ANTAGONISTS OF INTRACELLULAR PROTEIN-PROTEIN INTERACTIONS: A NEW CLASS OF TARGETED ANTICANCER AGENTS

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A deeper understanding of the molecular events leading to tumour formation, invasion, angiogenesis and metastasis has provided a new mechanistic basis for oncology drug discovery: targeted anticancer agents. By specifically blocking the molecular pathways implicated in the pathogenesis of cancer, targeted anticancer agents are expected to alter the natural course of the disease and, at the same time, offer an enhanced therapeutic index over traditional cytotoxic agents.

Over the last few years – in line with this new paradigm - a set of intracellular protein-protein interactions have been selected as candidates for drug discovery activities based on their association with dysfunctional signal transduction/cell cycle pathways. In spite of screening efforts, the identification and development of compounds capable of modulating specific intracellular protein-protein interactions in cancer cells has proven highly challenging, and consequently forced medicinal chemistry departments to re-think drug discovery strategies for this family of targets. In this regard, peptides and derivatives thereof are likely to play a major role in determining the biological functions of protein interactions and also in developing tool compounds or antagonists to further explore cancer biology as well as therapeutic intervention.

Drawing from my own research, I will present the application of peptide chemistry to three areas of drug discovery: the mapping of protein-protein interfaces, the development of structure-based design protein antagonists, and the validation of protein-protein targets in cellular settings.

STRUCTURE-ACTIVITY RELATIONSHIPS IN PEPTIDES: FROM MODELLING TO RATIONAL DRUG DESIGN

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Intensive efforts have been made to develop peptides or peptidomimetics that display more favourable pharmacological properties than their prototypes. Most of the researches concerns with the preparation of analogues with different chemical structure and possibly modified conformational preferences/stability, in order to induce changes in the biological response. Structural changes can be obtained by selectively substituting along the sequence specific residues with other coded residues with the same or with opposite chirality or with non-coded α-amino acid residues or peptidomimetics or by cyclising appropriately the peptide. Constrained non-coded α-amino acid residues are of particular interest as "building blocks", since their inclusion in a peptide sequence could maintain the pharmacological properties of the native peptide and possibly enhance resistance to biodegradation with improved bioavailability and pharmacokinetics.

On this basis we have investigated over the years the conformational behaviour of the symmetrical and unsymmetrical α,α-di-substituted glycines or peptides presenting D-residues. These modification have been incorporated in synthetic analogues to introduce predictable structural constraints in order to assess the biological active conformation and then to produce new peptide-based molecules to be used as drugs or diagnostics. With this knowledge we are now able to rationally design new peptides relevant to pharmacology and medicinal chemistry, which might mimic biological processes by enhancing or in general modulating their effects. The peptide pharmaceutical targets of these studies have been among others hormones, enzymes, transport systems, antibiotics, sweeteners, etc..

AN ADVENTURE IN PEPTIDE CONFORMATION
(JOSEPH RUDINGER MEMORIAL LECTURE)

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I will start my talk by giving appropriate credit to my two mentors, Prof. E. Scoffone and Prof. M. Goodman, and by briefly mentioning my personal recollections of Prof. J. Rudinger. Then, I will discuss the results I like most obtained in the first 15 years of my scientific adventure in the area of peptide conformation. I have decided to take this unique opportunity to focus on that period (1966-1980) because those findings have still implications on the conformational works going on today and because they are almost completely neglected by the authors of the current publications who usually do not go back more than 25 years in their literature search (a modest negative aspect of the otherwise extremely positive web modern technology).

(i) Post-doc and collaborative work with M. Goodman: critical main-chain lengths for alpha-helix and intermolecular beta-sheet formation; first CD and NMR spectra of monodispersed, folded, peptides; use of TFE as a secondary structure supporting solvent.

(ii) Independent work in Padova: stability rank order of protein amino acids for beta-sheet formation: parallel vs. antiparallel beta-sheet and intramolecularly H-bonded folded form tendencies of protein amino acids; CD of an unordered peptide conformation.

(iii) Collaborative work with M. Mutter: spectroscopy of PEG-bound hydrophobic peptides in aqueous solution; parallel effects of peptide main-chain length on conformation, solubility and reactivity; beta-sheet breaker role of a proline residue.

