

# Design, synthesis and structure of peptides and proteins



The design of ligands that recognise predefined 'patches' of the surface of a protein is highly relevant in biomedicine since this will allow future custom-designed modulation of protein-protein interactions. Our lab is working on several systems of therapeutic relevance, including inhibitors of beta-amyloid protein aggregation, 'chemical chaperones' for P53, angiogenesis inhibitors based on the molecular recognition of VEGF, and human brain prolyl oligopeptidase (POP) inhibitors. Although the design of these ligands is hampered by our poor knowledge, at a quantitative level, of non-covalent interactions, we have developed new methodologies that combine the automatic design *in silico* based on evolutionary algorithms with experimental 'validation' using a variety of spectroscopic techniques, in particular nuclear magnetic resonance (NMR; Pellecchia *et al*, 2008). Finally, it is necessary to emphasise the significant role that peptide 'shuttles' will play in the future. These shuttles will allow the new synthetic ligands to overcome physiological barriers, such as the plasmatic membrane or the blood-brain barrier (BBB), and reach their protein target.

## p53: Rescuing narcissistic protein-protein interactions

The stabilisation of protein-protein interactions holds potential as a therapeutic strategy, yet it has gained little attention. Our group has recently reported calixarene molecules that rescue and stabilise the functional tetrameric state of certain mutants of protein p53 (Gordo *et al*, 2008). Protein p53 is a transcription factor that is crucial for cell cycle and genome integrity. It is able to induce both cell arrest when DNA is damaged and the expression of DNA repair machinery. When the damage is irreversible, it triggers apoptosis. In its active form, p53 protein is a tetramer formed by four identical copies of proteins bound together. p53 has four domains with differentiated functions: activation of transcription, DNA binding, tetramerisation and regulation. The tetramerisation domain is responsible for stabilising the tetrameric structure.

More than 50% of cancer patients have mutations in the p53 gene. Although most of these are located in the DNA-binding domain, several mutations are found in the tetramerisation domain, thereby causing destabilisation of the entire protein with the consequent loss of activity. Two well documented examples of this kind of congenital predisposition are pediatric adrenocortical carcinoma and Li-Fraumeni syndrome. Therefore, the design of compounds with the capacity to stabilise the tetramerisation domain of p53 represents a new and attractive strategy for the development of anti-tumour drugs.

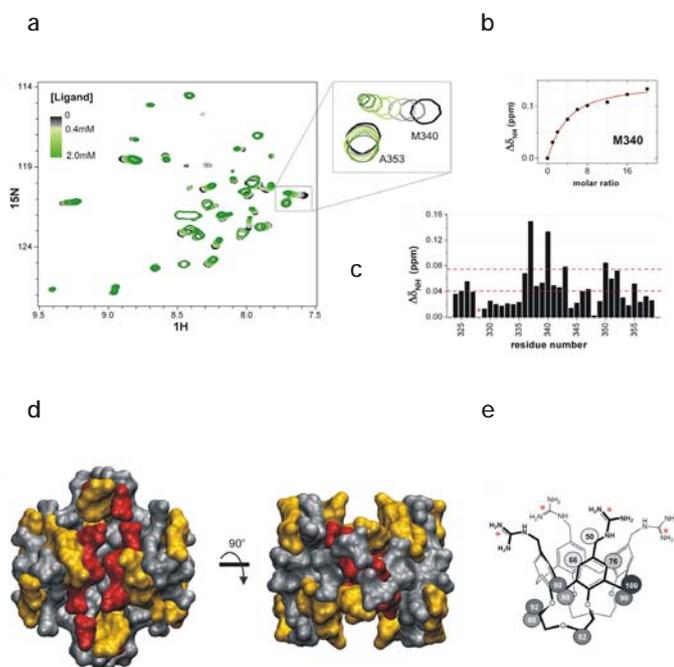
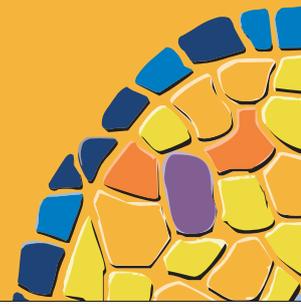


Figure 1. Interaction of a calyx[4]arene ligand with the tetramerisation domain of protein p53.

