

ABSTRACTS

ORAL

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Advanced glycan analysis using HPLC-Chip/MS technology

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Glycosylation is the primary cause of micro-heterogeneity in proteins (glycoisoforms). These reflect complexity at both molecular and cellular levels. There are many potential functions of glycosylation. For instance, physical properties include: folding, trafficking, packing, stabilization, protease protection, quaternary structure and organization of water structure. Properties relating to recognition and biological triggering are characterized by: weak interactions, multiple presentation and precise geometry. Changes in sugar prints may both reflect and results in physiological changes, e.g. cancer and other diseases. Hence it is necessary to understand the degree of glycosylation. Furthermore, free glycans in human milk are known to stimulate the innate immune system of infants.

In this presentation an update about the latest technical developments of the Chip-MS system for glycan analysis will be provided. Applications of the Chip-MS system to the study of all classes of glycans (free glycans, N-glycans, O-glycans and glycosaminoglycans) will be discussed. Furthermore a method will be discussed that allows the determination of N-glycan, O-glycans and their glycosylation site in a single experiment including the analysis of stereo-glycopeptide isomers.

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Polymer based intraocular lens adsorbome: a bottom up proteomics study

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The cataract is one of the leading causes of blindness in the world. The surgery is the only effective treatment to overcome the cataract and consists in removing the cloudy lens and replacing it by an artificial intraocular lens (IOL). Secondary cataract, or Posterior Capsular Opacification (PCO), is the most common postoperative complication of the cataract surgery. PCO is raised from the host response to the implant: the residual lens epithelial cells (LEC) proliferate, migrate, transdifferentiate to mesenchymal cells which form a cloudy layer and enclose the intraocular lens, causing patients to lose vision again. Following implantation of an IOL, the human host responses include protein adsorption, cell adhesion, inflammation, and wound healing. These foreign body reactions are all initiated from the first step of protein adsorption from aqueous humor or blood instantaneously after implantation of the IOL in the capsular bag. Numerous factors such as the chemical composition of the material, the design, the roughness, the bioadhesive character of the IOLs play a role in the appearance of these complications. In the present work, an optimized sample preparation protocol to identify and quantify the "adsorbomes" of hydrophilic and hydrophobic materials for IOLs known to

have a higher or a lower incidence of PCO, respectively, was obtained. Polymer disks incubated in a physiological environment simulating aqueous humor (diluted human serum) were used. Quantitative analysis of common proteins in all samples was made after trypsin digestion on disks and peptide analyses by mass spectrometry (UPLC-SYNAPT G2, MS^E-IMS acquisition). Albumin, immunoglobulins G, apolipoproteins and vitronectin are a first common protein signature of hydrophilic and hydrophobic acrylic materials. After quantification, it will be possible to correlate the relative intensities within protein signature and the surface properties, as well as low or high cell adhesion percentages of (LEC) on polymers.

1. Bozukova, D., C. Pagnouille, R. Jérôme and C. Jérôme (2010). "Polymers in modern ophthalmic implants—Historical background and recent advances." *Materials Science and Engineering: R: Reports* 69(6): 63-83
2. Christophe Pagnouille, Dimitriya Bozukova, Laure Gobin, Virginie Bertrand, Marie-Claire Gillet-De Pauw. Assessment of new-generation glistening-free hydrophobic acrylic intraocular lens material *J Cataract Refract Surg* 2012 1271-1277.

3

A Proteomic View of the Suppression of Host Cell Antiviral Responses by Respiratory Syncytial Virus

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Respiratory syncytial virus (RSV) is the most serious cause of lower respiratory tract infections in infants, young children and immunocompromised individuals. It is increasingly being recognised as a serious respiratory pathogen in the elderly. A hallmark of RSV is the lack of an adequate long lasting immune response to the virus, resulting in multiple infections throughout life. There are no vaccines or efficacious therapeutics for RSV. RSV encodes two small proteins which suppress antiviral responses of the host cell, including interferon induction and signalling. These proteins are termed non-structural proteins (NS1 and NS2) because they appear not to be packaged with budding virions. One of these proteins, NS1, appears to be the major culprit in terms of host cell response suppression, which it achieves through post-translational mechanisms. For instance it is proposed that NS1 acts as an E3 ligase. NS1 would appear to be a potential target for development of therapeutics for RSV. Accordingly, we have adopted a proteomic approach in conjunction with reverse viral genetics for identification of molecular targets for NS1 and to define its mechanism of action. Of interest is that NS1 appears to sustain the survival of proteins within the infected cell in order to suppress apoptosis on one hand whilst suppressing interferon responses on the other hand. These findings suggest that in part NS1 does not function to subject its targets to degradation. Processes used to achieve this novel marriage of reverse viral genetics and high-performance proteomic technologies will be presented in addition to the data supporting the observations cited above. In particular, a method for identification of protein regulation at a specific proteoform level will be presented.

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Progress, Problems, and Prospects

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Over the last 15 years, proteomics has evolved into a major field of biomedical/biological research, but one that is still well short of achieving its vast potential. Arising as a true paradigm shift from

protein chemistry, it is rooted in the determination of the human genome sequence (and a rapidly growing number of additional genome sequences) and three principal technological advances – 2D gels, protein and nucleic acid arrays and MALDI/ESI mass spectrometry. It has, as its generalized objective, the complete description of individual proteomes - derived from whole organisms to biologically defined subsets - and requires the identification/description of the structure/function of all proteins in the set including, where present, splice variants and post-translational modifications. It also requires the determination of the both the stable and transient protein-protein interactions that make up the highly complex cellular networks of even the simplest organisms. Achieving these objectives have been generally driven by large scale, 'shotgun' experiments. However, more recently the correlation with other 'omic data (genomic, transcriptomic and metabolomic), important for systems biology experimentation, has emerged as a major objective and have emphasized the necessity of quantitative data. This has led to the emergence of SRM/MRM/PRM-based methodology and the measurement of individual entities/modifications under normal and pathological conditions as an increasingly popular approach. While there have been notable advances in both types of efforts, providing some useful new insights, progress has been slow, particularly in translational applications, relative to the scope of what remains to be done. An overview of the accomplishments (both positive and negative) of the past decade and half, and some predictions about what the next decade and a half will bring, will be presented.

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Venomics Proteomics

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Malaysia is home for numerous venomous animals which are either terrestrial or marine. However, many of these venoms are not well studied and the active components of the toxins and venoms are remain biochemically and pharmacologically unknown. In recent years, advancement of proteomics technology has enabled rapid and massive discoveries in the composition of the venom of numerous animals. The technology could also help in understanding the venom and antivenom interaction and reactivity which could help the development of better antivenoms production. Novel bioactive discoveries made from utilization of proteomics technology not only enable the discoveries of various types of new novel toxin classes and isoforms of previously described toxins but also potentially new ones. In our laboratory, we are concentrating on the purification and characterization of new bioactive proteins and their isoforms for potential application not only in biomedical research but also in potential pharmaceutical and agricultural applications. There are five species of snakes of interest that currently being studied in our laboratory namely *Bungarus candidus*, *Bungarus fasciatus*, *Naja naja kouthia*, *Ophiophagus hannah* and *Callosellasma rhodostoma*. In order to selectively purify bioactive proteins of interest, we are using mass-spectrometry guided purification strategy to selectively purify bioactive proteins of interest. The purified bioactive proteins were then being characterized by using pharmacological and biochemical techniques to determine the activities such as neurotoxicity, cardiotoxicity, cytotoxicity and antibacterial properties. The bioactive proteins were analysed by using Q-TOF LCMS/MS for identification and de novo sequencing. By using this approach, we were successful in isolating several novel toxins which are currently undergoing characterizations. In conclusion, application of proteomics and mass spectrometry technologies provide a very rapid approach to purify known and identify unknown bioactive proteins from venoms. Application of these technologies could also help in understanding the composition and of venom and antivenom reactivity.

Pros and cons of inter-alpha trypsin inhibitor h4 fragment as cancer biomarker

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The potential use of a 35 kDa inter-alpha trypsin inhibitor H4 fragment (ITIH4f) as a cancer biomarker was unveiled when it was detected in sera of patients with breast cancer using gel-based proteomic analysis and a lectin that binds O-glycosylated glycoproteins. Subsequent studies showed that the serum ITIH4f was also of relatively higher abundance in patients with endometrial and ovarian cancer (germ-line and epithelial ovarian carcinoma) but not in patients with nasopharyngeal carcinoma, osteosarcoma (localized disease) and cervical cancer (squamous cell cervical carcinoma and cervical adenocarcinoma). One of the differences between the types of cancer that are associated with increased abundance of serum ITIH4f with those that did not is that the former is associated with enhanced levels of serum oestrogens. This suggests that release of ITIH4f is oestrogen-induced, and the hypothesis was further substantiated when sera of healthy pregnant women and patients with hydatidiform mole, whose serum oestrogen levels were elevated, were shown to contain relatively higher levels of serum ITIH4f than controls. In this seminar, the pros and cons of ITIH4f as a cancer biomarker will be discussed.

Quantitative Chemical Proteomics for the Identification of Specific Drug Targets – Application to the Study of Anticancer Mechanism of Andrographolide

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As most drugs exert pharmacological effects by interacting with their target proteins, identification of which is a critical step in unravelling the mechanisms of drug action. It is also imperative for our understanding of the pharmacodynamics of a known drug, suggesting the potentially unrevealed actions and thus refining its future clinical applications. However, current *in vitro* affinity chromatography-based and *in vivo* activity-based protein profiling (ABPP) approaches generally face difficulties discriminating specific drug targets from non-specific ones. We have come up with a novel approach combining isobaric tags for relative and absolute quantitation (iTRAQTM) with Clickable ABPP, named ICABPP, to specifically and comprehensively identify drug targets in live cells. This approach was applied to identify the protein targets of andrographolide (Andro), a natural product with known anti-inflammation and anti-cancer effects, in live cancer cells. A spectrum of specific targets of Andro was identified, revealing the mechanism of action of the drug and its potential novel application as the tumor metastasis inhibitor, which was validated through cell migration and invasion assays. Moreover, the target binding mechanism of Andro was unveiled with a combination of drug analogue synthesis, protein engineering and mass spectrometry-based approaches and the drug-binding sites of two protein targets, NF- κ B and actin, were determined.

Proteomics and Metabolomics – Thoughts Combine for Biomarker Discovery

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In the post-genome area, Proteomics has become a major tool for qualitative and quantitative analysis of complex biological systems and to understand the background of cellular functions and malfunctions. While mass spectrometric methods have reached the required level of sensitivity to identify proteins in biological systems, the vast complexity of protein modifications, isoforms,

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truncations or splice variants cannot be covered by today approaches. Recent publications have shown that each gene results in many different active forms of the protein are present in a cellular system. Protein-biomarker discovery is mainly based on the search for up- or down-regulated proteins, either by quantitative mass spectrometry comparing samples from healthy and diseased individuals or on extracting potential candidates from orthogonal data sets, as from Transcriptomics. This approach only takes increased or decreased levels of proteins into account, not looking at possible different post-translational modifications that affect the function of the protein and lead to diseases. Also gene-related modifications, as mutations, truncations, splice-variant, RNA-edited amino acid exchanges are not considered. Taking all the possible modifications into account, each protein covers a wide space in a three dimensional room with the dimensions: concentration, gene-related modifications and post-translational modifications. In this room some of the combinations will not affect the protein function in the cellular system, while others lead to malfunction and diseases. These would serve as an biomarker for diagnostics and to stratifying personalized medical treatment. The full analysis of each protein covering all of these dimensions is still not possible today.

A reverse Omics' approach may help to overcome these limitations. Most of the biological pathways are either directly or indirectly connected to metabolic processes, to the synthesis or conversion of small molecules. Even these are representing a vast complexity of different molecules, each of them represents a unique structure accessible by mass spectrometric techniques in combination with targeted sample preparation and extraction procedures. These metabolomic data enable the conclusion back to the disturbed pathway and a targeted analysis of the proteins and their mutations and modifications involved this pathway. Beside the potential use of the identified Metabolites as biomarkers, proteins of the disturbed pathway can be taken into account as biomarker candidates and submitted to verification using targeted mass spectrometry. A general approach for the combination of Proteomics and Metabolomics to detect potential biomarkers will be discussed.

Kinetics of antigen expression and epitope presentation during virus infection revealed by next generation proteomics

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Current knowledge about the dynamics of antigen presentation to T cells during viral infection is very poor despite being of fundamental importance to our understanding of anti-viral immunity. We have used advanced mass spectrometry to simultaneously quantify the presentation of vaccinia virus peptide-MHC complexes (epitopes) on infected cells and the amounts of their source antigens at multiple times after infection. The results show a startling 10,000-fold range in abundance as well as strikingly different kinetics across the epitopes monitored. The tight correlation between onset of protein expression and epitope display for most antigens provides the strongest support to date that antigen presentation is largely linked to translation and not later degradation of antigens. Finally, using data independent acquisition approaches (SWATH-MS) we have monitored all viral and host cell proteins providing a global view of viral antigen expression and host cell responses. This study highlights the complexity of viral antigen presentation and demonstrates the weakness of simple models that assume total protein levels are directly linked to epitope presentation and immunogenicity.

1. Croft, N. P., Smith, S. A., Wong, Y. C., Tan, C. T., Dudek, N. L., Flesch, I. E., Lin, L. C., Tschärke, D. C., and Purcell, A. W. (2013) Kinetics of Antigen Expression and Epitope Presentation during Virus Infection. *PLoS Pathog* 9, e1003129

Genome-wide proteomics: What have we learnt from the C-HPP initiative?

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We report progress assembling the parts list for chromosome 17 and illustrate the various processes that we have developed to integrate available data from diverse genomic and proteomic knowledge bases. As primary resources we have used GPMDB, neXtProt, PeptideAtlas, Human Protein Atlas (HPA), and GeneCards. All sites share the common resource of Ensembl for the genome modeling information. We have defined the chromosome 17 parts list with the following information: 1169 protein-coding genes, the numbers of proteins confidently identified by various experimental approaches as documented in GPMDB, neXtProt, PeptideAtlas, and HPA, examples of typical data sets obtained by RNASeq and proteomic studies of epithelial derived tumor cell lines (disease proteome) and a normal proteome (peripheral mononuclear cells), reported evidence of post-translational modifications, and examples of alternative splice variants (ASVs). We have constructed a list of the 59 'missing' proteins as well as 201 proteins that have inconclusive mass spectrometric (MS) identifications. In this report we have defined a process to establish a baseline for the incorporation of new evidence on protein identification and characterization as well as related information from transcriptome analyses. This initial list of 'missing' proteins that will guide the selection of appropriate samples for discovery studies as well as antibody reagents. Also we have illustrated the significant diversity of protein variants (including post-translational modifications, PTMs) using regions on chromosome 17 that contain important oncogenes. We emphasize the need for mandated deposition of proteomics data in public databases, the further development of improved PTM, ASV and single nucleotide variant (SNV) databases and the construction of websites that can integrate and regularly update such information. In addition, we describe the distribution of both clustered and scattered sets of protein families on the chromosome. Since chromosome 17 is rich in cancer associated genes we have focused the clustering of cancer associated genes in such genomic regions and have used the ERBB2 amplicon as an example of the value of a proteogenomic approach in which one integrates transcriptomic with proteomic information and captures evidence of co-expression through coordinated regulation.

Epitope binning assays on monoclonal antibodies using label-free biosensor

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In the discovery of therapeutic antibodies, finding an appropriate antibody/epitope pair is challenging because both the epitope and the antibody can have undesirable properties that frustrate the intended therapeutic application. Since an antibody's epitope and functional activity are correlated, it is helpful to organize a panel of antibodies into epitope families or "bins". This talk will describe how epitope binning assays can be performed on label-free biosensors.

Captive, Doped and Boosted: Enhancing capabilities for glycopeptide characterization with ETD/CID through electrospray supercharging

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Although the analysis of glycosylation patterns in glycoproteins has been well established, parallel analysis to localize glycosylation sites as well as determining the peptide amino acid sequences are also paramount in reducing analysis time and improving data mining. The generally lower ionization efficiency of glycopeptides compared with non-glycosylated peptides during mass analysis coupled with the high micro-heterogeneity in glycan structures however, have made the analysis of *N*- and *O*-glycopeptides largely a challenging subject. More recently, the combination of collision-induced-dissociation (CID) and electron-transfer-dissociation (ETD) techniques has been shown to be highly complementary and provides a more comprehensive characterization of glycopeptides. However, confident use of ETD has often been hampered by the lower charge states and the significantly increased molecular weights of glycopeptides. Herein, the application of a new spray source set-up utilizing a solvent-doped sheath gas has the capability to not only augment charge states, but improve overall signal intensities during mass spectrometry analysis is presented. We apply this pilot investigation on a model glycoprotein and report on the overall performance increase. We also observed that supercharging appears most pronounced on glycopeptides with larger glycans and/or higher degrees of sialylation. Based on this methodology, we demonstrate its successful application on the analysis of both *N*- and *O*-linked glycopeptides.

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Improved accuracy and sensitivity of large-scale labelled quantitation via multi-notch MS3 approach on hybrid quadrupole-Orbitrap-linear ion trap mass spectrometer

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The utility of multiplexed quantitation using isobaric tags (ITRAQ or TMT) has been well documented in the literature. Today such protein quantitation approach has become a first choice method in many proteomics laboratories due to its proven protocol and productivity to facilitate comparative study of up to 10 experiments. On a high resolution mass spectrometer, the quantitative data are obtained from the cyclic targeted MSMS process (data-dependent acquisition) which releases the associated reporter tags for the detected peptides. In most complex sample situation, isolations of target peptides during DDA have been challenged with co-isolation of other peptides within a 2-Da or 3-Da selection window. This invariably leads to unresolvable error in quantitation. A novel approach based on the new hybrid Orbitrap platform (Orbitrap Fusion) offers significant improvement in accuracy and sensitivity in complex labelled quantitation scenarios. This method involves a triple-stage HRMS measurements (MS3) using ion-trap based CID followed by HCD to generate the corresponding reporter tag ions.

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Old Proteins and Human Age-Related Disease. Understanding Processes using Spontaneous Peptide Degradation

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It is not widely known that there are a number of proteins in the body that are long-lived and some that exist for our lifetime. The list includes spectrin, elastin, collagen, crystallins and components of the nuclear pore complex. Over time, these old proteins become modified, however our knowledge of the molecular details, and which are of most importance, is poorly understood.

We have been characterizing some of these modifications using the human lens as a model system and characterising the reactions using synthetic peptides. Major modifications result from the intrinsic instability of some amino acid residues: serine, asparagine and aspartic acid. Quantitatively the most important reactions are racemisation, deamidation and spontaneous peptide bond cleavage. Unstructured regions of proteins are particularly susceptible to these posttranslational modifications. In the lens, the extent of these reactions increases over time and it is probable that they lead to protein denaturation. The ultimate result is that the lens becomes opaque and that age-related

cataract develops. It is likely that the gradual deterioration of long-lived proteins plays a role in other human age-related diseases.

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Diagnosing IBD? A quantitative proteomic view and marker validation study

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Disease-specific proteins, including low-mass peptides, can be difficult to detect amongst a diverse range of concentrations present for plasma proteins. Combined with the immense extent of human and disease variation and the challenges facing the development of sensitive and specific differentiators; developing diagnostics suitable for clinical use is a formidable task. Discovery phase quantitative approaches entail the differentiation of as many peptides as possible (rather than the identification of all proteins) from LC-MS experiments. The development of targeted, quantitative approaches that provide accurate and statistically reliable quantitative outcomes for multi-site studies may provide a critical bridge to establishing validity of individual or panels of biomarkers. Inflammatory bowel disease (IBD) is a life-long relapsing and remitting inflammatory disorder primarily affecting the gastrointestinal tract and is mainly subdivided into Crohn's disease (CD) and ulcerative colitis (UC).¹ Current treatment regimen aims to dampen the vigorous inflammation without a cure. The importance of an accurate diagnosis is paramount as the prognosis for CD and UC differ markedly, although a definitive diagnosis is difficult to achieve as clinical manifestations are very similar. Protein markers may strengthen the discrimination of IBD subtypes and provide prognostic information. This study has incorporated the techniques of low mass partitioning, LC-MS/MS and label-free quantitation for a discovery based survey of unique peptides and proteins able to distinguish between our phenotypes. Selected candidates have then been further evaluated in fresh cohorts comprising of 109 patients using multiple light and heavy synthetic peptides to determine absolute quantities within those phenotypes. Antibody detection using Westerns has also been carried out. Discussed will be the identification of unique differentiators for application to diagnosis of IBD's.

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Conotoxin evolution gets messy

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Cone snail venom comprises many 1000s of venom peptides that have evolved for prey capture and defence from a smaller set of transcriptomic sequences. Deep venomics has revealed surprising messiness in the venom at the proteomic level that arises from variable peptide processing by endoproteases. This "messy" processing generates ragged N- and C-terminal processing underlying much of the peptide diversity found at low levels using high sensitivity LC/MS techniques, in addition to a smaller number of predicted major cleavage products. Recently, we have uncovered another layer of venom peptide messiness seen at the transcriptomic level in cone snails. A surprisingly large number of conopeptide gene sequences were found to be expressed at low-levels, including a series of single amino acid variants, as well as sequences containing deletions and frame and stop codon shifts. Some of the toxin variants generated alternative cleavage sites, interrupted or elongated cysteine frameworks, and highly variable isoforms within families that could be identified at the peptide level. Together with variable peptide processing, this background biological messiness explains the hypervariability of venom peptides. Variable processing and transcriptomic messiness likely contributes to the rapidly evolution of venoms with new or altered function and might be exploited in non-venomous species and prove useful in expanding structure-function studies.

Peptide Tissue Imaging Mass Spectrometry in Ovarian Cancer

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In 1997, Caprioli et al detailed the novel application of MALDI-TOF MS directly on tissue for mapping of protein and peptide species [1]. Imaging Mass Spectrometry (IMS), is typically used to determine the distribution of proteins in fresh frozen tissue. In this respect, however, Tryptic Peptide Imaging has some advantages over imaging of intact proteins. These include access to higher molecular weight and hydrophobic proteins. Furthermore, peptide level analysis provides the possibility for identification by matching accurate m/z and in situ MS/MS to high quality LC-MS/MS data obtained through digestion of relevant laser dissected tissue. Finally, formalin-fixed paraffin embedded (FFPE) tissue can be analysed after antigen retrieval. FFPE archives have the additional advantage of providing large sample numbers complete with patient and disease specific annotations. Here we present the latest developments within our group, including up-to-date methods for analysis of formalin-fixed tissue (e.g. tryptic peptide MALDI-IMS), a method for linking LC-MS/MS data to MALDI-IMS data using internal calibrants as well as the generation of the first data for a MALDI-IMS patient and disease specific tryptic peptide database. This database will serve as a reference for tissue micro-array MALDI-IMS experiments, with the ultimate aim of extracting clinical information such as therapy response and patient outcome. The potential for tryptic peptide imaging on FFPE tissue as a diagnostic tool for ovarian cancer will be discussed using FFPE tumour tissue-micro-arrays from patients responding versus patients not responding to chemotherapy.

[1] Caprioli RM, Farmer TB, Gile J. Molecular imaging of biological samples: localization of peptides and proteins using MALDI-TOF MS. *Anal Chem.* 1997 Dec 1;69(23):4751-60

[2] J.O.R. Gustafsson, J.S. Eddes, S. Meding, T. Koudelka, M.K. Oehler, S.R. McColl, P. Hoffmann. Internal calibrants for high accuracy matching of tryptic peptide MALDI imaging features to peptides identified by LC-MS/MS. *Journal of Proteomics*, 2012 Aug 30; 75(16):5093-105

Targeted Quantitative Proteomics – an Evolving Tool Kit

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The goal of quantitative proteomics is to both identify and quantify a broad range of proteins and peptides. Targeted quantification continues to grow in importance as an increasing number of 'potential' protein biomarkers need to be verified / validated to confirm or refute their ultimate utility. This need for better quantification strategies drives technology development, pushing for increased throughput with increasingly lower detection levels. Increased throughput is required as larger sample numbers must be analyzed for verification / validation, which often means accelerated chromatography. This can increase the chance of interferences that could confound robust quantification which drives the need for higher selectivity strategies. Getting quantitative coverage of both high and low abundant precursors is a combination of both sensitivity and selectivity improvements as well as dynamic range increases. Recent MS innovations have also enabled a resurgence in data independent acquisition strategies which are proving to play an important role in targeted proteomics for early stage verification work. Technology progress in a variety of these key LCMS acquisition attributes will be explored.

Total Chemical Synthesis of Mirror Image Proteins

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All natural protein molecules are made up of L-amino acids and the achiral amino acid glycine.¹ The homochirality of natural proteins molecules is one of the most fundamental aspects of the biosphere. I will present case studies to illustrate the preparation by total chemical synthesis of unnatural 'mirror image' D-protein molecules made up of D-amino acids.² Novel features of these examples include facilitated determination of protein X-ray structures by racemic crystallography,³ and the systematic development of a D-protein antagonist for VEGF-A.⁴

1. 'Introduction to Stereochemistry' by Kurt Mislow, Dover Publications, New York, 2002
2. Total chemical synthesis of proteins. Stephen B.H. Kent, Chemical Society Reviews, 38, 338-51 (2009)
3. Racemic protein crystallography. Todd O. Yeates, Stephen B.H. Kent, Ann. Review Biophysics, 41, 41-61 (2012)
4. Chemical synthesis and X-ray structure of a heterochiral {D-protein antagonist plus VEGF-A} protein complex by racemic crystallography. Kalyaneswar Mandal, Maruti Uppalapati, Dana Ault-Riché, John Kenney, Joshua Lowitz, Sachdev Sidhu, Stephen B.H. Kent, Proc Natl Acad Sci USA, 109, 14779-14784 (2012)

Immune Therapies Targeted to HLA-Peptide Complexes

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West Nile Virus (WNV) was introduced into North America in 1999 where it has emerged as the most common cause of arboviral neuroinvasive disease. An estimated 2 million infections occurred between 1999 and 2013 in North America and there is no specific treatment or vaccine available. WNV-infected cells can be detected and destroyed by CD8⁺ cytotoxic T lymphocytes via the presentation of viral peptides by major histocompatibility complex class I molecules. Briefly, class I MHC molecules provide CD8⁺ cytotoxic T lymphocytes of the immune system with a "window" into the cell by sampling peptides from all protein constituents within a cell and then displaying these peptides at the plasmalemma. Currently, no one knows how many WNV derived peptides decorate the MHC of infected cells, nor do we know which viral proteins MHC molecules sample. Understanding the number and origin of viral peptides available for immune recognition is a key prerequisite in the development of immunotherapeutics that target intracellular disorders. Using secreted HLA class I molecules in a comparative proteomics system, we eluted peptide ligands from infected and uninfected class I molecules and compared these ligands by mass spectrometry. The resulting data demonstrate that different class I MHC molecules consistently prune a Flavivirus of

more than 3,000 amino acids down to a handful of peptides for immune recognition. We then extended this experimental protocol to HIV-1 infected cells, influenza infected cells, breast cancer cells, and ovarian cancer cells. In all instances we find a dozen or fewer MHC class I presented peptide ligands distinguish the diseased from the healthy cell. Finally, peptide based vaccines as well as antibodies specific for peptide/MHC complexes demonstrate the therapeutic potential of these peptide/MHC complexes during infection and cancerous transformation.

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How anti-Alzheimer's antibodies interact with the Abeta peptide

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Alzheimer's disease (AD) is the most prevalent neurodegenerative disease in humans with age being the biggest risk factor. The mechanisms by which the disease progresses to cognitive decline in the sufferer are complex and not fully elucidated. A defining pathological feature is the deposition of extracellular plaques composed primarily of misfolded amyloid beta (A β) peptide: a proteolytic breakdown product of the much larger Amyloid Precursor Protein. While A β peptides are the main constituents of amyloid plaques that burden the diseased brain, plaque burden correlates poorly with the severity of the disease. There is accumulating evidence that a prefibrillar or protofibrillar soluble form of A β can compromise neuronal functions and trigger cell death. Immunotherapy targeting Abeta is a promising direction in AD research with active and passive immunotherapies shown to lower cerebral A β levels and rescue cognitive function in animal models. Anti-A β immunotherapies are a significant class of AD therapeutics currently in human clinical trials.

We have been examining the molecular basis of antibody engagement of A β epitopes to inform the analysis of clinical trial data and to guide the engineering of anti-A β antibodies with optimised specificity and affinity. We have determined the structures of three different AD antibodies in complex with Ab peptides: (1) WO2, which recognises the N-terminus of A β , (2) Mab 2286, which like the AD immunotherapeutic Ponezumab (Pfizer), shows specificity for the C-terminus of A β 40 but has no significant cross-reactivity with A β 42/43, and (3) Bapineuzumab, a humanized antibody developed by Pfizer and Johnson & Johnson which recognises the N-terminus of A β but cannot recognize N-terminally modified or truncated A β peptides. All these studies reveal surprising aspects of A β peptide recognition by the antibodies and suggest new avenues for AD antibody development.

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A Semisynthetic Platform for the Site-Specific Conjugation of Lipid Adjuvants to Recombinant Protein Vaccines

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Immunization has proven to be one of the most effective means to prevent disease. Despite this success, effective vaccines are lacking for many important diseases (e.g. HIV & malaria), and the use of vaccines to treat disease (e.g. cancers & autoimmune disease) remains an exciting prospect that awaits its full exploitation. Developing new vaccines in many cases will require a movement from traditional whole organism approaches, towards chemically defined systems (e.g. subunit and DNA vaccines), and their administration with powerful immunostimulatory compounds (adjuvants). With relatively few safe adjuvants for human use, novel adjuvant systems are essential for producing new vaccines.

We have developed subunit vaccines incorporating defined peptide antigens attached to lipid-based adjuvants. The immune response against these vaccines was significantly enhanced compared to the administration of peptide antigens with the commercial adjuvant alum, and allows for stimulation of both antibody and cellular immunity, as well as mucosal "needle free" vaccine administration. The use of peptide antigens however imposes limits on the amount of antigenic information that can be

incorporated into a vaccine. We have therefore developed methods that enable the site-specific conjugation of lipid adjuvants onto expressed denatured or folded protein antigens. With recent advances in antigen identification providing numerous protective antigens suitable for subunit vaccine development, such an approach provides a more efficient means to develop lipid adjuvanted vaccines. These techniques have facilitated the simple and efficient production of homogeneous, chemically defined lipoprotein vaccines targeting group A streptococcus, incorporating combinations of conserved and protective peptide and protein antigens. These vaccines formed particles in the low nanometer size range (~ 40 nm), ideal for eliciting humoral immune responses. Following administration to mice, antigen-specific IgG antibodies were elicited against each incorporated antigen, without need for added adjuvant, or signs of toxicity.

Peptide-Polymer Conjugates as Therapeutic Vaccine against Cervical Cancer

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Cancer is the No. 1 killer of Australians. Cervical cancer remains third only to breast and ovarian cancer in terms of female cancer mortality worldwide. Only small increment in survival using chemotherapy has been witnessed. Immunotherapy has been proposed as one of the most attractive methods to reduce cancer mortality in human.¹ Among the various immunotherapeutic delivery systems, peptide-based vaccines are simple, stable, well tolerated and can be tailored to produce the desired immunogenic effects.² However such vaccines need delivery system and immunostimulant (adjuvant) to trigger desired immune responses.

We designed new vaccine delivery system-based on the polyacrylate polymer conjugated to peptide epitope. We applied this system to Group A Streptococcus (GAS) vaccine and have demonstrated that peptide subunit vaccine formulated into nanoparticle was able to induce desired humoral immune response in mice after single immunization when administered subcutaneously or intranasally without help of any external adjuvant.³⁻⁵

Recently, we applied this delivery strategy to create therapeutic peptide-based subunit anticancer vaccine. The synthesis of peptide epitopes was greatly improved by the change of standard SPSS procedure and application of the isopeptide method under microwave irradiation condition.⁶ The most promising antigens were identified, conjugated to polymer, and self-assembled into the particles. Modification of immunogenic epitope allowed the elimination of undesirable disulfide bond-based aggregation/polymerization of the peptide-polymers conjugates. Polyacrylate conjugated to the selected epitope produced excellent therapeutic effect against established tumour without help of any external adjuvant. This delivery system overcomes the lack of immunogenicity of peptide-based vaccines and common toxic side effect of external adjuvants. Noticeably, in contrast to many previously reported vaccine candidates, the conjugates demonstrated therapeutic effect after single immunization. Thus, we developed the first self-adjuvanting delivery system for the therapeutic vaccine against cervical cancer.

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MUC1 Glycopeptide Vaccine

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Due to its over-expression on almost all types of epithelial tumor tissues, the tumor-associated mucin MUC 1 is an attractive target antigen for a cancer immunotherapy. However, there also some problems to be solved. The first problem is tumor specific antigen. MUC1 is a member of transmembrane glycoprotein family, mucin. In normal tissues, MUC1 expresses on the free face of epithelial cells and complex glycosylated. But in tumor tissue, MUC1 protein over-expresses on all the faces of epithelial cells and specifically glycosylated, such as Tn, T, Sialyl-Tn and Sialyl-T due to alteration of enzyme activity. So 20-mer extracellular repeat glycopeptide sequence of MUC1 is an excellent target of vaccine development. The next problem is vaccine systems. Short peptides are always poor immunogenic, so vaccine systems are necessary to increase the immunogenicity of MUC1 glycopeptides. We synthesized MUC1 glycopeptides by solid-phase peptide synthesis with glycosylated threonines and serines as building blocks. And several systems were developed to load antigens. The first system is conjugates of glycopeptides and bovine serum albumin (BSA). This carrier protein elicited high level of antibodies¹. To simplify the structure, we conjugated T cell epitope or Toll-like receptor ligand to MUC1 glycopeptides, this kind of vaccines also elicited immune response²³. To simulate multivalent structure, we designed self-assembling vaccines, which aggregated in neutral condition and elicited immune system⁴.

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Poly-L-lysine Dendrimers as Vectors for Targeted Drug Delivery

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Dendrimers are dendritic or branched polymers that can be synthesised to provide macromolecular constructs with hydrodynamic radii in the low nanometer size range and with good control over size and surface properties. As such dendrimers show increasing promise as drug delivery vectors, and have particular application in cancer therapy. In common with most potential nanomedicines, the in vivo distribution properties of dendrimers are highly dependent on molecular weight and surface character. Derivatisation of the dendrimer surface with polyethylene glycol (PEG), for example, can maintain concentrations of dendrimer based drug delivery systems in the plasma for several days and

in doing so promote passive uptake into sites with increased vascular permeability. PEGylation also promotes absorption from subcutaneous injection sites and appears to preferentially redirect a proportion of the administered dose into the lymphatics and lymph nodes. These delivery properties are highly consistent with the use of dendrimers as drug delivery vehicles and when coupled with the use of tumour-specific drug release mechanisms have the potential to significantly enhance therapeutic benefit. Here, the impact of modification of the dendrimer surface with a PEG on dendrimer absorption, clearance and tumour targeting is described and the implications of these findings for delivery system design discussed. Examples of PEGylated and drug conjugated dendrimers as enhanced anti-cancer nanomedicines will be subsequently described and in particular recent data suggesting utility in the targeted treatment of lymph-resident and lung-resident cancer metastases.