From 1980 on, a new adventure (conformation of Aib peptides) took off in my laboratory in collaboration with Prof. E. Benedetti…, but this is another story.

Finally, adequate acknowledgements will be presented to my coworkers and external collaborators, and to the supporting Company, all essential for my overall positive adventure in peptide conformation.

OR001

A 1, 2-AMINO ALCOHOL DERIVATIVE OF FMOC AMINO ACIDS: A MASKED ALDEHYDE FOR USE IN SEQUENTIAL CHEMOSELECTIVE LIGATION

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Chemoselective ligation between C-terminal peptide aldehydes and peptides containing an N-terminal hydroxylamine or hydrazine functionality can be used to assemble peptides and proteins that can not be synthesised as a single chain by solid phase peptide chemistry(1). If more than two peptide fragments are to be joined, selective masking of one reactive group is required, allowing fragments to be sequentially ligated. Masking a C-terminal aldehyde as a 1, 2-diol has been employed in the sequential chemoselective ligation of several long peptide fragments(2), but was limited by low level diol substitution on chlorotrityl-resin. 1, 2-amino alcohols are an alternative masked aldehyde to 1, 2-diols, and the amino functionality is significantly more reactive towards chlorotrityl-resin than an alcohol. We therefore have developed a synthetic route for the preparation of 1, 2-amino alcohol derivatives directly from Fmoc protected amino acids, avoiding the need to change the protecting groups of either the amino or side chain of the amino acid. Using these derivatives, synthesis of peptides bearing a C-terminal amino alcohol can be achieved in high yield. Unmasking of the amino alcohol to aldehyde by periodate oxidation proceeds smoothly to produce C-terminal peptide aldehydes. In summary the 1, 2-amino alcohol derivative of Fmoc-amino acids provide access to the chemical synthesis of large peptides and proteins via sequential chemoselective ligation.

(1) S.B.H. Kent, P. Dawson, Annual Review of Biochemistry 2000 69 925-962
(2) R. J Broadbridge, C. E Winsor and R. P Sharma Proceedings of the 7th International Symposium, Southampton 2001 33-38

OR002

EVALUATION OF A STEROIDAL SCAFFOLD FOR THE CONFORMATIONAL RESTRICTION OF PEPTIDES IN THE DEVELOPMENT OF PEPTIDE VACCINES

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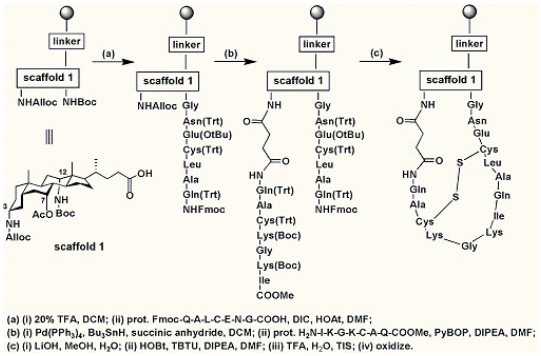
Despite efficient vaccines, measles continues to cause considerable morbidity and mortality worldwide. Improved vaccination strategies may well be required to achieve the World Health Organization goal of measles eradication.
This project aims at using the dipodal steroid-based scaffold 1 as an attachment point for the Hemagglutinin Noose Epitope (HNE) to give the latter the necessary preorganisation for it to adopt an analogous loop-like conformation to the one it has in the H-protein. From a synthetic point of view, the obvious strategy to follow is solid phase peptide synthesis. Peptides can either be coupled stepwise or in a convergent way. Cyclisation can be carried out at various points along the peptide chain.



(a) (i) 20% TFA, DCM; (ii) prot. Fmoc-Q-A-L-C-E-N-G-COOH, DIC, HOAt, DMF;
(b) (i) $Pd(PPh_3)_4$, Bu_3SnH, succinic anhydride, DCM; (ii) prot. H_2N-I-K-G-K-C-A-Q-COOMe, PyBOP, DIPEA, DMF;
(c) (i) LiOH, MeOH, H_2O; (ii) HOBt, TBTU, DIPEA, DMF; (iii) TFA, H_2O, TIS; (iv) oxidize.

