Design, synthesis and structure of peptides and proteins

Ernest Giralt

Improving our knowledge of the rules that govern molecular recognition of how molecular recognition is regulated is behind all our endeavours in this field. The study of protein-protein interactions in general and protein self-assembly in particular affords many opportunities to improve our understanding of molecular recognition. Of greater importance still, from a more applied perspective, these studies provide numerous possibilities for drug discovery. Many additional unknowns have yet to be addressed. To make good progression in this field, several of these unknowns are also studied by our group. How can we design a peptide to ensure efficient cellular uptake? Is it possible to achieve remote control of the disruption of amyloid fibrils? Can we use peptides to shuttle drugs across the blood-brain barrier? Finally, methodological improvements are constantly required in all scientific activities. This is the focus of our more recent work in using NMR to study protein-ligand and protein-protein interactions, improving solid-phase methods for peptide and protein synthesis and developing computational evolutionary algorithms for structure-based drug design.

Intracellular delivery by cell-penetrating peptides

Attaining satisfactory intracellular delivery is crucial for many drugs. A successful delivery strategy is the use of peptide sequences that have the capacity to translocate through the cytoplasmic membrane, the so-called cell-penetrating peptides (CPPs). The most common feature of CPPs is the presence of positive charges, from arginines or lysines.

A CPP class of particular interest comprises amphipathic proline-rich peptides (Pujals, 2008), derived from the N-terminal domain of □-zein, a maize storage protein. On the basis of the observation that a Pro content of 50% is enough to maintain PPII structure, we transformed a PPII helix into an amphipathic sequence by placing hydrophobic residues at positions i/i+2, i+6/i+8, etc, and hydrophylic amino acids at i+3, i+7, etc. Several peptides with the structure $(VXLPPP)_n$ were synthesised, where X=His, Arg or Lys and n=1-3. The hydrophobic residues Val and Leu were chosen in an analogy with the aforementioned N-terminal domain of □-zein. Evaluation of the internalisation properties of the distinct compounds by plate fluorimetry showed CF-$(VRLPPP)_3$ (where CF=5(6)-carboxyfluorescein) to be the peptide most efficient at crossing the cellular membrane.

This new family of intracellular vectors holds several advantages: non-viral origin, high solubility in aqueous media, and absence of cytotoxicity at very high concentrations. The best candidate, (VRLPPP)3, was named SAP (Sweet Arrow Peptide) for the aforementioned reasons.

Efforts have been made to improve the cell penetration capacity of SAP by increasing its hydrophobicity and amphipathicity, and developing fatty acyl derivatives (Fernández-Carneado *et al*, 2005) and a silaproline derivative (Pujals *et al*, 2006).

The mechanism by which SAP is internalised in the cell has been examined (Pujals *et al*, 2008) through colocalisation studies and experiments with ATP depletion. This study concluded that the internalisation mechanism is lipid raft- or caveolae-mediated endocytosis. The most relevant property of lipid rafts and caveolae is that during transport, unlike in internalisation via clathrin-mediated endocytosis, the pH remains neutral and no degradation of cargo occurs, ie, late endosomes do not fuse with lysosomes. This is crucial for carrying cargo susceptible to proteases or nucleases inside the cell. Furthermore, a totally human-serum-stable version of SAP built with non-natural D-amino acids (D-SAP) has been developed (Pujals *et al*, 2008). The enantiomer retained the non-cytotoxic and translocation properties of SAP and was stable to high concentrations of trypsin and in 90% human serum.

The toxicity of D-SAP was also evaluated in Balb/c mice over one week (Pujals *et al*, 2007). The com-

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pound was administered on alternate days, and the most relevant blood biochemical parameters, as well as weight, were measured. No significant differences were found between treated and untreated animals. We then performed *in vivo* internalisation studies using the carboxyfluoresceinated version of D-SAP. The uptake of D-SAP in white blood cells was monitored by flow cytometry, which showed that internalisation was time-dependent, with great fluorescence 1 and 4 h post-injection and low fluorescence at 24 h. Confocal laser scanning microscopy showed distribution of fluorescence signal in the kidney, heart, spleen and liver.