Cell penetrating peptides for skin delivery: enhanced skin permeation and efficacy of diclofenac

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The aim of the study was to assess the skin permeation and distribution of a novel cell penetrating peptide (CPP), diketopiperazine (DKP) and HIV-1 Trans-Activator of Transcription (TAT). The effect of DKP and TAT on the skin permeation, distribution and efficacy of diclofenac was assessed.

CPPs were applied as physical admixtures with diclofenac and conjugated to diclofenac. DKP alone, TAT alone and chemical conjugates of DKP-diclofenac (1:1 molar ratio) and TAT-conjugates were synthesized. Ex vivo permeation across human epidermis was assessed (300 μ L at 500 μ g/mL DKP or TAT alone, physical admixture of DKP and diclofenac, and DKP-diclofenac or TAT-diclofenac conjugates). The content of DKP, TAT, diclofenac, and conjugates in the receptor and epidermal membrane samples was determined using HPLC. COX-2 inhibition by DKP-diclofenac conjugate and diclofenac alone was assessed using a COX fluorescent inhibitor assay concentration range up to 0.3 mM).

The cumulative amount of DKP, TAT and diclofenac permeating human epidermis over 24h was 10.46, 3.43, and 4.06 μ g/cm² respectively. The physical admixture with DKP enhanced permeation of diclofenac (7.61 μ g/cm²; >2-fold increase). Chemical conjugation with DKP or TAT enhanced the permeation of diclofenac (7.82 and 7.92 μ g/cm² respectively). The amount of DKP, TAT, diclofenac, DKP-diclofenac and TAT-diclofenac recovered from the epidermal membrane at 24h was 10.43, 13.2, 8.58, 138.07 and 20.25 μ g respectively. There was no significant difference in COX-2 inhibition was seen between diclofenac alone and DKP-conjugate.

CPPs enhanced the permeation of diclofenac into and through human epidermis. Diclofenac permeation through the epidermis was enhanced to a similar extent by conjugation to DKP and TAT (\approx 2-fold). However DKP significantly increased the deposition of diclofenac within the epidermis (\approx 16-fold). This novel skin permeation enhancement approach may be useful for targeting delivery within the skin.

Xentry, a new class of cell-penetrating peptide for delivery of drugs

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Cell-penetrating peptides (CPPs) offer new opportunities for the delivery of drugs as they overcome the impermeability of the plasma membrane, and can be readily modified to confer tissue-specific targeting. We have discovered an entirely new class of cell penetrating peptide represented by a 7-amino acid peptide designated "Xentry" derived from an N-terminal region of the X-protein of the hepatitis B virus. Xentry permeates adherent cells using syndecans as a portal for entry, but is uniquely restricted from entering certain non-adherent cells, such as resting blood cells. Intravenous

injection of Xentry alone or conjugated to beta-galactosidase led to its delivery to most tissues in mice, except circulating blood cells. Thus, it may have a therapeutic advantage as it is not taken up and diluted by blood cells. It has a predilection for uptake by epithelia, becoming concentrated in the epithelia of major organs, including the epithelial lining of the lungs and intestine. Xentry is able to deliver a variety of different biologically active cargo types to cells including peptides, proteins, antibodies, and siRNA. Anti-B-raf antibodies and siRNA linked to Xentry were capable of killing B-raf-dependent melanoma cells. Xentry represents a new class of CPP with properties that are potentially advantageous for life science and therapeutic applications.

Future of Peptides as Blood-Brain Barrier Shuttles

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Over recent years we have worked extensively on the use of peptides as BBB-shuttles to carry drugs that cannot cross the blood-brain barrier (BBB) and therefore cannot reach the brain unaided. The capacity of a drug to cross the BBB is crucial, as several major diseases require brain treatment. These include neurodegenerative disorders such as Parkinson's and Alzheimer's, but also central nervous system (CNS) diseases, such as schizophrenia, epilepsy and bipolar disorder. Cerebral cancer, HIV, and some aspects of obesity can also be included as pharmaceutical targets inside the brain.¹

Initially, we focused our efforts on passive diffusion as a transport mechanism.^{2,3,4} In these studies, we achieved molecules with 2-4 amino acids that act as passive BBB-shuttles and are efficient at carrying drugs such as L-Dopa, Baicalin, GABA, Nipecotic and Aminolevulinic acids. In some cases, the shuttle plays a dual role and once inside the brain acts as an enzyme inhibitor.⁵ In spite of their potential use for small molecules, passive diffusion shuttles have limitations for transporting macromolecules (proteins, mAbs, nanoparticles). This prompted us more recently to focus on the use of peptides recognized by receptors as actively transported BBB-shuttles^{6,7} and study their ability to carry antibodies through this barrier.

In our quest for novel BBB shuttle candidates, we have been interested in studying animal venoms that have been reported to affect the CNS without disrupting the BBB. Designing non toxic analogs of these peptides that have the BBB transport capacity but are non toxic is one of our recent sources of BBB-shuttles that we are developing.

In this communication, we will review our latest results of peptides as passive BBB-shuttles and present our unpublished results on peptide-shuttles that use active transport to cross the blood-brain barrier.

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Bioportides: Bioactive Cell Penetrating Peptides

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Many polycationic cell penetrating peptides (CPPs) have been effectively utilised as intracellular delivery vectors. Common CPP sequences such as *Tat*, *Penetratin* and *Transportan-10* are highly

efficient vectors that can access intracellular compartments with minimal cellular toxicity when employed at concentrations achievable *in vivo*. Thus, CPPs can be engineered to enhance the intracellular delivery of a range of bioactive cargoes which vary in size from small drugs to whole proteins.

It is likely that intrinsic cell penetrating sequences, alternatively described as transduction domains, could enable structurally diverse proteins to translocate biological membranes - a mechanistic process analogous to the intercellular shuttling of transcription factors. Furthermore, the sites of protein-protein interactions (PPIs), often containing polycationic micro-domains, represent a viable therapeutic modality. PPIs are considered intractable to small drugs but can be effectively targeted with more extended peptides including helical sequences. Thus, there is tremendous scope to develop bioactive CPPs or biportides that can selectively modulate the activities of intracellular proteins. One common approach is to utilise a synthetically-organised chimeric peptide consisting of a distinct CPP vector extended with a known bioactive sequence. Other biportides present multiple pharmacophores for cellular penetration and biological activities that are discontinuously organised within the primary structure. The term rhegnylogic is a useful descriptor to delineate this latter class of biportide. Thus, recent years have witnessed a tremendous increase in the description of biportides that can modulate cellular dynamics. Methodologies to target CPPs and biportides to therapeutic sites include the application of receptor ligands and other homing sequences.

This presentation will provide a succinct overview of developments that support the contention that biportides exhibit tremendous potential for the development of research tools and potential therapeutics.

Molecular Obesity, potency and other addictions and challenges drug discovery, with a special focus on PPI and peptide related issues

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This presentation reflects on some of the learnings of recent years in relation to the causes of attrition in drug discovery and looks at the particular challenge of Protein-Protein Interactions and Peptides as sources of inspiration for and translation into drugs.

Despite the increase in global biology and chemistry knowledge, the discovery of effective and safe new drugs seems to become harder rather than easier. Some of this challenge is due to increasing demands for safety and novelty, but some of the risk involved in this and the consequential attrition should be controllable if we had more effectively learnt from our failures. The term Molecular Obesity has been introduced to describe the tendency to build potency into molecules by the inappropriate use of lipophilicity which leads to the premature demise of drug candidates. The challenge of PPI and peptide derived drugs is further compounded by the need for cellular access of many targets of interest which again is in conflict with the size of molecules typically active in this domain of drug discovery. The emergence of and role of indices to help in drug discovery will be discussed as will ways of understanding the chemical tractability of targets. The challenge of understanding the concentration of drug molecules at the site of action will also be discussed as this is another critical issue for understanding dose related and hence ADMET issues.

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Activation of g-protein-coupled receptors (gpcrs) by peptide ligands

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A fundamental issue in molecular pharmacology today is defining, at the molecular level, how G-protein-coupled receptors (GPCRs) are activated. Despite being activated by a wide variety of stimuli from photons to large glycoproteins, these receptors exhibit a conserved protein architecture comprising a bundle of seven transmembrane (TM) helices linked by extracellular loops (ECLs) and intracellular loops. Defining differences in the mode of binding exhibited by agonists and antagonists within this receptor structure will aid rational drug design and will provide insight into understanding their agonist-induced activation processes.

Our studies have addressed GPCRs for peptide ligands (peptide-GPCRs) and have focussed on the receptors for the neurohypophysial peptide hormones vasopressin and oxytocin in particular. Using systematic mutagenesis we have identified motifs and individual residues that are critical for high affinity agonist binding and receptor activation but not antagonist binding. Using a combination of peptide chemistry, site directed mutagenesis and molecular modelling, we have identified key agonist-specific contacts established between the receptor and ligand.

New insights into the molecular mechanisms underlying the formation and function of caveolae

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Caveolae are abundant cell-surface pits that have been implicated in lipid regulation, signal transduction, and endocytosis. Caveolins, the major membrane proteins of caveolae, play a crucial role in the formation of caveolae. Mutations in caveolins are associated with breast cancer and with a number of muscle diseases, including limb girdle muscular dystrophy. We have studied how caveolin-lipid interactions generate the unique architecture of the caveolar domain by studying caveola formation in a model prokaryotic system. Vesicle formation is induced by expression of wild-type caveolins, but not caveolin mutants defective in caveola formation in mammalian systems. In addition, cryo-electron tomography shows that the induced membrane domains are equivalent in size and caveolin density to native caveolae and reveals a possible polyhedral arrangement of caveolin oligomers. The caveolin-induced vesicles form by budding in from the cytoplasmic membrane, generating a membrane domain with distinct lipid composition. We propose a model in which caveolin oligomers expand the cytoplasmic leaflet and generate membrane curvature. Our recent studies have identified a new family of coat proteins, termed cavins, that regulate caveola formation in vertebrate cells. Study of the cavins and their dynamics of caveola association/dissociation provides new insights into the role of caveolae in mechanosensation.

Stem Cells: Changing Cell Biology and Evolving New Medicine

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CIRM is the \$US 3 billion stem cell initiative that was founded by Proposition 71 in 2004 by the State of California. These funds have been used to establish the institutional and intellectual infrastructure that has resulted more than 1300 publications (27% in high impact factor journals), 12 new stem cell institutes and a wave of new investigators from within and from outside California, moving into stem cell science and translational medicine. CIRM has established 23 national and international collaborative funding agreements now involving 27 projects and >\$75M from the collaborating partners.

The basic research program has led to transformational discoveries such as the establishment of “disease in the dish” models of human disease; the direct transdifferentiation of adult cells in vitro and in vivo; repurposing tumor suppressor genes for regeneration; determination of gene regulatory mechanisms and robust pathways in differentiation and maturation of cell function; and discoveries in many other new areas of biology and medicine. CIRM has also initiated the formation of academic-industry research teams focused on translational medicine to prepare the way for clinical trials for a wide variety of diseases and injuries. These include treatment for macular degeneration (loss of central vision); type I diabetes; stroke; targeting cancer stem cells in; glioblastoma, leukaemias, solid tumors; cure for HIV/AIDS; and correction of genetic diseases such as sickle cell disease, β thalassemia and epidermolysis bullosa. CIRM is establishing a major new center of excellence in genomics/stem cell biology to transform genetics in stem cell medicine and biology and will be creating a major network of alpha new stem cell clinics in California to deliver clinical benefits to patients⁷ in the next 2 years.

Novel Neuroendocrine Peptide Analogues in Reproductive Hormone Modulation for Hormone-Dependent Diseases

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The reproductive hormone cascade is driven by the brain decapeptide, gonadotropin-releasing-hormone (GnRH), which in turn stimulates pituitary gonadotropins which regulate ovarian and testicular sex hormones and gamete production. Total ablation of GnRH actions through desensitization with GnRH agonists or inhibition with GnRH antagonists reduce sex steroids to castration levels. Substitution of glycine in position six with a D-amino acid enhances a beta II turn which increases binding affinity, reduces degradation and metabolic clearance.

These analogues are extensively used to treat prostatic cancer and other hormone-dependent diseases.

However, reduction of sex steroids to castration levels results in unwanted side effects such as bone loss and flushing. To ameliorate this bifunctional GnRH-steroid molecules have been developed as well as orally-active GnRH analogues which can be dose adjusted to partially inhibit sex steroids.

Recently, novel neuropeptides, kisspeptin (Kp) and neurokinin B (NKB) were discovered as regulators of GnRH secretion. Their cognate receptors have therefore become a target for the partial inhibition of GnRH and ensuing sex steroids. Kp antagonists inhibit GnRH neurone firing, GnRH secretion, gonadotropin secretion and sex steroids. They delay progress through puberty and prevent the ovulatory surge of LH gonadotropin but do not lower basal LH and steroids. Hence they have promise as novel female contraceptives and the treatment of a number of hormone-dependent diseases, such as endometriosis, uterine fibroids, polycystic ovarian disease and benign prostatic hyperplasia, without causing bone loss and flushing.

Design, synthesis and biological evaluation of dual opioid agonists - neurokinin 1 receptor antagonists: Modulation of opioid and NK1 activity through structural modification

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Bifunctional peptides possessing both neurokinin 1 receptor (NK1R) antagonist and opioid agonist properties have shown advantages over currently used opioid analgesic drugs (e.g. a potent analgesic effect in neuropathic pain states, suppression of tolerance).² In this study, compact opioid agonist-NK1R antagonists were prepared, based on a constrained azepinone scaffold. By framework combination of an active in-house opioid tetrapeptide and a newly designed NK1R antagonist, a chimeric ligand with the desired dual activity, H-Dmt-D-Arg-Aba-Gly-NMe-3,5-(CF₃)₂Bn, was obtained.¹ This compound underwent in vivo bio-evaluation. Because of its compact and constrained character this peptide did cross the blood-brain barrier (BBB) and was able to induce a potent antinociceptive response after systemic administration. Transport through the BBB represents a major hurdle for CNS drugs. Unfortunately, after repetitive administration and in analogy with morphine, tolerance did emerge. Hence, efforts were dedicated to improve both opioid and NK1 activity within this lead structure.

By structural modification of the lead structure, crucial features were identified for both activities. As such, the interpharmacophore distance was adapted by replacement of the glycine unit by a beta-alanine, a modification that resulted in a significant enhancement of opioid potency. A second modification that boosts the opioid potency is realized by a switch to D-Cit in position 2, whereas the removal of the amide N-methyl and trifluoromethyl groups reduce NK1R antagonism. Next, these compounds were tested in vivo. Upon comparison of in vivo and in vitro results, it became clear that the challenge herein does not only consist of finding high potency. One also has to consider the detrimental impact on BBB transport by subtle shifts in molecular flexibility. In conclusion, this structure-activity study can be used to modulate the relative potency in the investigated ligands and shows the importance of flexibility in these hybrids.

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Novel roles and regulation of the vasopeptidase, ACE2

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Angiotensin converting enzyme-2 (ACE2) acts as the protective arm of the renin-angiotensin system (RAS). ACE2 counterbalances the actions of ACE by metabolising its catalytic product, angiotensin II (Ang II), to Ang-(1-7). ACE2 also metabolises a restricted number of other bioactive peptides, including apelin. Enhanced ACE2 expression may be protective in disease states including diabetes, cardiovascular disease, fibrosis and cancer. ACE2, like ACE, also plays important non-catalytic roles ranging from acting as viral receptor, as integrin receptor, and in regulation of amino acid transport. Relatively little is known, however, about the specific physiological factors regulating ACE2 and few

transcriptional regulators have been identified. Here we show that ACE2 expression is increased under conditions of cell stress including hypoxic conditions, cytokine treatment and treatment with the AMP-mimic AICAR via mechanisms involving the NAD-dependent deacetylase SIRT1. Chromatin immunoprecipitation analysis demonstrated that SIRT1 was bound to the ACE2 promoter. AICAR treatment increased SIRT1 binding to the promoter. Inhibition of SIRT1 activity ablated the AICAR-induced increase in ACE2. As such it was established that the expression of the ACE2 transcript is controlled by the activity of SIRT1 under conditions of energy stress. ACE2 is also subject to microRNA regulation. These novel factors regulating ACE2 expression and functional activity may represent novel sites for modulating the angiotensin peptide levels, and other bioactive peptides, in cardiovascular and other diseases.

Substrate Specificity of Lysine Deacylases In Vitro

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Histone deacetylase enzymes (HDACs and sirtuins) have received considerable attention due to their potential as targets for therapeutic intervention in a variety of disease states, including several types of cancer. Methods for accurate biochemical profiling of putative drug candidates are therefore desirable. Furthermore, recent reports in the literature have shown that acyl groups other than acetyl may also play important roles in cellular signaling, which considerably broadens the scope of this class of ϵ -*N*-acyllysine posttranslational modifications.¹

Here, the evaluation of a series of diverse fluorogenic substrates against the panel of human zinc-dependent HDACs 1–11 as well as the NAD-dependent sirtuins (SIRT1–7), will be discussed. Kinetic parameters describing a selection of the enzyme–substrate interactions in further detail were performed, and a selection of inhibitors were evaluated. Our investigations show noteworthy trends in certain ligands' mechanism-of-inhibition across different enzyme isoforms, which is of importance for correct selectivity profiling.

Additionally, efficient new fluorogenic substrates for SIRT5² and SIRT6 screening will be presented, and finally, we have shown that HDAC3 harbors lysine decrotonylase activity in addition to deacetylase activity,³ albeit at a considerably lower rate than its deacetylase activity.

Generally, our synthetic chemical peptide tool approach holds promise for discovery and investigation of yet undiscovered enzyme function related to this class of posttranslational modification.

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Redesigning Insulin

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The determination of the key molecular determinants of insulin function is important not only for examining the downstream pathways leading to its physiological effects but also the development of new, higher performing clinical compounds. Insulin's bridging cystine residues serve a structural role in maintaining the receptor-binding domain in its correct conformation, yet less is known about other possible functional roles including disulfide exchange at the receptor binding site, proteolytic degradation and/or formulation stability. It is now very clear that these bridges play a significant role in regulating insulin's activity.

This paper will discuss the design, synthesis and biological evaluation of dicarba insulin analogues. Using a homogeneous catalysis approach, three dicarba insulin analogues (two unsaturated (C=C, *E* and *Z* isomers) and one saturated (CH₂-CH₂)) can be generated from a single linear precursor. Not all linear peptides, however, undergo facile metathesis and performing metathesis on insulin sequences was found to be particularly troublesome. During the course of our study, the application of microwave irradiation, chaotropic salts, turn-inducing pseudoproline residues, interrupted SPPS-catalysis methods, and tandem catalysis were all investigated to overcome competing aggregation phenomena and low metathesis/hydrogenation yields.

This work culminated in viable syntheses of target dicarba-insulin analogues. The insulin receptor competition binding and receptor kinase activation assays which followed showed that many of the dicarba insulin analogues possessed identical metabolic activity to insulin. Several of the analogues were also equipotent in [³H]-2-deoxyglucose uptake assays and *in vivo* glucose tolerance tests. However, unlike insulin, and its short and long acting variants, several of the dicarba analogues possess significantly lower mitogenic potency as measured in DNA synthesis assays.

The generation of diaminosuberic acid analogues of cystine-containing peptides *via* homogeneous catalysis provides a powerful tool for examining structure-activity relationship. This presentation highlights how homogeneous catalysis can be used to solve complex biological problems.

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Understanding the structure/activity relationships of the iron regulatory peptide hepcidin

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The peptide hormone hepcidin is a key homeostatic regulator of iron metabolism and involved in pathological regulation of iron in response to infection, inflammation, hypoxia and anaemia. It acts by binding to the iron exporter ferroportin, causing it to be internalised and degraded; however, little is known about the structure/activity relationships of the interaction of hepcidin with ferroportin. Here we show that there are key residues within the N-terminal region of hepcidin that influence its interaction with ferroportin, and we explore the structure/function relationships at these positions. We found that the interaction is primarily hydrophobic with critical stereochemical requirements at positions 4 and 6. In addition, a series of hepcidin mutants in which disulfide bonds had been replaced with diselenide bonds showed no change in biological activity compared to native hepcidin. We have also explored the effect of backbone cyclisation on stability and biological activity. The results provide mechanistic insight into the interaction between hepcidin and ferroportin and identify important constraints for the development of hepcidin congeners for the treatment of hereditary iron overload.

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A novel non-animal collagen-like protein functionalized using expressed protein ligation

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Collagen is the most abundant structural protein in vertebrates being a substantial part of the extracellular matrix. The protein is built of three polyproline-II-like polypeptides which supercoil forming a triple-helix. These triple-helices form bundles and fibres to form larger tissue structures.¹

Collagen is used in a variety of medical products, but there is significant concern about possible transfer of diseases due to extraction from animal sources. Cost-effective production of recombinant collagen is desirable.² For animal collagens, the requirement of co-expression of prolyl-4-hydroxylase (P4H), which enables secondary modification of certain proline residues to give hydroxyproline, makes fermentation cost intensive and complex.² An alternative approach uses stable collagen-like triple-helical sequences, with glycine as every third residue and high proline content, which have been found in various bacteria, and which do not require co-expression with P4H.³

The bacterial collagen V-CL(V: globular domain, CL: collagen-like domain) examined in this work was fermented giving yields up to 19 g/L lacking biological activity, making it a versatile template for further modifications.⁴ To enhance the properties of this collagen-like protein, expressed protein ligation (EPL) is used. This method enables a number of novel materials to be produced. A key aspect of this approach is the possibility to modify the protein after expression by functionalising its C-terminal in many potential ways only at one specific site. For example, new functionality can be easily introduced by ligation of biologically active peptides with different cell specificities. In particular, this allows the introduction of unusual amino acids, cyclic peptides, sugars, molecules to enhance solubility, cross-linking, etc., that cannot normally be introduced by recombinant approaches. Alternatively, constructs bearing an N-terminal Cys residue can be ligated to the protein via its C-terminal thioester yielding a native peptide bond and forming dimers or higher polymers.

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Post-antibody medicines “*MicroAntibodies*”: Generation of molecular-targeting peptides by directed evolution

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Use of antibody medicines has been limited due to the biophysical properties, immunogenicity, non-cell permeability, high cost to manufacture, and so on. To enable new applications where antibodies show some limitations, we have developed an alternative-binding molecule with non-immunoglobulin domain. The molecule is a helix-loop-helix peptide, which is stable against natural enzymes in vivo and is too small to be non-immunogenic. In our previous work, we have succeeded to develop a directed evolutionary process for improving efficiency of catalytic antibodies.¹ Here, we apply our technology to construct a phage-displayed library of the helix-loop-helix peptides² and then screened the library for G-CSF receptor. Finally, the screened binding peptides were cyclized by introduction of a disulfide-bond linkage into the N- and C-termini. The cyclic peptide showed strong binding affinity (K_d : 4 nM) to the receptor, an enzyme-resistant property (half-life: 15 days in mouse sera), and non-immunogenicity. This peptide is named “*microAntibodies*” due to having the same properties as those of antibodies. The semi-rational strategy, which combines directed evolution with *de novo* design, provides a new way to generate structured functional peptides as alternatives to antibody medicines.

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Exploring bioactive peptides as pharmacological tools for oxytocin and vasopressin ligand design

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The diversity in nature has long been and still is one of the biggest resources of pharmaceutical lead compounds and many natural products often exhibit biological activity against unrelated biological targets,¹ thus providing us with starting points for pharmacological analysis. Natural peptides of great number and diversity occur in all organisms from plants to microbes to man. Examples for such rich and yet largely untapped libraries of bioactive compounds are animal venom peptides, invertebrate peptide hormones or plant defense peptides. Our goals are to discover and characterize novel bioactive peptides,⁴ screen their pharmacological activity, determine their structure-activity relationship and synthesize optimized peptide compounds to study ligand-receptor signaling.³

As proof-of-concept we have used a genome-mining approach or mass spectrometry to determine the occurrence and molecular structure of naturally-occurring oxytocin-like peptides and we have investigated their pharmacological profile on human oxytocin and vasopressin receptors. Plant cyclotides, insect inotocins² and marine cone-snail conopressins⁵ have been identified as pharmacological probes to study receptor-subtype selectivity. Combining structure-activity analysis and peptide chemistry, we are aiming to generate selective, potent and stable peptide ligands that are potentially oral bioavailable and may be useful for the treatment of a wide range of challenging, but yet untreated diseases.

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Total Chemical Synthesis of the antimicrobial peptides Snakin 1 and 2

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Snakin 1¹ (63 residues) and Snakin 2² (66 residues) are disulphide rich antimicrobial proteins isolated from *Solanum tuberosum* (potato) tubers. Both are active (1-20 µM) against several fungal and bacterial plant pathogens but due to a lack of readily available material a detailed investigation of their structure and structural requirements for their biological activity remains undetermined. We outline the first synthesis of these intriguing peptides using native chemical ligation techniques and describe the oxidative folding of the 12 cysteines present in the linear polypeptide to afford the postulated 6 disulphide bonds. Both synthetic Snakin 1 and 2 demonstrated full biological activity when compared to natural Snakin 1 peptide. Furthermore, comparison by spectroscopic methods of the folded synthetic Snakin 2 to that obtained from natural sources suggested that these were otherwise identical.

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Downsizing proteins into short water-stable alpha helices that maintain biological properties

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Recombinant proteins are important therapeutics due to potent, highly specific, and nontoxic actions in vivo. However, they are expensive medicines to manufacture, chemically unstable, and difficult to administer with low patient uptake and compliance. Small molecule drugs are cheaper and more bioavailable, but less target-specific in vivo and often have associated side effects. Here we combine some advantages of proteins and small molecules by taking short amino acid sequences that confer potency and selectivity to proteins, and fixing them as small constrained molecules that are chemically and structurally stable and easy to make. We show that short peptides, corresponding to helical epitopes from viral, bacterial, or human proteins, can be strategically fixed in highly α -helical structures in water. These helix-constrained compounds have similar biological potencies as proteins that bear the same helical sequences. Examples are (i) a picomolar inhibitor of Respiratory Syncytial Virus F protein mediated fusion with host cells, (ii) a nanomolar inhibitor of RNA binding to the transporter protein HIV-Rev, (iii) a submicromolar inhibitor of *Streptococcus pneumoniae* growth induced by quorum sensing pheromone Competence Stimulating Peptide, and (iv) a picomolar agonist of the GPCR pain receptor opioid receptor like receptor ORL-1. (vi) Agonists of AP-1 transcriptional regulator This approach can be generally applicable to downsizing helical¹ regions of proteins with broad applications to biology and medicine

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In vitro selected cyclic peptides for the cocrystallization and inhibition of a transmembrane multidrug transporter

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Transmembrane proteins are challenging crystallization targets. Their ample hydrophobic surfaces create the need for restricted buffer conditions and undermine specific protein-to-protein interactions that lead to lattice formation. Cocrystallization proteins, such as antibodies, have been employed in the past to help solubilize the target protein, mediate lattice formation, and increase the chances of quality crystallization. In search of a new ligand scaffold to expand the cocrystallization ligand toolbox, we used the random non-standard peptide integrated discovery (RaPID) system to identify high-affinity, target-specific cyclic peptides and evaluated their efficacy as cocrystallization ligands. *Pyrococcus furiosus* multidrug and toxic compound extrusion (PfMATE) transporter was used as a model protein in our proof-of-concept experiment demonstrating the efficacy of cyclic peptides as ligands in cocrystallization. Not only does PfMATE have a copious amount of hydrophobic surface but is also proposed to be highly dynamic according to its putative proton-driven antiporter mechanism. Therefore, even highly purified samples of PfMATE could exist as a heterogeneous mixture of protein in various conformational states. The cyclic peptides identified by the RaPID system did facilitate crystallization and structure elucidation to 2.5-3.0 angstrom resolutions, but not by mediating lattice formation.¹ The 17-residue macrocyclic peptide, MaL6, was found to bind to the cleft on the extracellular side between the N-terminal and C-terminal lobes. Lariat-shaped peptides MaD3S and MaD5, bearing identical 7-residue minicycle heads, were found in the substrate-binding pocket of PfMATE. Both methods of binding appear to lock PfMATE into the outward-facing conformation.

Additionally, it was found that the one of the transmembrane helices, TM1, interconverted between a kinked and a straight state as part of the drug extrusion mechanism. The pocket-binding peptides would also lock this helix into the straight conformation, which further increases the homogeneity of the cyclic peptide-protein mixture.

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Proline-rich antimicrobial peptides of the apidaecin and oncocin family – in vivo therapeutic potential and pharmacokinetics in mice

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Serious infections by multi- or pan-resistant bacteria are causing more and more increasing concern in clinical microbiology, hospital hygiene and antibiotic management. Antibiotic resistance does therefore not only develop in hospitals and the community, but arises in parallel from excessive prophylactic antibiotic treatments in livestock farming. Uncontrollable spread in the environment and horizontal gene-transfer by bacteria multiplies this challenge frighteningly. In contrast, the pharmaceutical pipeline is almost empty with colistin being the last approved antibiotic that often represents the final treatment option of infections caused by multi-resistant Gram-negative *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* pathogens.

In the last 20 years research focused on natural antimicrobial peptides (AMP) to investigate their clinical potential as a novel class of antibiotic drugs. Among the different AMP families proline-rich peptides like apidaecins and oncocins appear especially interesting as they act intracellularly on specific bacterial targets, e.g. heat shock protein DnaK. This class of AMPs does not penetrate eukaryotic membranes and does therefore not exhibit cytotoxic potential, as confirmed in vitro against eukaryotic cell lines and by hemolysis assays.

The optimized apidaecin derivatives Api88 and Api137 and oncocin derivatives Onc72 and Onc112 show minimal inhibitory concentrations in the low micromolar range and half life times of more than six hours in serum. Tolerance testing with NMRI mice treated four times intraperitoneally with a total daily dose of at least 160 mg/kg body weight revealed no adverse effects.

The in vivo antibacterial activity was examined using three different systemic intraperitoneal infection models with *Escherichia coli* and *Klebsiella pneumoniae*. When dosed intraperitoneally three times, the mean ED50 was around 1.25-2.5 mg/kg body weight. Pharmacokinetics in mice was investigated after single intraperitoneal and intravenous injections by a quantitative LC-MS/MS method from plasma, kidney and liver, linking the determined levels of the peptides to their therapeutic effect.

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De Novo Design Natural Xanthone Derivatives Based Peptidomimics to Mimick the Topology of Cationic Antimicrobial Peptides with Potentials for Topical Application

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Methicillin-resistance *Staphylococcus aureus* (MRSA) is a predominant source of infections associated with the blood, skin and soft-tissue. In our series of studies, alpha-mangostin, a natural xanthone, which was extracted and purified from pericarp of *Garcinia mangostana*, has shown good activity against Gram-positive bacteria, direct interactions of α -mangostin with the bacterial membrane are responsible for the rapid concentration-dependent membrane disruption and bactericidal action; however, it has low selectivity between bacteria and mammalian cells, and less action against Gram-negative organisms [1]. A new family of xanthone derivatives was designed using alpha-mangostin-based peptidomimics to mimick the topology of cationic antimicrobial peptides. AM-0016 showed the most potent antimicrobial activity amongst the series of semi-synthetic alpha-mangostin based dicationic molecules. AM-0016 has broad spectrum antimicrobial activity with improved potency against 15 isolates of MRSA with MIC99 in the range of 0.39-1.56 μ g/ml (0.59-2.35 μ M), no in vivo cytotoxicity at 50 μ g/ml on corneal wound healing, hemolytic activity with HC50 of 20 μ g/ml tested with rabbit red blood cells, and increased selectivity index of 25-100. AM-0016 exhibited rapid in vitro bactericidal activity (3-log reduction within 10-20 minutes). In a multistep (20 passage) resistance selection study using MRSA DM21455 (source: eye), *Enterococcus faecalis* ATCC29212 and VISA, AM-0016 showed less than a 4-fold increase in MIC. [2] Biophysical studies together with molecular dynamics demonstrated that the amphiphilic AM-0016 was a membrane targeting antimicrobial against MRSA, disrupting bacteria membrane leading to leakage of intracellular contents within 20-30 minutes.[2] New lead compound shows excellent selectivity and in vivo activity with potential for topical application [3]. The design principle and approach provided a powerful platform for design of membrane targeting antibiotics by fine tuning the hemolytic and antimicrobial activities of xanthone based peptidomimics with potentials for topical and even systemic application to treat the infection caused by MRSA.

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Macrocyclisation of Peptides by Novel Stable Selenoether Bridges

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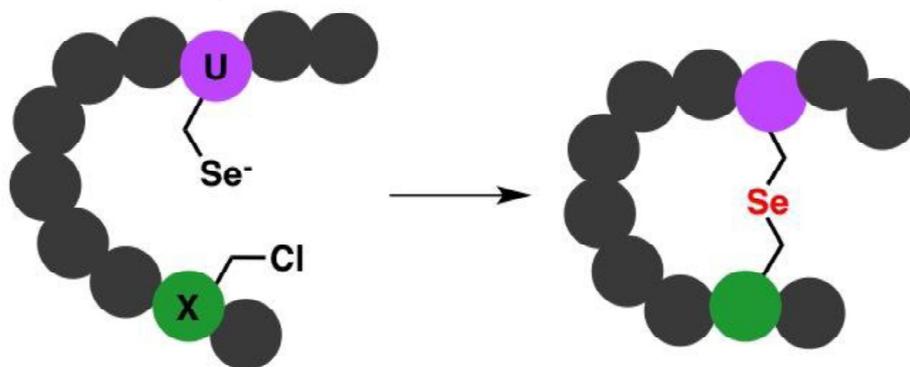
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In the search for more potent, selective and less toxic drugs, medicinal chemists have turned their interest into peptides as drug candidates instead of small molecules. Nevertheless, the proteolytic instability of peptides still presents a limitation for the widespread utilization of these molecules as

therapeutics. One common strategy to improve bioactivity and stability of peptides is to cyclise the linear peptide into a rigid, stable conformation.

Here we report a novel approach for macrocyclisation of peptides based on selenoether crosslinking. Selenoethers, like thioether linkages, are promising surrogates of disulfide bonds with the advantage of being stable to reducing environments. However, thioether macrocyclization of peptides by intramolecular thiolation of cysteine is often difficult, but can be overcome by replacing cysteine with the more reactive isosteric selenocysteine amino acid. This simple synthetic strategy provides an easy way to cyclize peptides with short non-reducible linkages.

The linear peptide is assembled on solid support and equipped with selenocysteine and halogenated residues. Cyclisation is carried out under mild conditions in solution via Se-alkylation of the selenocysteine by the halogenated amino acid. The selenide closure is chemoselective and can be applied to unprotected peptides in aqueous media at room temperature without a catalyst. We have recently applied the new methodology to construct seleno-analogs of naturally occurring lantibiotic peptides in an unprecedented simplified fashion.¹ We now describe biologically active analogues of the neuropeptide oxytocin, where the stable selenoether bridge replaces the native disulfide bond with minimal structural perturbations.



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Natural Sources for Oxytocin and Vasopressin Ligand Design

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Oxytocin (OT) and arginine-vasopressin (AVP) were the first peptides to be discovered, synthesized and characterized over 60 years ago, yet today we still lack a complete toolbox of selective agonists and antagonists for the four known receptor subtypes. Considering the ubiquitous presence of OT and AVP receptors in the periphery and CNS in addition to their role in a multitude of important physiological processes, it is central to have a plethora of selective ligands for concluding studies. Ideally, these ligands should not only be selective, but should also preserve their selectivity in different species allowing for translational studies important for drug development. While there has been great medicinal chemistry conducted over the last decades on shaping such ligands, we

decided to take a different approach, taking advantage of the fact that the OT/AVP signaling system has been well preserved in the kingdom of life for millions of years, and started exploring these natural sources for novel ligands.