TOTAL CHEMICAL SYNTHESIS OF INSULIN VIA BIOMIMETIC FOLDING

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Several insulin analogues have recently been introduced clinically for improved treatment of diabetes. Industrial productions of such insulins are based on microbial expression systems, which are highly efficient, but generally limited to the 20 proteogenic amino acids. Also, some sequences form inclusion bodies or fail to express. The total chemical synthesis of insulin in research scale was a landmark achievement in peptide science. However, the most commonly used method relies on recombination of A- and B-chains under "random" folding and pairing of the three disulfide bridges. This folding/oxidation step is difficult and low yielding. A general approach using a removable auxiliary which can direct correct formation of disulfide bridges is highly desirable.

In the pancreas as well as in microbial expression systems, insulins are prepared and folded as single chain precursors, with a C-peptide connecting the A and B-chains. The C-peptide helps direct the orientation of A and B-chains in obtaining the correct disulfide pairing and overall peptide folding. Upon folding, the C-peptide is removed enzymatically.

We report here a new method for total chemical synthesis of insulin by use of Fmoc-based step-wise solid-phase synthesis of single-chain precursors followed by C-peptide directed folding and cleavage of C-peptide, thereby allowing total chemical synthesis of novel insulins with unnatural substitutions.

OR004

DIRECTED FOLDING OF ALPHA-CONOTOXINS USING SELENOCYSTEINE

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Many biologically active peptides from animal venoms contain multiple cystine links that are crucial for their biological activity. One of the smallest members of this group are the α-conotoxins which target the nicotinic acetylcholine receptor [1]. They are relatively small peptides (from 8-16 residues) with 4 cysteine residues forming 2 disulfide bonds (1-3; 2-4), which maintain the globular conformation. The other two possible isomers, the ribbon isomer (1-4; 2-3) and the bead form (1-2; 3-4) are generally of significantly lower activity. Current synthetic approaches to the alpha-conotoxins take advantage of the tendency of this conotoxin class to prefer the globular fold. By contrast, access to the two other folds is limited unless chemoselective approaches to disulfide bond formation are incorporated in the synthetic scheme [2].

We describe here a novel approach that takes advantage of the isosteric nature of sulfur and selenium by regiospecifically incorporating pairs of protected selenocysteine (Sec) residues into the synthetic scheme. The different physiochemical properties between Sec and Cys such as its low pKa (5.2 Sec vs. 8.5 of Cys) and the low redox potential (-381 mV Sec vs. -180 mV of Cys) facilitate a directed oxidative workup of the reduced conotoxin via simple pH control. This is illustrated through regioselective syntheses of potent α7 nAChR preferring conotoxins.

References:
1.M. L. Loughnan, P. F. Alewood, European Journal of Biochemistry 271, 2294 (2004).
2.C. J. Armishaw, P. F. Alewood, Current Protein and Peptide Science 6, 221 (2005).

OR005

NEUROPROTECTIVE PEPTIDES DERIVED FROM AMYLOID PRECURSOR PROTEIN AS TOOLS IN ALZHEIMER RESEARCH

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The amyloid cascade hypothesis is the most accepted explanation for the pathogenesis of Alzheimer disease (AD): the accumulation of β-amyloid peptides (Aβ) in the brain is a central event leading to the development of AD. Proteolytic cleavage of the amyloid precursor protein (APP) by the α-secretase within the amyloid-β peptide (Aβ) sequence precludes formation of amyloidogenic peptides and leads to a release of soluble N terminal APP fragments of (APPsα). Secreted APPsα appears to have beneficial properties, evoking coordinated responses in neuronal and some peripheral target cells.The existence of a membrane receptor for α-secretase-generated APPsα has been postulated for a long time, but at present the receptor protein is completely unknown. The aim of our work is to identify a receptor responsible for the neuroprotective effect of APPsα. For this purpose we synthesized 10- and 20- amino acid peptides with sequences from C-terminus of APPsα, which discriminate APPsα from APPsβ. The latter lacks neuroprotective function. We have examined the neuroprotective properties of these peptides against the toxicity of 6-hydroxydopamine (6-OHDA) and Aβ25-35 in human neuroblastoma cells (SH-SY5Y) by viability assays. Our results show that C-terminal APPsα-peptides protect cells in a similar way as APPsα against toxicity induced by 6-OHDA and Aβ25-35. In contrast, C-terminal truncated APPsα lacks neuroprotective properties in both assays. For APPsα receptor identification tritium-labelled peptide ligands have been synthesized and a classical ligand-binding assay in plasma membranes prepared from neuronal cells and from rat brain has been established.