Design and synthesis of □-amyloid aggregation inhibitors

One hundred years after the discovery of Alzheimer's disease (AD), current treatments offer only symptomatic benefits to patients. Although this is discouraging, during these one hundred years, basic research has led to the identification of many of the pathways that contribute to this devastating disease, thereby providing an excellent opportunity to develop new treatments that target the root causes (Carulla *et al*, 2006).

AD is associated with aggregation of the β-amyloid (Aβ) peptide, a 39- to 42-residue peptide. Soluble Aβ converts into insoluble amyloid fibrils or plaques that accumulate in the brain of patients suffering from AD (Figure 1). Thus, one strategy to defeat AD involves the design of compounds with the capacity to interfere with and/or prevent Aβ aggregation. Within this context, our laboratory addresses the design of peptide-based Aβ aggregation inhibitors. We have used the self-recognition property of Aβ, that is to say its capacity to bind to itself, and employed a central Aβ fragment, Aβ(16-20) as the recognition element.

The end products of Aβ aggregation are amyloid fibrils, which are stabilised by intermolecular β-sheets with hydrogen bonds expanding in the direction of the fibril growth. Our inhibitor peptides contain an N-methyl amino acid, which allows the inhibitor peptides to present two unique hydrogen-bonding faces when arrayed in an extended, β-strand conformation. One face of the inhibitor peptide has normal hydrogen bonding capacity, which allows interaction of the inhibitor with the Aβ molecules. The other face has an N-methyl group in place of a backbone amide proton, which prevents hydrogen bond formation precluding further Aβ molecules from binding to the inhibitor and thus capping fibril growth.

Peptide drugs offer high activity, high specificity and low toxicity, but are susceptible to proteolytic deg-

radation. A strategy to overcome this problem is to produce peptides using non-natural D-amino acids. Although all-D peptides are not recognised by proteases, they may be less active than their corresponding all-L peptides because of differences in the orientation of amino acid side-chains. To preserve the orientation of the side-chains of the parent L-peptide in a D-analogue, the D-amino acid residues can be linked in the reverse order to that of the parent peptide in order to produce a retro-enantio peptide. This approach emphasises the maintenance of the topological native orientation of the side-chains but not of the backbone.

After considering the pros and cons of an all-D peptide and a retro-enantio peptide versus an all-L peptide, we chose to study the use of the retro-enantio approach in the development of peptide-based Aβ aggregation inhibitors for the treatment of AD. We have worked with three inhibitors, inL (an all-L peptide), inD (an all-D peptide), and inrD (a retro-enantio peptide). inD and inrD were designed to improve proteolytic stability since they are made of D amino acids. The work with inrD allowed us to test the utility of the retro-enantio approach to obtain peptides able to interact with β-sheet proteins. Using biophysical methods, we evaluated the capacity of the three inhibitors to interfere with Aβ aggregation.

Furthermore, we have screened their capacity to inhibit Aβ cytotoxicity in neuroblastoma cell cultures. The inhibitor peptides have been subjected to trypsin and cerebrospinal fluid (CSF) to evaluate their stability to proteases.

Finally, we have modelled the interaction of the inhibitor peptides with an Aβ fibril model to examine their possible mode(s) of action. From these studies, we have learned that inrD, the one designed using the retro-enantio approach, is the peptide with the highest capacity to interfere with Aβ aggregation and decrease Aβ cytotoxicity, while being stable to proteases (Figure 1; Grillo-Bosch *et al*, submitted for publication). Given that during Aβ aggregation β-sheet formation is one of the predominant conformations sampled and inrD interferes with Aβ aggregation, we consider the use of retro-enantio peptides a valuable approach for obtaining peptides able to interact with β-sheet proteins.

This observation may have major implications for the design of bioactive peptides for myriad therapeutic indications, including those targeted at disrupting protein-protein interactions. As a result of the success of this study and with the idea to go one step further in our goal towards developing drugs to treat AD, we have initiated a collaboration with Isidre Ferrer (Institut de Neuropatologia de Bellvitge) to study the effect of inrD in transgenic AD mouse models.