The venom of the cone snail and the genome of social ants turned out to be such natural sources, both yielding excellent lead compounds for the design of receptor selective ligands. Here we would like to discuss the rationale behind our approaches, the techniques involved and how structure-activity relationship studies of these lead peptides influenced our ligand design, eventually leading to the identification of an agonist to antagonist switch for AVP, the development of a selective hOTR agonist that retained its selectivity profile in mice and humans, the identification of exceptionally potent peptides for zebra fish social studies and the development of stable ligands towards the treatment of chronic abdominal pain.

***In Silico* platform for predicting and designing therapeutic peptides for drug delivery**

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Many therapeutic molecules like DNA, proteins, and small molecules do not reach clinical trials due to poor delivery and low bioavailability. However, small peptides known as cell-penetrating peptides (CPPs) have remarkable ability to traverse biological membranes without significant membrane damage. In addition, CPPs are capable of transporting a wide range of therapeutic molecules into the cell interior, thus offering a great potential as versatile drug delivery vehicles. Keeping in mind the huge therapeutic importance of CPPs, we have developed a database of CPPs-“CPPsite”, a unique repository of its kind, which provides comprehensive information about experimentally validated 843 CPPs. A wide range of user-friendly tools like searching, browsing, analyzing, and mapping tools have been incorporated in CPPsite. Next, we have developed a support vector machine (SVM) based algorithm-“CellPPD”, which is a very useful platform for predicting and designing CPPs. Preliminary analysis revealed that certain residues are dominated, and few residues (e.g. Arg, Lys, Pro, Trp, Leu, and Ala) are preferred at specific locations in CPPs. Therefore, SVM models were developed using amino acid composition, dipeptide composition, and binary profiles as input features. In addition, we have identified various motifs in CPPs, and used this information for developing a hybrid prediction model (Motif + SVM). All models were evaluated using five-fold cross-validation technique. We have achieved maximum accuracy of 97.40% using the hybrid model. CellPPD is the first web server in the public domain where users not only can predict CPPs but also can design better CPP analogs with desired physicochemical properties. CellPPD will generate all possible substitution mutants of each submitted peptide with SVM scores and physicochemical properties and allows user to select the best analogues. In conclusion, both CPPsite and CellPPD will be helpful in designing efficient CPPs for delivery of therapeutic molecules.

Availability:

CPPsite: <http://crdd.osdd.net/raghava/cppsite/>¹

CellPPD: <http://crdd.osdd.net/raghava/cellppd/>²

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Surface-Bound Peptides for Biosensing and Cell Biology

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Peptides on surfaces have many applications in biosensing and biomaterials, both in influencing the affinity for biomolecule and cells for a surface and in providing that surface with the ability to determine the activity of protease enzymes in a biological medium. In this presentation we will discuss some of our work on using peptides on surfaces for biosensing and cell biology applications. We will initially start with an electrochemical biosensors for glycosylated haemoglobin, referred to as HbA1c and an important biomarker of the glucose levels in the blood over the preceding 3 months. This biosensor employs a surface-bound glycosylated pentapeptide to ensure specific binding of anti-HbA1c antibodies to the surface. The presentation will next cover using peptide modified photonic crystals as a means to monitor the release of matrix metalloprotease enzymes, MMP2 and MMP9, from live macrophage cells. In this work surface chemical techniques to allow different peptides to be immobilised on the exterior and interior walls of mesoporous silicon photonic crystals is outlined. In later work we demonstrate the ability to give these photonic crystals specificity for a given protease enzyme using a custom synthesized polymer cross-linked with appropriate peptide sequences. Finally the talk will cover work on modifying flat surfaces with different densities of RGD peptides on a surface and showing how the surface density of peptides influences cell phenotype and behaviour. The influence of the peptide density on cell behaviour is ascertained using electrochemical impedance, cell migration studies and western blots for monitoring signalling proteins.

Homopolysilaproline a new peptide with PPII structure

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The importance of the left-handed polyproline II (PPII) helical conformation has recently become apparent. This conformation generally is involved in two important functions: protein-protein interactions and structural integrity. The PPII helix is believed to be the dominant conformation for many proline-rich regions of peptides and proteins. In particular PPII are major features of collagens.

In our laboratory, an original proline analogue has been synthesized: the 4,4-dimethylsilaproline, denoted silaproline (Sip)¹. The presence of dimethylsilyl group confers to silaproline a higher lipophilicity as well as an improved resistance to biodegradation.

Recently we developed a gram scale synthesis of enantiomerically pure Sip, requiring resolution of the racemate by chiral high performance liquid chromatography (HPLC)².

With this new starting material, our aim was to construct polysilaproline polymers to investigate physico chemical properties and modifications of PPII structure.

In this study monodisperses homopolypeptides have been synthesized by peptide coupling in solution, polydisperses homopolypeptides have been prepared by ring opening polymerization of *N*-carboxyanhydrides (ROP). These peptides have been characterized by RMN, MALDI-Tof and circular dichroism.

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Modulation of nano-structure properties by peptide design

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The modification of surface properties of metal and semiconductor surfaces is important to tailor and modify the in vitro and in vivo interaction of these materials with biological systems, e.g. for implants, for diagnostics and control of cellular growth on such surfaces. We immobilize complex and bioactive peptides on such surfaces in a highly controlled manner by application of click chemistry. Methodology for reliable silanization to produce complete and flawless monolayer coverage by reaction with azidopropyl triethoxysilane will be presented. All reactions performed on the support were thoroughly characterized by fluorescence labeling and imaging. We show that elaborate washing procedures are required for proper production of covalently bound monolayers. Similar experiments were carried out, both with silanization and with thiolate modification of InAs wafers, often used in growth of Nanowires. The modifications of Nanowire arrays were compared to modification of the planar surfaces. CuAAC click reactions¹ were optimized using model reactions on these types of substrates. The optimized conditions were used in CuAAC immobilization of adhesion peptide k-l-h-r-v-r-a-Pra-a-OH,² a metabolically stable D-amino acid peptide that promote cell adhesion, proliferation and spreading of cells. The interaction of the membrane of the cells with the modified surface was investigated by high resolution fluorescence microscopy.

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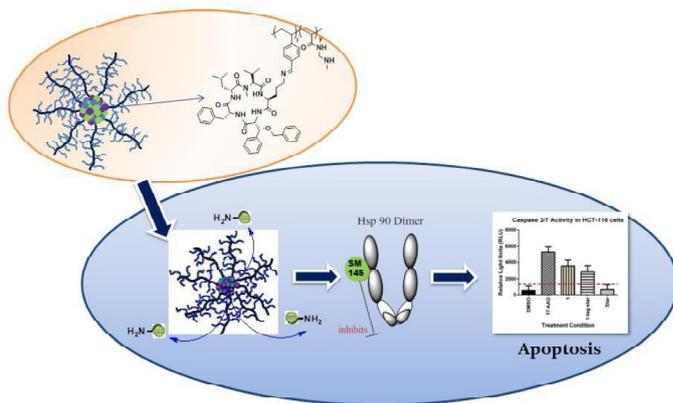
Effectively delivering a drug using nanoparticles: Improving solubility of a unique C-terminal modulator of Hsp90 inhibitor

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We have identified a novel molecule, SM 145, which is a peptide natural product derivative that inhibits heat shock protein 90 (Hsp90). Clinically relevant Hsp90 inhibitors target the ATP-binding pocket within the N-terminal domain of Hsp90, and these molecules do not inhibit binding interactions between co-chaperone proteins and Hsp90's C-terminal domain. Previously we have shown that SM 145 inhibits the formation of ionic interactions between the basic residues of client proteins and Hsp90's acidic MEEVD region. Although these data suggest that SM 145 will overcome the current limitations of existing Hsp90-targeted drugs, the hydrophobicity of SM 145 has limited its potential as a viable clinical candidate. Drug delivery of this unique molecule via nanoparticles is one approach that could enhance the efficacy of SM 145 by delivering the molecule to the cell via endocytosis instead of passive diffusion. Herein we report the synthesis of a novel Hsp90 inhibitor conjugated to a star polymer. Using RAFT polymerization, we prepared star polymers comprised of PEG attached to a pre-designed functional core with vinyl benzaldehyde (VBA) and crosslinker. Disulfide crosslinker generates nanoparticles that are easily degraded by the body. Star polymers are non-toxic, they reliably enter cells, and the drug payload is easy to calculate. The Hsp90 inhibitor, SM 145, release was studied at pH=5.5, and 7.4; conditions representative of endosomal and extra cellular environments. We show via cytotoxicity studies and confocal analysis that using this nanoparticle delivery system improves the effectiveness of this Hsp90 inhibitor by five-fold. Further, like the parent compound, we showed that conjugating SM145 with star polymers induce apoptosis by a caspase 3/7 dependent pathway. These studies show that we can deliver SM145 into cells effectively using nanoparticles, and lower the dose of compound required to induce apoptosis.



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Tyrosine Mediated Ultrathin 2D Peptide Assembly

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Tyrosine is one of the most significant amino acids. They can store the mechanical energy by protein folding and also facilitate proton-mediated electron transport in photosystem II. In our study, we systematically introduced repeating tyrosine units into peptides of various lengths to study the impact of the peptide sequence on self-assembly. The ordering of peptides investigated here has a strong driving force that overcomes the large surface tension of water. We have identified specific sequences of tyrosine containing peptides that can afford densely packed 2D film structure at air/water interface and the resulting structure can withstand the surface tension of water and modify the intrinsic curvature of water droplet. The atomic force microscopy (AFM) analysis along the film edges shows the evidence of film stacking of multiple nano sheets, whose minimum thickness is 1.4 nm. Additionally, the peptide interface presented here provides a tunable platform to template 2D hybrid materials. Consequently, highly ordered 2D assemblies of polypyrrole that cannot be formed by using conventional methods were created for the first time

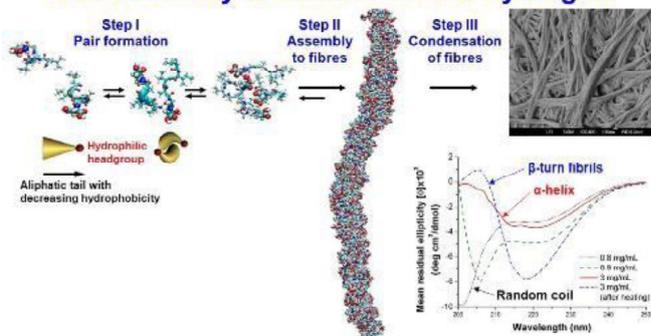
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Self-assembling aliphatic ultrashort peptides: A model systems for understanding and preventing amyloidosis

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Self-assembly to nanofibers and hydrogels



Institute of
Bioengineering and
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A*STAR
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We have designed a new class of aliphatic peptides made of just 3-6 amino acids that self-assemble in water via α -helical intermediates to amyloid β -type fibers. While core sequences of 4-7 residues that form amyloid fibrils have been identified within natural proteins, the mechanism of aggregation remains unclear. We compared the self-assembly of our designed peptides with core sequences in Amyloid-beta, Amylin and Calcitonin using a multimodal approach. A common feature was the appearance of α -helical intermediates before the final β -turn structures. Another amyloid-beta core sequence containing the diphenylalanine motif was chosen to evaluate the role of aromatic residues in self-assembly. The repeated occurrence of aromatic residues in core sequences has led to widespread conclusions about their key role in driving self-assembly. Surprisingly, the diphenylalanine-containing sequence did not form cross- β aggregates or involve the α -helical intermediate step. Our study puts forth a new, simplified model system to study amyloidosis and indicates that aromatic interactions are not as important as previously postulated. The results provide valuable insight into the early intermediates and factors driving self-assembly, which is necessary for developing small molecule therapeutic drugs that prevent amyloidosis.

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Supramolecular Self-Assembly of *N*-Acetyl Capped β -Peptides Leads to Nano- to Macroscale Fibre Formation

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Supramolecular self-assembly represents a powerful approach to the design of functional nanomaterials. Peptide self-assembled systems offer significant advantages including biological compatibility, ease of synthesis, low toxicity and functionalisability. However, the control over essential features such as chemical, structural and metabolic stability, the scale and relatively slow rate of self-assembly remain significant challenges. Using peptides consisting of only β^3 -amino acids offers the means to overcome these limitations. β^3 peptides exhibit high metabolic stability. Structurally, they adopt stable, helical conformations in solution. 14-Helical β^3 -peptides in particular contain exactly 3 residues per complete turn of the helix (ie $n = 3$) and thus form cylindrical molecules with perfect longitudinal alignment of residues. This provides extraordinary opportunities for designing new materials with functionality located along these faces. Perhaps even more significant, is that the perfect pitch offers the opportunity to design a supramolecular self-assembly motif to link the monomers in a highly symmetrical manner reminiscent of one dimensional crystallization.

We hypothesized that a H-bonding motif similar to that required for 14-helical stabilization would, if propagated intermolecularly, mediate axial head-to-tail self-assembly, and ultimately promote fibre formation. *This mode of fibre self-assembly contrasts with existing approaches which predominantly exploit lateral interactions, rather than axial, assembly motifs.* Indeed we have now found that 14-helical, *N*-acetyl β^3 -peptides spontaneously self-assemble in a unique head-to-tail fashion to form fibres from solution. Fibre size can be controlled from the nano- to macro scale.^[1] The inherent flexibility in design and ease of synthesis provide powerful new avenues for the development of novel bio- and nanomaterials via supramolecular self-assembly.

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Self-Assembling Peptide Materials for Cell Engineering

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Self-assembly of biomolecules has attracted increasing interests for fabrication of nano- and micro-scaled biomaterials. In particular, designed peptides that self-assemble into nanofibers can form hydrogels and be utilized as biomaterial scaffolds for cell engineering with biocompatibility and biodegradability. Designed peptides have advantages in modulating molecular assembly and introducing functionality.¹

We have found that *de novo* designed short peptides, Y9 (Ac-YKYEYKYEY-NH₂), self-assembled into highly-networked fibrillar structures with beta-sheet conformations in aqueous solution. In addition, we have constructed self-assembling peptide nanofibers with responsive ability to calcium ion (Ca²⁺) to control assembled structures. A various number of Glu residues were conjugated to the N termini of Y9 as a Ca²⁺ responsive site (EnY9). Moreover, the cell-adhesive and -differentiation peptide units (RGDS and IKVAV) were introduced to the C termini of E1Y9 to functionalize as cell-adhesive scaffolds (E1Y9-RGDS/-IKVAV). In the presence of CaCl₂, EnY9 and E1Y9-RGDS/-IKVAV peptides assembled into wider structures than Y9, and formed stable hydrogels. On the hydrogels composed of E1Y9, E1Y9-RGDS/-IKVAV, 3T3-L1/PC-12 cells were cultured more than 7 days without any toxicity. The self-assembling peptide materials based on the Y9 peptide are useful for cell engineering as novel scaffolds.

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Homo- and mixed oligomers based on a constrained bicyclic β -amino acid to support helical diversity

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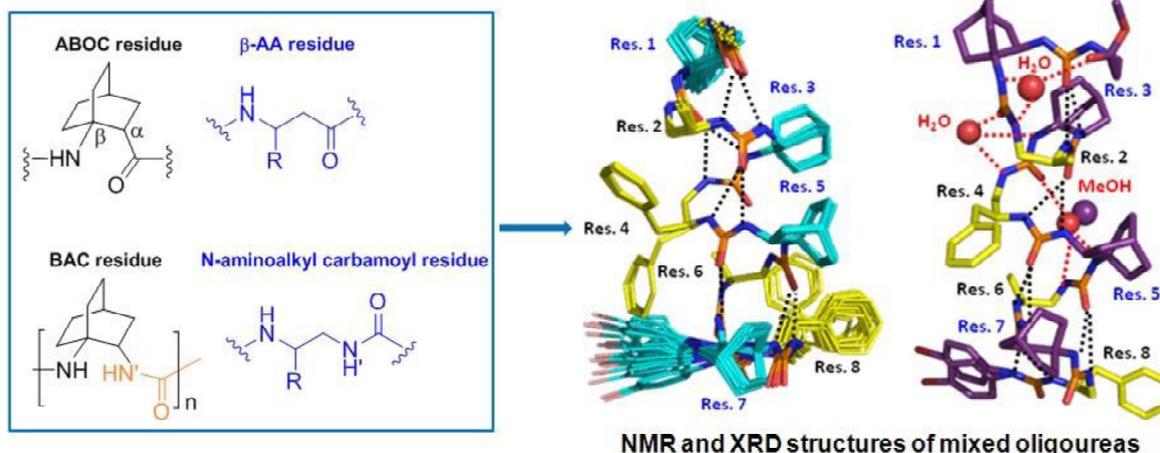
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Foldamers are non-natural oligomers able to adopt well-defined conformations, stabilized by non-covalent interactions, often inspired by natural compounds.¹ By mimicking the shapes of natural polymers such as peptides, proteins or nucleic acids, they became attractive tools for designing molecules with specific function and potential biomedical applications. For example, they can be putative cell penetrating compounds, protein-protein interactions modulators or can present antimicrobial activities. In this context, derivatives of α -amino acids such as β - and γ -amino acids have received much attention for the construction of artificial architectures able to display a large diversity of helical structures (*i.e.* 14-, 12, 10/12-helix,...). These helical systems were substantially enlarged with the use of homo- and heterochiral constrained cyclic derivatives and/or with the combination of α -, β - and γ - amino acids to synthesize α/β , α/γ , β/γ sequences of particular folding.²

In this work, we explored the ability of homo- and mixed oligomers incorporating a highly constrained bicyclic β -amino acid [(S)-aminobicyclo[2.2.2]octane-2-carboxylic acid] [(S)-ABOC],^{3,4} to induce a broad range of stable and predictable helical structures.

Different oligomers using amide or urea linkages and various alternating α -amino-acid and β^3 -homo-amino acid sequences have been generated.⁵ Their synthesis and their structural characterizations conducted by circular dichroism (CD), X-ray diffraction crystallography (XRD), nuclear magnetic resonance (NMR) and molecular modeling will be presented.



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The Evolution of Anticancer Peptides from Self-Assembled Hydrogels

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We have designed a class of hydrogels from self-assembling beta-hairpin peptides that enable the direct three-dimensional encapsulation and subsequent localized delivery of small molecules, proteins or cells to tissue. Although cytocompatible towards mammalian cells¹, we found, through a serendipitous discovery, that some of these hydrogels kill a broad spectrum of drug-susceptible bacteria on contact. Through *de novo* peptide design, we have been able to prepare next generation gels that have potent activity against drug-resistant strains of bacteria including MRSA and *P. aeruginosa*, a multi-drug resistant bacterium.^{2,3} Based on our understanding of the mechanism of antibacterial action and the fact that bacteria and cancer cells share similar traits with respect to their cellular membranes, we have gone on to design a new class of non-gelling, soluble peptides that show anticancer activity against a broad array of cancer cells.⁴ These peptides adopt an ensemble of random coil, bio-inactive conformations in solution. However, when they encounter cancer cells, they bind to, and fold at, the cell's surface, adopting a highly lytic conformation capable of killing the cell. This surface-induced folding mechanism takes advantage of the aberrant lipid composition that

characterizes many cancer cell types and allows these peptides to exert their action preferentially against cancerous as opposed to non-cancerous cells.

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Tunable Peptide Dendrimers as Bioactives

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Peptide dendrimers are branched polypeptides and represent a cost-effective approach to design bioactive peptides by amplifying peptides of various lengths. They have the advantages in the ease of synthesis and in the increase of binding affinity to protein targets by virtue of their multivalency. Here we report on the development of peptide dendrimers as anti-infectives and anti-proliferatives. For anti-proliferatives, we have designed tunable peptide dendrimers that are membrane-permeable and are capable to inhibit intracellular protein-protein interaction. We were able to develop such a design by combining both intracellular delivery and functional roles in a single moiety with high efficiency and specificity. An advantage of the dendrimer peptide is that it avoids the conventional design of a bipartite construct consisting of a functional cargo and a cell penetrating peptide (CPP) as transportant. An example of our design is D4R which contains four tetrapeptide (RLYR) strands linked to a branched trilycine scaffold. Modification of D4R at its N- or C-terminus with small-molecular chemicals showed extraordinary specificity of delivery to subcellular compartments. Epi-fluorescence microscopy showed that they can penetrate into the most densely-packed cellular compartments such as the nucleolus and the mid-body during mitosis in HeLa or MCF-7 cells. Cold treatment and ATPase inhibitor showed that their translocation is direct and independent of both endocytosis and ATP-dependent process. When coupled to a 19-residue p21 antitumor peptide, D4R also served effectively as a transportant in cargo delivery to enhance apoptosis. Combining peptide dendrimers with small chemical moieties could lead to an approach to develop useful organelle-specific cell-permeable intracellular inhibitors for laboratory and therapeutic uses.

Total synthesis and biological studies of thiazole/thiazoline-containing marine peptides

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Marine cyanobacteria have produced a wide variety of peptides, many of which have demonstrated antiproliferative activity, acute cytotoxic activity, or have specific neurotoxic activity. Their diversity in both biological activity and in structural complexity has made these marine peptides the focus of much work in recent years. In a large number of marine peptides, thiazole/thiazoline heterocycles are found as key structural features. These heterocycles rigidify otherwise flexible backbones, contribute to lipophilicity, and their presence frequently correlates with DNA, RNA, and protein binding properties. The recently isolated thiazole/thiazoline-containing marine peptides included largazole,

grassypeptolides, hoiamides and bisebromoamide. The small quantities of peptides that are produced by many marine cyanobacteria have proven to be a severe obstacle for the development of promising new leads for the development of novel drugs. In order to verify the primary structure proposed on the basis of studies of the marine peptides, and modify the structure, with the ultimate aim of improving activity or the physicochemical/biological properties of the lead molecule, we have undertaken extensive programs towards the synthesis of thiazole/thiazoline-containing marine natural products. The presentation will focus on two aspects: (1) Synthetic studies towards the total synthesis of these thiazole/thiazoline-containing peptides, which included the strategic design of the synthetic approaches, problem-solving, confirmation of structures of largazole, grassypeptolides, hoiamides, and revision of stereochemistry of bisebromoamide. (2) Biological studies of largazole and bisebromoamide in treatment of liver fibrosis through inhibition of both fibrogenesis and angiogenesis.

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Flipping the life/death switch with peptide-based antagonists of the Bcl-2-regulated apoptosis pathway

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Disruption of protein:protein interactions can often be achieved with synthetic peptides which are capable of binding their targets with high affinity and specificity. As such, synthetic peptides have long been considered for clinical applications to antagonise or activate cell signaling pathways. In this presentation, our efforts to target the cell death or apoptosis pathways regulated by interactions between the pro-death and pro-survival members of the Bcl-2 family of proteins are reported. As this pathway is frequently dysregulated in cancer, new strategies to activate the pathway in a highly specific manner provide a potential avenue for therapeutic application of Bcl-2 antagonists. Accordingly, through rational design as well as screening of large peptide libraries, we have developed highly potent peptide-based ligands that can specifically target particular pro-survival Bcl-2 proteins¹. We will present data on how viral delivery of such ligands can be used to effectively kill tumour cells lines (e.g. acute myeloid leukaemia cells) as well as primary tumour cells from patients², providing proof-of-principle for similar-acting drugs. Moreover, modification of the peptide sequences through incorporation of non-natural amino acids, specifically beta-amino acids, renders them highly stable compared to their natural BH3 peptide counterparts, an important first step in their direct clinical application³. High resolution X-ray crystallographic structural data on how such peptides engage their targets will be presented^{4, 5, 6}, together with how this information can be used to rationally design new sequences with altered binding profiles⁶. These data have wider application for the design of unnatural peptide ligands for other cellular targets.

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Structural capacitance in protein evolution and human diseases

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2. *Monash University, Clayton, VIC, Australia*

Canonical methods of protein evolution include diversification of folds through genetic and epigenetic alterations including point mutations, silencing, deletions and duplication. Following a survey of the entire human mutation database, we describe a new mechanism 'structural capacitance' that enables the *de novo* generation over rapid timescales of new peptide microstructures in previously disordered regions. These new elements of peptide microstructure are functionally implicated in the pathogenesis of a wide range of human diseases. The finding has implications for the ancestral diversification of protein folds, the engineering of highly evolvable proteins, and the identification and selective targeting of human disease epitopes.

NextGen Venomics: Natural, virtual and synthetic venoms for peptide drug discovery, target deorphanization and lead optimization

Reto Stöcklin¹

1. *Atheris Laboratories, Geneva, Switzerland*

The animal kingdom includes 200'000 venomous species that developed over several hundred million years of evolution. Each venom typically consist of a unique cocktail of hundreds different molecules, mostly highly potent and selective bioactive ingredients falling within the class of well-structured medium-sized peptides. These biomolecules naturally possess a number of key drugability requirements: while poorly immunogenic, they are highly potent, selective, stable and soluble. In addition, they are a proven source for screening pharmaceutical targets that are difficult to address using standard medicinal chemistry approaches such as ion channels, transmembrane or circulating proteins. Dozens of venom-derived mini-protein drug candidates have been taken into drug development and six of these made it to clinical use^{1,2}.

Usually, the first step in the search for new compounds is a bioassay, which is followed by a deconvolution process leading to isolation and characterization of the bioactive substance. While these conventional bioactivity-guided strategies have a proven track record, they are time and sample consuming. Today, the "Venomics" strategy that we pioneered³ combines state-of-the-art peptide synthesis, proteomics⁴, transcriptomics⁵ and post-genomics technologies with specialized bioinformatics tools⁶. This generates an abundance of valuable data in a very short period of time and using much smaller amounts of natural products.

We will present an innovative NextGen Venomics platform that is aimed at providing access to yet impossible to explore samples in an unprecedented manner through a combination of natural, synthetic and virtual venoms:

- Pre-fractionation of venoms to produce libraries of **natural venoms** for HTS
- Massive venom gland NextGen mRNA sequencing
- Bioinformatics to extract sequences and produce the **first virtual venoms**
- Original software to streamline the transcriptomics-assisted deconvolution process
- An integrated *in silico* platform to screen virtual venoms and design optimized leads
- Large-scale multiplex synthesis of peptides as **first synthetic venoms libraries**

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Structure based design Hepatitis C Virus immunogens

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It is estimated that more than 170 million people are infected by HCV worldwide. HCV infection is considered a silent killer because it can be transmitted to new hosts unnoticed and can take up to 20-40 years before severe clinical symptoms develop. To solve the global HCV problem, more effective and affordable drugs against HCV, as well as a vaccine are needed. The extreme genetic diversity of circulating HCV is a major roadblock to a HCV vaccine. HCV isolates from different genotypes can differ by as much as 35% in sequence, consequently, any vaccine based on a single isolate is unlikely to be effective. To overcome this challenge, a broadly effective vaccine must target conserved B and T cell epitopes. We are working towards immunogen development using the epitope vaccine strategy: that a broadly effective HCV vaccine can be attained by targeting conserved neutralizing epitopes on the virus. We have designed peptides to mimic the conformational epitopes observed in the crystal structure between broadly neutralizing HCV antibodies and fragments of the HCV E1 and E2 coat proteins. The design, synthesis and structural properties of these peptides will be discussed as well as our progress towards developing these peptide mimics as immunogens capable of inducing a neutralizing response to HCV

Extending chemical ligation

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Native chemical ligation is a remarkable reaction that enables the chemoselective coupling of two unprotected peptides. This technology has found numerous applications in chemical biology beyond its original purpose for the synthesis of small proteins. However, there are two major limitations to its use, the requirement for a peptide thioester, not straightforward by Fmoc solid phase peptide synthesis, and the absolute requirement for an *N*-terminal cysteine. Recently we reported that a peptide-*N*-methylsulfonamide synthesised by Fmoc chemistry using the popular sulfamylbutyryl linker can participate directly in a ligation reaction with no need for prior conversion to a peptide thioester.¹ We report recent developments in ligation with latent thioesters using simple Fmoc chemistry and their use in tandem with ligation auxiliaries to extend the synthetic reach of chemical ligation.

Burlina F, Morris C, Behrendt R, White P, Offer J. 2012. Simplifying native chemical ligation with an *N*-acylsulfonamide linker. *Chem Commun*; 48:2579-81

Different binding modes of antagonists and agonists at the relaxin-3 receptor RXFP3

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The neuropeptide hormone relaxin-3 mediates its actions through activation of the relaxin-family peptide receptor-3 (RXFP3). Current evidence confirms an involvement of the RXFP3/relaxin-3 signalling system in the central regulation of feeding, arousal/sleep, emotional memory, and metabolism/neuroendocrine function. Thus RXFP3 represents a promising new target for various mental disorders. However the development of lead molecules for this receptor is non-trivial. Relaxin-3 is a large, complex, two-chain peptide and poorly selective for RXFP3 over related receptors. In addition any drug targeting RXFP3 is required to be able to pass the blood-brain barrier after systemic delivery, a considerable challenge. A detailed picture of how ligands interact with RXFP3 is therefore desirable for the rational design of novel analogues.

We have through extensive mutational efforts generated structure-activity relationships both for ligands and the receptor identifying key features of the interaction. These data have been used to generate models of the complex using the program package HADDOCK. Our data suggest that a recently developed single-chain antagonist, R3 B1-22R, which is a truncated variant of the relaxin-3 B-chain binds strikingly different to native relaxin-3. These data are highly relevant for the future development of this antagonist towards the clinic.

A strategy for stabilizing weak interactions of posttranslationally modified proteins

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Survival of motor neuron (SMN) protein is part of the SMN complex that mediates assembly of ribonucleoprotein complexes¹. SMN protein contains a central Tudor domain that recognizes symmetrically dimethylated arginine residues (sDMA), leading to transient interactions with Sm proteins. Due to the weak but specific interaction of SMN Tudor with sDMA ($K_D \sim 0.5$ mM) a large excess of free sDMA had to be used in previous structural studies of the SMN Tudor-sDMA complex². Such conditions are not ideal for structure analyses and covalent coupling of the two interacting moieties can shift the equilibrium to the bound state by lowering the entropic cost of binding.

Here sDMA is covalently linked to SMN Tudor via expressed protein ligation, using a flexible glycine linker³. NMR analyses and ligation yields allow assessment of the ideal linker length and enable structural analysis of a weak interaction at moderate concentrations. The described strategy can help to overcome current challenges in structural analysis of such weak, transient interactions often mediated by posttranslational modifications.

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Bioorganic-inorganic materials and polymers from hybrid alkoxysilane-peptides building blocks

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The mild conditions used in sol-gel process allow the preparation of hybrid materials containing peptides. In most of reported cases, peptides are either non-covalently entrapped or grafted after the synthesis of the material¹, requiring surface treatment and chemoselective ligation. In contrast, we designed a new family of *bioorganic-inorganic hybrid structured materials* relying on the hybrid peptide-trialkoxysilyl building blocks, synthesised in Fmoc/tBu SPPS, that can be engaged directly in a sol gel process.

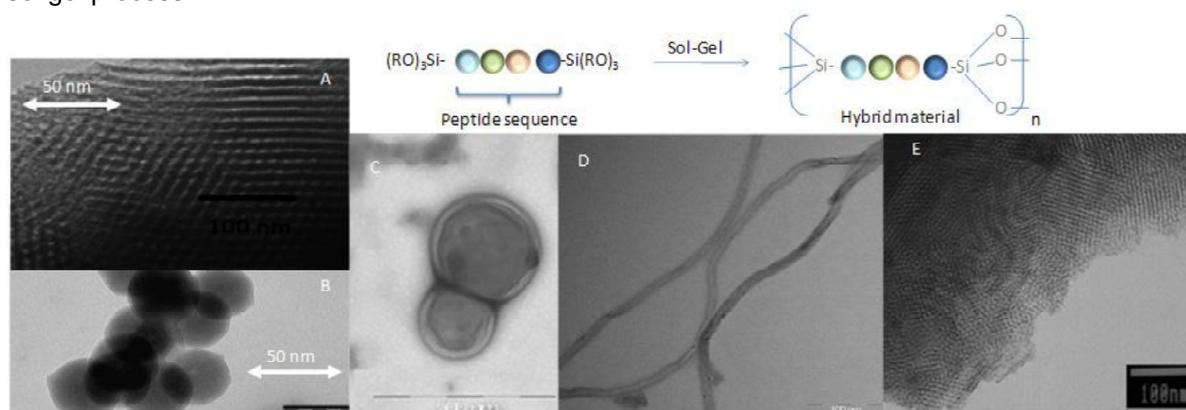


Figure 1. A Hybrid peptide ordered mesoporous silica catalyst, B Nanoparticles grafted with bioactives hybrid peptides, C,D,E Structures obtained by self assembly of hybrid peptides sequences derived from collagen.

This bottom up approach is flexible enough that any type of functionalities, biological and physicochemical properties afforded by the peptide unit can be introduced on the material either by grafting or by direct synthesis. Several examples of hybrid peptide materials will be presented (i.e. multifunctional nanoparticles, peptide-catalytic mesopous materials²³, anti fouling glass surfaces). At last, the self-assembling properties of the peptide unit can be exploited to yield a range of bio-inspired nanostructured materials

Biocompatible, bioinert and biodurable, silicons have been widely used since the 60's as biomaterials for medicine for orthopedics, catheters, drains, shunts, extracorporeal equipment, implants and as injectable fluids for soft-tissue augmentation. On the other hand, peptide-based hybrid copolymers⁴ are a class of biomimetic materials that significantly become important due to their bioactive properties. To the best of our knowledge, only silicones incorporating random homopolypeptide sequences obtained by ring opening polymerisation of amino-acids NCAs or from hydrolysates of proteins are described so far. In this context, we first describe a *new class of silicon-peptides biopolymers* obtained by mild hydrolytic polymerization using well defined chlorosilyl hybrid peptides as monomeric units, synthesised in solution or on solid support⁵.

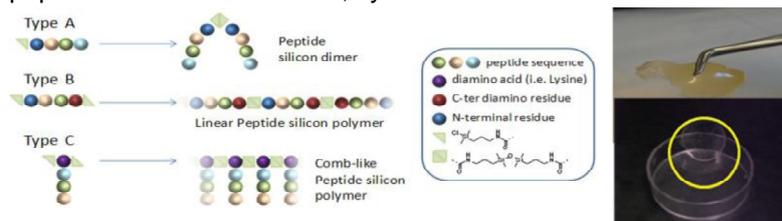


Figure 2. Types of peptide-silicon hybrids

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Glycopeptide and lipopeptide derivatives with potent activity against multi-drug resistant pathogenic bacteria responsible for UTI, cSSSI and CAP/NP infections

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The lack of treatments available for multi-drug resistant bacteria is one of the most pressing health issues currently facing humankind. We have designed lipopeptide and glycopeptide derivatives selective for bacterial membranes. We are optimising and developing these for the treatment of community-acquired and nosocomial infections; complicated skin and skin structure (cSSSI), urinary tract, and pneumonia (CAP/NP).