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In collaboration with de Mendoza's group (Institute of Chemical Research of Catalonia, Tarragona), we have designed, synthesised and studied a compound with the capacity to interact with the p53 tetramerisation domain. This new compound is a conical calix[4]arene with four cationic guanidiniomethyl groups at the wider edge (upper rim) and hydrophobic loops at the narrower edge (lower rim) that fits nicely and cooperatively into the hydrophobic clefts between two of the monomers at each side of the protein and keeps the tetrameric structure, like molecular templates, by both ion-pair and hydrophobic interactions.

We have used a variety of biophysical tools to thoroughly characterise the interaction between the ligand and wild-type p53. These include <sup>1</sup>H saturation transfer difference NMR, advanced mass spectrometry, differential scanning calorimetry, and circular dichroism. More importantly, we have shown how this new rationally designed molecule is capable of holding together the

four monomers of the mutated p53-R337H protein, thereby recovering the tetramer integrity as in the wild-type structure. Furthermore, we have found a good agreement between the structure of the complex and the nature of the interactions predicted by molecular dynamics calculations.

Looking to the future, this is the first proof of concept on the way towards the design of a new class of drugs based on the use of small molecules that could act as moulds or tethers to preserve the active form of protein tethers in order to stabilise the native forms of proteins or to recover/rescue disease-related mutated ones.

### Jumping hurdles

The adage that 'good fences make good neighbours' is perhaps nowhere better illustrated than in the human body, which

encompasses various gas- and fluid-filled sacs, (eg, the lungs, colon, and stomach) separated by specialised tissues (uterus, brain capillaries) that act as barriers, regulating the passage of molecules from one compartment to another (Teixidó *et al*, 2007). Several diseases require treatment of the brain. These include neurodegenerative diseases 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 in the brain. In many cases, there are promising compounds for their treatment. However, because of problems associated with crossing the BBB, more than 98% of these compounds do not reach the drug development stage (Teixidó *et al*, 2008).

Prolyl oligopeptidase (POP) is a cytosolic serine peptidase that hydrolyses proline-containing peptides at the carboxy terminus of proline-residues. POP has been associated with schizophrenia,

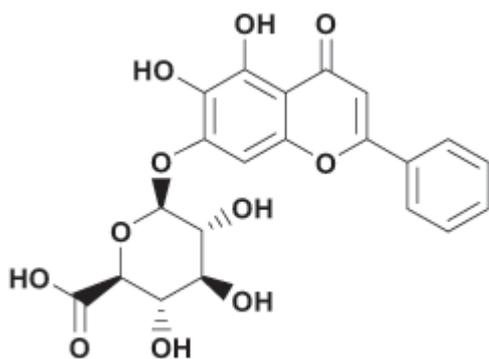


Figure 2. The chemical structure of baicalin.

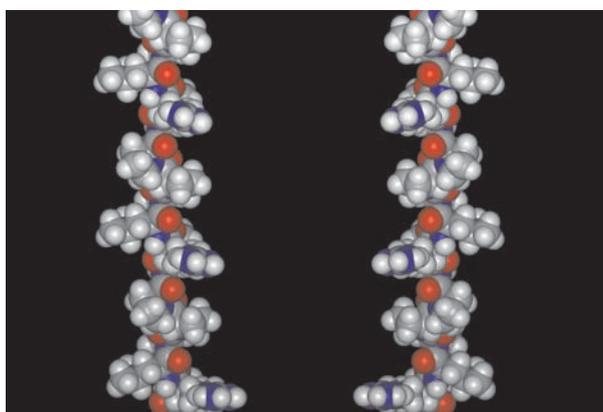


Figure 3. D-SAP: a new, non-cytotoxic and fully protease-resistant cell-penetrating peptide.

bipolar affective disorder and related neuropsychiatric disorders and therefore may have important clinical implications. In previous work, we used <sup>19</sup>F NMR to search for new POP inhibitors from a library of plant extracts used in traditional Chinese medicine and identified several extracts as powerful inhibitors of this peptidase. We have recently discovered (Tarragó *et al*, 2008) that the alkaloid baicalin, which we isolated as the active component of an extract of *Scutellaria baicalensis* roots, exhibits POP inhibitory activity. Baicalin inhibited POP in a dose-dependent manner. Inhibition experiments using baicalin analogues showed that the sugar moiety was not required for activity. The IC<sub>50</sub>s of baicalin and its aglycone derivative baicalein were similar, thereby suggesting that the sugar moiety was not involved in the interaction of baicalin with POP. These results were confirmed by saturation transfer difference NMR experiments.