EXPLORING THE RELATIONSHIP BETWEEN TURN GEOMETRY AND ALLOSTERIC ANTAGONISM OF PEPTIDE MIMIC LIGANDS FOR A PROSTAGLANDIN RECEPTOR

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Prostaglandins are involved in a large number of biological activities mediated by their G-protein coupled receptors (GPCRs). The prostaglandins PGF2 alpha receptors are found specifically in uterine muscle, where they initiate parturition and labor. The PGF2 alpha receptor plays a key role in preterm labor, for which medical and social costs are estimated at $ 9 billion per year in the USA (the highest per patient cost of any disorder). Peptide mimics have been developed in our laboratory(1,2), that serve as allosteric antagonists of the PGF2 alpha receptor. The importance of the turn geometry of the central residue in these peptide mimics has been investigated using enantiomeric indolizidin-2-one beta-turn mimics which can respectively induce type II and II' geometry. Our presentation will discuss the synthesis and biology of these novel allosteric modulators of prostaglandin PGF2 alpha receptor activity.

1. Peri, K.G.; Quiniou, C.; Hou, X.; Abran, D.; Varma, D.R.; Lubell, W. D.; Chemtob, S. "THG113: a novel selective FP antagonist which delays preterm labor" Seminars in Perinatology 2002, 67, 389-397.
2. Peri, K.G.; Polyak, F.; Thouin, E.; Lubell, W.D., Chemtob, S. PCT Int. Appl. serial No. 60/387, 424, June 11, 2002.

OR007

MECHANISM OF THE INTERACTION BETWEEN HIV-1 INTEGRASE AND LEDGF/P75

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The integrase protein (IN) of HIV-1 catalyses the integration of the viral genome into the host chromosome. IN binds the viral DNA as a high-order oligomer. The cellular protein LEDGF/p75 binds IN and stimulates its activity. Our research aim is: (1) To reveal the active oligomerization state of IN, and how LEDGF/p75 affects it (2) To characterize IN binding to the viral DNA and to LEDGF/p75 (3) To reveal what is the mechanism in which LEDGF/p75 enhances IN activity.

Based on the IN-LEDGF/p75 crystal structure complex, we have synthesized a set of fluorecein-labeled peptides derived from the IN binding loops of LEDGF/p75 and tested their IN binding using fluorescence anisotropy. IN bound the LEDGF361-370 peptides with Kd=3microM. Binding was highly cooperative, with Hill coefficient around 4, indicating tetramerization of IN upon peptide binding. IN did not bind a peptide from the second LEDGF loop. Binding of IN to 36-bp viral LTR DNA was cooperative with a Kd=37nM, and, Hill coefficient of 2, indicating binding of IN dimer to the DNA. A truncated IN mutant, IN52-288, did not bind the LEDGF peptides or the DNA, indicating involvement of IN1-51 in the binding process. LEDGF peptides did not alter the affinity of IN to DNA. Analytical gel filtration showed that free IN is highly aggregated, and binding to the LEDGF peptides or to DNA forces it out of the aggregate into its active dimeric or tetrameric form. Our results shed light on the molecular mechanism of IN-LEDGF/p75 binding.

OR008

USING NOVEL BENZOPHENONS TO STUDY MICROBIAL SUGAR RECOGNITION AND TRANSLOCATION BY PHOTO-AFFINITY LABELING / PHOTOLABELING

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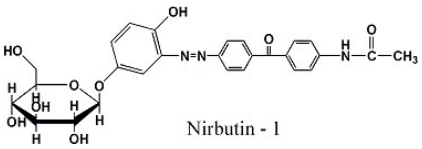
The BglF protein of Escherichia coli is a beta-glucosides permease of the phosphoenolpyruvate- dependent PhosphoTransferase System (PTS). The PTS is a multi component system that catalyzes vectorial phosphorylation of various sugars. It couples sugar transport to phosphorylation driven by the high phosphate transfer potential of phosphoenolpyruvate. The PTS components are involved in complex signal transduction pathways including virulence. PTS is ubiquitous in bacteria but is not found in animals and plants. Its uniqueness and pleiotropic functions make the PTS an attractive target for the development of novel antibacterial drugs.