In the Gram -ve active lipopeptide series, we have identified compounds active against multi-drug resistant *E. coli*, *P. aeruginosa*, *K. pneumoniae*, and *A. baumannii*, including colistin-resistant and NDM-1 strains. In the Gram +ve active glycopeptide series, compounds are 20- to 100-fold more active (MIC <3 ng/mL) than vancomycin (MIC ~ 1 ug/mL), and are bactericidal. They have excellent plasma and microsomal stability, show minimal inhibition of CYP450 enzymes, are clear in Ames mutagenicity testing, and show no cytotoxicity in mammalian cell MTT assays at concentrations exceeding 1000 times the MIC. Mouse pharmacokinetic profiles are consistent with once daily dosing in man, and the compounds show equivalent or superior efficacy to the reference antibiotics vancomycin and daptomycin in multiple mouse models, including MRSA and *S. pneumoniae* thigh infection, blood bacteremia, sepsis, and lung infection models. For example, potent activity is observed in the MRSA thigh infection model with a single sub-cutaneous 10 mg/kg dose, showing equivalent efficacy as 200 mg/kg vancomycin, with greater than 100,000-fold reduction in bacterial load (cfu/thigh) at 24h. The compounds are also active in lung infection models (where daptomycin fails), with 100% survival in a lethal dose study with a MDR *S. pneumoniae* strain.

Is there a future for peptide mimetics as anti-microbial agents?

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Background: In an era of questionable antibiotic stewardship there is a increasing need for new antimicrobials as bacteria and viruses become resistant to commonly used drugs. Peptide mimetics have recently been added to our weaponry for the treatment of infectious diseases and they offer some advantages against resistance.

Methods: We have developed peptide mimetics and dominant negative decoy proteins (dNdP) that can block the replication of specific viruses and bacteria by mapping binding domains of essential protein binding partners for selected targets of Influenza virus (virus replicase PA/PB1, PI3 kinase p85 β , and TRIM25), Respiratory Syncytial Virus (RSV) (polymerase Phosphoprotein subunit), *Chlamydia trachomatis* (type III secretion proteins LcrH1, CopD/B, and Cpn0585) using Pepscan epitope mapping and synthesized peptide mimetic inhibitors (10-20mers). A cell penetrating peptide (11-mer) was conjugated to peptide mimetics and dNdP which were expressed in *E. coli* or yeast as fusions with either maltose binding protein (MBP) human serum albumin (HSA) as the scaffold.

Results: Peptide mimetics and dNDPs inhibited replication of influenza and RSV when added to MDCK cells 10 minutes prior to infection with virus. These antiviral peptides had ID50 values ranging from 10-20 μ M. Anti-chlamydial peptides inhibited chlamydial infection of HeLa cells even when added 6 hours post infection. We have recently genetically engineered probiotics and commensal bacteria to synthesize and secrete antimicrobial proteins and are presently testing these antimicrobials in animal models.

Conclusion: We have developed novel antimicrobials including peptide mimetics and dNDP that inhibit both viruses and bacteria. These proteins have been engineered onto a human serum albumin scaffold to increase half life and allow synthesis in gram quantities using commercially available yeast expression systems. These proteins are presently being tested in animal models and if they demonstrate good pharmacological profiles will be candidates for human phase I trials.

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Functional designed peptides: Antimicrobials and enzymes

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De novo designed active peptides are of significant interest. Such peptides can be potentially utilized as novel therapeutics and catalysts. Further, designing folded peptides allow us to examine fundamental problems of protein structure and folding. We are developing two groups of peptides: broad-spectrum antimicrobial/anti-endotoxic and membrane protein mimics. Antimicrobial peptides (AMPs) have high potential as novel antibiotics active against multiple drug resistance (MDR) pathogens. Among drug-resistant bacteria, Gram-negative ones are of significant threats due to the lack of potent antibiotic in drug discovery pipelines. The outer membrane or lipopolysaccharide (LPS) of Gram negative bacteria acts as a permeability barrier against antimicrobial agents. LPS or endotoxin is also well known for sepsis mediate mortality. In our studies, we have developed peptides those act through LPS-outer-membrane. These peptides adopt boomerang like beta-structures in complex with LPS lipids. Further, we have observed that hybrid peptides obtained from boomerang motif conjugated with inactive (against Gram negative bacteria) AMPs are highly potent against drug-resistant Gram negative bacteria. In another project, we have designed beta-hairpin peptides (IV8) with heme binding and peroxidase activities in lipid membrane. NMR structure of IV8 has been determined in detergent micelles. This research has implications in membrane protein design with novel functions.

1. Beta-Hairpin peptides: heme binding, catalysis and structure in detergent micelles.. *Angewandte Chemie* (published On-line).
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3. Designed beta-boomerang antiendotoxic and antimicrobial peptides: structures and activities in lipopolysaccharide. Anirban Bhunia, Harini Mohanram, Prerna N. Domadia, Jaume Torres, and Surajit Bhattacharjya. *Journal of Biological Chemistry* (2009), 284, 21991-22004.
4. Designed beta-boomerang antiendotoxic and antimicrobial peptides: structures and activities in lipopolysaccharide. Anirban Bhunia, Harini Mohanram, Prerna N. Domadia, Jaume Torres, and Surajit Bhattacharjya. *Journal of Biological Chemistry* (2009), 284, 21991-22004.

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Sub3, an antimicrobial peptide with cell-penetrating properties

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The discovery of new antimicrobials that use novel modes of action and avoid antibiotic resistance is crucial to fight microbial infections. Cationic antimicrobial peptides (AMPs) have been regarded as promising antibiotic candidates and knowledge on their mode of action is important for further improvement. Here we investigate the mechanism of action of Sub3, an AMP with high activity against Gram-negative and Gram-positive bacteria and low cytotoxicity. Using model membranes and bacterial cells with fluorescence spectroscopy, flow cytometry, Zeta potential and atomic force microscopy imaging we found that the action of Sub3 involves both membrane and intracellular targeting. At lethal concentrations Sub3 targets the bacteria membrane, through electrostatic attractions, and internalizes inside the bacteria without permeabilizing the cell membrane. In addition, Sub3 can also internalize into human cells without being toxic to them. The observation that Sub3 is able to internalize into host cells, is of major relevance because in addition to its potent activity against bacteria, Sub3 has promise for treating locally-infected mammalian cells which are difficult to treat.

Development of first-in-class adiponectin receptor agonist and antagonist peptides

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The adipose tissue participates in the regulation of energy homeostasis as an important endocrine organ that secretes a number of biologically active adipokines, including adiponectin. The research of adiponectin and its receptors is hampered owing to the difficulties in handling these proteins. The C-terminal half of adiponectin protein representing the globular domain (gAd) exhibits potent metabolic effects in various tissues. In addition, epidemiological studies found an inverse association between circulating adiponectin levels and several obesity-related malignancies, including cancers of the breast. In spite of the clinical need, before our studies adiponectin receptor response modifier peptides were not available.

Recently we developed and characterized a first-in-class adiponectin receptor agonist. This 10-amino acid residue multiply modified peptide has attractive physical properties and was indeed used to describe a series of cellular processes *in vitro* and investigate the potential of adiponectin-based therapies *in vivo*. Peptide ADP355 inhibits AdipoR-dependent cancer cell growth at 100 nM – 1 μ M, frequently better than gAd. In *scid* mice carrying MCF-7 orthotopic xenografts, ADP355 treatment moderately reduces the growth of established tumors. Similarly modest effects were observed on preservation of body mass and fat pad weight in lipodystrophic mice suggesting that the structure needs further refinements to warrant further preclinical development efforts. Nevertheless, the peptide fully restores fear conditioning in lipodystrophic animals.

Based on the sequence of the AdipoR agonist, a novel receptor antagonist was designed according to general rules of agonist – antagonist conversions. This peptide counteracts 100 nM ADP355-mediated breast cancer cell growth inhibition in a concentration-dependent manner. The antagonist itself slightly promotes cell growth, perhaps due to antagonizing endogenous adiponectin or acting as an inverse agonist.

Engineering stable disulfide-rich, cyclic scaffolds

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1. *Institute for Molecular Bioscience, University of Queensland, Brisbane, QLD, Australia*
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3. *Novo Nordisk A/S, Måløv, Denmark*
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7. *Institute of Laboratory Medicine, University Hospital, Ludwig-Maximilians-University Munich, Germany, Europe*

Gene-encoded cyclic peptides were originally thought to be anomalies in the peptide world, but are now known to be widespread throughout nature, with examples in plants, bacteria, fungi, and mammals. The evolution is not well understood but recent studies have provided fascinating insights into how these cyclic peptides are made. The intrinsic stability associated with the cyclic backbone has piqued the interest of researchers and has led to the hypothesis that these cyclic peptides can be used as scaffolds in drug design. Gene-encoded cyclic peptides from plants have been of particular interest for drug design. The potential of plant-derived disulfide-rich cyclic peptides in the design of novel drug leads for a range of therapeutic applications, including cancer, will be discussed.

Towards the development of rxfp1 receptor specific minimised relaxin analogue

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3. *School of Biomedical Sciences, the University of Queensland, Brisbane, Australia*

Since its discovery in 1926, human relaxin-2 (H2 relaxin) has undergone several clinical trials. It has finally passed the last Phase (Phase III) of the trials late last year for the treatment of acute heart failure. The indications are that H2 relaxin will enter into the clinic by the end of this year. However, this drug faces some limitations that need to be addressed in order to maximize its translational potential:

1. *Cross-reactivity with other receptor:* H2 relaxin exerts its biological function through its own receptor, Relaxin Family Peptide Receptor 1 (RXFP1; also known as LGR7). However, it also activates other receptors, including RXFP2 (native receptor for the related insulin-like peptide 3, INSL3). Thus, it would be beneficial to have a highly selective RXFP1 analogue to avoid potential undesired side effects mediated through RXFP2.

2. *Large size and complex structure:* The size (53 amino acids) and complex structure (two chains, A and B, and three disulfide bonds) of H2 relaxin represent a considerable challenge for its synthesis. This is a major limiting factor for the exploration of modifications of this peptide to optimize efficacy and stability. Thus a simpler H2 relaxin analogue that would be easier to prepare and modify is desirable.

Our recent structure-activity relationship (SAR) studies^{1,2,3} on H2 relaxin provide new insights into the mechanism of interaction of RXFP1 and RXFP2 by H2 relaxin, leading to a potent and RXFP1-selective minimized analogue.

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Medicinal Chemistry of Peptidic Compounds for the Treatment of Muscular Diseases

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Muscular diseases occur in all age groups and can cause serious physical disabilities. Duchenne muscular dystrophy (DMD) is one of the most severe forms of dystrophinopathy that occurs in younger age groups. The current trends among aging populations in developed countries indicate that sarcopenia, which is a muscular weakness involving muscular atrophy, will continue to be a serious problem in elderly persons. Our research has focused on identifying peptide therapeutics for these muscular diseases.

Two therapeutic approaches have significantly progressed in this field. The first approach centers on a mutation in the dystrophin gene that codes for an important structural protein within muscle tissue, dystrophin. This mutation accounts for about 20% of the congenital disease cases and is caused by nonsense mutations that introduce a premature termination codon (PTC) into the mRNA sequence. We have examined the utility of a dipeptidic antibiotic (+)-negamycin **1**,¹ which promotes PTC readthrough activity, and recently succeeded in developing more potent derivatives of **1** that displayed low levels of antimicrobial activity.² These derivatives may be effective for the treatment of other genetic diseases caused by nonsense mutations. In an effort to increase the muscular mass in patients, we have focused on myostatin (growth differentiation factor 8, GDF-8), a member of the TGF- β protein family, which inhibits muscle differentiation and growth. Inhibitor peptides with about 20 amino acids in length have been developed as a second therapeutic approach. These peptides selectively inhibit myostatin signaling in a cell-based reporter assay and have resulted in improved muscular mass in mice upon direct intramuscular injection. These peptides could potentially enhance the muscular mass of elderly persons, treat muscular dystrophy, and improve cachexia in patients with cancer, chronic obstructive pulmonary disease (COPD), metabolic syndromes, and the muscular strength of astronauts.

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Self-assembling cages from de novo designed peptide modules

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Encapsulation and compartmentalization are key features of living systems. An ability to mimic this via designed peptides would provide a unique route towards new materials for delivery, and the development of encapsulated complex systems. Recently, we have made use of the coiled coil; a fold in which two or more α -helices self-associate about a central hydrophobic core. As one of the most well-understood protein folds, we utilized a number of design rules¹ to produce a pair of robustly-folded assemblies: a parallel *homotrimer*; and a *heterodimer*. Tethering these together in a back-to-back fashion produces two 6-stranded hubs which, when combined, produce a network of tessellated hexagons. Moreover, owing to the symmetry of this system, any inherent curvature (as indicated by molecular dynamics simulations) would result in enclosed objects. Indeed, when the *homotrimer*—*heterodimer* peptides were mixed a fine precipitate was observed, which, following Scanning Electron Microscopy was shown to be composed of uniform spherical objects 97 ± 19 nm

($n=135$) in diameter. Further analysis by Atomic Force Microscopy confirmed the spherical nature of these objects in the hydrated state, which were seen to collapse to ~ 9 nm in height when dehydrated providing evidence for hollow structures. Startlingly, images obtained using Lateral Molecular Force Microscopy revealed details of a hexagonal surface structure consistent with the design. More recently, we have explored factors contributing to the formation of the cages: by making single point mutations to our coiled-coil building blocks we can affect both curvature and the stage at which closure occurs, furnishing populations of larger and smaller nanocages, respectively. This work has recently been published² and reviewed³.

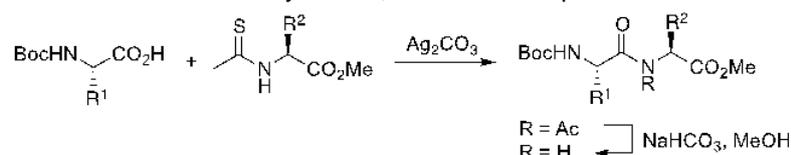
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New methods for amide bond formation

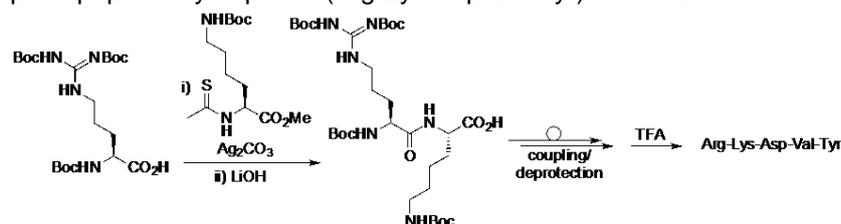
James R Cochrane¹, Aysa Pourvali¹, Craig A Hutton¹

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In recent years a range of novel amide ligation strategies have been developed, many incorporating thioacids or thioesters as carboxylic acid surrogates.¹ Most of these methods also require either a thiol-functionalised amine coupling partner, or an amine surrogate. We will present investigations into the scope and limitations of the reaction of amino acid thioamides with carboxylic acids in the presence of silver(I) to generate imide and amide adducts, based on the work of Palacios.² This transformation represents an umpolung approach to amide bond formation that does not require pre-activation of the carboxylic acid, and as such epimerisation levels are very low.



The application of this methodology to the solution-phase, N \rightarrow C direction synthesis of the pentapeptide thymopentin (Arg-Lys-Asp-Val-Tyr) will be described.



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Utility of Peptide Ligation Chemistry for the Chemical Synthesis of Post-Translationally Modified Peptides and Proteins

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Glycopeptides and glycoproteins are ubiquitous in nature and possess a range of biological activities that make them attractive targets as novel therapeutics and for applications as biomaterials. The

difficulty associated with isolating these complex biomolecules in pure form from nature has led to significant research effort directed toward the total chemical synthesis of homogeneous glycopeptides and glycoproteins for biological study.^{1,2} This talk will highlight the use of novel solid-phase methodologies and chemical ligation strategies for the total chemical synthesis of a range of homogeneous glycopeptides and glycoproteins (up to 19.5 kDa in size). The preparation of homogeneous fish-derived antifreeze glycoproteins,³ chemokine receptor domains⁴ and potent anti-thrombotic glycoproteins will be described. Structure-activity data has provided important information on the role of glycosylation on the activity of the peptides and proteins and will also be discussed.

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Development of a Universal Influenza Synthetic Peptide Vaccine

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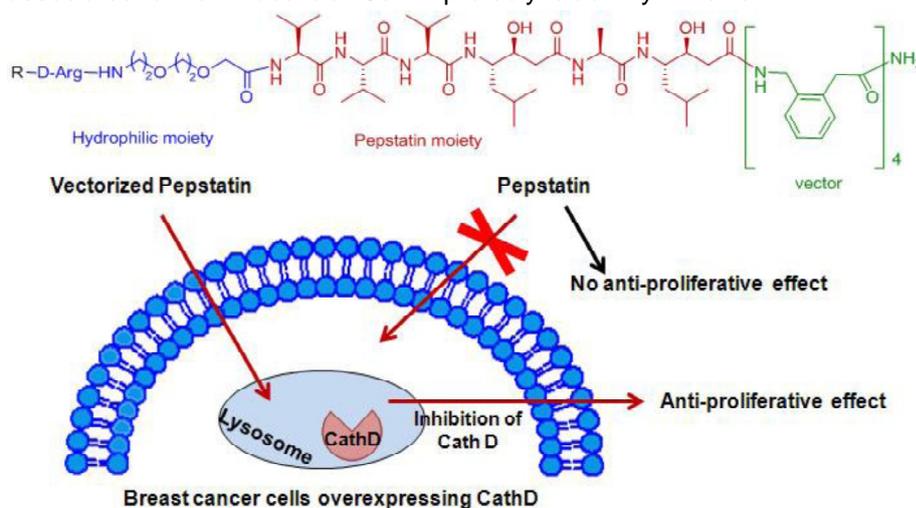
Influenza A virus infects billions of people worldwide resulting in 500,000 deaths every year. Current vaccines target the receptor-binding domain (RBD) of the viral surface hemagglutinin (HA) protein. This region is antigenically dominant and is prone to mutations that leave current vaccines ineffective at neutralizing virus, thus requiring the design of new vaccines every year. Furthermore, antibodies generated to the RBD of HA from one strain of influenza typically lack the ability to cross-react with and neutralize infection by other strains of influenza A. We have designed a platform technology to elicit protective antibodies against class-1 viral fusion proteins *in vivo*. These antibodies target conserved regions of the viral HA protein that are involved in large conformation changes during the infection process. Therefore, any mutations in this region that may produce escape mutants are also likely to produce inactive viruses that are unable to infect host cells. Since this region is conserved across multiple strains of influenza, the antibodies derived from our immunogen exhibit enhanced cross-reactivity. Our platform technology consists of a synthetic two-stranded alpha-helical coiled-coil peptide template with Ile and Leu residues buried in the hydrophobic core **a** and **d** positions of the repeating heptad sequence (**abcdefg**)_n to provide maximum stability to the coiled-coil structure. The resulting peptide immunogen is further stabilized with an interchain disulfide-bridge. An alpha-helical sequence from a native protein of interest is inserted into the template to expose the helical surface of the native protein (positions **b**, **c**, **e**, **f** and **g**). Antibodies to our lead peptide possess broad cross-reactivity among distant strains of influenza virus (Group1 H1, H2 and H5 and Group2 H3 and H7). Hence, this technology is a robust means of generating cross-reactive antibodies *in vivo* and should protect against any new pandemic strains.

Intracellular delivery of Cathpsin D inhibitors by dipeptide mimetic oligomers: a target for cancer cell proliferation inhibition?

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The identification and the use of tumour progression key modulators, an overexpressed peptidase for example, constitutes a relevant approach to decrease the side toxicity of standard chemotherapies. In this work we targeted Cathpsin D (CathD), a lysosomal aspartyl protease, overexpressed and abnormally secreted by a large number of malignancies.¹ This protease is involved in tumor progression and metastasis development. Moreover, several evidences involve proteolytic activity of the intracellular CathD in tumor growth and progression.^{2,3} Therefore, CathD inhibition represents an attractive approach for the development of potential anti-cancer agents.⁴ In this context, pepstatin, the most potent inhibitor of CathD so far identified has been evaluated. However, this inhibitor was showed to be inactive on cancer cell growth and this inefficiency was mainly attributed to a problem of cellular internalization. In this work, we developed cell penetrating CathD inhibitors by combining the potent pepstatin inhibitor with new vectors of cell penetration. This cell penetrating compounds were constructed by oligomerization of constrained dipeptide mimetics and they specifically targeted the endolysosomal compartment where the CathD activity is mainly localized.^{5,6} We showed that these conjugates were able to cross the cell membrane and exhibited a potent anti-proliferative activity associated to the intracellular CathD proteolytic activity inhibition.



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Rational Design of Substrate-Based BCR-Abl Kinase Inhibitors for the Treatment of Chronic Myeloid Leukemia

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The constitutively active tyrosine kinase BCR-Abl has been implicated as the underlying cause of chronic myeloid leukemia (CML). Current treatments for CML patients focus on the long-term use of imatinib, a small-molecule tyrosine kinase inhibitor (TKI) targeting the ATP binding site of BCR-Abl, but the response is not sustained for a number of patients due to point mutations that develop in the drug-binding region. Second- and third-generation ATP-competitive inhibitors have been developed to override imatinib resistance, but none of them exhibits inhibitory activity against T315I mutated BCR-Abl (Quintas-Cardama et al, 2007). Therefore, therapeutic approaches that target regions distant from the ATP cleft may be a promising strategy to overcome the emerging drug-resistance. In this study, a new class of substrate-based Abl kinase inhibitors targeting the ligand binding site was developed by grafting modified substrates onto the cyclotide scaffold to improve the stability and therapeutic efficacy of the inhibitor. Disulfide-rich cyclic peptides within the cyclotide family have been shown to possess extraordinary stability against enzymatic, chemical and thermal degradation (Colgrave & Craik, 2004). The exceptional stability combined with the possibility to accommodate different loop sequences without disturbing the overall peptide fold point towards potential pharmaceutical applications of cyclotides. A cell-penetrating cyclotide, MCoTI-II (Cascales et al, 2011; Greenwood et al, 2007), was employed as the scaffold for our grafting study. One of the grafted peptides showed significant Abl kinase inhibition in the low micromolar range *in vitro*. More importantly, the MCoTI-II mutant displayed enzymatic stability and cell penetrating properties inherited from its parent scaffold. The results suggest that this approach can be applied to develop stable, non-toxic peptide-based kinase inhibitors towards various disease-linked kinases.

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Membrane interactions of modified helical cell-penetrating peptides

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Cell penetrating peptides (CPPs) are able to translocate across cellular membranes and transport various cargoes, but the uptake mechanisms are yet not fully understood. Our goal is to determine the membrane binding properties and conformational features of two different types of CPPs, in order to correlate them with the uptake efficiency and toxicological effects. On the one hand, we compare stapled amphiphilic α -helical CPPs with their linear analogues. Furthermore, we have designed amphiphilic α -helical CPPs with a "charge zipper" motif that utilizes intramolecular ladders of salt

bridges for compensating the net charge. Circular dichroism (CD) is used to determine the extent of helicity in different lipid vesicles, and Oriented CD reveals the helix alignment in the membrane. A high-throughput screening in model membranes is used to measure the uptake efficiency of the CPP and leakage of the lipid vesicles simultaneously. These experiments allow us to detect any aggregation tendency of the CPPs and at the same time provide insight in the correlation between the uptake efficiency with peptide structure, lipid composition, and membrane curvature.

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Novel polymeric excipients for prolonged residence time

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The aim of this study was to develop and evaluate a more effective mucoadhesive thiomers for prolonged applications. Therefore, 2-iminothiolane was covalently attached to chitosan backbone. Following, a preactivation step followed, mediated by 6,6'-dithionicotinamide (6,6-DTNA), thiol groups were modified by disulfide bonds formation. Mucoadhesion studies were performed on mucosa. Additionally, water binding capacity, disintegration and cytotoxicity studies were accomplished. Chitosan-thiobutylamidine-mercaptanotinamide (Ch-TBA-MNA) displayed 1.8-fold higher stability and 1.6-fold higher mucoadhesive properties, respectively. Due to these results preactivated thiomers exhibits an improved stability and enhanced mucoadhesive properties. Therefore, preactivated chitosan seems to be advantageous administrations with prolonged residence time compared to corresponding thiomers and chitosan.

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PepFect15, a novel endosomolytic cell-penetrating peptide for oligonucleotide delivery via scavenger receptors.

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Gene-regulatory biomolecules such as splice correcting oligonucleotides and anti-microRNA oligonucleotides are important tools in the struggle to understand and treat genetic disorders caused by defective gene expression or aberrant splicing. However, oligonucleotides generally suffer from low bioavailability, hence requiring efficient and non-toxic delivery vectors to reach their targets. Cell-penetrating peptides constitute a promising category of carrier molecules for intracellular delivery of bioactive cargo. In this study we present a novel cell-penetrating peptide, PepFect15, comprising the previously reported PepFect14 peptide modified with endosomolytic trifluoromethylquinoline moieties to facilitate endosomal escape. Pepfect15 efficiently delivers both splice-correcting oligonucleotides and anti-microRNA oligonucleotides into cells through a non-covalent complexation strategy. The peptide and its cargo form stable, negatively charged nanoparticles that are taken up by cells largely through scavenger receptor type A mediated endocytosis.

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Cell-penetrating peptide-mediated delivery of telomerase inhibitors

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Targeting telomerase presents several key advantages compared to most other cancer targets in its relative universality, criticality and specificity for cancer cells including cancer stem cells. At present, there are several therapeutic approaches for targeting telomerase in tumors. One in particular, currently undergoing clinical trials, is based on synthetic lipid-modified oligonucleotide antagonists aimed at inhibiting the ribonucleoprotein subunit of human telomerase¹. However, while enabling efficient uptake, the lipid modifications reduce the potency of the therapeutic oligonucleotides compared to non-modified oligonucleotides². Non-covalent complexation strategies for cell-

penetrating peptide-mediated delivery presents an option to circumvent the need for potency-reducing modifications while allowing for a highly efficient uptake, and could significantly improve the efficiency of telomerase-targeting cancer therapeutics.

The aim of the current work is to assess the capacity for a cell-penetrating peptide designed in our lab, PepFect15³, to efficiently deliver and potentially improve upon the potency of telomerase-inhibiting oligonucleotides.

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D-PYC98, a novel substrate-selective peptide inhibitor of c-Jun N-terminal Kinase (JNK)

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Protein kinase have remained attractive drug targets in the treatment of various pathological conditions with recent attention moving from ATP-competitive inhibitors towards the discovery and development of peptide inhibitors targeting the protein kinase substrate-binding site. Initially discovered in yeast two-hybrid screen, PYC98 is a JNK inhibitory peptide with a 5-fold increased potency to inhibit c-Jun phosphorylation in its D- retroinverso form. *In vitro* JNK activity assays revealed that D-PYC98 inhibited phosphorylation of an EGFR-derived peptide substrate, transcription factors c-Jun and ATF2, and the microtubule-regulatory protein DCX (doublecortin), but not phosphorylation of the transcription factor Elk1 or the microtubule-destabilising proteins SCG10 and Op18 (Stathmin). Surface Plasmon Resonance analysis confirmed D-PYC98 binding to both non-phosphorylated (inactive) and active JNK1, but not the substrates c-Jun and Elk1. Further biochemical analyses to determine the kinetics of inhibition revealed the non-ATP competitive mechanism of action of D-PYC98. The targeting the common docking site of JNK1 by D-PYC98 was further confirmed by BIAcore competition assay in presence of TIJIP and the failure of JNK1 common docking site mutants (JNK1-R127A-E329A) to bind D-PYC98. Lastly, in evaluating the efficacy of this peptide to act as a substrate competitive inhibitor in cell culture system, we observed the action of a cell-permeable version of D-PYC98 (D-PYC98-TAT) to inhibit c-Jun phosphorylation at Ser63 under hyperosmotic stress conditions. Overall, D-PYC98 is a novel substrate-selective, non-ATP competitive inhibitor of JNK, and in its modified form as D-PYC98-TAT represents an effective JNK-inhibitor suitable for use as a probe to discern particular biological functions of this important stress-responsive kinase in cellular systems.

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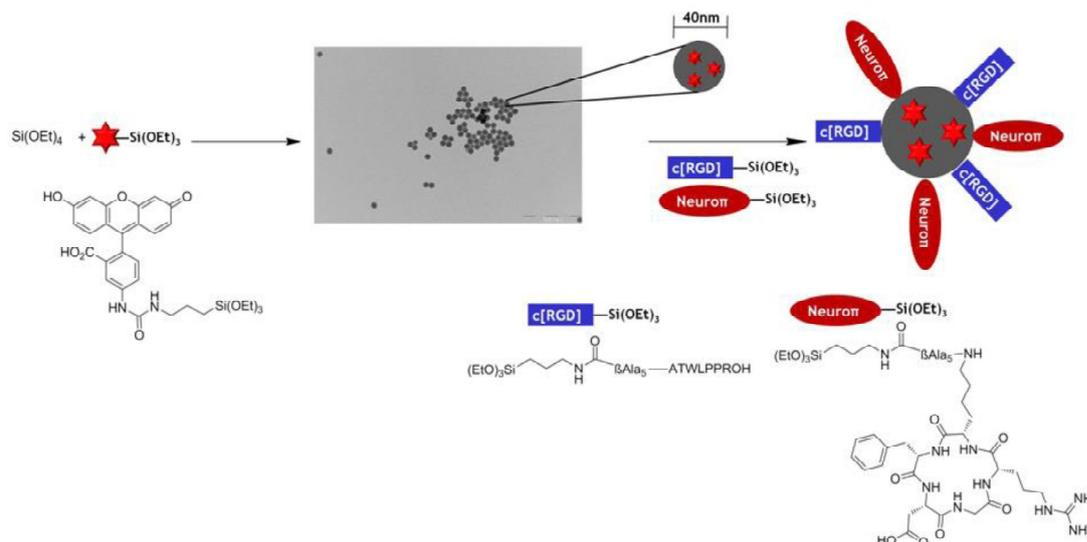
Multifunctional silica nanoparticles obtained in one step with hybrid trialkoxysilyl-peptides

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Drug delivery systems have continuously evolved to improve the efficiency of the cancer treatment. In this field, silica nanoparticles (SiNPs) are a class of promising nanomaterials.¹ Their easy synthesis by sol-gel condensation, tuneable size control, high surface areas, chemical stability, functionalizable surfaces and biocompatibility³ make SiNPs adequate tools for theranostic applications. In this context, we develop an efficiency strategy to obtain in a single step, multifunctional silica nanoparticles. It relies on the use of trialkoxysilyl-peptide hybrid building blocks developed in our laboratory⁴². Noteworthy, the use of the hybrid peptides do not require any prior functionalization of the silica surface, nor chemoselective reaction, and proceeds at RT in water.



Noteworthy, the derivatisation of fluorescein with trialkoxysilane moiety, enables the direct synthesis of fluorescent NPs, avoiding any unwanted bleaching.

The synthesis of hybrid alkoxy silane bioactive peptides and enzyme-sensitive sequences for a releasing system based on shell degradation is reported. At last, the simple grafting of SiNPs using sol-gel process will be presented.

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More Light, Greater Sensitivity - Revolutionary New LVF Monochromator™ Technology in the CLARIOstar® Microplate Reader

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Monochromators are wavelength selection devices that can be used in a microplate reader. With monochromators, users can conveniently type in any wavelength for excitation or emission, whereas with filters, it is necessary to have the correct hardware. A major drawback to conventional monochromators, however, is that they are not sensitive enough to perform all of the leading microplate reader applications, like filters.

In this poster, BMG LABTECH introduces a new, advanced type of double monochromator to microplate reading instrumentation, the LVF Monochromator™. Able to use adjustable bandwidths up

to 100 nm, LVF Monochromators™ can have significantly greater light transmission than conventional monochromators. In addition, this new monochromator technology is based on linear variable filters, which avoid the inherent stray light encountered with conventional monochromators. The result is more light transmission and greater sensitivity in all fluorescence and luminescence assays.

A new strategy for on line sequencing of peptides bearing disulfide bridges

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The characterization of peptides bearing multiple disulfide bridges requires sequence and a correct assignment of cysteine pairings. One major goal of the European Project Venomics is to develop a reliable high throughput method to fully characterize peptides in complex mixtures such as raw venoms. A specific emphasis is given to the determination of cysteine pairings. Ion mobility may bring an important contribution. It separates mass selected conformers on the basis of differences in drift time in the presence of collision gas. Those differences result from differences in collision cross sections that can be represented by the rotationally averaged projection of the ionic volume on a plane. Each “shape separated” peptide is then activated by CID and sequenced using MS/MS.

We modified the method to assign cysteine pairing inducing differences in cross section using electron transfer reduction. This step consists in statistically opening only one disulfide bond¹. As verified by molecular modeling, once a disulfide bridge is opened, the structure rapidly expands. CID MS/MS of the opened moiety becomes more efficient. In the case of peptides bearing two disulfide bridges, two arrival time distributions can be observed for the singly reduced species, each corresponding to a specific connectivity. On the contrary to chemical reduction in solution, disulfide bond scrambling is less likely to occur. Differences in MS/MS spectra allow assigning the S-S pairing.

Applied to crude venoms, this LC/MS-ETD-IMS-CID-MS is a promising new fast online full characterization strategy, including SS pairing assignment. Figures of merit and limitations will be commented on the basis of examples involving species of increasing mass ranging from small toxins (<4kDa) in *Conus* snails type venoms to larger peptides (>4kDa) from snake venoms.

1. Ion Mobility Mass Spectrometry as a Potential Tool To Assign Disulfide Bonds Arrangements in Peptides with Multiple Disulfide Bridges J. Echterbille, L. Quinton, N. Gilles, and E. De Pauw Anal. Chem., 2013, 85 (9), pp 4405–4413

Cyclotide Discovery by Peptidomics and Transcriptome-Mining

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Cyclic cysteine-rich plant peptides (cyclotides) are found in many plant species of the *Rubiaceae*, *Fabaceae*, *Violaceae*, *Cucurbitaceae*, *Solanaceae* and *Poaceae* families. Despite promising applications as drug templates, little is understood in cyclotide's distribution, evolution and variability in plants and of in planta bioprocessing. Widely recognised identification criteria in peptidomics for cyclotide research are their strong retention in reversed phase chromatography, a molecular mass of 2500-4000 Da, a head-to-tail cyclized backbone and a knotted arrangement of six conserved cysteines, nevertheless a valid identification relies on *de novo* sequencing of at least two adjacent inter-cysteine loops of the peptide¹. However, cyclotide screening is prone to false-negative or false-positive hits. Hence, more robust criteria for large scale screening studies are of interest in cyclotide research². Here we present a combined peptidomics/transcriptomics approach to overcome limitations in conventional cyclotide discovery³. Applying this refined identification workflow, ~300 species of >40 plant families with focus on *Rubiaceae* species were evaluated. Altogether six full

sequences, ten partial sequence tags and nine new cyclotide expressing families all from the *Palicoureeae* tribe were identified for the first time. Two novel sequences from *Carapichea ipecacuanha* lack of the highly conserved glutamic acid, thus our finding underlines the little understood plasticity of these plant peptides. In particular transcriptome mining provides the full primary sequence and eliminates false-negative hits. Besides phylogenetic insight into cyclotides distribution in *Psychotria* species, comparison of cyclotide precursor sequences amongst the *Rubiaceae* family revealed a moderate variability for several amino acid residues on the proto-N and proto-C-termini previously described as highly conserved due to their involvement in planta bioprocessing. In conclusion, combined peptidomics/transcriptome analysis is a powerful tool to shed light on the distribution, evolution and diversity of circular, disulfide-rich peptides within the plant kingdom and enables targeted screening to accelerate peptide discovery.