To further elucidate the absorption and transport mechanisms of baicalin and baicalein, we evaluated their transport *in vitro* through the gastrointestinal tract and the BBB using a parallel artificial membrane permeability assay. The molecule which potentially crosses both barriers was identified as baicalein, the aglycone moiety of baicalin. Our results show that baicalin is a new prodrug with POP inhibitory activity. As baicalin is a natural compound with a long history of safe administration to humans, it is a highly attractive base from which to develop new treatments for schizophrenia, bipolar affective disorder and related neuropsychiatric diseases.

For drug targets located inside the cell, attaining satisfactory intracellular delivery is crucial. Unfortunately, most drug candidates are unable to cross the cytoplasmic membrane alone. Hence, several drug delivery strategies have been proposed, including microinjection, electroporation, liposomal formulation and the use of viral vectors. However, each of these has its respective problems in terms of toxicity and therapeutic feasibility. An alternative delivery strategy is the use of peptide sequences that can translocate across the cytoplasmic membrane (Pujals *et al*, 2008).

Our main contribution to this field during the last five years has been to show that amphipathic Pro-rich peptides are a promising source of cell-penetrating peptides (CPPs). When compared to the principal families of CPPs, they exhibit very low cytotoxicity but much lower uptake. The search for non-cytotoxic derivatives with much more efficient translocation is still underway. In the meantime, we have recently prepared a fully protease-resistant CPP. Proteolytic stability was obtained by chiral inversion of the residues of a known self-assembling CPP from all L-amino acids to all D-amino acids and then assessed against trypsin and human serum. Circular dichroism studies confirmed the enantiomeric structure of the analogue, and transmission electron microscopy (TEM) studies indicated that the new inverse analogue retains the capacity of the original peptide to self-assemble. The results of uptake experiments indicate that the protease-stable (that is, D-amino acid) analogue of the peptide is internalised by cells to the same extent as the protease-susceptible (that is, L-amino acid) parent peptide. The all D-amino analogue has also proven to be non-cytotoxic and successfully distributed among several organs in a preliminary *in vivo* study (Pujals *et al*, 2008).

## Publications

Araya E, Olmedo I, Bastus NG, Guerrero S, Puntos V, Giralt E and Kogan MJ. Gold nanoparticles and microwave irradiation inhibit beta-amyloid amyloidogenesis. *Nanoscale Res Lett*, 3(11), 435-43 (2008)

Foerg C, Weller KM, Rechsteiner H, Nielsen HM, Fernandez-Carneado J, Brunisholz R, Giralt E and Merkle HP. Metabolic cleavage and translocation efficiency of selected cell penetrating peptides: a comparative study with epithelial cell cultures. *AAPS J*, 10(2), 349-59 (2008)

García-Martin F, Cruz LJ, Rodríguez-Mias RA, Giralt E and Albericio F. Design and synthesis of FAJANU: a *de novo* C(2) symmetric cyclopeptide family. *J Med Chem*, 51(11), 3194-02 (2008)

Gordo S, Martos V, Santos E, Menéndez M, Bo C, Giralt E and de Mendoza J. Stability and structural recovery of the tetramerization domain of p53-R337H mutant induced by a designed templating ligand. *Proc Nat Acad Sci USA*, 105(43), 16426-31 (2008)

Jiménez JC, Lépez-Macia A, Gracia C, Varon S, Carrascal M, Caba JM, Royo M, Francesch AM, Cuevas C, Giralt E and Albericio F. Structure-activity relationship of kahalalide F synthetic analogues. *J Med Chem*, 51(16), 4920-31 (2008)

Madurga S, Sánchez-Céspedes J, Belda I, Vila I and Giralt E. Mechanism of binding of fluoroquinolones to the quinolone resistance-determining region of DNA gyrase: towards an understanding of the molecular basis of quinolone resistance. *ChemBioChem*, 9(13), 2081-86 (2008)

Malakoutikhah M, Teixidó M and Giralt E. Toward an optimal blood-brain barrier shuttle by synthesis and evaluation of peptide libraries. *J Med Chem*, 51(16), 4884-89 (2008)

Olmedo I, Araya E, Sanz F, Medina E, Arbiol, Toledo P, Alvarez-Lueje A, Giralt E and Kogan MJ. How changes in the sequence of the peptide CLPFFD-NH2 can modify the conjugation and stability of gold nanoparticles and their affinity for beta-amyloid fibrils. *Bioconjug Chem*, 19(6), 1154-63 (2008)