The nature of the sugar translocation channel is still unknown. To advance understanding of sugar translocation, we synthesized βglucoside sugars that contain a benzophenone photoactive group, termed Nirbutins, to be used for photoaffinity labeling of BglF. The expected advantages of Nirbutins as photoprobes are: (i) chemical stability. (ii) manipulated in ambient light and activated at 350-360nm. (iii) efficient covalent bonding to macromolecules. (iv) site specificity.

Nirbutin-1 was demonstrated by us to be a recognized and transported by BglF, as well as utilized by E. coli cells expressing BglF. We are currently synthesizing bi-functional Nirbutins that can bind to two sites in the permease concomitantly. We intend to use the Nirbutin series: (i) mapping the sugar binding sites, (ii) studing the route that the sugar traverses through the membrane, and (iii) investigate the sugar-induced conformational changes.



Nirbutin - 1

OR009

MODIFYING PROTEIN P53 TETRAMERIZATION BY DESIGNED CALYX[4]ARENE COMPOUNDS

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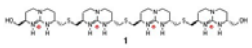
Protein p53 is one of the main targets in anticancer research due to its high mutation frequency in human cancers. Most of their mutants affect its central DNA binding domain, resulting in non-functional or oncogenic species. Some other mutants are found within the tetramerization domain (p53TD), compromising the ability to form the active tetramer. Molecules that modulate the stability of the oligomeric structure could thus be helpful tools for anticancer therapy.

We previously reported how the tetraguanidinium ligand 1 was able to recognize the solvent exposed anionic patch of the p53TD surface. Based on the p53TD structure and taking into account the strong interaction established between the guanidinium groups of 1 and the carboxylate anions of the protein, a family of calyx[4]arene ligands (such as 2) was computationally designed.

NMR studies show that the calixarene ligands cause spectacular changes in the p53TD structure. These same changes are also found in the p53TD mutant R337H. These ligand-protein systems have been further characterized by circular dichroism, microcalorimetry and analytical ultracentrifugation.



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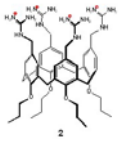

2

OR010

FENTEPHALINES: DIMERIC BIOACTIVE PEPTIDES BASED ON AMINO ACIDS COUPLED TO 4-ANILINO-N-PHENETHYL-PIPERIDINE

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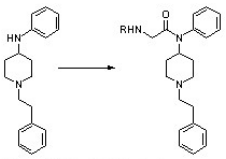
Design of chimeric molecules based on two distinct classes of opioid ligands, the peptide portion derived from enkephalins and the non-peptide moiety from a 4-anilidopiperidines series, holds numerous possibilities in the area of drug design, and their medicinal applications. Fentanyl related compounds are the most powerful drugs in modern medicine for relief of severe acute and chronic pain, but their use is limited due to side effects. The inherent m-opioid receptor selectivity of fentanyl and its analogues allows introduction of m-selectivity into corresponding opioid peptide analogues. The designed analogues (Scheme 1) were prepared in moderate to good yields by solution and solid phase synthesis using Fmoc/Boc chemistry and tested for in vitro and in vivo opioid activity. The results of our biological tests show that substitution of the propionyl moiety of fentanyl with opioid peptide ligands leads to compounds possessing high affinity for opioid receptors. The dimeric nature of fentephalines is responsible for their extraordinary pharmacological profile. The work is supported by grants from the US Public Health Service, National Institute of Drug Abuse.



Scheme 1. R: Tyr-*D*-AA-Gly-Phe-Leu, *D*-AA: *D*-Ala, *D*-Pro, *D*-Abu, *D*-Phe.