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Doping control analysis of bioactive peptides in horse urine and plasma by liquid chromatography – high resolution mass spectrometry

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The abuse of performance-enhancing peptides is a threat to the integrity of both equine and human sports. Last year, a large number of post-race samples from different horse racing jurisdictions in the USA were reported positive for dermorphin (aka “frog venom”), a potent painkiller¹. Besides, the Australian Crime Commission has recently reported the widespread use of peptides and hormones by professional athletes in Australia². In order to tackle this challenge, the author’s laboratory has established a method to detect seven bioactive peptides and their *in vitro* metabolites in horse plasma³. This method has recently been expanded to cover a total of 16 bioactive peptides (namely, AOD9604, -casomorphin, bremelanotide, CJC-1295, dermorphin, desmopressin, GHRP-1, GHRP-2, GHRP-6, hexarelin, hyp⁶-dermorphin, ipamorelin, morphiceptin, N-acetylated LKKTETQ (active ingredient of TB-500), selank, and triptorelin). In addition, another method has been developed to detect 15 of the above peptides (excluding CJC-1295) and -casozepine in horse urine. This paper describes these two methods for the simultaneous screening of 16 bioactive peptides and some of their *in vitro* metabolites in horse urine or plasma. Urine and plasma extractions were performed using different mixed-mode ion exchange solid-phase extraction cartridges. The final extracts were subject to ultra-high performance liquid chromatographic separation on a C18 column and then analysed with LC/HRMS in positive ESI mode. The limits of detection for the target peptides were within 50 – 200 pg/mL in urine or plasma. The applicability of these multi-analyte methods was demonstrated by the identification of N-acetylated LKKTETQ and/or its metabolites in urine and plasma obtained after subcutaneous administration of TB-500 to two thoroughbred geldings. Method validation with respect to specificity, precision, recovery and ion suppression effect will be presented. A comprehensive *in vitro* study to identify potential metabolites useful for the control of these bioactive peptides will also be described.^{βα}

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Multi-state Membrane Perturbation Mechanisms of Antimicrobial Peptides

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Multi-drug resistance (MDR) bacterial infections represent a major global health problem with increasing rate of mortality. Antimicrobial peptides (AMPs) targeted to destroy bacterial membranes have great potential as effective antimicrobial agents against MDR infections. Due to our limited understanding of the precise mechanism of action of AMPs required for improving therapeutic efficacy, no antimicrobial peptide has been approved as a drug against bacterial infection in over a decade. While alteration of the molecular organisation of lipid molecules is the major effect of AMPs resulting in loss in membrane functionality, this phenomenon is rarely measured. We have developed membrane chip technology combined with dual-polarisation interferometry (DPI) to simultaneously measure the mass bound to and the structural ordering (birefringence) of the membrane. The interaction characteristics of melittin, magainin and its analogue, HPA3, aurein 1.2, maculatin1.1 with supported planar DMPC, DMPC/DMPG (4:1), POPC, and POPC/POPG (4:1) bilayers show various degrees of disordering at different levels of bound peptide. These data allow us to determine the P/L ratio for critical threshold events corresponding to distinctive membrane structural changes related to surface binding, insertion and disruption. Further analysis of the real-time binding profiles using our recently-developed "combined mass and structural multi-state kinetics program", reveal a three-state binding process with bilayer expansion on a POPC bilayer while at least a two-state binding process for DMPC bilayers. Overall, studying the perturbation mechanisms induced by peptides over a range of mass-density loadings provides further understanding of the mechanisms of membrane-active peptides. We also show for the first time multi-state kinetic models for simultaneous changes in bilayer structure and membrane-bound peptide which can be used to map the route of membrane destabilisation required for the rational design of membrane-destructive antimicrobial agents.

ISD high-throughput platform for the sequencing of animal peptide toxins

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Animal venoms are complex mixtures, comprising a wide range of biologically active reticulated peptides that target with high selectivity and efficacy a variety of membrane receptors. Assuming the fact that each of the 170,000 venomous species reported can produce more than 250 bioactive toxins, at least 40,000,000 bioactive peptides/proteins may be discovered. Following this idea, a FP7 European Project called VENOMICS (<http://venomics.eu/>) was proposed in November 2011. Its goal is to construct a bank of 10,000 toxin sequences generated from the study of 200 selected venomous species. This bank will be exploited to discover new peptides that can potentially be used as human drugs. High-throughput toxin sequencing represents one of the main bottlenecks of the project.

This work shows the strategy designed to generate high-throughput accurate sequence tags from crude venoms. The sequencing strategy rests on a chromatographic purification of the venoms followed by MALDI-In-Source Decay fragmentation. ISD has already been demonstrated efficient for toxin sequencing¹, and especially when using 1,5-DAN as a reducing matrix. Even if this technique requires simple peptide mixtures, reached by LC-MALDI, ISD yields to sequence tags that cover more

than 70% of the full toxins, even for high molecular mass toxins (>6500 Da). Additionally, ISD spectra are simpler to interpret than classical MS/MS ones. Indeed, when using 1,5-DAN, ISD generates intense singly charged c-type ions that can be easily exploited to sequence unknown peptides. This property permitted the development of in-house software, dedicated to the automated spectra interpretation. The software firstly treats the spectra with a smart smoothing, then normalizes the peaks and finally analyzes the spectra to propose relevant sequence tags. A score is calculated for each tag, on the basis of the intensity of the fragments and on the difference between the measured amino-acid masses and the theoretical ones.

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Cyclization of the antimicrobial peptide gomesin with native chemical ligation: Influences on stability and bioactivity

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Gomesin is an antimicrobial peptide which contains two disulfide bonds (CysI-CysIV and CysII-CysIII) in a beta-hairpin structure.¹ Poor stability often hinders potential peptides such as gomesin for further development into drug candidates. There are many ways to improve peptide stability, one possible way is through backbone cyclization. An example on using this approach is a study based on a small bioactive peptide, Vc1.1, which derived from the cone snail venom.² Improvement on stability and bioactivity were observed after cyclization of Vc1.1. Given the close proximity of the N and C termini of gomesin, cyclization approach was utilized in this study to assess if similar improvements could be observed. Cyclization was done by substituting the pyro-glutamic acid residue in the sequence with a glycine residue as a linker and using native chemical ligation³ method to link both N and C termini. A range of in vitro assays including membrane binding study were carried out to compare the bioactivity of the linear and cyclic forms of gomesin. Structural studies by NMR were also carried out to characterize the cyclic form of gomesin. In summary, this study has provided additional insights into the range of improvements in bioactivity and stability that can be achieved as a result of backbone cyclization of peptides. P. I. Silva, Jr., S. Daffre, P. Bulet, *J. Biol. Chem.* 2000, 275, 33464-33470.

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Identification of Novel Anti-Viral Peptides Against Dengue Virus Serotype 2 By Using Peptide Phage Display Technique.

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Dengue is an important tropical and subtropical viral disease caused by the infection of any one of the 4 dengue virus (DENV) serotypes (DENV 1, 2, 3, and 4). In human, DENV infection causes a

spectrum of illness ranging from mild symptoms to life threatening dengue shock syndrome^[1]. Treatment for DENV however remains limited to supportive care with emphasis on fluid therapy and management of its complications. No specific anti-viral drug is currently available to treat dengue infection^[2]. In the current study, peptide phage display methodology was applied to screen for novel anti-viral peptides against DENV. This technique allows high throughput screening billions ($10^6 - 10^{11}$) of components with a fast selection procedure to identify novel lead candidates. In this study, DENV was cultured in Vero cells and purified for peptide screening assay. A random dodecapeptide and loop-constrained random heptapeptide library displayed on filamentous bacteriophage M13 were interacted with the purified DENV. 3 rounds of biopanning procedure were carried out and blue plaques were selected. A total of 18 different gene sequences from blue plaques were obtained and analyzed. Further studies were carried out to test for cytotoxicity and antiviral properties of the 18 different peptide conjugated phages by using WST-1 assay and plaque reduction assay, respectively. Phage that showed highest inhibitory property was subsequently synthesized. Synthesized peptide (SP) was tested for cytotoxicity (WST-1 assay) and antiviral properties (plaque reduction assay and real-time pcr). No significant cytotoxicity was observed up to 1mM while 78% viral inhibition was achieved at 50 μ M. It is hoped that continuation and further optimization of current study would reveal potent peptides with anti-viral property for the development of new drugs for the control of DENV.

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Proteomic analysis of the venom from the giant ant *Dinoponera quadriceps*: A comparative study and characterization of the major components of the venom derived from 4 distant areas of Brazil

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Ants from the genus *Dinoponera* are believed to be the world's largest living ants with a body length of 3cm. Their occurrence is restricted to tropical areas of South America. Despite the noxious effects inflicted by its envenomation, the information about the biological properties and composition of their venom is still limited. In this work, we study the venom of the giant *Dinoponera quadriceps* ant collected in 4 different regions of Brazil. By using combinatorial mass spectrometric approaches, we aim at: (i) characterizing the venom composition of these ants; (ii) establishing a comparative analysis of the venom from the 4 different regions. The ants were captured in the surroundings of Contendas, Manoel Vitorino, Caetite and Feira de Santana (Brazil). Venom was extracted by mechanical stimulation and then dried. An aliquot of each sample was analyzed by MALDI-TOF/TOF and nanoLC-ESI-Q-TOF. The combinatorial mass spectrometry analyses demonstrate that ant venom is a copious source of new compounds. Several new peptides were identified and selected for "de novo sequencing". Since most of our new peptides showed similarities with antimicrobial peptides (AMPs), antimicrobial assays were performed with the purpose of evaluating their activity. The biological assays were carried out on a broad spectrum of microorganisms, including Gram-positive and Gram-negative bacteria, yeasts and fungi. In regard to the comparative study, we observed considerable differences in the venom composition which could be linked to an adaption to the environment. Concerning the biological assays, the peptides called Dq-3162 and Da-3177 showed a wide-ranging antimicrobial activity. In an increasing antibiotic resistance era, the description and characterization of new AMPs with a broad spectrum of activity and different scaffolds may aid scientists to design new

therapeutic agents and understand the mechanisms of those peptides to interact with microbial membranes. The results obtained pinpoint the biotechnological potential of ant's venom.

Novel antibacterial peptide against oral pathogens isolated from *Jatropha curcas* L.

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The increasing tendency of microbial infections, rapid emergence of drug-resistant to recent antibiotics and quick evolution through mutation are of great threats to control of microbial infection. It has generated the urgency to develop new class of antibiotics. Antimicrobial peptides (AMPs) are the most important and effective antibiotics to combat the increasing emergence of drug resistant bacteria. In plants, several molecules are responsible for defense having known function as antimicrobial peptides (AMPs). The plant species used in this study is *Jatropha curcas* L., the twigs of *J. curcas* are used for tooth brushing from the ancient times. A novel antimicrobial peptide isolated by RP-HPLC from *Jatropha curcas* L and molecular weight was determined in MALDI-ToF mass spectrometry, designated as *Jc*-AMP. It has excellent activity against *S. mutans* and *S. epidermidis*, human oral pathogenic bacteria. The MIC and MBC values of the *Jc*-AMP were 2 and 4 µg/mL, respectively. *Jc*-AMP showed antimicrobial activity by increasing the membrane permeability through the degradation of cell membrane of *S. epidermidis* and *S. mutans*. It also inhibits the biofilm formation of *S. mutans* and has no adverse effects on red blood cells. *Jc*-AMP showed a hydrophobic ratio of 64% and a boman index of -1.03 kcal/mol. Electron microscopy and membrane permeability assay (FACS) confirm the formation of pores or channels in the bacterial cell membrane through hydrophobic surface of the peptide.

Key words: Antibacterial peptides, *Jatropha curcas*, hydrophobic, membrane permeability.

Better activity of shorter cathelicidin-derived peptide LL-31 against *Burkholderia pseudomallei*

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Burkholderia pseudomallei (BP) is causative agent of melioidosis, a disease endemic in tropical area such as in Southeast Asia and Northern Australia. Previous reports showed that cathelicidin-derived peptides LL-37 and its truncated LL-31 could inhibit growth of BP and LL-31 showed better activity than LL-37. This study aimed to elucidate the mechanism of action that allow better killing activity of LL-31 when comparing to LL-37 on live BP and membrane model, LL-31 showed better killing activities on BP than LL-37. In LPS-binding assay, LL-31 exhibited 9 to 14% displacement of PMB-BY whereas LL-37 exhibited only 3 to 5%. For the membrane permeabilization study, LL-31 showed slightly stronger in both outer and inner membrane permeation than LL-37, as observed with NPN-uptake assay and SYTOX[®] green influx in both cases. In addition, testing membrane fluidity by change in TMA-DPH and DPH anisotropy, results of this study showed that LL-37 could induce a change in membrane fluidity more effective than that of LL-31 through observed change in TMA-DPH anisotropy and DPH anisotropy. The leakage activity of ANTS/DPX liposome induced by LL-37 and

LL-37 were about 55% and 50%, respectively. The hydrophobicity (H) and hydrophobic moment (μH) of LL-31 were more than those of LL-37. Taken together, we demonstrate better activity of LL-31 was through its better permeabilizing ability due to more hydrophobic content.

Additive inhibitory effect of combined peptides with ATPase inhibitor, membrane permeabilizing agent, or detergent against *Burkholderia pseudomallei*

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Burkholderia pseudomallei (BP) is the causative agent of melioidosis, the endemic disease causing septic shock in Southeast Asia and northern Australia. BP is intrinsically resistant to conventional antibiotics, resulting in difficulty in treatment. Antimicrobial peptides (AMPs) are alternative to classical antibiotics and does not cause resistance due to different mode of action. Combination effect between AMPs and other agents have been proved to increase the bacterial inhibitory effect. This study aimed to determine antimicrobial activity of AMPs alone and combination of AMPs with ATPase-inhibiting agent (NaN₃) or detergent (TritonX-100 and Tris) on live BP. The selected peptides were bactenecin (RLCRIVVIRVCR-NH₂), BMAP-18 (GRWKRWRKKWKKLWKKLS-NH₂), CA-MA (KWKLFKKGKFLHSAKKF-NH₂), and RTA3 (RPAFRKAAFRVMRACV-NH₂). When testing AMPs without combination, bactenecin exhibited strongest inhibition as seen in percentage of killing. When combining peptides with NaN₃ or Tris, all combination exhibited stronger inhibitory effect than treating with peptides or the agents alone. However, the only CA-MA combining with TritonX-100 exhibited stronger effect than the peptides or the agent alone.

Fatty acid chain length determines antimicrobial activity of the lipopeptide C_x-KYR

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The increasing resistance of microbe to available antibiotic is a major concern, leading to seek for a promising new generation of antimicrobial compounds. Alternatives to classical antibiotics are needed and peptide antibiotics are promising as one of the choices. Two promising families of drugs that meet these criteria are host-defense cationic antimicrobial peptides (AMPs) and lipopeptides. Most lipopeptides consist of a short linear or cyclic peptide sequence, with either a net positive or negative charge and a long fatty acid (8-18 carbon atoms) attached at the N-terminus with covalent bond. Many studies have shown that an amphipathic structure and hydrophobicity of the peptide are important for the biological function of antimicrobial peptides. However, the factors that dictate their cell selectivity are not yet clear. In the present study, we investigate the activity of KYR sequence and the attachment of fatty acid with different lengths as decanoic acid (C₁₀), lauric acid (C₁₂), myristic acid (C₁₄) and palmitic acid (C₁₆) at the N-terminus of the KYR sequence to compare effect of aliphatic acid length and addition of hydrophobicity of ultrashort peptide activity. The results reveal that: (i) increase in chain length of C_x-KYR increased lytic activity against different type of microbes; (ii) In the time killing kinetic assay, C₁₆-KYR at 20 μM could completely inhibit the growth of *Staphylococcus aureus* 1466 and *Escherichia coli* O157:H7 within 1 hour; (iii) All lipopeptides could depolarize the membrane of bacteria.

Competitive lipid membrane environments to better mimic the *in vivo* situation

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By combining vesicles of a particular lipid composition that contain the fluorescent dye calcein with calcein-free vesicles, the activity of an antimicrobial peptide having a known pore-forming activity has been investigated within a competitive lipid environment. This novel approach allowed demonstrating the AMP's preferential affinity for a negatively charged membrane, such as bacterial membranes, than for a neutral membrane, such as prokaryotic cell membranes, in a single experiment despite the AMP was dramatically more active in eukaryotic-like membrane environment only than prokaryotic-like only. This approach can also be used to probe the binding strength in a competitive lipid environment. By pre-incubating the peptides with a calcein-free lipid environment and then introducing another lipid environment containing the dye, the pore-forming AMP was shown to traffic from the eukaryotic-like membrane interface to the prokaryotic-like interface, although it had formed a pore in the previous environment.

This result questions the value of the therapeutic index, which are often measured in infected and healthy cells separately. It also opens further possibilities in testing for instance the impact of membrane curvature in modulating membrane-active peptides or proteins activity.

Chemical interference with HLA-peptide binding explains the majority of immune mediated adverse drug reactions

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Human leukocyte antigens (HLAs) are highly polymorphic proteins that initiate immunity by presenting pathogen-derived peptides to T cells. HLA polymorphisms mostly map to the antigen-binding cleft, thereby diversifying the repertoire of self-derived and pathogen-derived peptide antigens selected by different HLA allotypes. A growing number of immunologically based drug reactions, including abacavir hypersensitivity syndrome (AHS) and carbamazepine-induced Stevens-Johnson syndrome (SJS), are associated with specific HLA alleles. However, little is known about the underlying mechanisms of these associations, including AHS, a prototypical HLA-associated drug reaction occurring exclusively in individuals with the common histocompatibility allele HLA-B*57:01, and with a relative risk of more than 1,000. We show that unmodified abacavir binds non-covalently to HLA-B*57:01, lying across the bottom of the antigen-binding cleft and reaching into the F-pocket, where a carboxy-terminal tryptophan typically anchors peptides bound to HLA-B*57:01. Abacavir binds with exquisite specificity to HLA-B*57:01, changing the shape and chemistry of the antigen-binding cleft, thereby altering the repertoire of endogenous peptides that can bind HLA-B*57:01. In this way, abacavir guides the selection of new endogenous peptides, inducing a marked alteration in 'immunological self'. The resultant peptide-centric 'altered self' activates abacavir-specific T-cells, thereby driving polyclonal CD8 T-cell activation and a systemic reaction manifesting as AHS. We also show that carbamazepine, a widely used anti-epileptic drug associated with hypersensitivity reactions in HLA-B*15:02 individuals, binds to this allotype, producing alterations in the repertoire of presented self peptides. Our findings simultaneously highlight the importance of HLA polymorphism in the evolution of pharmacogenomics and provide a general mechanism for some of the growing number of HLA-linked hypersensitivities that involve small-molecule drugs.

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Evidence for the Antagonistic Form of CXCL10 in Ovarian Cancer

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Patients with high grade, serous epithelial ovarian carcinoma (HGSOC) are generally diagnosed with extensive peritoneal metastases, and exhibit 5-year survival rates <30%. A subset of these tumours over-express mRNA encoding the T-cell-recruiting chemokine CXCL10. Whilst tumour infiltrating CD4⁺/CD8⁺ T-cells are generally associated with good prognosis, patients diagnosed with HGSOC typically exhibit poor survival. Recently an "antagonistic" CXCL10 variant - modified by the enzyme Dipeptidyl Peptidase 4 (DPP4) - was identified as an *in vivo* inhibitor of leukocyte recruitment (*J. Clin. Invest.* 121, pp.308-317). We hypothesized that some HGSOC's might also express antagonistic CXCL10, interfering with leukocyte recruitment and contributing to poor patient prognosis.

We analyzed the expression, localization and association of CXCL10 with CD3+ T-cells in HGSOC tissues grouped according to pathology, grade and FIGO stage at diagnosis. CXCL10 was increased in a subset of HGSOC samples, and correlated with CD3+ tumour infiltrating T-cells in benign disease but not in malignancy. Immunoprecipitation and *de novo* sequence analysis of CXCL10 from patient samples identified the N-terminally cleaved, "antagonistic" variant in malignant tumours only. Furthermore, the cleaved variant was localized to the tumour epithelium by Imaging Mass Spectrometry. The presence of cleaved CXCL10 in tumour epithelium also correlated well with the presence of DPP4.

Our data demonstrate that antagonistic CXCL10, known to inhibit (rather than promote) leukocyte recruitment, is present in serous ovarian tumours and is associated with decreased leukocyte infiltration. We suggest that represents a previously unrecognized mechanism by which tumours might partially attenuate the early anti-tumour immune response, and is a plausible explanation for the commonly poor prognosis experienced by these patients. This finding provides new insight into ovarian tumour progression, and may lead to novel therapeutic interventions.

Immunosuppressive mechanism and properties of circular plant peptides

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The immune system is in a stringent vigilance recognizing and defending endogenous and exogenous threats. In case of an over-reactivity, as in autoimmunity, self-reactive T-cells or antibodies target the body's own cells and tissues and may cause severe organ damage even leading to death. Therefore several immunosuppressive treatment options are available to medicate autoimmune diseases. One of these inhibitory agents is cyclosporine A, a naturally-occurring circular depsipeptide, which interferes in interleukin-2 signaling in T-lymphocytes to prevent proliferation. However this and other treatment options often produce severe and life-threatening side-effects.

Recently we identified circular peptides from plants, which exhibit promising anti-proliferative effects towards T-cells¹. These so-called cyclotides are composed of about 30 amino acids with six conserved cysteines that are arranged in a typical cyclic cystine-knot motif, which confers them with resistance to enzymatic, chemical or thermal degradation, making them attractive pharmaceutical tools². The aim of this study is to investigate the immunosuppressive properties of native and synthetic cyclotides and its underlying mechanism in human and mouse immune cells *in vitro* and to compare their mode-of-action to known immunosuppressive drugs. Furthermore we want to examine a proliferation inhibition *in vivo* using a murine autoimmune model.

We were able to demonstrate an interleukin-2 specific anti-proliferative effect of cyclotides towards human activated lymphocytes *in vitro*³. This significant inhibition was dose-dependent without inducing cytotoxicity in the active concentration range. To test the therapeutic impact *in vivo* an experimental autoimmune encephalomyelitis mouse model was performed. Here a significant delay

and minor symptoms of disease in mice treated with cyclotides were observed. This anti-proliferative effect could additionally be confirmed in isolated restimulated mouse splenocytes *in vitro*. Plant cyclotides, due to their unique structural topology, have great potential as peptide-based drugs and display promising immunosuppressive properties towards lymphocytes *in vitro* and *in vivo*.

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A robust affinity chromatography for antibody purification based on IgG Fc-binding peptide

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Use of antibodies is rapidly increasing in therapy and diagnosis and technology relevant to antibody production and purification is of great academic and industrial interest. Although bacterial protein A and protein G have been widely used as affinity ligands for antibody purification, use of these proteins has many disadvantages leaving much room for the development of small ligands. We have previously described an IgG Fc domain-binding cyclic peptide conjugate (FcBP) consisting of 13 amino acids and a soluble surface linker, which was used for the efficient capture of antibodies on a surface plasmon resonance (SPR) chip. In the present study, the peptide was immobilized on Sepharose resin and packed into columns, and the use of such columns for antibody purification was investigated.

All four columns packed with FcBP, reduced FcBP (FcBP-Red), protein A, or protein G showed good binding capacities for a human IgG, Herceptin. Of these columns, FcBP-Red allowed antibody purification at a less acidic pH (pH 2.8) than was required by the protein affinity columns (pH 2.1 and 2.3 for protein G and protein A columns, respectively). The use of FcBP column also purified antibody of swine serum to a level as pure as that obtained from the protein columns, and the regenerated FcBP column operated 30 runs without loss of efficiency, which is the maximum number performed in the present study. These results demonstrate that FcBP, used as a robust affinity ligand for antibody purification, is a viable alternative compensating the disadvantages associated with protein affinity ligands such as protein G and protein A.

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PK20, a novel enzymatically stable hybrid compound that induces antinociceptive responses by bearing a full agonist activity at both opioid and neurotensin receptors

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The clinical treatment of various pain states relies upon opioid analgesics. However, most of them produce – apart from strong antinociceptive effect – several side effects such as sedation, euphoria, constipation, and finally development of dependence and addiction¹⁻³. One strategy to overcome these major side effects and to prolong the antinociceptive efficiency of the applied drugs involves the creation of multifunctional compounds, which contain hybridized structures^{4,5}.

Neurotesin (NT)-induced antinociception is not mediated through the activation of the opioid system which makes it a potentially interesting target in pain research. Additionally, hybridizing neurotensin with an opioid element may result in a potent synergic antinociceptor⁶.

Herein, we would like to present the first hybrid compound containing both opioid and neurotensin pharmacophores, PK20. This chimera was shown to induce a long-lasting and time- and dose-dependent analgesic action when administered centrally (i.t) as well as peripherally (i.v.). The high antinociceptive effect of the investigated compound was proven by comparing the potency of PK20 injected at a much lower dose than morphine (0.02nM/rat vs. 3nM/rat, resp.). At a time-point of 60min post-injection, the administered dose of PK20 induced an almost equipotent antinociceptive effect to morphine, injected at the 150-fold higher dose⁶. Interestingly, the opioid-neurotensin chimera PK20 showed full agonism at both MOP receptor (pKi of 7.09 and EC₅₀ of 79 nM) and neurotensin NTS1 receptor (pKi of 6.66 and EC₅₀ of 217 nM,) when performing the [³⁵S]GTPγS binding assay⁷.

These results give hope that more efficient opioid analgesics are accessible through the combination of the agonism at opioid and NT receptors.

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Effects of the hybridization of opioid and neurotensin pharmacophores on neurotoxicity in hippocampal organotypic cultures

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Neurodegenerative diseases are defined as hereditary and sporadic conditions which are characterized by a progressive loss of a structure or functions of neurons. In such disorders neuronal destruction is caused mainly by an abnormal elevated level of one of the primary excitatory neurotransmitters in the mammalian CNS – glutamate¹. This prolonged and excessive accumulation of glutamate causes over-stimulation of its receptors, especially of the *N*-methyl-D-aspartate (NMDA) receptor, which in consequence leads to excitotoxicity, a process well seen in CA1 area, which is known to be the hippocampal region most sensitive to excitotoxicity. According to that, several endogenous peptides have been studied for their neuroprotective profile.

The tridecapeptide neurotensin (NT) is known from the ability to induce naloxone-insensitive analgesic responses after brain injection², which makes it a promising target in treatment of various pain states. Unfortunately, NT plays an important role as a neurodegenerative factor by significantly increasing glutamate release in discrete rat brain regions and intensifying NMDA-mediated glutamate

signaling³. In contrary, our as well as others recent study regarding an opioid peptide indicated that opioids have neuroprotective behavior following NMDA exposure^{4,5}.

This information prompted us to combine an opioid and neurotensin into one entity hoping that an opioid pharmacophore may make a modulatory impact on neurotensin's neurodegenerative activity. This step seems to be understood according to our previous study reporting that a chemical hybridization of opioid and neurotensin pharmacophores resulted in hypersynergism between both parts of the novel chimera⁶.

The aim of the presented study was to investigate the possible neuroprotective effects of PK20 opioid-neurotensin hybrid peptide on glutamate-mediated CA1 region cell death in comparison with its opioid element as well as with neurotensin.

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The “Hop-step-and-jump” of Scaling Up from Analytical to Process Scale HPLC

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What works in analytical HPLC is not necessarily feasible on larger scale separation. A whole new set of considerations are necessary to perform the “hop-step-and-jump” to bridge the gap between the two HPLC techniques. The presentation aims to give practical guide, ample examples for crucial differences.

While on analytical scale almost all octadecyl bonded silica based stationary phases are practically the same, the small dissimilarities add up and make decisive difference in the economics of a real purification process. Comprehensive explanation on how bonding style and density affects the retention time resulting in different stationary phase characteristics. Mechanical strength of the silica is coming into focus in larger processes. The chromatographer faces new challenge: The necessity of self- packing the big columns on site. Large scale process HPLC users demand guidelines for CIP (Cleaning In Place) or regeneration. Regulatory issues come to play. The presentation highlights the new challenges and provides guide to successful scale up to process scale HPLC purification.

PeptiSil 10: The New Bulk Silica for Peptide Purification

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Large scale peptide production is the second biggest market for HPLC grade bulk spherical silica based stationary phases. (The biggest application is insulin manufacturing.) Reversed phase HPLC purification of peptides is a widely used technique in the purification process. Peptides with less than 6000 dalton mol. weight are purified best on 100 – 120 Angstrom pore size silica, the usual choice is 10 micron particle size with ODS (C18) modification. PeptiSil 10 is a 100A 10 micron C18 modified phase featuring extremely high alkaline resistance (it makes stronger cleaning or re-generation steps) and super mechanical strength. These two features combined promise longer life time of the silica.

High loadability makes this phase a good choice for better process economy. The creation of this new phase from concept to experimental is detailed demonstrating an example of the holistic design approach.

APR-1 and ASP-2 protein-based subunit peptide based vaccine against hookworm parasite

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Hookworm is a blood feeding helminthes that infects more than 700 million people worldwide, predominantly in regions with low socioeconomic conditions.¹ Hookworm moulds into 5 different stages in its life cycle. The different stages resulted in difficulties in selection of specific vaccine target against the parasite due its biological reformations. Thus, it is preferably to develop a vaccine that can target at least two of the five hookworm stages to combat the hematophagous parasite.

Two critical hookworm enzymes were found to be essential in feeding and growth. Hookworm larvae (L3) utilizes aspartic enzyme (ASP-2) during its growth into adult hookworm while adult hookworm exploits aspartic enzyme (APR-1) for haemoglobin digestion during feeding (Figure 1). Vaccine candidates based on these two proteins resulted in reduced faecal egg counts^{2,3}; however, APR-1 autodegrades rapidly³ while ASP-2 vaccine trial to individuals previously infected by hookworm resulted in immediate-type hypersensitivity.⁴ To overcome these problems, subunit peptide based vaccine approach utilizing minimal peptide sequences from the proteins, showed promising results.⁵

With the aim to conserve the subunit peptides' secondary structure, a series of subunit peptides vaccines based on APR-1 and ASP2 were designed. To enhance the antigen's efficacy, peptide sequence modifications and conjugations to lipid core peptide (LCP) delivery system were performed. The peptide series were designed using Peptide Secondary Structure Prediction tools and homology test were performed using UniProt Blast software. LCPs and peptide epitopes were synthesized via solid phase peptide synthesis (SPPS). Products were analysed using transmission electron microscopy (TEM), dynamic light scattering (DLS), and circular dichroism (CD). We demonstrated that these vaccine candidates were able to self-assemble to form particles. The peptides were also verified to have the desired conformation upon conjugation to the LCP system.

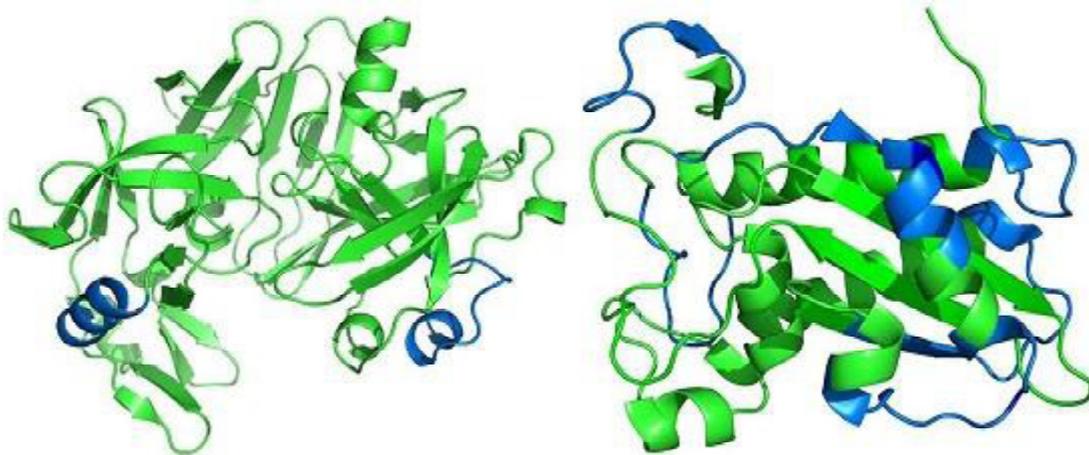


Figure 1: APR-1 (left) and ASP-2 (right) proteins used to design the vaccines. Highlighted in blue are regions corresponding to selected peptide epitopes.

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Peptide-based antibody delivery into live cell

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Antibody has become a crucial tool for the detection and regulation of protein functions. It serves as a key element in immunodetection for disease diagnosis or laboratory sensing of biomarkers. However, limited cell permeability of antibodies restricts their usage towards experiments involving cell surface, body fluids or cell lysate.

Antibody delivery into live cells can be effective tools for live cell imaging and into-cell delivery technique. Though live cells do not uptake the antibody easily, several methods for internalization of antibody into live cells have been developed including electroporation, microinjection, and transfected gene mediated intracellular expression of antibody and each method is not without some drawbacks. We have developed new antibody delivery methods using cell-penetrating peptides (CPPs) and IgG Fc domain-binding cyclic peptide conjugate (FcBP). CPPs have been employed for the cellular delivery of proteins, DNA or nanoparticles in the form of complexes. Several CPPs have been reported including penetratin, Arg8, HIV-1 TAT, MPS, etc. FcBP is a peptide ligand comprising a soluble surface linker and 13 amino acids, which specifically binds to Fc domain of IgG, and it has

been utilized for capturing antibodies on various solid surfaces. In this work, we constructed several antibody carriers, placed FcBP and CPPs via protein expression technique or chemical method using an adequate linker, and tested their usage for transduction of fluorescence-labeled antibodies. This represents the first peptide reagents enabling targeted antibody delivery into live cells avoiding any detergents or other physical methods.

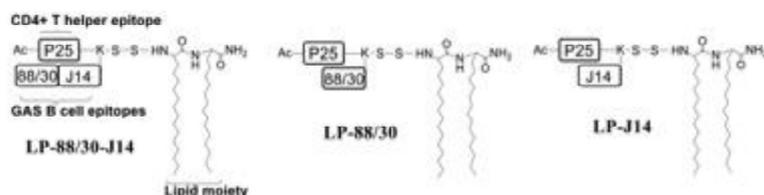
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Lipopeptide containing B-cell and T-helper epitope as intranasal vaccine candidates against Group A Streptococcus (GAS)

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Group A streptococcus (GAS) infections result in a number of human diseases, including potentially life-threatening postinfectious sequelae. To protect against infection with multiple GAS serotypes, we designed and synthesized a self-adjuvanting lipopeptide (LP)¹ GAS vaccine constructs. Each lipopeptide was composed of GAS B-cell epitopes (J14 or 88/30 or incorporating both epitopes), a universal CD4+ T-cell helper epitope (P25) and an immunostimulant lipid moiety^{2,3}. The lipopeptides were synthesized using Boc-solid phase peptide synthesis (SPPS) and were self-assembled into nanoparticles⁴. The immunogenicity of the nanoparticles was tested in mice and antibody titres were analyzed using ELISA. Systemic IgG antibody response was elicited in outbred swiss mice after intranasal immunization⁵. In comparison, 88/30 specific IgG response was higher in the analogue containing both 88/30 and J14 epitope (LP-88/30-J14) than LP containing only 88/30 epitope (LP-88/30). Physico-chemical characterization was carried out including dynamic light scattering (DLS), transmission electron microscopy (TEM) and circular dichroism spectroscopy (CD) studies. Studies exhibited that LP-88/30-J14 formed nanoparticles of smaller size (10 nm) than LP-88/30 (100 nm). CD spectroscopy was performed to analyze the peptide conformation. Results showed that none of the tested LPs formed a typical conformational secondary structure. The current study showed that the incorporation of multiple copies of GAS peptide epitopes into a single LP construct enhanced systemic IgG antibody response to epitopes of interest. Size of the lipopeptides and immune response is mediated by the choice of peptide epitopes. These findings could provide an optimal strategy for the development of a GAS vaccine.



