clues of the peptidase specificity. Altogether, these results provide the basis for the rational design of low molecular weight enzyme inhibitors.

Acknowledgements

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P77

Interplay of peptide bond geometrical parameters in non-globular proteins

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Several investigations performed in the last two decades have unveiled that, in protein and peptide structures, peptide bond geometrical parameters are highly correlated. Initial studies have shown that peptide valence bonds strongly depend on the local conformation. [1] Similar dependencies are also shown by peptide bond distortions and bond distances. [2-6] Although these studies have provided interesting insights into one of the fundamental aspects of protein structure, these analyses have been conducted either on globular protein or on small peptide models. We here report a detailed investigation of the correlation of peptide bond geometrical parameters in non-globular proteins and in amyloid-fiber forming peptides. Our analysis clearly indicates that the valence bond dependency on the conformation and the correlation between the dihedral angles ψ and ω are also detected in the structures of membrane proteins. Moreover, such trends also hold in the structure of fibrous proteins such as collagen triple helix. Interestingly, the interplay of these parameters is also observed in the tightly packed steric zipper structure formed by amyloidogenic peptides. Collectively, these analyses demonstrate that the trends reported for globular proteins are of general validity, even in cases in which the peptide bond is located in a completely different environment. The implications of these findings are discussed.

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Computational approaches for unveiling the molecular basis of amyloid-related diseases

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The definition of the molecular basis of human diseases is one of the most important goals of structural biology. Computational methodologies, which include popular techniques such as molecular modeling and dynamics, statistical surveys of databases, and molecular design, represent valuable tools for coping with these challenging projects. We have recently applied computational techniques to obtain insight into the molecular mechanisms of diverse pathologies of the nervous system. In particular, we have designed new compounds which are effective as neurotrophin agonists in chronic pain models. [1,2] We have also undertaken molecular dynamics (MD) simulations on several amyloid-like peptides arranged in a cross-β spine structure, which are linked to the occurrence of severe and widespread neurodegenerative diseases. The analysis of these models has provided information on the structural properties of amyloid fibers [3-6] and of their small soluble precursors, that are the actual toxic species.[7-8] On these basis, we have related the toxicity of these oligomers with some specific structural features such as the exposure of sticky β-strands. We here report a detailed MD characterization of novel peptide model systems that are able to form distinct structure assemblies. The stability of these distinct assemblies corroborate the idea that they may be representative of different disease *strains*.

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New cosmeceutical agents from bioactive compounds by peptide conjugation

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Tyrosinase is a multifunctional copper-containing oxidase, and is considered as the key enzyme orchestrating mammalian melanogenesis. This enzyme catalyzes two distinct reactions in the biosynthesis pathway of melanin; the hydrozylation of monophenol (tyrosine) and the conversion of an *o*-diphenol (dopa) to the corresponding *o*-quinone (dopaquinone). A multitude of different tyrosinase inhibitors have been developed so far to treat epidermal hyperpigmentation conditions, but without rational design. To investigate the most effective tyrosinase inhibitor structure, we synthesized series of kojic acid-peptide or amino acid conjugates and studied their structure related tyrosinase inhibitory activity. During this study, we have discovered that aromatic ring structure next to kojic acid plays an important role to increase tyrosinase inhibitory activity, and confirmed this by a docking study of inhibitors at the tyrosinase active site. Furthermore, we modified the structure of kojic acid-amino acid amide and optimized the structure of tyrosinase inhibitor, which afforded the highest tyrosinase inhibitory activity. Based on this study, we further applied this concept to hydroxyphenolic acid-amino acid conjugates to develop a potent tyrosinase inhibitor. The present studies suggest new strategies to develop dermal treatment agents.

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NMR structural characterization of two mutants (C27D and H42A) of the protein ROS87 from *Agrobacterium tumefaciens*

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Ros, a 15.5 kDa protein encoded by the chromosomal gene *ros*[1] in *Agrobacterium Tumefaciens,* is the first classical zinc-finger containing protein identified in the prokaryotic kingdom. We have structurally characterized Ros DNA-binding domain (Ros_{56-142}) and shown that the prokaryotic Cys_2His_2 zinc-finger domain possesses a novel protein fold. The NMR structure of Ros87 consists of a globular domain (from Pro9 to Tyr66) of 58 amino acids, in which the zinc ion is tetrahedrally coordinated by Cys-24 and Cys-27 and by His-37 and His-42, and two disordered tails at the N- and C-terminal regions[2,3]. Ros87 globular fold is arranged in a βββαα topology and it is stabilized by an extensive hydrophobic core of 15 amino acids[3]. These new features define a novel fold never found in literature[3,4].

Here, we report the study of two point mutants of Ros87: C27D (in which the second coordinating cysteine is mutated to aspartic acid) and H42A (in which the second coordinating histidine is mutated to alanine). They are still able to ccordinate the zinc ion and to bind the same DNA sequence of Ros*WT*[4]. NMR experiments demonstrated that in H42A the zinc ion is tetrahedrally coordinated by Cys-24, Cys-27, His-37 and His-41 mutant while in C27D mutant there is a novel zinc coordination zinc ion sphere composed by Cys-24, Asp-27, His-37 and His-42[2,4]. The structural characterization through NMR spectroscopy of C27D and H42A mutants will allow to elucidate the zinc coordination properties of this new protein fold.

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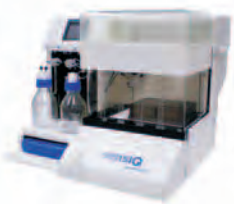
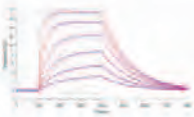
Biomolecular Interaction Analysis





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