Pellecchia M, Bertini I, Cowburn D, Dalvit C, Giralt E, Jahnke W, James JL, Homans SW, Kessler H, Luchinat BM, Oschkinat H, Peng J, Schwalbe H and Siegal G. Perspectives on NMR in drug discovery: a technique comes of age. *Nature Rev Drug Discov*, 7(9), 738-45 (2008)

Pujals S, Fernández-Carneado J, Ludevid MD and Giralt E. D-SAP: a new, noncytotoxic and fully protease resistant cell-penetrating peptide. *ChemMedChem*, 3(2), 296-01 (2008)

Pujals S and Giralt E. Proline-rich, amphipathic cell-penetrating peptides. *Adv Drug Deliv Rev*, 60(4-5), 473-84 (2008)

Tarragó T, Kichik N, Claassen B, Prades R, Teixidó M and Giralt E. Baicalin, a prodrug able to reach the CNS, is a prolyl oligopeptidase inhibitor. *Bioorg Med Chem*, 16, 7516-24 (2008)

Tarragó T, Masdeu C, Gómez E, Isambert N, Lavilla R and Giralt E. Benzimidazolium salts as small, nonpeptidic and BBB permeable human prolyl oligopeptidase inhibitors. *ChemMedChem*, 3(10), 1558-65 (2008)

Teixidó T and Giralt E. The role of peptides in blood-brain barrier nanotechnology. *J Pept Sci*, 14(2), 163-73 (2008)

## Research networks and grants

*Ajuts per potenciar i donar suport als grups de recerca*  
Generalitat de Catalunya, 2005SGR00663 (2005-2008)  
Principal investigator: Ernest Giralt

*Design of peptide ligands for protein-surface recognition*  
Spanish Ministry of Science and Innovation, BIO2005-00295 (2005-2008)  
Principal investigator: Ernest Giralt

*Design, synthesis and structural studies of new VIH protease dimerisation inhibitors*  
FIPSE-Foundation for AIDS Research and Prevention in Spain, 36606/06 (2006-2009)  
Principal investigator: Ernest Giralt

*Estudis estructurals i dinàmics d'espècies oligomèriques i fibril·lars de beta amiloide. Experiment d'intercanvi protodeuteri analitzat per ressonància magnètica*  
'La MTV3' Foundation, BM05-60-0 (2006-2008)  
Principal investigator: Ernest Giralt

*Nanotechnologies in biomedicine (Nanobiomed)*  
Spanish Ministry of Science and Innovation, CONSOLIDER-INGENIO 2010 (2006-2010)  
Principal investigator: Ernest Giralt

*Novel nanobiomaterial development: Modification of autoaggregation and protein conformation to reduce toxicity*  
Spanish Agency for International Cooperation (AECID), A/010967/07 (2008)  
Principal investigator: Ernest Giralt

*Structural and dynamic characterisation of  $\alpha\beta$  aggregation. Examination of  $\alpha\beta$  aggregation peptide inhibitors*  
'La MTV3' Foundation, 092 (2006-2009)  
Principal investigator: Ernest Giralt

*Structure and dynamics of  $\beta$ -amyloid oligomeric and fibrillar species. Hydrogen/deuterium exchange experiments analysed by nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS)*  
'La Caixa' Foundation, BM05-60-0 (2006-2008)  
Principal investigator: Ernest Giralt

*Studies of neurosecretion by remote control of exocytosis and endocytosis (OpticalBullet)*  
European Commission, ERC-StG Starting Grants, ID 210355 (2008-2013)  
Principal investigator: Ernest Giralt

*Use of peptides for intracellular nanoparticle delivery (NANOFAR)*  
Spanish Ministry of Science and Innovation, NAN2004-09159-C04-02 (2006-2008)  
Principal investigator: Ernest Giralt

## Collaborations

*Applications of the Suzuki reaction to the synthesis of conformationally constrained peptides*  
Paul-Lloyd Williams, Organic Chemistry Department, 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 (Tarragona, Spain)

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

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

*Synthesis and structural studies of  $\beta$ -peptides*  
Rosa M<sup>a</sup> Ortuño, Chemistry Department, Autonomous University of Barcelona (Barcelona, Spain)

#### Awards

Ernest Giralt, appointed member of the Royal Academy of Sciences and Arts of Barcelona (2008)