Lipopeptide GAS vaccine candidates. The lipopeptides incorporate a helper T-cell epitope (P25), GAS B-cell epitope (J14 or 88/30), and an adjuvant

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Thioether-conjugated glycopeptide lipopeptide antitumor vaccines

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Mucin MUC1, a large membrane glycoprotein, is expressed on almost all epithelial tissues. In tumor cells, MUC1 is over-expressed and aberrantly glycosylated. So MUC1 glycopeptide is considered as promising antigen for the development of tumor vaccine. MUC1 glycopeptides have weak immunogenicity. To overcome this problem, extra stimulant is needed to induce strong immune response. And a novel thioether-ligation method for the synthesis of glycopeptide vaccines containing T-cell epitopes and the Pam3Cys lipopeptide was developed. These vaccines can induce strong immune responses and produce high levels of IgG1, IgG3, and IgG2a. Moreover, the antibodies have strong affinity to MCF-7 cells and can initiate the killing of the tumor cells through activation of the complement-dependent cytotoxicity (CDC) of rabbit.

Acknowledgments: This work was supported by the National Natural Science Foundation of China (20825206 and 21028004) and by the Sino-German Center for Research Promotion (GZ561).

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Synthesis and characterization of HIV1-Tat and bovine antimicrobial peptide indolicidin conjugated to levofloxacin

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There is a need for new drug compounds or new ways in delivering a drug to its target due to the rising trend in drug resistance¹. Levofloxacin is a potent broad-range antibiotic belonging to the class of fluoroquinolones currently used as a first-line therapy for community-acquired pneumonia²³. However, levofloxacin resistance occurs worldwide. We developed a new drug delivery method to combat antibiotic resistance by attaching peptides to the antibiotic. The peptides; indolicidin and Tat were chosen for this study as they have been known to assist in drug delivery by increasing membrane permeability. Indolicidin peptide has antimicrobial properties, however; it is also haemolytic⁴. The parent peptides, indolicidin and Tat, were synthesised using Fmoc SPPS. Subsequently, either glycine or glycolic acid linker was attached to the peptides. Conjugation of levofloxacin to the peptide via glycine linker was performed using HBTU/DIPEA. An additional catalyst, DMAP, was used alongside HBTU/DIPEA for the conjugation of levofloxacin to the peptide via ester linker. Conjugates were characterised using ESI-MS and analytical HPLC prior purification by preparative HPLC. Haemolytic assay was carried out following conjugation. The parent peptides (indolicidin and Tat) and final conjugates (levofloxacin-glycine-indolicidin,

levofloxacin-glycolic acid-indolicidin, levofloxacin-glycine-Tat and levofloxacin-glycolic acid-Tat) were successfully synthesised. The conjugation methods were optimised to attain the final compounds with moderate to high yields (44-80%) and excellent purity ($\geq 95\%$). Haemolytic assay was performed on all the 7 compounds including levofloxacin. Both indolicidin-levofloxacin conjugates were haemolytic ($11\% \pm 0.05$ and $13\% \pm 0.01$) at $100\mu\text{M}$. However, these conjugates were not haemolytic at lower concentrations while both Tat-levofloxacin conjugates were not haemolytic ($\leq 0.4\% \pm 0.01$) even at high concentrations of $100\mu\text{M}$. These new antibiotic derivatives are currently under biological evaluation to determine their antimicrobial activity. The current study has paved a novel method for drug delivery.

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N-terminal Racemisation: A New Peptide Modification

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Background

Understanding peptide stability is a key factor underpinning the use of peptides as drugs and for following changes to long-lived proteins in the human body. Physiologically, polypeptides are subject to a range of non-enzymatic modifications including truncation, deamidation and racemisation, however, it is likely that other modifications remain to be elucidated.

Methods

Peptides were exposed to prolonged incubation at 37°C , as well as to elevated temperatures, at pH 7 and the products characterised using MALDI and ESI mass spectrometry and NMR spectroscopy.

Results

Incubation of peptides resulted in racemisation of the N-terminal amino acid. The rate varied depending on the N-terminal amino acid with 38% racemisation of PFHSPSY occurring after 2 weeks at 60°C compared with 25% for SFHSPSY and 5% for AFHSPSY. Incubating PFHSPSY at 37°C resulted in $\sim 5\%$ racemisation after 8 weeks [1].

The penultimate residue also influenced racemisation, with the extent varying between 15 to 40%. A mechanism is proposed to explain this modification that involves formation of a Schiff base intermediate which may decompose via one of two pathways: one yielding a diketopiperazine and one producing a racemised N - terminus.

Conclusions

This study reports for the first time, spontaneous racemisation of the N-terminal amino acid in a peptide. While the implications for peptide structure are unknown, it is likely that having a D-amino acid at the N-terminus of long-lived proteins will inhibit cleavage by exopeptidases

Given the decades that some long-lived proteins such as those found in the brain, heart and lens are exposed to in a similar environment, it is proposed that this is a new modification to which long-lived proteins are subject. Analysis of one such long-lived protein (aquaporin 0) from aged human lenses revealed 28% conversion of the N-terminal amino methionine to the D-form.

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Evaluation of the YB-1 protein as a prognostic biomarker in breast cancer by immunohistochemistry

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The Y-box binding protein 1 (YB-1) is a multifunctional cold shock domain protein which has physiological roles in growth and development, transcription, DNA repair and translation. However, YB-1 is also widely reported to be involved in tumorigenesis with roles in tumor growth, metastasis and chemoresistance. The aim of this study is to determine the association of the expression of YB-1 in invasive ductal carcinoma tissue samples with clinicopathological parameters of breast cancer patients. Immunohistochemical staining of the YB-1 protein was performed on breast cancer tissue microarrays. The intensity of YB-1 staining and its percentage were scored and immunoreactivity score (IRS) was computed. Chi-square or Fisher's Exact Test was used to determine the association of IRS of YB-1 with parameters such as age, tumor size, mitotic index, lymph node metastasis and estrogen receptor status. The specificity of the antibody was verified by Western Blotting. Cytoplasmic staining of the breast cancer cells was observed and a higher expression of YB-1 was significantly associated with higher grade of tumor, increased mitotic index and estrogen receptor negative status. There was also an association between the expression of YB-1 and tumor size with borderline significance. Since mitotic index and tumor size are proliferation parameters, the correlation of YB-1 with a proliferation marker, proliferating cell nuclear antigen (PCNA) was evaluated. There was a significant correlation between YB-1 and PCNA. Multivariate analysis revealed that a higher expression of YB-1 increased the risk of having higher mitotic index by 4.5 times. The results showed that YB-1 is a potential biomarker for breast cancer and further studies done on this protein could give rise to a promising target for the prognosis and treatment of breast cancer.

General mechanism of matrix-assisted laser desorption/ionization in-source decay mass spectrometry: Toward a novel method for *De Novo* sequencing of peptides and proteins

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MALDI in-source decay (MALDI-ISD) is a fragmentation that occurs in the MALDI source. It has already been successfully used for the de novo sequencing of peptides and proteins, including the localization of post-translational modifications. MALDI-ISD involves the hydrogen attachment to peptides, leading the N-C α bond cleavage. The source of the hydrogen radical involved in MALDI-ISD was investigated by Takayama using deuterium-labeled peptide and/or matrix. The result clearly shows that MALDI-ISD is initiated by the hydrogen transfer from the matrix to the peptide. However, the mechanism of fragmentation that follows this transfer is not fully understood. During our investigations, we observed that the formation of ISD ions was suppressed by using ionic liquid and amorphous structure of matrix whereas strong signal were observed on sharp crystals. This seems to indicate that the initial step of MALDI-ISD forming hydrogen-abundant peptide radical mainly occurs on the matrix crystal during the dissipation of the laser energy. Resulting hydrogen-abundant peptide radical fragments into c'/z \bullet fragments pair by radical-induced N-C α bond cleavage located on the right side of radical site. Subsequently, z \bullet radicals evolve by reacting with a matrix molecule or by losing their side-chain. These two reactions competitively occur and are controlled by the collision rate in MALDI plume. This work led to new insights in the hydrogen-abundant radical formation, N-C α bond cleavage and further radical reaction processes during MALDI-ISD that will be helpful to interpret MALDI-ISD spectra of peptides and proteins.

Large Scale Targeted Protein Quantification Using HR/AM Selected Ion Monitoring with MS/MS Confirmation on a Novel Hybrid, Q-OT-qIT Mass Spectrometer

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A highly sensitive and selective data independent acquisition (DIA) workflow for large-scale targeted protein quantification was developed on the new Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer. For data independent acquisition set-up, three high-resolution, accurate-mass (HR/AM) selected ion monitoring (SIM) scans (240,000 FWHM) with wide isolation windows (200 amu) were used to cover all precursor ions of 400 – 1000 m/z. In parallel with each SIM scan, 17 sequential ion trap MS/MS with 12 amu isolation windows were acquired to cover the associated 200 amu SIM mass range. Quantitative information for all precursor ions detected in three sequential SIM scans is recorded in a single run. All MS/MS fragment information was recorded for sequence confirmation of any peptide of interest by querying specific fragment ions in the spectral library. The quantitative performances and throughput of this new approach were evaluated using various samples. Ten (10) attomole limits of detection (LODs) were observed for the isotopically labeled standards spiked in 500 ng E. coli digest matrix. Four (4) orders of linear dynamic range was observed with good precision. Highly reproducible and complete quantitative results were achieved by applying a targeted data extraction strategy after the data independent acquisition.

Analysis of protein changes during oil palm fruit ripening by 2-DGE and MALDI-ToF

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The oil palm (*Elaeis guineensis*) is the highest yielding crop in the world. The fruit is a drupe whose thick fleshy mesocarp is exceptionally rich in oil (80% dry mass). Many studies have focused on the biochemical and dynamic changes of mRNA levels of oil palm fruit ripening and this study focuses on profiling of mesocarp proteins using two-dimensional gel electrophoresis (2-DGE) and mass spectrometry approaches. Oil palm fruits were collected at various stages starting 10 week after anthesis (WAA) to complete ripe stage of 20 WAA. Total proteins were extracted using TCA-acetone precipitation and individual proteins were separated by 2-DGE. In this study, changes in the oil palm mesocarp proteins expression between 18 and 20 WAA were reported. More than 60% of up- and down-regulated proteins were successfully identified and majority of the proteins were linked to carbohydrate, amino acid and xenobiotics metabolisms. Fewer proteins related to energy, lipid, secondary; and cofactors and vitamins metabolisms. Further analysis is being conducted for a better understanding on the regulatory mechanisms that function during the maturation and ripening in the oil-rich mesocarp.

Elucidating the envenomation strategy of cone snails through integrated Venomics

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Cone snails are renowned for their deadly envenomation strategy where small neuro-active peptides called conotoxins are injected into prey and/or predators producing rapid paralysis. Increasingly

sensitive proteomics techniques have revealed they can produce thousands of conotoxins along their venom duct, with different sections expressing different toxins. However, the injected venom represents only a small subset of the total venom complement. Therefore, understanding envenomation strategy requires characterisation of the injected venom in conjunction with duct venom. Here we present for the first time, the proteomic and transcriptomic characterisation of the injected venom of two worm-hunting cone snails *Conus vexillum* and *Conus capitaneus* of the Rhizoconus clade through the integration of highly sensitive LC-MS and LC-MS/MS data with RNA-Seq powered by 2nd generation sequencing on the 454 platform. Surprisingly, the injected venom was the simplest discovered thus far, being dominated by conotoxins belonging to the D-superfamily that are active on mammalian nicotinic acetylcholine receptors. We also report the presence of a peptide belonging to the con-ikot-ikot superfamily that was previously discovered in fish-hunting cone snails found to be active at the AMPA receptors. This illustrates the ability of cone snails to exquisitely target receptors that have vital physiological functions in prey to induce rapid paralysis with both nAChRs and AMPA type glutamate receptors shown to have important functions in worms. Interestingly, the injected venom peptides were predominantly expressed within the proximal sections of the venom duct along with lower levels of numerous other peptides. Thus, despite the expression of hundreds of peptides within the venom duct only a few were injected, suggesting the existence of a hitherto undescribed toxin selection mechanism(s).

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Analysis of Differentially Expressed Proteins During Early Oil Palm (*Elaeis guineensis* Jacq.) Fruit Development

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The fruit is a highly specialized plant organ as it provides suitable environment for seed maturation and also dispersal mechanisms. Fruit development involves fruit set, cell division and establishment of tissue identity, cell expansion, maturation and ripening. These processes occur in a successive way suggesting an underlying developmental program. One of the critical stages to fruit development is the fruit set which determine the fruit developmental program to continue or abort. Oil palm (*Elaeis guineensis* Jacq.) is an economically important crop and accumulates up to 90% of oil in its fruit. Despite its scientific and economic interests, information about how the fruit develop and the molecular, cellular and physiological events of its growth are becoming available more recently. To add to the array of information, a protein approach was undertaken to study proteome changes during early oil palm fruit development. In this study, differentially expressed proteins between early fruit tissues of oil palm (*Elaeis guineensis* Jacq.) at stages 2, 5 and 8 weeks after pollination (WAP) were identified and characterized. Proteins were extracted using the phenol-based method and subjected to two-dimensional gel electrophoresis. Extracted proteins were resolved by using two-dimensional gel electrophoresis in the 4 to 7 pH range and stained with Coomassie Brilliant Blue-250. The differentially expressed proteins were excised and digested with trypsin and then identified with the matrix-assisted laser desorption ionization-time of flight tandem mass spectrometry (MALDI-TOF/TOF-MS). The identified proteins were mostly involved in metabolism, growth and developmental processes, cellular biogenesis and response to stimuli. This proteome analysis provides some information on early oil palm fruit development that could be linked up with previously reported transcriptome study.

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Methodical Guide for Stationary Phase Selection for Peptide Purification

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Peptide purification on ever larger scale is a challenge for API manufacturers. Effective removal of commonly generated by-products during assembly and cleavage is essential (deletion peptides, Cis-Trans isomers of Pro, Succinimide from Asp, degradates of Trp generated by acid cleavage, Met-

sulphoxide). Another indicator of the worthiness of stationary phases is the separation effectiveness of oligopeptides especially di-, tri-, penta-, and octapeptides. The major application is the final purification of recombinant human insulin from its highly similar analogs. Taking a systematic approach for phase screening for these purposes is essential. A simple step-wise guide is provided to choose the best matching stationary phase for any given purification challenges, because always the stationary phase should be chosen for the process and not the process optimized for the stationary phase.

Oil palm root proteome during *Ganoderma boninense* infection

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Ganoderma boninense is a fungus which causes basal stem rot disease in oil palm and affects the palm oil productivity. Fungal attack on oil palm results in numerous host-specific biochemical responses. The activation of some of them is critical for the oil palm's survival. The oil palm may release some compounds which may act as a defence mechanism during the fungal attack. Many efforts have been made to control the basal stem rot disease spread. However, the infected palm did not show any early symptoms, which aggravates the control measure action as early as possible. Biochemical study via gel-based proteomic was conducted to identify the differentially expressed proteins during artificial infection of *Ganoderma boninense* on oil palm at day 7. Control palms were also prepared to get rid of background effect. The oil palm was subjected to two different treatments (infected and control). Artificial infection was conducted via root-inoculation technique, which the primary root was inserted into a rubber wood block filled with *Ganoderma* inoculum. The respected roots were taken for phenol-based protein extraction method (Sheffield *et al.*, 2006). Proteome maps with different molecular masses and pH, ranged between 10 to 100 kDa and 3 to 11 respectively, were successfully dissolved. In total, 450 protein spots per gel were emerged and only 25 protein spots were determined as highly expressed in the infected root at day 7. MALDI-TOF MS analysis was carried out and the mass spectra were searched against in-house database. About 12 protein spots with significant MOWSE score was also searched through other databases (NCBI, Swiss Protein, InterProScan, Pfam, TAIR) for confirmation. These spots gave high identities to proteins that involved during fungal infection in other plants.

Time and space monitoring of surfactin secretion by *Bacillus amyloliquefaciens* colonizing plant roots, revealed by MALDI Mass Spectrometry Imaging

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One of the greatest challenges of the next century is to develop new eco-friendly weapons to fight against crop diseases by limiting the input of chemicals. During the past decades, biological control through the use of natural antagonistic microorganisms has emerged as a promising alternative. In that context, plant beneficial rhizobacteria are good candidates, especially members of the *Bacillus* genus. Indeed, some *Bacillus amyloliquefaciens* isolates may devote up to 8% of their genetic equipment to the synthesis of antibiotics including cyclic lipopeptides (LPs). Among these LPs, surfactins play a key role in plant-*Bacillus* interactions since, on one hand, they contribute to the rhizosphere fitness of the producing strain by favoring motility and biofilm formation and on the other hand, they act as elicitor of the host plant immunity. Nevertheless, little information is available on the *in situ* production of such lipopeptides, allowing to better understand their spatio-temporal accumulation in the root microenvironment.

In this work, we took advantage of the versatility and sensitivity of MALDI mass spectrometry imaging to analyze the metabolites that are released by bacterial cells following their inoculation on roots of tomato plantlets. We developed specific bioassays for time-course monitoring by MALDI MSI. First analyses revealed an efficient secretion of surfactin by *Bacillus* cells after 7 days when colonization as biofilm-structured populations is well established. Looking at earlier time-points, we observed that the lipopeptide starts to accumulate in the medium surrounding roots as early as 24-48h post-inoculation. However, such a time-period is not sufficient for significant biomass development, suggesting that the surfactin biosynthesis machinery may be up-regulated in *Bacillus* in the earliest hours following recognition of root cells. Preliminary data on gene expression support this hypothesis and the role of surfactin as keyword in the molecular dialogue between plants and their root-associated *Bacillus* communities will be discussed.

Radiosynthesis and preliminary PET study of an ^{11}C -labelled RGD peptide containing [^{11}C]1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid

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Molecular imaging is an emerging technology that allows for the visualization of the interactions between molecular probes and biological targets. Positron emission tomography (PET), in particular, is a useful modality that enables *in vivo* biological information to be obtained in a noninvasive manner using a variety of PET radiopharmaceuticals.¹ Radiolabeled peptides are becoming increasingly important in nuclear oncology, where they are used in the diagnosis and treatment of several different cancers. Cyclic RGD peptides, such as cyclo[Arg-Gly-Asp-D-Tyr-Lys], are potent antagonists for the $\alpha\text{v}\beta 3$ integrin receptor.² A variety of different cyclic RGD peptides conjugated to a radioactive tracer have been reported for the PET imaging of tumors that over-express the $\alpha\text{v}\beta 3$ integrin receptor.³

We reported here radiosynthesis and preliminary PET study of a ^{11}C -labelled peptide containing [^{11}C]1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid ([^{11}C]Tpi), from corresponding cyclo[Arg-Gly-Asp-D-Tyr-Lys(Trp)] hydrochloride via a Pictet-Spengler reaction by using [^{11}C]formaldehyde. We proceeded to investigate the remote-controlled radiosynthesis of cyclo[Arg-Gly-Asp-D-Tyr-Lys([^{11}C]Tpi)] ([^{11}C]1) using an automatic production system to generate the [^{11}C]CH₃I. From a starting point in the range of 21.0-22.2 GBq for the [^{11}C]CO₂, [^{11}C]1 was obtained at the end of synthesis in the range of 0.8-1.4 GBq. The average time required for the synthesis was found to be 35 min from the end of the bombardment. The radiochemical purity of [^{11}C]1 was found to be greater than 98% and its specific activity was 85.7 \pm 9.4 GBq/ μmol .

The usefulness of our synthesis was demonstrated PET studies using [^{11}C]1 for the mouse bearing MIAPaca-2 and Bx-PC3, a pancreatic cancer cell line. The Bx-PC3 cell-to-muscle and MIAPaca-2 cell-to-muscle of [^{11}C]1 at 22 min were retained at 2.8 and 1.3, respectively. Further details about the tumor imaging studies by PET will be reported.

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Dicarba α -conotoxin Vc1.1 analogues with differential selectivity for nicotinic acetylcholine and GABA_B receptors

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The α -conotoxin Vc1.1 is a peptide extracted from the venom of the cone snail *Conus victoriae*¹. Specifically, Vc1.1 is a 16-residue peptide with an amidated C-terminus and four cysteine residues which form two cysteine bridges with the connectivity CysI-CysIII, CysII-CysIV². It acts both as antagonist on the $\alpha 9\alpha 10$ subtype of nicotinic acetylcholine receptors (nAChRs) and as an agonist on γ -aminobutyric acid (GABA_B) G protein-coupled receptors showing a potent analgesic activity^{3,4}. The mechanism by which Vc1.1 exerts its analgesic effect has not been fully elucidated yet.

In the present study, each disulfide bond of Vc1.1 was replaced with an unsaturated dicarba bond using a technique successfully developed within our group and applied to several peptidomimetic studies^{5,6,7}. This mutation is an invaluable tool in determining whether a disulfide bond within a peptide plays a functional or structural role. Four dicarba Vc1.1 analogues were obtained: *cis*-[2,8]-dicarba Vc1.1, *trans*-[2,8]-dicarba Vc1.1, from the substitution of the CysI-CysIII bridge, and *cis*-[3,16]-dicarba Vc1.1 and *trans*-[3,16]-dicarba Vc1.1 from the replacement of the CysII-CysIV bridge.

NMR spectroscopy showed that the structures of *cis*-[2,8]-dicarba Vc1.1, *cis*-[3,16]-dicarba Vc1.1 and *trans*-[3,16]-dicarba Vc1.1 are almost identical to the structure of native Vc1.1³ confirming that the dicarba mutation is well tolerated by the peptide.

The activity of the dicarba analogues was assessed *in vitro* on $\alpha 9\alpha 10$ nAChRs and the GABA_B receptor³. The two [2,8]-dicarba Vc1.1 analogues were both only active on GABA_B receptor, conversely the two [3,16]-dicarba Vc1.1 analogues were both only active on the $\alpha 9\alpha 10$ nAChRs. These findings highlight the importance of the disulfide bonds in regulating structure and function of the native Vc1.1 peptide.

Selected dicarba analogues are currently being assessed in animal models of pain to gain further insights into their analgesic properties. These results will be presented in this paper.

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Peptidomimetic inhibitors of the West Nile virus NS2B-NS3 protease

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The West Nile virus (WNV) is a member of the *Flaviviridae* family of viruses transmitted by infected mosquitoes.¹ Originating from Africa, it has spread to humans in Asia, southern Europe, the Middle East and North America.² Between 1999 and 2011, the United States Center for Disease Control reported more than 23,000 human infections in North America, resulting in 1,251 fatalities.³ The most recent outbreak that occurred in North America resulted in 1,118 human infections and 41 fatalities in 2012.⁴ There is currently no vaccine or specific drug therapy to treat WNV infections, highlighting the urgent need to develop an anti-viral against this disease.

A potential drug target is the viral NS2B-NS3 trypsin-like serine protease due to its crucial role in viral replication.⁵ Herein, we describe the structure-activity relationships of dipeptides containing a C-terminal agmatine (decarboxylated arginine) moiety which were found to inhibit the viral protease at low micromolar IC₅₀s.^{6,7} We believe our results will facilitate the design of new peptide-based antivirals against WNV infections.

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2-Oxoamides based on dipeptides and pseudodipeptides as inhibitors of Ca²⁺-independent phospholipase A₂

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Phospholipases A₂ (PLA₂) hydrolyze phospholipids to release free fatty acids and lysophospholipids, some of which can mediate inflammation and demyelination, two hallmarks of the autoimmune disease multiple sclerosis. We have developed polyfluoroketones which are selective inhibitors of Ca²⁺-independent phospholipase A₂ (GVIA iPLA₂)¹ and we have shown that such selective inhibitors reduce the severity and progression of experimental autoimmune encephalomyelitis (EAE).² Most recently, the interactions between a polyfluoroketone inhibitor and the active site of GVIA iPLA₂ have been studied.³ Our studies have shown that an aromatic ring attached to a four-carbon linker fits very well to one of the binding sites of GVIA iPLA₂. To increase the binding of the inhibitor to the target enzyme, we decided to develop new 2-oxoamides containing an aromatic ring attached to four-carbon linker in combination with a small peptide chain. Thus, a series of 2-oxoamides based on dipeptides and pseudodipeptides have been synthesized and their activities towards the two human intracellular GIVA cPLA₂ and GVIA iPLA₂ and the human secreted GV sPLA₂ have been evaluated. *tert*-Butyl esters of 2-oxoamides based on either Nle-Gly or a corresponding ether pseudodipeptide were found to present interesting inhibition of GVIA iPLA₂. This work was supported by the European Social Fund and National Resources Herakleitos II.

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Approach of peptide as a drug in medical science

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Rational drug design solves the problem through the use of structural biology combined with many other disciplines to speed up the development process. The structure based drug involves the selection of target proteins based on their involvement in the biological pathway integral to the course of a disease, *in-vitro/ in-vivo* studies and uses the structural information as a blueprint to design drug candidate. Once the target molecule is characterized the specific ligands are required to be designed to interact with it for the inhibition of its function leading to the design of specific therapeutic agents. Peptides are usually highly specific and therefore exhibit relatively low systemic toxicity. They do not accumulate in the body as they have relatively short half lives. They are easily acceptable by body; side effects will be very low. We have targeted PLA2, COX-2, LOX, p38a, p38b, for different cancers. The expression level of these enzyme in the patients serum level were estimated and specific peptides against different enzymes were tested for inhibition studies. The biochemical assay was done for determining the K_i , IC_{50} and K_D and very significant result were obtained which were comparable with the known drugs. The specific peptides also inhibited the proteins in serum in the presence of respective substrates, which were also confirmed on various cell lines. Hence, peptide can be considered to be potent therapeutic agent for different cancers.

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Diffusion-limited kinetics and membrane domain heterogeneity facilitate sustained excitation of sympathetic neurons by angiotensin ii: single photon imaging and fluorescence correlation spectroscopy

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Sympathetic neurons lie outside the blood-brain barrier and are exposed to circulating regulatory peptides. Angiotensin II (AngII) produces sustained increases in sympathetic excitability via AT1 receptors, with little desensitisation. Therefore, we used a Leica SP5 confocal microscope with photon-counting avalanche photodiodes and a resonant scanner with capture time of 0.5 μ s/pixel to image AngII linked to Alexa-647 fluorophore (AngII-A647) or Alexa-488 (AngII-A488) binding to AT1 receptors expressed by sympathetic neurons from guinea-pig coeliac ganglion. We also used fluorescence correlation spectroscopy (FCS) to measure AngII-A647 diffusion through extracellular space and close to neurons expressing AT1 receptors. AngII-A647 or AngII-A488 (10-100nM) binding to sympathetic neurons occurred in distinct membrane microdomains. After 60 minutes continuous exposure to the peptide, little internalisation was observed, and most binding microdomains remained fixed in their location. Sequential exposure of neurons to AngII-A488 following AngII-A647 revealed that some microdomains binding AngII-A647 also bound AngII-A488, some did not, while some AngII-A488 bound to domains not previously labelled by AngII-647. These inaccessible domains presumably had flipped rather than fully internalised. FCS showed that diffusion coefficients varied from 120 μ m²/s in free space to less than 1 μ m²/s at binding domains. Optically-measured binding kinetics around binding domains were no faster than those in extra-cellular connective tissue, indicating that receptor-ligand binding is significantly limited by slow peptide diffusion. Our observations suggest that diffusion-limited kinetics combined with rapid local turn-over of membrane receptor domains facilitate prolonged excitation of sympathetic neurons by AngII.

The effect of Antioxidant Peptides UPF1 and UPF17 on the Intracellular Localization of Transcription Factor NRF2 and on the activation of Antioxidative Response Element

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Excessive oxidative stress has been considered as one of the key factors in development of several neurodegenerative conditions, cancers and cardiovascular diseases. A battery of protective enzymes, such as glutathione S-transferases and NADPH quinone oxidoreductases has evolved in order to combat the excessive oxidative stress. Antioxidant response element (ARE) is a conservative domain found in the promoter region of majority of genes responsible for expression of protective enzymes. The expression of the genes that contain ARE is regulated by the transcription factor NRF2 (NFE2L2) which in turn is regulated by Keap-1 protein.

UPF1 and UPF17 are glutathione (GSH) analogues, developed recently by Ehrlich *et al.* which outperform free radical scavenging properties of GSH, while not showing any toxicity to the cells [1]. Although similar in structure, UPF1 and UPF17 show diverse effects on the Antioxidant Defense System as shown by Kairane *et al.* in Human Erythroleukemia Cells K562 with UPF1 increasing the cellular GSH levels, while UPF17 having the opposite effect [2].

In the present study we investigated if the increase and decrease of the cellular GSH levels induced by the UPF peptides are related to the intracellular localization and expression of the ARE activating transcription factor NRF2. We found that while UPF1 and UPF17 have negligible effect on the mRNA expression in human HepG2 cells, the peptides have diverse effects on the intracellular localization of the NRF2. UPF1 increased the NRF2 levels in the nucleus after 15 min incubation with peptide, while UPF17 after 15 min lowered the nuclear NRF2 levels.

Together our data suggests that GSH analogues UPF1 and UPF17 have effect on the activation of ARE when affecting the intracellular localization of its transcription factor NRF2.

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A novel tripeptide that suppresses PGE2 formation in rat mesangial cells

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Cytosolic phospholipase A₂ (cPLA₂) and secreted phospholipase A₂ (sPLA₂) play a major role in the production of arachidonic acid and various eicosanoids, for example PGE₂, in cells. We have recently reported that a long chain oxoamide based on α-(S)-leucine (GK126) presents interesting inhibition of sPLA₂.¹ In addition a simple amide derivative of γ-(R)-norleucine (GK115) showed inhibition of sPLA₂.² The purpose of the present work was to study the effect of these two sPLA₂ inhibitors on the suppression of PGE₂ formation in mesangial cells and to identify novel small peptides that will be able to cause such an effect. Cultures of renal mesangial cells were stimulated for 24 h with interleukin 1 plus forskolin to trigger a huge increase of PGE₂ synthesis. Cells were treated in the absence or presence of increasing concentrations of sPLA₂ inhibitors. Supernatants were collected and taken for a PGE₂-ELISA to quantify PGE₂ released from the cells. Both sPLA₂ inhibitors GK126 and GK115 presented interesting suppression of PGE₂ formation. After these promising results, we continued our project carrying out molecular docking studies to understand if small peptide derivatives based on either α-leucine or γ-norleucine may interact with sPLA₂. Some of the peptide derivatives that presented interesting binding score were synthesized and tested for their effect on rat mesangial

cells. We were pleased to find that benzoyl-glycyl-glycyl- γ -(R)-norleucine showed an interesting effect on the suppression of PGE2 formation. Thus, we have identified a lead for the development of novel agents that can be used to treat inflammatory diseases.

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Peptides Derived from the Transmembrane Domain of Bcl-2 Proteins as Potential Mitochondrial Priming Tools

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The Bcl-2 family of proteins is crucial for apoptosis regulation. The Bcl-2 proteins are synthesized in cytosolic ribosomes before reaching the lipid bilayer, where they insert through a specific C-terminal anchoring transmembrane domain. The members of this family of proteins target distinct intracellular membranes, including the mitochondrial outer membrane where they interact in order to determine cell fate. While the mitochondrial membrane has been proposed to actively participate in these protein-protein interactions, the influence of the transmembrane domain in the membrane-mediated interactions is poorly understood. The putative transmembrane domain (TMD) of anti-apoptotic (Bcl-2, Bcl-xL, Bcl-w and Mcl-1) and pro-apoptotic (Bax, Bak) members were selected and synthetic peptides (TMDpepts) derived from the respective domains were synthesized and characterized. TMDpepts showed higher affinity to interact with mitochondria-like than with plasma membrane-like model bilayers and higher binding correlated with greater membrane perturbation. These TMDpepts were effective as tools to prepare mitochondria for apoptosis by sensitizing human cervix adenocarcinoma cells to chemotherapeutic agents.

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Selectivity in LIM-binding peptides

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LIM-only (LMO) and LIM-homeodomain (LIM-HD) proteins play important roles in cell specification and cell development in many different tissues. Several members of this family also have causative roles in diseases including T-cell leukaemia and breast cancer, and are potential target for inhibitor development. These proteins share a common motif of two tandemly arrayed LIM domains at, or near, their C-termini, which mediate interactions with other proteins, including the LIM domain binding protein 1 (Ldb1). My laboratory has shown that a short intrinsically disordered peptide region in Ldb1, the LIM interaction domain (LID), binds in an extended fashion across the same face of both LIM domains, forming a tandem β -zipper interface in which short β -strands in the LID peptide augment β -hairpins in the LIM domains. We have now established that a number of other proteins use similar peptide regions to target the same binding face in the same manner - despite low sequence homology in the binding peptides. However, whereas all 4 LMO and 12 LIM-HD proteins (from mammals) bind Ldb1, these other binding peptides have higher levels of selectivity. We are investigating the basis for selectivity in this system and are using a range of protein engineering approaches to try and develop peptides with tailored binding properties for specific LIM targets.

Role of amyloid precursor protein intracellular domain (AICD) peptide in transcriptional regulation of peptide clearance proteins

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Proteolytic cleavage of the amyloid precursor protein (APP) by the successive actions of β - and γ -secretases generates several biologically active metabolites including the Alzheimer's disease amyloid- β peptide (A β) and the APP intracellular domain (AICD), a 50-59 amino acid peptide. The latter, as we have demonstrated, regulates expression of the metallopeptidase neprilysin (NEP) which, in turn, degrades the A β peptide. This up-regulation of NEP requires binding of the AICD peptide to the *NEP* promoter while down-regulation of *NEP* gene involves histone deacetylase occupancy at the promoter site. Functionally active AICD was found to be preferentially generated from the amyloidogenic processing of the neuronal APP₆₉₅ isoform. We have examined whether differences exist between the various APP isoforms in the production of AICD and have recently confirmed that AICD action is modulated via MED12, a subunit of the RNA polymerase II transcriptional Mediator complex. Since AICD is rapidly degraded in the cell and seldom detected we have developed several techniques to manipulate AICD peptide levels in order to study its function and established that γ -secretase inhibitors and hypoxic conditions reduce AICD levels, whereas the tyrosine kinase inhibitor, Gleevec, and alkalization with ammonium chloride increase AICD levels in the cells. We have shown that in SH-SY5Y-APP₆₉₅ cells expression of several other genes is also changed. However, unlike NEP, AICD appeared to down-regulate expression of the EGFR gene. Chromatin immunoprecipitation experiments also confirmed the presence of AICD on the promoter region of the transthyretin (*TTR*) gene in APP₆₉₅ over-expressing cells. Like NEP, TTR is also involved in A β peptide clearance from the brain. This study clearly demonstrates an important role of APP and AICD in epigenetic regulation of various neuronal genes and especially of those participating in peptide metabolism and clearance. Supported by MRC, ARUK, Programme of RAS "Fundamental Sciences to Medicine", RFBR 13-04-00388.