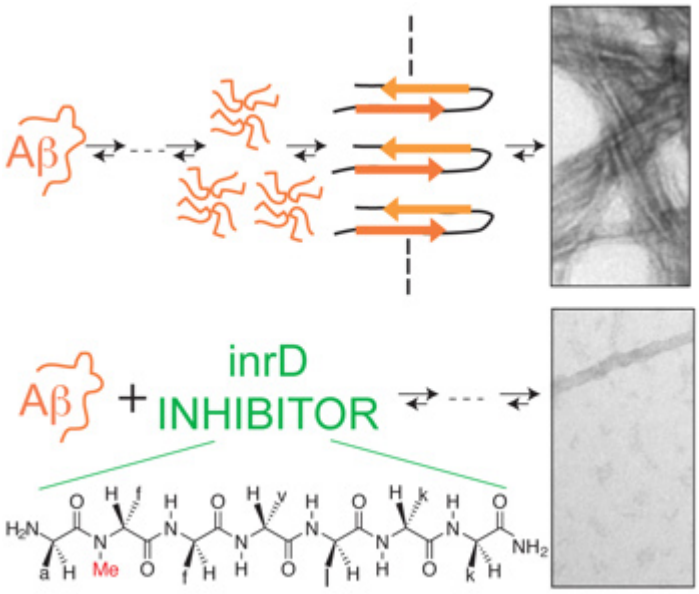

***Figure 1**. Schematic representation of the effect of inrD on Aβ aggregation. (Top) Schematics of a possible mechanism for Aβ aggregation and amyloid fibril formation. Monomeric Aβ is converted into amyloid fibrils through a series of steps. At the end of Aβ aggregation, electron micrographs show the presence of abundant Aβ amyloid fibrils. (Bottom) Schematics of Aβ aggregation in the presence of inrD. The electron micrograph reveals the presence of very few protofilaments as well as spherical oligomers in the βA samples incubated with inrD. The amino acid sequence of inrD is depicted in the figure, all the amino acids are in the D configuration and the N-Me group is shown in red.*

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Research Networks and Grants

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Research Director: Ernest Giralt

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Research Director: Ernest Giralt

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Research Director: Ricardo Ibarra

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Research Director: Ernest Giralt

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Fundació La Marató TV3, 092: 2006-2009
Project Coordinator: Ernest Giralt

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Ministerio de Educación y Ciencia, Programa Hispano-Brasileño de Cooperación Interuniversitaria, estancias de movilidad, PHB2005-0068-PC: 2006-2007
Research Director: Ernest Giralt

Collaborations

Applications of the Suzuki reaction to the synthesis of conformationally constrained peptides
Paul-Lloyd Williams, University of Barcelona (Barcelona, Spain)

Cyclodepsipeptides as potential anticancer agents
Ricardo Pérez-Tomas, Bellvitge Hospital, University of Barcelona (Barcelona, Spain)

Design of HIV-1 protease dimerisation inhibitors
Michele Reboud-Ravaux, CNRS-University of Paris (Paris, France)

Design, synthesis and study of P53 ligands
Javier de Mendoza, Institute of Chemical Research of Catalonia -ICIQ (Tarragona, Spain)

Remote manipulation of protein aggregation
Marcelo Kogan, University of Chile (Santiago, Chile)

Synthesis and conformational analysis of cyclodepsipeptides of marine origin
Fernando Albericio, IRB Barcelona (Barcelona, Spain)

Synthesis and structural studies of □-peptides
Rosa Mª Ortuño, Universitat Autònoma de Barcelona (Barcelona, Spain)

Awards

Peptide Idol Award for young investigators, American Peptide Society, 20th American Peptide Symposium (Canada, 2007).
Awardee: Meritxell Teixidó

Patents

Compounds as blood brain barrier shuttles and shuttle-cargo constructs
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Patent application number: PCT/ES2007/0401
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