OR011

ISOLATION AND IDENTIFICATION OF PHOSPHOPEPTIDES FROM K562 CELLS

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Protein phosphorylation is a common posttranslational protein modification (up to 30% of the proteome) used to modulate cellular protein activity. Deregulation of protein kinase activity may lead to abnormal events such as inflammation, intracellular infection or malignant transformation. Phosphorylated peptides have been detected on cell surface MHC class I molecules and have also been shown to modulate the immune response. Chronic myeloid leukaemia (CML) is a neoplastic disorder characterised by the presence of the fusion of two genes abl and bcr to give the fusion bcr/abl oncogene. The gene products of abl, bcr and bcr/abl are protein kinases.

MHC class I peptides were eluted from the surface of HLA-A3 transfected K562 cells using a buffer which minimised cell lysis and retained IMAC compatibility. The peptides were chemically modified to reduce non specific interactions with the (Ga3+) IMAC resin. The bound peptides were subjected to dot blots and peptide PAGE to confirmed the presence of phosphorylated peptides. Further analysis and identification was achieved with RP-ES/MS/MS.

OR012

A NOVEL PEPTIDE VECTOR FOR EFFICIENT INTRACELLULAR DELIVERY OF PROTEINS

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Most of the known cell penetrating peptides (CPP) are needed to be fused or chemically linked to a cargo molecule to deliver it into cell. Recently described peptide Pep1 has been shown to be able to transport proteins and peptides across cell membrane without the necessity of covalent coupling. Here we report about a new gramicidin A analogue C-terminaly modified with a short cationic sequence. This peptide (P10C) forms noncovalent complexes with proteins and promotes their effective delivery in different cell types. The optimum P10C/protein ratio was shown to be 10 times less than that of the Pep1. Cellular uptake studies revealed both temperature-dependent and temperature-independent modes of complexes uptake. To study P10C-membrane interactions, we performed biophysical assays on planar bilayer and liposomes. The data suggest that P10C can cross synthetic bilayers spontaneously in vitro and form large unselective pores. The systematic study around P10C provided insight into the effects of modifications in hydrophobic and cationic parts on the structural properties of the peptide and their relationships to protein binding and cell penetrating activity. Taken together these data suggest P10C to be a competitive protein delivery system as well as a useful tool in biological research and a potential instrument for the protein and peptide pharmaceuticals intracellular delivery.

OR013

CELLULAR UPTAKE OF S4(13)-PV CELL PENETRATING PEPTIDE: AN ENDOCYTOSIS-INDEPENDENT PROCESS FOLLOWING PEPTIDE CONFORMATIONAL CHANGES INDUCED BY PEPTIDE-MEMBRANE INTERACTIONS

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Cell penetrating peptides have been successfully used to mediate the intracellular delivery of a wide variety of molecules in vitro and in vivo, although the mechanisms by which cellular uptake occurs remain unclear and controversial.

In face of accumulating reports demonstrating that uptake of some cell penetrating peptides occurs through previously described endocytic pathways, or is a consequence of fixation artifacts, we conducted a detailed and systematic analysis of the mechanism responsible for the cellular uptake of the S4(13)-PV cell penetrating peptide.

We report that the S4(13)-PV peptide accumulates inside cells very efficiently through a rapid, dose-dependent and non-toxic process, independently of cell fixation. Studies addressing the effect of several drugs, as well as experiments involving overexpression of a dominant-negative mutant of dynamin, consistently exclude endocytosis as the mechanism responsible for the cellular uptake of this peptide. Analysis of peptide uptake by mutant cells lacking heparan sulfate proteoglycans demonstrates that the presence of cell surface proteoglycans facilitates peptide uptake. Finally, we demonstrate that, upon interaction with model membranes, the S4(13)-PV peptide undergoes sequence-dependent conformational changes, consistent with the formation of helical structures. Such conformational changes occur concomitantly with penetration of the peptide into the lipid bilayer, indicating that the resulting helical structure is crucial for the non-endocytic cellular uptake of the S4(13)-PV peptide.

Overall, our data support that rather than endocytosis, the cellular uptake of the S4(13)-PV cell penetrating peptide is a consequence of its direct translocation through cell membranes following conformational changes induced by peptide-membrane interactions.