Identification of virtual bicyclic, membrane permeable cyclic peptide inhibitors of peptidyl arginine deiminase 4.

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The RaPID (random non-standard Peptides Integrated Discovery) system is a powerful methodology for the identification of bioactive cyclic and bicyclic peptides, and has been previously used to great effect to find both inhibitors and agonists of a wide range of protein targets. However, peptides in general as well as those discovered by the RaPID system often suffer from poor permeability across biological membranes, diminishing their utility for intracellular targeting. We reasoned that cyclic peptides containing two non-canonical long aliphatic side chains may be able to adopt both hydrophobic (*i.e.* side chains outwards) and hydrophilic (*i.e.* side chains inwards forming a virtual bicyclic structure) conformations, thus facilitating membrane permeability. Using genetic reprogramming combined with mRNA display, we screened a library of greater than 10^{13} such bicyclic peptides for binding to the human histone-modifying enzyme peptidyl arginine deiminase 4 (PAD4). In this manner, two virtual bicyclic peptides were identified, which exhibited very strong binding affinities for, and moderately strong inhibitory activities against, PAD4. Moreover, one of these peptides exhibited membrane permeability in cell culture studies, demonstrating the utility of this scaffold for the isolation of membrane permeable inhibitors of target proteins.

Bifunctional vs. Bivalent Opioid Receptor Ligands: Facts and Fiction

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There is a strong pharmacological rationale for the development of mixed opioid μ agonist/ δ antagonists as analgesics with no or low propensity to produce tolerance and dependence. Bifunctional compounds with this profile would interact with distinct non-interacting μ and δ opioid receptors and the lack of tolerance/dependence development would be due to interactions at the systems level. Alternatively, a bivalent ligand containing a μ agonist and a δ antagonist component connected via a linker of the correct length could simultaneously interact with μ and δ binding sites in a putative μ/δ receptor heterodimer. However, on the basis of simple thermodynamic considerations, the modest binding affinity increases seen with such "bivalent" μ/δ ligands as well as with "bivalent" ligands proposed to interact with other G-protein-coupled receptor heterodimers do not support a bivalent binding mode. Bifunctional compounds may contain integrated, overlapping or distinct pharmacophores. As expected, a μ agonist/ δ antagonist tetrapeptide of the integrated pharmacophore type, DIPP-NH₂[Ψ], given i.c.v. produced potent antinociception in the rat tail-flick assay, little tolerance and no physical dependence. In an effort to develop a μ agonist/ δ antagonist capable of crossing the blood-brain barrier (BBB), a series of chimeric peptides containing the μ agonist [Dmt¹]DALDA linked to a δ antagonist of the TIPP family were synthesized. In these bifunctional compounds [Dmt¹]DALDA plays a dual role as potent analgesic in both acute and neuropathic pain models^{1,2} and as vector for carrying the entire peptide construct across the BBB. Several of these compounds showed the expected μ agonist/ δ antagonist profile in vitro. One of them given s.c. was a potent centrally acting analgesic and, in comparison with morphine, was longer acting and produced less tolerance. These results indicate that systemically active, bifunctional opioid peptides of this type look promising as drug candidates for chronic pain treatment.

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Discovery of the myostatin inhibitory peptide from myostatin prodomain

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Transforming growth factor- β (TGF- β) superfamily consists of more than thirty members that are TGF- β s, bone morphogenic proteins (BMPs), growth differentiation factor (GDFs), activins and others¹. Among them, GDFs mainly regulates cartilage and skeletal development, and one of the GDF proteins, GDF-8, which is designated as myostatin, functions as a negative regulator of skeletal muscle volume. In the previous studies, inactivation of the myostatin gene in knockout mice significantly increased muscle volume up to 2-fold and systemic myostatin-overexpression induced cachexia in adult mice². It is also known that the activity of myostatin is physiologically regulated in several ways. One of which is the inactivation by myostatin prodomain. Lee, et al., reported that a cleaved *N*-terminal prodomain sequence of myostatin precursor interacted with the mature myostatin at the *C*-terminal part³. Hence, in the present study, we have focused on the *N*-terminal region of mouse prodomain sequence to discover a short peptide that selectively binds to human myostatin, resulting in the inhibition of myostatin function. Using a reporter assay with pCAGA₁₂-luc, which is activated *via*

the intracellular Smad signaling pathway lying downstream of the myostatin receptor, we screened synthetic peptide fragments and found a selective myostatin inhibitory peptide (24 AA, named as “mighty peptide 1”) with an IC₅₀ value of 4.1 μM. Phosphorylation of Smad2 protein was also inhibited by the treatment with the peptide 1. Moreover, intramuscular administration of 1 in the groin of mice notably increased the femoral muscle volume. Therefore, peptide 1 would be a significant starting point for the development of more potent inhibitors, which may contribute to the treatment of muscular diseases in a near future.

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An approach for formulation of self-assembled amphiphilic peptide containing-somatostatin sequence

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The noncovalent self-assembly of molecules into nanostructures is used as target selective drug carriers, or contrast agents in MRI. For example, amphiphilic molecules, comprised of hydrophobic and hydrophilic blocks, self-assemble to form micelles that solubilize hydrophobic drugs under aqueous environments, thus improving their bioavailability. On other hand, somatostatin-14 (SS-14) related peptide, octreotide (cyclic 8-amino acid peptide) is successfully applied for preparation of nanoparticles targeting SS-14 receptors (SSTRs) overexpressed on cancer cells. Added to octreotide, a lot of efforts have been made to develop SS-14 analogue with clinically useable anti-tumor activity. Keri et al. reported that TT-232 [H-D-Phe- α (Cys-Tyr-D-Trp-Lys-Cys)-Thr-NH₂], exhibited a potent antiproliferative activity without antisecretory action through SSTRs [1]. Based on its sequence, we synthesized H-Tyr-D-Trp-NH-1-(Ada; Adamantane) (YO-14) and reported YO-14 had potent antiproliferative activity on human colon carcinoma (HCT116) cells. A structure-activity relationship analysis revealed that the hydrophobicity of YO-14 could be responsible for its antiproliferative activity.

In this presentation, we described design of amphiphilic peptides and their self-assembly formulation. Amphiphilic peptides consist of a charged amino acid sequence (Tyr-D-Trp-Lys or TT-232 itself) covalently attach to hydrophobic alkyl chain (Stearic acid or palmitic acid) through a variety of linkers. YO-135, in which a stearic acid bound to the Tyr-D-Trp-Lys, through the linker, (AdOO; 8-amino-3,6-dioxaoctanoic acid)₂-Gly, showed both antiproliferative activity on HCT116 cells and DNA polymerase inhibitory activity at 100 microM. YO-136, in which Tyr-D-Trp-Lys sequence was substituted with TT-232, showed more potent antiproliferative activity than YO-135. Furthermore, in the aqueous YO-135, the critical micelle concentration (cmc), 1.1×10^{-4} M, was obtained, suggesting that YO-135 could self-assemble to form micelles. Physicochemical properties and anti-cancer activity of the self-assemblies will be discussed.

Next generation microwave SPPS – 4 minute cycle times, scalable, and 90% waste reduction

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A new process for SPPS is presented that allows for cycle times of only 4 minutes along with up to 90% reduction in total chemical waste compared to existing methods. In all cases this new process has met or exceeded the maximum purity we have observed using either conventional or standard microwave assisted SPPS with close scrutiny of well-known side reactions such as aspartimide formation and epimerization. Substantial economic and environmental value is realized from major solvent reduction, replacement of the controlled substance piperidine with a novel piperazine deblocking cocktail, use of a low cost enhanced carbodiimide based activation, and the ability to use high substitution resins (> 0.6 mmol/g). Recently developed derivatives for cysteine and histidine were also explored and will be discussed.

The application of these new methods is demonstrated on a new automated microwave peptide synthesizer which allows the total synthesis and cleavage of average length peptides to be as short as standard purification methods. The scalability of this new process was then demonstrated utilizing a new automated 2.5 liter microwave peptide synthesizer. This new process for SPPS will have a major impact on synthesis of difficult peptides, high throughput peptide production, and larger scale peptide production as it offers improvements in speed, chemical usage, and synthesis efficiency.

Angiotensin I- converting enzyme inhibitory activities of bioactive peptides derived from *Stichopus horrens*

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Hypertension, a non-communicable disease affecting over one third of the world population, is a risk factor for heart diseases. Thus developing functional foods with ability of alleviating hypertension is of great importance to maintain body health in a natural way. Sea cucumbers, belonging to the class of Holothuroidea, are rich sources of nutrients and bioactive compounds and there is a strong belief upon their various health benefits. Angiotensin-I converting enzyme (ACE) is a key enzyme of the renin-angiotensin-aldosterone system, and responsible for the elevation of the blood pressure. Therefore, production of natural, safe and effective ACE inhibitors is a tall order. Due to the advantages of food-derived bioactive peptides, this work was aimed at generating bioactive peptides through enzymatic proteolysis of *S. horrens* flesh using different proteases. The proteolysate was further purified and characterized for ACE-inhibitory activity. Among the six (6) different proteases employed, alcalase exhibited the highest ability to proteolyse and generate ACE-inhibitory peptides followed by flavourzyme with IC₅₀ values of 0.41 and 2.24 mg/mL, respectively. Fractionation of the effective peptides was carried out based on their hydrophobicities and isoelectric points. The most effective fraction was subjected to UPLC-MS/MS for peptides identification. A total of 24 peptides were identified showing 4 to 16 amino acid residues (molecular weights between 453.4 and 1771.9 kDa) with IC₅₀ values in the range of 0.05-8.8 mM. The Kinetics study of the peptides revealed a mixed-mode inhibition pattern. This study has demonstrated *S. Horrens* as a suitable marine source for ACE-inhibitory peptides generation.

Novel multifunctional bioactive peptides derived from *Actinopyga lecanora*

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Actinopyga lecanora, a type of sea cucumber commonly known as stone fish with relatively high protein content, was used as raw material to generate bioactive peptides using six commercial proteases. Among the enzymes used, bromelain hydrolysate showed the multifunctional bioactivities including antimicrobial, antioxidant, ACE inhibitory and NO production inhibition. Therefore, the bromelain protein hydrolysate was fractionated based on the hydrophobicity and isoelectric point of their peptides content. The total, twelve peptides were identified using UPLC-MS/MS system, synthesized and their biological activities were evaluated. Among the peptides identified three novel bioactive peptides revealed multifunctional activities including antibacterial against *Pseudomonas aeruginosa* (48.64-65.5%), *Pseudomonas* sp. (40.26-51.32%), *Escherichia coli* (61.76%) and *Staphylococcus aureus* of (45.30-50%); antioxidative (68.4-93.3%); ACE inhibitory (59.9-98.12%); and ability to inhibit LPS-induced NO production in RAW 264.7 cells (56.32-69.94%). These multifunctional bioactive peptides would be used in the formulation of various functional foods, and nutraceuticals.

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The Potential of Green Soybean (*Glycine max*) as a Source of Angiotensin I-converting Enzyme Inhibitory Peptides

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The rising global prevalence of hypertension sees no sign of abating in the near future, and the common use of synthetic antihypertension drugs causes undesirable side effects. Hypertension has been identified as one of the main risk factors leading to mortality. Therefore, there is considerable interest in developing alternative means of lowering blood pressure through consumption of functional foods. Green soybean is mostly cultivated as a vegetable crop, but other than that, it has limited use. Nonetheless, green soybean seeds have been found to be a good source of protein containing all of the essential amino acids that cannot be produced by the human body, as well as phytochemicals, vitamins and minerals. The green soybean protein was subjected to partial enzymatic hydrolysis (DH up to 50.1%) under optimized conditions to yield a mixture of unique smaller peptides in the hydrolysate, possessing strong antihypertensive effect with IC₅₀ values ranging from 0.140 mg/ml to 3.160 mg/ml. The hydrolysate was fractionated via high performance liquid chromatography and further separated according to their isoelectric points. A total of 6 novel peptides were identified, and were able to exert significant antihypertensive activity with IC₅₀ values ranging from 0.307 mM to 1.01 mM. These peptides may potentially be incorporated into various types of beverages and food which confers them with the ability to lower blood pressure through a normal diet. The use of green soybean protein as a source of antihypertensive peptides is advantageous as it is easily cultivated, provides essential amino acids and does not cause any adverse side effects when compared to synthetic antihypertensive drugs. It may also open up new opportunities in the production of high value-added products based on green soybean constituents.

Evaluation of anticancerous activity of sandalwood peptide using silk based 3D breast cancer model

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Development of novel anti-cancer peptides requires a rapid screening process which can be accelerated by using appropriate *in vitro* tumor models. Breast carcinoma tissue is a three dimensional (3D) microenvironment, which contains a hypoxic center surrounded by dense proliferative tissue. Biochemical clues provided by such 3D cell mass can not be recapitulated in conventional 2D culture systems. In this experiment, we evaluate the efficacy of the sandalwood peptide on established *in vitro* 3D breast cancer model using invasive MDA-MB-231 cell line. The anti-proliferative effect of the peptide on 3D silk tumor model is monitored by alamar blue assay, with conventional 2D culture as control. Apoptotic cell morphology, DNA fragmentation assay and flow cytometry are investigated. Further investigations involve the combinatorial use of this peptide with doxorubicin; resulting decreased IC₅₀ compared to the peptide alone. Overall, these findings indicate the role of sandalwood peptide as a promising anti-cancer therapeutics.

Diversity of conotoxin gene superfamilies in *Conus victoriae*

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Animal venoms represent a vast library of bioactive peptides and proteins with proven potential not only as research tools but also as drug leads and therapeutics. This is illustrated elegantly in cone snails (genus *Conus*). *Conus* venom consists of a mixture of hundreds of peptides (conotoxins) with a diverse assortment of molecular targets including voltage- and ligand-gated ion channels, G-protein coupled receptors and neurotransmitter transporters. Several conotoxins have found use as important research tools while some are being used or developed as therapeutics. The primary objective of this study was the discovery of novel conotoxin sequences from the venom gland of *Conus victoriae*. To this end, a combination of state-of-the-art techniques in molecular biology and bioinformatics was utilized; including cDNA library normalization, high-throughput 454 sequencing, *de novo* transcriptome assembly and annotation with BLAST and profile hidden Markov models. Subsequent matching of the transcriptome to a mass spectrometry profile of the crude venom was used to interrogate venom peptide composition and confirm post-translational modifications present in the mature venom peptides.

We report the discovery of over 100 unique conotoxin sequences from 20 gene superfamilies. Many of the sequences identified are new members of known conotoxin superfamilies, some will help to redefine these superfamilies and others represent altogether new classes of conotoxin. This work paints a comprehensive portrait of the molecular diversity present in *Conus* venom and demonstrates how an animal venom gland can be efficiently mined to generate a library of sequences encoding bioactive peptides.

A Proteomic Analysis of low molecular weight Excretory/Secretory components of the blood-feeding hookworm *Ancylostoma caninum*

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Hookworms secrete a barrage of Excretory/Secretory molecules (ES) into the mammalian host's gut mucosa to assist in blood-feeding and evasion of the host's immune response. ES products of the hookworm, *Ancylostoma caninum*, are therefore of interest as a source of therapeutic molecules for treating inflammatory disorders [1]. To date, hookworm ES proteins >10 kDa have been characterized using proteomics techniques,[2] and have displayed efficacy in suppressing inflammation associated with mouse models of colitis[3]. Some of the ES proteins responsible for suppressing inflammation have been identified and shown to protect against chemical colitis[4]. Much of the amino acid content of hookworm ES products is associated with as yet characterized low molecular weight peptides that pass through 10 kDa cut-off membranes but this material has not yet been characterized at the molecular level. Small peptides and compounds of less than 10 kDa are capable of acting in roles as diverse as signal transduction, immunomodulation and hormone-driven processes. In this investigation we carried out a shotgun proteomic analysis of the low molecular weight ES products secreted by *A. caninum* and show that defined fractions separated by RP-HPLC protect mice against TNBS colitis. Future work will identify the protective moieties and produce them in synthetic form for further trials. We have shown that hookworm secreted peptides possess anti-inflammatory properties and reveals their potential as a source of novel therapeutic compounds.

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Generation of multifunctional bioactive peptides from winged bean [*Psophocarpus tetragonolobus* (L.) DC.] seeds

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Winged bean seeds is a rich source of proteins with amino acid composition comparable to soybean. In this study, winged bean seed was proteolyzed using papain enzyme under optimum pH and temperature conditions. The crude proteolysate was determined for *in vitro* ACE inhibitory and antioxidative activities. The ACE inhibitory, DPPH• radical scavenging and metal ion chelating activities were found to be 87.2%, 65.0% and 65.7% respectively. The proteolysate was further characterized using a two-step purification, which consisted of reverse phase-high performance liquid chromatography (RP-HPLC) and isoelectric focusing. Fractions showing ACE inhibitory activity

between 50.1 and 87.2% with reasonable peptide content were subjected to peptide sequencing. Three multifunctional bioactive peptides were successfully identified, namely YPNQKV, FDIRA and VSARDLVG. The physical and biochemical properties of the peptides were compared.

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Towards engineering greater Na_v selective ligands: rational mutagenesis of a spider venom peptide

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Because of their key role in the transduction and transmission of nociceptive stimuli, voltage-gated sodium (Na_v) channels represent an essential therapeutic target for the treatment of multiple neuropathic conditions. However, with nine Nav isoforms – each of which displays a distinct functional profile and tissue-specific expression pattern – subtype-selectivity is of the utmost importance for both research and therapeutic purposes. To date, current therapeutics targeting Na_v channels critically lack subtype selectivity and as such exhibit a range of harmful side-effects.

The high specificity towards ion channels, inherent selectivity, and ability to act as gating modifiers through state-dependent interactions make spider venom peptides an excellent starting point for the engineering of selective Na_v modulators. Of these, β/δ-TRTX-Pre1a was isolated from the tarantula *Psalmpoaeus reduncas* and is part of the recently classified Na_v spider toxin Family 1, sharing high sequence similarity and structural homology in the form of an inhibitory cysteine knot (ICK) motif. Pre1a exhibited neuronal selectivity and unique pharmacological characteristics, acting as a sub-micromolar inhibitor of peak current to Na_v1.2 and Na_v1.7 and also an inhibitor of fast inactivation for Na_v1.3. Further, Pre1a demonstrated structural heterogeneity by exhibiting multiple, identifiable conformations in solution. To study the pharmacology and structure in more detail, a rational mutagenesis was pursued in combination with the standard Ala substitution. Residues of interest were decided using publicly available data on known peptides of similar sequence and function.

The work presented here details the results of functional mutations at a single residue of Pre1a, K₃₄, and how these changes strongly affected Na_v selectivity towards specific isoforms.

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Multifunctional peptides generated from palm kernel cake proteins

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The aim of this study was to generate multifunctional bioactive peptides from palm kernel cake (PKC) protein. Extracted PKC protein was hydrolyzed using different proteases. Antioxidant activity and degree of hydrolysis (DH) of each hydrolysate were evaluated using DPPH• radical scavenging activity assay and O-phthalaldehyde spectrophotometric assay, respectively. The results revealed a strong correlation between DH and radical scavenging activity of the hydrolysates, where among these, protein hydrolysates produced by papain after 30 h hydrolysis exhibited the highest DH (91 ± 0.1%) and DPPH• radical scavenging activity (73.5 ± 0.25%) compared to the other protein hydrolysates. The papain generated proteolysate was further fractionated based on hydrophobicity and isoelectric point to separate the peptides using RP – HPLC and IEF methods, respectively. Consequently, nine peptides were identified and their respective antioxidant activities were evaluated. All of the nine peptides showed multifunctional activity. The antioxidant activity as measured by radical scavenging activity and chelating activity assays were found to be in the range of 31-71% and 38-56% while those of ACE inhibitory activity and antibacterial were in the range of 40-100% and 10-78%, respectively. Fractionation of the most effective proteolysate by reverse phase high performance liquid chromatography indicated a direct association between hydrophobicity and radical scavenging activity. Isoelectric focusing tests also revealed that proteolysates with basic and neutral isoelectric point (pI) have the highest radical scavenging activity. The peptides derived from PKC

proteolysate were more potent and distinctive compared to those previously reported from the other protein sources.

Research on the Aggregation Kinetics of Human Amylin

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Small changes in the terminal region can result in significant differences in protein folding and aggregation for several important proteins. In this work, we described the impact of the C-terminus on determining the assembly process of amylin peptides via Thioflavin T (ThT) fluorescence assay, photo-induced cross-linking of unmodified proteins (PICUP) and conducted SDS PAGE-silver staining experiments. It has been proved that Amylin37-CONH2 aggregated remarkably faster than Amylin37-COOH and there are more LMW oligomers during the aggregation process for Amylin37-COOH. From the molecule dynamics simulation of amylin37-COOH and amylin37-CONH2 dimers, we found that the amylin37-CONH2 dimers are more stable than amylin37-COOH, which may promote the aggregation process. This work provides us new clues to understanding the underlying mechanism of peptide aggregation and the development of toxicity induced by terminal differences and will be helpful for future work involving mutations and modifications at specific residues within these peptides.

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Neurotensin analogue design: Stability and affinity improvement for in vivo analgesic activity

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Neurotensin (NT) is a tridecapeptide, which was first isolated from bovine hypothalamus¹. This molecule exerts a variety of physiological effects as hypothermia, analgesia or antipsychotic properties. Structure-activity relationship studies showed the minimal active sequence was the C-terminal fragment called NT(8-13). As many peptides, neurotensin has a short half-life time due to enzyme degradation. Indeed, electrophoresis analysis and plasmatic stability studies highlighted that specific enzymes affect three out of the five peptide bonds.

To overcome neurotensin instability, we developed several NT analogues using different approaches including unnatural amino acid incorporations², peptide bond modifications and cyclisation³. One of our objectives was to target the NT-induced analgesic effect. Among the three receptor subtypes, Sarret et al⁴ showed that selectivity toward NTS2 receptor is a parameter that has to be considered in the analogue design strategy. Molecular modelling calculations have been instigated to assist the understanding of NTS2 selectivity and help the design of new analogues.

A strategical approach was initiated to study structure-activity relationship of the active NT(8-13) fragment and offered an access to bioactive and resistant NT-analogues. Synthesis, binding affinity as well as *in vivo* analgesic activity on acute and neuropathic pain in rats will be presented.

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Prospection of Bioactive Molecules for G Protein Coupled Receptors: Mass spectrometry as a tool to explore new specific ligands

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G-protein-coupled receptors (GPCRs) constitute the largest family of transmembrane proteins. They control almost all physiological processes in humans and consequently emerge as a molecular target of around 40% of the commercially available drugs. However, most of the GPCRs subtypes are still unexplored, mostly due to the lack of specific ligands. Since the number of bioactive compounds found in animal venoms can reach up to 40 millions, the use of this natural library as a source for new ligands and pharmacological tool discoveries comes to light. This work aimed the development and the improvement of a new MS based technique able to identify new ligands for GPCRs. Vasopressin type 2, human Endothelin (ET-B) and human Muscarinics M₁ receptors were incubated with their respective ligands (vasopressin, endothelin-1, and MT-7 toxin) plus a simple mix of known peptides. After the incubation, two fractions were obtained: one containing the peptides that bound to the receptors ('bound fraction') and the second, the peptides that did not bind ('free fraction'). Both fractions were analyzed with a MALDI-TOF/TOF. The second phase of this work is linked to the incubation of receptors with crude (or fractions of) animal venom to ensure the workability of the protocol when applied for a complex mixture of unknown compounds and to discover new specific ligands. The MS analyses showed the presence of the non-specific peptides in the free fraction, while the ligands were just detected in the bound fraction. These results demonstrate the feasibility of the protocol in fishing for specific ligands. The presented work developed and improved a new methodology to screen for specific ligands for GPCR in either a simple mixture or a complex mixture; in consequence, this may assist the discovery of new ligands or even characterize orphan receptors, since the protocol can be adapted for other GPCR.

Convenient synthesis of phosphopeptides and sulfonopeptides

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Phosphopeptides and sulfonopeptides are important phosphorus and sulfur analogues of naturally occurring peptides. They have been widely used as stable mimetics of tetrahedral transition states for the hydrolysis of peptides and proteins in recent years. The phosphopeptides and sulfonopeptides as transition state analogues have also been exploited as enzyme inhibitors and as haptens for catalytic antibody research.

Recently, we developed a new and effective one-pot approach to synthesizing phosphopeptides, phosphonopeptides, phosphinopeptides, and phosphinonopeptides using the Mannich-type pseudo four component condensation reaction of carbamates/amino amides, aldehydes, and dichlorophosphite/aryldichlorophosphines, followed by aminolysis, alcoholysis, and hydrolysis. This is an economic and convergent synthesis of phosphopeptides.

For synthesis of sulfonopeptides with various functionalized side-chains, an efficient and convenient method was developed. We synthesized O-ethyl S-(2-N-protected aminoalkyl) xanthates via the radical addition of various xanthates to N-allylphthalimide. The synthesis of N-protected α -substituted β -aminoalkanesulfonopeptides was realized via NCS/HCl oxidative chlorination of xanthates or

thioacetates to the corresponding sulfonyl chlorides followed by reaction with amino esters. It is a useful and efficient strategy for the synthesis of sulfonopeptides with diverse functionalized side-chains.

In summary, efficient strategies for synthesis of phosphonopeptides and ulfonopeptides have been provided.

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Synthesis of covalent RANTES-PF4 heterodimers using an optimized oxime strategy

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Atherosclerosis is caused by chronic inflammation of the arterial wall leading to serious complications such as myocardial infarction and stroke. Inflammation is driven by recruitment of mononuclear cells to the vascular wall, a process that is exacerbated by platelet-dependent deposition of chemokines CCL5 (RANTES) and CXCL4 (platelet factor 4: PF4) on the inflamed endothelium. Recently it was found that heterodimer formation of RANTES and PF4 promotes monocyte recruitment to the vascular wall and might thus be a very suitable clinical target.

In order to study the mode of action of the RANTES-PF4 heterodimer bridging the monocyte-endothelium interactions, synthetic access to a homogenous covalently linked RANTES-PF4 molecule was needed. Molecular dynamics simulations and HSQC chemical shift mapping show that non covalent RANTES-PF4 interactions are occurring in the *N*-termini of the chemokines¹. However, as RANTES interacts with its receptor via the *N*-terminus it was hypothesized that a coupling of the two termini would not lead to an active dimer. Based on the molecular dynamics, positions for covalent bond formation between the two proteins were identified that will not influence chemokine activity. The covalent linkage will be made with an oxime bond using novel oxime formation chemistry. The usual introduction of a ketone functionality through levulinic acid led to low yields in a model setup. It was found that cyclization leads to a side product that is unable to react with the aminoxy modified protein. A mechanism for cyclization of levulinic acid was proposed based on NMR measurements. The oxime formation was optimized using alternative keto-acids; kinetics of the oxime formation were characterized. Both PF4 and RANTES were synthesized in three parts and ligated using native chemical ligation. PF4 and RANTES were modified with ketone and aminoxy moieties, respectively. Results of the heterodimer synthesis with optimized oxime chemistry will be presented.

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The cyclic cystine ladder in theta-defensins is a structured and stable scaffold for the design of peptide therapeutics.

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Many disease processes are mediated by protein-protein interactions that involve large molecular surfaces and highly specific contacts, making them difficult to disrupt using small organic molecules. Peptides are able to fill the size and specificity requirements needed to target protein-protein interactions, and cyclic peptides have the additional advantage that they have the stability needed to withstand protease-rich biological environments. Theta-defensins are cyclic peptides from mammals and have inherent antimicrobial and immunomodulatory properties, and low cytotoxicity. In addition to these inherent properties, we have demonstrated that the core structural motif of theta-defensins, the cyclic cystine ladder, can also be used as a structured and stable scaffold for the design of peptide

therapeutics. We explored the role of the cyclic peptide backbone and three disulfide bonds comprising the cyclic cystine ladder and showed that one or two disulfides can be removed without compromising the thermal or serum stability. Inclusion of an integrin-binding RGD motif in either or both of the turn regions demonstrated that a desired bioactivity can be imparted to the cyclic cystine ladder, and also highlighted the potential for designing dual-functionalised theta-defensin analogues. This study demonstrates that the cyclic cystine ladder of theta-defensins is a versatile scaffold for providing stability and conformational restriction to bioactive epitopes in a beta-strand or hairpin conformation and has the potential to be applied as a scaffold for targeting protein-protein interactions involved in a variety of diseases.

Development of stabilized helical peptides for vitamin D receptor-coactivator interaction inhibitor

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Antagonistic vitamin D receptor (VDR) ligands are considered to be useful for the treatment of conditions involving hypersensitivity to $1\alpha,25\text{-dihydroxyvitamin D}_3$ [$1\alpha,25(\text{OH})_2\text{D}_3$], such as Paget's disease of bone, which results in an abnormal bone architecture. To date, several secosteroidal VDR antagonists have been developed. In addition, small molecules and peptides containing the consensus sequence LXXLL [L: leucine (Leu), X: any amino acid residue] have been demonstrated to inhibit VDR-coactivator interactions. Therefore, they are also considered to be drug candidates for reducing VDR-mediated transactivation. In order to bind to the VDR, small molecules/peptides must contain three Leu residues or Leu mimics. Furthermore, small peptides have to have an α -helical structure to efficiently interact with the VDR. It is sometimes difficult to form stable α -helices in short peptides. However, α,α -disubstituted α -amino acids and a covalent cross-linking system have been demonstrated to be useful for stabilizing the helical structures of such peptides. Here, we developed several stabilized helical peptides containing the LXXLL motif and evaluated their ability to inhibit VDR-coactivator interactions. The peptides were prepared by solution-phase methods using EDC and HOBt as coupling reagents. These peptides were examined the inhibition assay by human VDR on receptor cofactor system, and some of them showed moderate-to-strong activities ($\sim 3.2 \mu\text{M}$).

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Gp10 based-thioetherification (10BASE_d-T) on a displaying peptide of the bacteriophage T7

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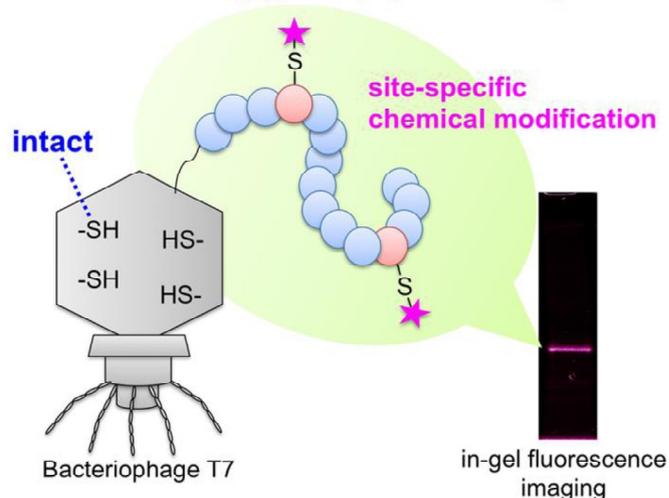
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Here, we report that site-specific introduction of haloacetamide derivatives into designated cysteines on a displaying peptide at a capsid protein (gp10) of a bacteriophage T7 has been achieved. This easiest gp10-based thioetherification (10BASE_d-T) undergoes almost quantitatively in one-pot without side reaction or loss of phage infectivity. Construction of such peptide-based library possessing non-natural structure will be useful for future drug discovery. For this aim, we constructed a tetramethylrhodamine-conjugated peptide library and isolated a glutathione S-transferase binder with a dissociation constant of $3.6 \mu\text{M}$. Furthermore, we successfully constructed stapled peptide libraries. Affinity selection against target proteins with the peptide library is currently underway, and we have

already obtained several candidates. Functional analysis of the peptides *in vitro* and *in vivo* is also in progress.

In conclusion, we established a general and instant method of post-translational chemical modification for peptide library on T7 phage.

TMR-conjugated peptide library



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Fmoc-Cys(Ddm) and Fmoc-Cys(MBom) prevent the risk of racemization in solid phase peptide synthesis

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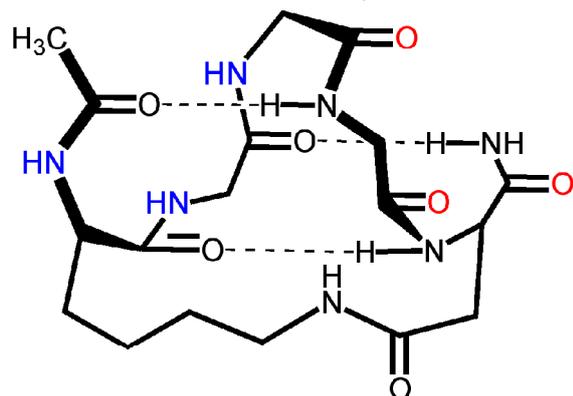
In Fmoc chemistry, the Trt group is widely accepted as offering protection for Cys. However, the use of Fmoc-Cys(Trt) in peptide synthesis occasionally entails serious obstacles: (1) racemization of Cys arising during its incorporation mediated by phosphonium or uronium reagents such as PyBOP or HBTU, respectively, and (2) racemization of the C-terminal Cys esterified to the solid support during repetitive base treatment. These may hamper purification of the products including those obtained after the NCL and disulfide formation reactions. Therefore, the carbodiimide-mediated coupling method has been recommended to reduce the racemization rate to acceptable levels (<1.0%), although the DIC/HOBt method is considered to have no advantage in terms of coupling efficiency over that using PyBOP/HBTU. In addition to this, racemization of the C-terminal Cys likely to occur even when employing a Trt-type resin with the aid of its steric hindrance. In the present study, we reviewed the acid-labile protecting groups on Cys from the perspective of suppressing racemization both when Cys incorporation is conducted with PyBOP/HBTU and when Cys is linked to a resin via ester linkage. The base-catalyzed racemization of Cys is considered to proceed via enolization due to stabilization of the carbanion formed on α -proton abstraction. To destabilize this enol form, therefore, an S-protecting group that possesses an electron-donating and/or a sterically-hindered effect(s) would be essential to prevent racemization of Cys. As had been expected, the 4-methoxybenzyloxymethyl (MBom) and 4,4'-dimethoxydiphenylmethyl (Ddm) groups were found to effectively prevent the risk of Cys racemization during its incorporation and that of the C-terminal Cys esterified to the solid support. Even when performing the 1-min preactivation procedure of coupling with Fmoc-amino acid/HCTU/6-Cl-HOBt/DIEA (4/4/4/8 equiv) in DMF, Fmoc-Cys(Ddm) and Fmoc-Cys(MBom) caused a significant reduction in the level of racemization (0.6% and 0.4%, respectively) while Fmoc-Cys(Trt) led to a considerable level of racemization (8.0%).

Cyclic Pentapeptides As Alpha Helix Nucleators In Unstructured Peptides

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The shortest known α -helix in water is a pentapeptide stabilized by cyclisation.¹ Here we closely compare the alpha helicity of several cyclic pentapeptides and then examine one as an alpha helix nucleator appended to the N- versus C-terminus of short unstructured peptides. The structures of cyclic pentapeptides Ac-(cyclo-1,5)-[KAAAD]-NH₂ (**1**), Ac-(cyclo-1,5)-[DAAAK]-NH₂ (**2**) and Ac-(cyclo-1,5)-[EAAAK]-NH₂ (**3**) were compared by CD and 2D proton NMR spectroscopy, with (**1**) being the most alpha helical in water. However, detailed NMR studies revealed a small defect from idealised alpha helical geometry near the C-terminal Asp residue in (**1**), due to torsional strain imposed by the K_(i)D_(i+4) lactam bridge. The more water-soluble Ac-(cyclo-1,5)-[KARAD]-NH₂ (**4**), being identical to (**1**) in helicity and structure, was appended to the N- or C- or N- and C- termini of an unstructured palindromic peptide ARAARAARA ($\leq 5\%$ helicity), resulting in 65%, 90% and 100% alpha helicity respectively in water (10 mM phosphate buffer, pH 7, 25°C). Detailed analysis by 1D and 2D ¹H NMR spectra, including multiple solution structure determinations, Ramachandran plots and molecular dynamics simulations highlighted torsional effects imposed by the K_(i)D_(i+4) lactam bridge and explained why Ac-(cyclo-1,5)-[KARAD]-NH₂ is more effective as an alpha helical nucleator when appended to the C-terminus of a peptide sequence. Finally, the capacity of (**4**) to nucleate alpha helicity was investigated through attachment to extended 10-25 residue palindromic peptide sequences and it was found that 13 amino acids, or 4 helical turns, were maintained α -helical in water before the helix structure dissipated.



H-bond Donors H-bond Acceptors

Figure 1. A model cyclic pentapeptide as N- and C-termini helical inducer caps. The dotted lines are represented H-bonds and atoms colored blue and red are possible H-bond donors and H-bond acceptors respectively.

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A New Class of Aggregation Inhibitor of Amyloid β Peptide based on the O-Acyl Isopeptide

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Inhibition of the aggregation process of amyloid β peptide (A β) would be a promising target for the development of an anti-Alzheimer's disease agent. In this context, a number of fragment peptides of A β ,^[1] in many cases with modifications (e.g., *N*-methylation, replacement with D-amino acid), have been reported as aggregation inhibitors of full-length A β . The amino acid sequences in any of the inhibitors correspond to β -strand-forming regions of A β , which are crucial for self-recognition of A β to progress packings into fibrillar aggregates.

We previously reported that an O-acyl isopeptide of A β 1–42 (**1**), in which a Gly²⁵–Ser²⁶ amide bond of A β 1–42 was isomerized to an ester bond at the β -hydroxy group of Ser²⁶, exhibited considerably lower aggregation potency than A β 1–42.^[2] Here, we demonstrate that **1** also has a capability of inhibiting fibril formation of A β 1–42 at equimolar ratio. In addition, the inhibitory activity was retained in the *N*-Me- β -Ala²⁶ derivative **2**, in which the ester of **1** was replaced with *N*-methyl amide to avoid O-to-*N* acyl rearrangement under the neutral pH conditions, verified by the measurements of fluorescence anisotropy, western blot analysis and atomic force microscopy. The results would provide a valuable insight into the design of new class of aggregation inhibitor of A β , with a key modification at Gly²⁵–Ser²⁶, towards an anti-Alzheimer's disease agent.

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Amino Acids and Peptides as Efficient Catalysts for Various Asymmetric Organic Transformations

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Over the past decade several peptides have emerged as efficient organocatalysts for various asymmetric transformations.¹ Within our project dedicated to the development of organocatalysts based on amino acids and peptides, we have reported that primary amine-thioureas based on di-tert-butyl aspartate are excellent organocatalysts for the Michael reactions.² Herein, we present our work based on simple amino acids and peptides that can be efficiently employed as catalysts for the aldol reaction, the Michael reaction and the epoxidation reaction. Tripeptide and tripeptide mimetics based on proline have been employed for the aldol reaction leading to high yields (up to 99%) and selectivities (up to 99% ee).³ More recently, we have disclosed the use of either phenylalanine or aspartate derivatives as catalysts for the Michael reaction between aldehydes and maleimides leading to substituted succinimides in high yields and selectivities with low catalysts loading (1 mol%).⁴ Aspartate derivatives were also found to catalyze efficiently the asymmetric α -amination of α,α -disubstituted aldehydes leading to products that can be transformed to enantioenriched α,α -disubstituted amino acid derivatives.⁵ Finally, our efforts have been focused on the design and synthesis of tripeptides bearing thioureas that can be employed as catalysts for the asymmetric epoxidation reaction of olefins. Acknowledgment. The author gratefully acknowledge the Operational Program "Education and Lifelong Learning" for financial support through the NSRF program "ENIΣXYΣH METAΔIΔAKTOPQN EPEYNHTQN" (PE 2431) co-financed by ESF and the Greek State

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Synthesis of BVD15 Analogues as Models for Radioligands in Tumour Imaging

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Breast carcinomas are prevalent in females around the world. However, current diagnostic and therapeutic measures are limited by low sensitivity and specificity. Breast carcinomas are found to over-express neuropeptide Y (NPY) Y₁ receptors, whose functional roles in tumour growth and metastasis have been demonstrated.¹ Therefore, Y₁ receptor is a valuable target for diagnosis and treatment of breast carcinomas. Here we describe the synthesis of 38 truncated NPY analogues as models for Y₁-specific peptidic radioimaging agents used in positron emission tomography. These sequences are based on the previously reported Y₁ antagonist BVD15 scaffold.² Different strategies to improve Y₁ affinity and metabolic stability were investigated. We found that a basic amino acid residue at position 4 improved Y₁ affinity, and Arg⁴ substituted analogues possessed excellent tolerability to *N*-terminal aryl capping groups. While various conjugations at Lys⁴ ε-amine were tolerated, a further *N*-terminal aryl group sacrificed affinity. Replacing Asn² by a Lys retained affinity, and a NOTA conjugation at its ε-amine was well tolerated. Modifications to position 5 were limited to small prosthetic groups. Finally, incorporation of D-amino acids for stability enhancement caused loss of Y₁ affinity.

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Synthesis of cyclic α,α-disubstituted amino acids bearing a pendent chiral center and conformational analysis of heteropeptides containing their amino acids

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Cyclic α,α-disubstituted amino acids, in which the side chain becomes a cyclic structure, have attracted considerable attention because of their characteristic properties, such as chemical stability, metabolic stability, and conformational restriction. We recently reported that side-chain chiral centers of amino acids affect the secondary structures of their peptides. Herein, we wish to report the synthesis of chiral cyclic α,α-disubstituted amino acids; (S)-Pip^{Me,Ph} and (S)-oPip^{Me,Ph}, and also the preparation of heteropeptides containing them in dimethylglycine sequence. Amino acids Cbz-(S)-Pip^{Me,Ph}-OMe and Cbz-(S)-oPip^{Me,Ph}-OMe were prepared using dimethyl malonate and 2-bromomethyl-1,3-dioxolane as starting materials. The synthesis of heteropeptides was carried out according to solution-phase methods by using EDC•HCl and HOBt as coupling reagents. FT-IR and

NOESY ^1H NMR spectra indicated that (S)-Pip^{Me,Ph} heteropeptide preferentially formed a 3_{10} -helical structure in CDCl_3 solution. X-ray crystallographic analyses indicated that (S)-Pip^{Me,Ph} heteropeptide formed both right-handed (*P*) and left-handed (*M*) 3_{10} -helical structures, while (S)-oPip^{Me,Ph} heteropeptide formed (*P*) 3_{10} -helical structure.

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PepFect 20-28; a series of novel amphipathic cell-penetrating peptides

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Cell penetrating peptides (CPPs) are peptides capable of crossing the cellular membrane and transporting cargoes into the cell. In most cases CPPs are cationic peptides, 8-30 amino acids long and commonly either primary or secondary amphipathic.

CPPs have gained considerable interest as potential delivery systems for drugs and oligonucleotide therapies; most of this has been focused on peptides derived from naturally occurring proteins or chimeric combinations of protein-derived and synthetic sequences. Purely synthetic CPPs have also been reported, in many cases synthetic peptides are simple poly-amino acid sequences such as polyarginines, but there are also examples more complex synthetic structures.

In a recent study, we have developed a series of novel cell-penetrating peptides using a combination of the model amphipathic peptide (MAP) sequence and PepFect strategies. Stearyl-MAP and a series of stearyl modified MAP analogues were designed and synthesized. The novel peptides were found to form non-covalent peptide-plasmid complexes by co-incubation of peptides and plasmids in water solution, the complexes were characterized by dynamic light scattering and cellular uptake of the complexes was studied in a luciferase based plasmid transfection assay. A quantitative structure-activity relationship (QSAR) model of cellular uptake was developed and experimental uptake data correlated to QSAR predictions. The predicted biological effects obtained from the model correlated well with experimental data. This QSAR model could potentially be used to understand structural requirements for cell penetration, or to predict novel CPP sequences.

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Semi-enzymatic cyclisation of disulfide-rich peptides using Sortase A

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Cyclotides are head-to-tail backbone cyclized peptides with knotted topology brought about by three conserved disulfide bonds¹. Kalata B1 was one of the first cyclotides discovered and is now well-characterized in terms of its structure and activity². Due to its remarkable stability at high temperature and on exposure to proteases it has been considered as a potential drug scaffold^{3,4}. To date, the most commonly used method for peptide head-to-tail cyclization has been native chemical ligation⁵. However, enzyme-mediated cyclization has been emerging rapidly in recent years because of several attractive properties, including efficiency, safety and cost-effectiveness. Sortase A is a bacterial enzyme with transpeptidase activity that recognizes the penta amino acid motif "LPXTG" and cleaves the peptide bond between Thr and Gly⁶ following attack by a poly-glycine sequence resulting in an amide bond formation. Here, we present the cyclization of the disulfide rich kalata B1 by sortase A as a proof of concept of disulfide rich peptide cyclization by enzymes. The successful cyclization of kalata B1 with LPVTG motif in loop 6, its structure and activity will be discussed.

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Organic Peptides - New Technologies for a bio-based Production

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Peptides are a very attractive and promising class of biomolecules combining high specificity and activity, low toxicity and unique characteristics. Previously mainly applied in oncology, the peptide market has broadened and nowadays peptides are considered in various fields – for example metabolic diseases, obesity, as antimicrobials, surfactants or bio-glues. The global market is continuously growing and reached 14 billion US \$ in 2014, considering only therapeutic peptides. Above 70 therapeutic peptides are currently on the market and a tremendous progress in this field is ongoing reflected by 270 peptides in the clinical and 400 peptides in the preclinical phase of development.

Despite the obvious demand, major challenges are limiting the sustainable growth of the peptide market. A major challenge is the manufacturing process of peptides, especially in large scales. Currently, more than 90 % of peptides are produced by chemical synthesis (solid-phase or solution-phase peptide synthesis). Suitable for many peptides up to 30 amino acids, these techniques are challenged with the production of long, hydrophobic or repetitive peptides. Moreover, they are rather expensive, difficult to up-scale and generate huge amounts of chemical waste. In contrast, the bio-based, recombinant production of peptides is currently very restricted and no suitable platform-technologies exist. Here, the major limitations are 1. the expression of peptides *per se*, 2. the intrinsic instability of peptides directing them to proteolytic degradation and 3. the tendency of peptides to form insoluble aggregates.

Here, we present new bio-based approaches for the efficient production of peptides. These technologies tackle limitations of chemical synthesis and allow the recombinant production of peptides in high yields and in a cost-efficient and an organic way.

Effect of amino acid side chain length on the pH and temperature responsiveness of acidic β -hairpin peptide hydrogels

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Hydrogels is a class of material that composed of heavily hydrated network. It has been studied and used in various biomedical applications including drug delivery, cell therapy and tissue engineering. In our lab, we are interested in generating an acidic peptide-based hydrogel for cellular delivery and tissue engineering. Here, β -hairpin peptides capable of folding and self-assembly affording self-supportive hydrogels were rationally designed. Then, acidic amino acids with different side-chain length were incorporated into each peptide to investigate the folding, self-assembling and subsequently rheological behavior of the hydrogels. The resulted peptides are VX, VE and VD, composed of amino adipic acid, glutamic acid and aspartic acid as one of the building blocks respectively. Each peptide undergoes gelation in response to changes in environmental pH and temperature. The side-chain length of amino acids dictates the conditions at which folding and self-assembly is permitted. For examples, at 50°C pH 7.0, VX folds and self-assemble affording self-supporting hydrogel while VD remains unfold as shown by circular dichroism spectroscopy and oscillatory rheology. All VX, VE and VD display similar network morphologies as revealed by transmission spectroscopy even though the building blocks are different. To generate acidic hydrogels that can form under physiological conditions, VE3 and VEQ1 were then designed by controlling

peptides' charge state. Both gels can be used to directly encapsulate cells providing homogeneous cellular distribution. The shear-thinning and recovery property as well as the cytocompatibility of the VE3 and VEQ1 gels make them promising cell carriers and scaffolds for tissue engineering applications.

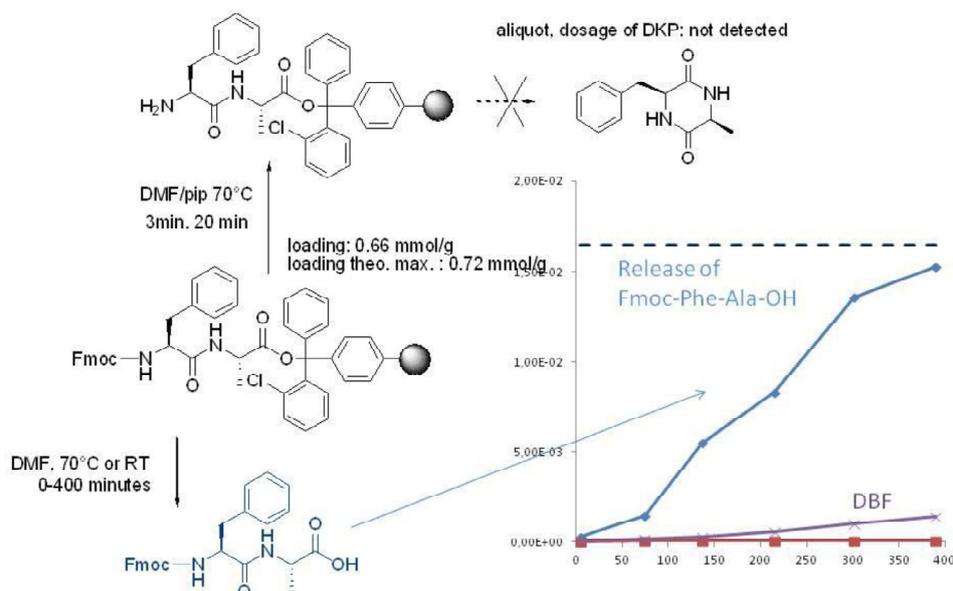
Heating and Microwave assisted SPPS of C-terminal acid peptides: The truth behind the yield

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Microwave (MW) irradiation is now accepted as an alternative and efficient way of heating, speeding up organic reaction. Applied to SPPS, the thermal effect of MW¹² enable the synthesis of a 20-mer in few hours. However, despite correct purity of crude peptides prepared in Fmoc/tBu microwave assisted SPPS³, surprisingly, lower yields than those expected were obtained while preparing C-terminal acid peptides on trityl resin. This could be explained by cyclisation/cleavage through diketopiperazine formation during the second amino acid deprotection and third amino acid coupling. However, we provide here evidence that it is not the case and that this yield loss was due to high temperature promoted cleavage of the 2-chlorotrityl ester, releasing the growing C-terminal acid peptides in solution through a S_N1 hydrolysis during MW-assisted SPPS. This mechanism is simiral to which was observed to explain trityl acetate hydrolysis in solution.⁴



Three different peptide models (comps 1 2 and 3) were synthesised with and without heating, examining the yields and the side products. Moreover, a tripeptide model was prepared to perform kinetic studies at different stages of the MW assisted SPPS cycle.

Cmpd #	sequence	MW (g/mol)	Yield (%) (RT)	Yield (%) (70 °C)
1	H-EKRYCS-OH	784.8	64.6	7.2
2	H-GSNKGAIIGLM-OH	1060.3	95	2.6

At last we demonstrated that it is possible to get advantage of the thermal instability of trityl-peptidyl resin to cleave in neutral conditions a fully protected peptide. However, due to long reaction times that could be required (> 48 hours), a careful monitoring of the released peptide has to be set up to ensure that side chain protecting groups are not partially removed.

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Using the SFTI scaffold to deconstruct and redirect the Laskowski mechanism of protease inhibition.

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Laskowski inhibitors play vital roles in development, cell differentiation and death by reversibly inhibiting proteolytic enzymes. In nature, these inhibitors masquerade as efficient substrates but they avoid the fate of genuine substrates by way of a unique religation reaction that restores the reactive site bond following cleavage^{1,2}. Understanding this mechanism is imperative when adapting these molecules for new protease targets. Here, we use the cyclic peptide, sunflower trypsin inhibitor-1 (SFTI-1)³, as a model system to explore the molecular basis for Laskowski inhibitor potency and specificity. We demonstrate that pre-organization of the inhibitory loop confers rapid binding association while coordinated inter- and intramolecular interactions promote religation and slow release. By applying these concepts to SFTI-1, we engineered a series of potent inhibitors tailored for selective or broad-range inhibition. Analyzing fold-divergent inhibitor families revealed that these features are generally applicable to Laskowski inhibition, providing new insights on protease inhibitor function and design.

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Synthesis of potent multivalent oxytocin dendrimers via “click” chemistry”

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Peptide dendrimers as therapeutics have potential use in different biotechnological applications.¹ Nevertheless, the efficient conjugation of peptides containing disulfide bonds to a core dendron may be challenging due to the steric hindrance.^{2,3} In this study we describe the synthesis, structure, functional activity and anti-nociceptive properties of dendrimeric oxytocin (OT) agonists containing one disulfide bond. Dendrimeric peptides with up to 16 copies of OT agonists were successfully synthesised via copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction based on the biodegradable, alkyne modified, oligolysine dendron and azido-PEG(9)-[Lysine]⁸-OT. Detailed NMR analysis of the OT dendrimers suggests that each attached OT molecule is identical with negligible structural perturbation as compared to OT in the monomeric form. The motion of OT dendrimers in solution was studied by diffusion ordered spectroscopy (DOSY), indicating that the arms of the dendrimers are disordered allowing each OT monomer to rotate freely about its own axis. Functional activity was demonstrated on human cell lines containing the OTR receptor. Interestingly, the OT dendrimers inhibited colonic nociceptors in a mouse model of chronic abdominal pain.

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Inhibitor peptide design – improving affinity without losing specificity

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A challenge in the development of inhibitors of intracellular targets is to achieve specificity and high affinity for those targets. Here peptides, that can mimic specific protein-protein interaction surfaces, can provide the answer, so long as cell permeability and stability issues are addressed. Here we describe progress in developing a specific inhibitor of the Grb7-SH2 domain involved in cancer progression. Grb7 is an adapter protein, aberrantly co-overexpressed with *erbB-2* and identified as an independent prognostic marker in breast cancer. Grb7 signals the activation of *erbB-2* and *erbB-3*, which play key roles in dysregulated cell growth in cancer. Grb7 also mediates signalling pathways from another tyrosine kinase, focal adhesion kinase (FAK) exacerbating cell migration and the metastatic potential of cells. It is thus a prime target for the development of novel anti-cancer therapies. We have characterised a non-phosphorylated cyclic peptide (G7-18NATE) that is a specific inhibitor of Grb7 and inhibits cellular growth and migration in cancer cell lines. X-ray crystallographic structure determination of the G7-18NATE/Grb7-SH2 domain complex and binding studies using surface plasmon resonance have revealed the basis of affinity and specificity of the peptide¹⁻³. Here we describe how this information has been used to successfully design second generation bicyclic peptides that show enhanced binding without loss of specificity. These peptides can be coupled to cell penetrating peptide sequences to be taken up by cells, lowering cell growth and migration. We anticipate that these Grb7 inhibitor peptides will form the basis of novel therapeutics that can be used in conjunction with existing therapies against breast cancer.

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Synthesis of single chain Nitro-Tyrosine B16 des Thr-B30

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A new class of human insulin analogues has been developed and produced by biosynthetic and semi synthetic methods. These analogues are characterized by the replacements or deletion of single amino acid residue to the C-terminal of the β chain. The analogues have been evaluated by measuring the in vitro biological potency in mouse fat cells, by osmometric determination of association state in solution at natural pH and the blood glucose lowering effect found after subcutaneous injection in pigs.

Our plan is to synthesis Nitro-Tyrosine (B16)-insulin as single chain with deleting of Thr-B30

Systematic procedure is as follows

- Zinc free insulin and enzymatic cleavage of B23-30 to give des-octa peptide (DOP) insulin
- Sulfitolysis of (DOP) insulin to give A(SSO₃)₄+B(SSO₃)-DOP to give A(SS)₂+B(SS)₂-DOP after reduction-oxidation
- Synthesis of heptapeptide B23-29 by automatic peptide synthesizer
- Cleavage of heptapeptide B23-29 from resin, purification, and protection of the N-terminal heptapeptide by Boc-group
- Coupling of Boc-B23-29 to A(SS)₂ by chemical coupling with Dicyclohexyl carbodiimide (DCCI) method
- Nitration of Tyr(B16)DOP insulin with tetranitromethan
- Protection of Boc-B23-29 A(SS)₂ (cleavage of Boc group)
- Coupling of NO₂-Tyr(B16)DOPBSS+B23-29-A(SS)₂ to give single chain as des Thr-B30 insulin by using trypsin as single chain des Thr-B30 insulin

Characterization of the single Des (Thr-B30) insulin NO₂-Tyrosine was held by HPLC, capillary electrophoresis amino acid analysis

Heteroatom-contained Peptide Side-chain Formation Method Development

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Peptides as drug candidates have the prior of biocompatibility (compare with small molecular) and easy manual synthesis (compare with protein), while peptide also have the shortcomings of short half-life, conformation flexible, poor cell permeability. To conquer these many peptide modification methods were developed. Side-chain cyclization developed in the past several decades was widely used now days and have been proved can neutralize these shortcomings. While till now only a few methods such as RCM was widely used for the side-chain formation. For variable side-chain modifications new facile side-chain formation methods were needed. Our group has developed several methods which can easily insert heteroatom on most of the position of a hydrocarbon side-chain. And we have proved through serum and mouse digestion that this kind of side-chain on short peptide can highly enhance peptide's stability and CD result show this method also have some efficacy in peptides alpha-helix conformation stabilization.

Evaluation of cytotoxic effect of peptides derived from ninety-four medicinal plants against a gastric carcinoma cell line

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Medicinal plants have been widely used in the complementary and alternative medicine. In addition to various bioactive organic substances, the efficacy of bioactive peptides released from the plant proteins by the digestive enzymes was become a research interest. In this study, crude protein extracts of ninety-four medicinal plants were subjected to hydrolysis by pepsin. Each plant hydrolysate was filtered through a 3kDa molecular weight cut-off membrane. The peptide mixture from each plant was then evaluated for cytotoxic effects against human gastric carcinoma (KATO-III, ATCC No.HTB103) cell line by MTT assay. Only the peptides from three medicinal plants including rhizome of *Gynura pseudochina* var. *hispida*, stem bark of *Streblus asper* Lour and seed of *Piper nigrum* showed potent cytotoxicity against the cancerous cell line with no effect to African green monkey kidney cell line (Vero, ATCC No. CCL 81) at 100 ug protein/ml. The peptides from rhizome of *Gynura pseudochina* var. *hispida* showed the highest cytotoxicity with an IC₅₀ value of 55.28 ug protein/ml. Isolation and identification of the peptide responsible the cytotoxic effect is on going.

Antimicrobial Peptide from the Crab Hemolymph *Eurypanopeus Orientalis* (Sakai, 1939)

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Marine organisms seem to contribute remarkable drugs for several diseases. Many antimicrobial agents have been characterized from the marine organisms which defend against several opportunistic pathogenic infections. Antimicrobial peptides are a major component of the innate immune defence system in marine invertebrates. In such a way an antimicrobial peptide from the hemolymph of a marine crab *Eurypanopeus orientalis* was isolated for the first time and it seems to have an immense antimicrobial effect against the bacteria *Salmonella typhi*. A maximum antimicrobial effect of 17mm was recorded against these pathogens. The preliminary analytical screening of active crab hemolymph with thin layer chromatography assured the presence of peptides in it. The exact peptide which possesses the antimicrobial effect from the hemolymph was then isolated by purification through RP-HPLC. The active peptide fraction was then quantified using SDS PAGE analysis and ¹H-NMR and ESI-MS studies for their determination of molecular weight. The antimicrobial peptide isolated was a 16 k Da peptide and its mass range was quantified to be 431 m/z range. Further the 3 dimensional structure prediction of the isolated antimicrobial peptide through X-ray crystallography is in progress for molecular docking studies. The present study indicates that the hemolymph of *E.orientalis* crabs may possess potential antibiotics.

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Hunting for Cystine Knot Peptides in Seeds

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Plant-derived cystine knot peptides – unique peptides sharing a disulfide knotted core, which endows them high stability against enzymatic, chemical and thermal degradation – have promising use in medicine and agriculture. To fully exploit cystine knot peptides for these purposes, it is imperative to understand their diversity (inter-relationship between sequence, structure, function) and distribution (origin, localization, evolution); knowledge grounded on the discovery of cystine knot peptides. At times, discovery efforts are hampered by the availability of plant materials. However, current screening strategies have not taken into account the efficiency of extraction methods and availability of plant materials. Therefore, the development of an efficient screening strategy is vital. Here, we evaluate five methods commonly used to extract cystine knot peptides: acetonitrile/formic acid¹, boiled water, dichloromethane/methanol², ammonium bicarbonate³, and sodium acetate/acetone⁴, using small amounts of *Momordica cochinchinensis* (gac) seeds as the plant material. The relative abundances of nine known cystine knot peptides, i.e. MCoTI-I, II, IV, V, VI, VII, VIII, Mco-1 and Mco-2, inferred from their extracted ion chromatogram peak intensities, revealed large differences in extraction yield with the acetonitrile/formic acid method having an average of 42% higher yield than the second best (sodium acetate/acetone). Having established the best extraction method, the next challenge is sequencing the cyclic version of cystine knot peptides in crude extracts; given the complexity of fragments that could be generated by enzymatic digestion. Therefore, ongoing work attempts to develop a one pot reduction-alkylation-cleavage to produce full-length linearized cyclic cystine knot peptides in crude extracts for tandem MS sequencing. These data may then be interrogated against transcriptomic data, which would only require relatively small amounts of plant materials to generate, to confirm the peptide sequences and thus providing a new approach for the discovery of cystine knot peptides from rare samples.

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Optimization of the fermentation of okara by the lactic acid bacteria.

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Okara, also named soy pulp is the by-product of the soy milk and tofu production process. Because the production of soy-texturized proteins from pilot plant will generate a lot of wastes, an attempt to make the value added from okara should be considered. However, the wastes contain high amount of proteins that can be useful for further food product development. Since the *Lactobacilli* (lactic acid bacteria) possess the ability to digest large protein molecules into small peptide chains and the bacteria are generally used in the food processing products. It would be thus interesting to investigate

the digestibility of the Lactobacilli to okara. In this study, three species of the lactic acid bacteria (*L. brevis*, *L. fermentum*, and *L. rhamnosus*) were selected. Each of the *Lactobacilli* was cultured in the yeast-extract depleting MRS media and okara was used as nitrogen source instead. All cultures were performed at 37 °C for six different fermentation times (0, 3, 6, 9, 12 and 24 hours). No inoculation set was used as a control. The amount of proteins was extracted from the supernatants, and quantitatively measured the Quant-iT Protein Assay Kit. The results showed that the fermentation with *L. brevis* for 9 hours gives the highest amount of total proteins (5.12 mg/g of dried okara) when compared to those from the other two. On the other hand, after processing through the 3 kDa-MW cut-off ultrafiltration, the fermentation with *L. fermentum* for 12 hours gives the best yield of the low molecular weight peptide (2.26 mg/g of dried okara). The supernatant containing small peptide chains from the fermented okara by *L. fermentum* were subsequently analyzed by the HPLC/MS to estimate their mass. This optimization process will be applied for the semi-pilot scale in development of food processing products containing okara derived small peptides.

Environment-Dependent Conformational Switching in a Designed Peptide: a Molecular Dynamics Study

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Intrinsically disordered peptides (IDPs) defy conventional views of the protein/peptide structure-property paradigm, by conferring function in the absence of a well-defined secondary or tertiary structure.¹ An interesting sub-class of IDPs comprise peptides that can reversibly switch conformation from random-coil to a well-defined structure, in the presence of an external stimulus.² To understand this behavior, bioinformatics approaches have sought to correlate sequence motifs and individual residues of IDPs to their structural preferences based on environmental influences.^{3,4}

JAK1 is a de novo designed IDP, comprising Ala-rich segments that favour helical structure at lower temperatures⁵ as well as the incorporation of Gla (gamma carboxy-glutamic acid), a strong chelator to calcium. It has been proposed that the Gla-rich regions, with their highly-charged side chains, make helical structures unfavourable in the absence of calcium ions. Based on circular dichroism spectroscopy (CD) JAK1 presents a random coil structure in Ca²⁺-free solution but an alpha helical structure when either adsorbed at the aqueous hydroxyapatite (HA) interface, or in the presence of a strong concentration of free Ca²⁺ ions in solution. A control peptide, cJAK1 (where Gla → Glu), showed no such behavior, and did not bind to HA. Molecular simulation can provide complementary, in-depth molecular-level insights into this reversible switching mechanism.⁶ Here, we present results of large-scale Replica Exchange with Solute Tempering (REST) molecular dynamics simulations of JAK1 and cJAK1 in buffer solution, as well as in Ca²⁺ saturated solution.⁷ Using Ramachandran plots to investigate secondary structure preferences, and residue-residue contact maps to determine key intra-peptide interactions, we provide molecular level insight into how environmental influences can mediate the conformational switching of JAK1 through side chain interactions.

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Peptides and nanotechnology

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Remarkable progress has been made to date in the discovery of material binding peptides and their utilization in nanotechnology, which has brought new challenges and opportunities. Nowadays phage display is a versatile tool which has been adapted over the past decade to select material-specific peptides. Screening and selection of such phage displayed material binding peptides has attracted great interest, in particular because of their use in nanotechnology. Phage display selected peptides are either synthesized independently or expressed on phage coat protein. Selected phage particles are subsequently utilized in the synthesis of nanoparticles, in the assembly of nanostructures on inorganic surfaces, and oriented protein immobilization as fusion partners of proteins. The use of proteins to build artificial supramolecular nanostructures has advanced with the development of a computational method to design peptides that will self-organize into specific supramolecular structures on a given surface. Specifically, peptides that assemble on the surface of carbon nanotubes have been designed. Engineering structures on the smallest possible scales — using molecules and individual atoms as building blocks — is both physically and conceptually challenging. Recently, a method of computationally selecting the best of these blocks, drawing inspiration from the similar behavior of proteins in making biological structures has been developed. Proteins that can wrap around single-walled carbon nanotubes have been achieved. An algorithm that sifts through hundreds of thousands of atomically detailed actual and potential protein structures has been designed and can be compared with the structural parameters of the desired scaffolding. This algorithm can be used to design a protein that would not only stably wrap around a nanotube in a helix but also provide a regular pattern on its exterior to which gold particles could be attached. In this paper, we present an overview on the research conducted on this area.

Evaluating Peptide Interactions at the Aqueous Titania Interface Using Molecular Dynamics Simulations

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Understanding the recognition and interactions at the interface between titania and peptides is crucial for numerous applications including the development of biocompatible materials for medical implants and the design and fabrication of nanomaterials for biotechnological and nanotechnological applications¹⁻⁴. Despite the considerable efforts that have been directed toward a general understanding of peptide recognition and binding to inorganic materials, a deep understanding of the molecular-level features of recognition and binding is far from being accomplished⁵. Although several MD simulation studies of peptide adsorption at titania interface have been reported,^{3,4} efficient conformational sampling of adsorbed peptides at the interface remains a great challenge. In this work, we meet this challenge by using a recently-developed state-of-the-art technique, Replica Exchange with Solute Tempering (REST)^{6,7}. Using this approach, for the first time, we studied the binding and interaction of two titania-binding dodecameric peptides at the negatively-charged rutile titania (110) surface, under aqueous conditions. Metadynamics simulations were used to calculate the free energy of adsorption of the peptides to titania. The results reveal critical insights into the role of peptide sequence and conformation in the binding to titania surface.

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Materials-Selective Binding of Peptides on Inorganic Substrates using Molecular Simulation

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Understanding how to control the interaction of biomolecules with noble metal (Ag/Au) and oxide (quartz) surfaces and/or nanoparticles, at the molecular level, will find widespread use in areas including biosensing and nano-medicine. Harnessing the capability of biomolecule-directed assembly of both metallic and non-metallic components may be crucial for realizing hierarchical spatial control in multi-materials assembly. Pivotal to success in this area is the exploitation of materials-selective binding of peptides (*i.e.* preference for a given sequence to stick to one material over another), under aqueous conditions.

As a first step to gaining the in-depth knowledge required for predictably controlling compositionally-selective peptide-materials binding and assembly, we use molecular simulations, in partnership with experiment. Molecular simulations give complementary information relative to experimental characterization, providing a detail of the peptide-materials interface at the atomistic level. Here, I report our findings for the peptide-quartz^{1,4}, peptide-gold^{1,2} and peptide-silver³ interfaces, that we are studying for the purposes of creating self-assembled Au/Ag and Au/SiO₂ nanoparticle arrays with controllable spatial distribution. Our approach described can be generalized to a wide range of biomolecules and inorganic materials.

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Design and Synthesis of Chloroalkene Dipeptide Isosteres as Ground State Peptidomimetics

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Peptide isosteres have emerged as important peptidomimetics for chemical biology and medicinal chemistry. In particular, alkene-type dipeptide isostere is one of the ideal ground state mimetics of peptide bonds. Recently, we have designed a novel peptide isostere possessing an *E/Z*-chloroalkene unit as the surrogate functionality of a peptide bond, which is designated as a chloroalkene dipeptide isostere. In this study, practical and divergent methodologies for the synthesis of chloroalkenes dipeptide isosteres have been developed. A key to our approach is the use of organocopper-mediated

single electron transfer (SET) reduction of allylic gem-dichlorides, which allows the gram-scale synthesis of Xaa-Gly-type chloroalkene dipeptide isosteres. In complementary work for the preparation of Xaa-Yaa-type isosteres, we have found that the stereogenic center corresponding to the side chain of Yaa can be controlled by 1,4-asymmetric induction derived from the tert-butylsulfonylamide group, which enables the stereospecific construction of (L,D)-type isostere framework in high to excellent yields. Design concept, synthetic methods, structural information of chloroalkene dipeptide isosteres and the unique reactivity of allylic gem-dichlorides will be discussed.

Global in-depth quantitative proteomic analysis of HIV infected cells using a novel Q-OT-qIT mass spectrometer

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Quantitative changes in the phosphoproteome of human cell lines following infection by the human immunodeficiency virus (HIV) were analysed by hybrid Orbitrap-based mass spectrometry. Global identification and quantification of changes in human phosphoproteome in response to HIV was performed using stable isotope labeled (SILAC) Jurkat cells and HILIC/TiO₂ fractionation and enrichments using the new Orbitrap FusionTM TribridTM mass spectrometer and EASY-ETDTM source. More phosphopeptides were identified using a combination of ETD and CID fragmentation in a single run (via novel data dependent decision tree with dynamic scan management) than when using CID fragmentation alone which permitted precise localization of phosphorylation more frequently.