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Design of Protein–Protein Interaction Inhibitors Based on Protein Epitope Mimetics

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How can you design synthetic molecules to inhibit protein–protein interactions (PPIs)? What mechanistic principles can you use as an aid in inhibitor design? Unfortunately, the underlying mechanisms of protein–protein, or more generally protein–ligand recognition are still not very well understood. The basic physicochemical properties of proteins, together with the special solvent they are dissolved in (water), cannot yet be described accurately enough to allow rational de novo ligand design. The situation is not quite so bad for the case of enzymes; here mechanism-based approaches to inhibitor design are well established. These exploit knowledge of substrate and transition-state structures, and the fact that enzyme active sites are typically found in deep pockets, which have evolved under intense selection pressure in Nature to bind reaction transition states with very high affinity (typically $K_a = 10^{16\pm4}\,\text{M}^{-1}$).[1] Although we might not fully understand the origins of enzymic catalysis, even a poor transition-state mimic might still bind and inhibit an enzyme in the nanomolar range. In comparison, PPIs have typically evolved in vivo to affinities only in the range $K_a = 10^{7\pm3}\,\text{M}^{-1}$. Protein–protein interfaces also tend to be relatively flat and rather large (700–1500 Å^2 per protein),[2] and the surfaces of proteins often display complex dynamical behavior,[3] all of which greatly complicates ligand design.[4] Nevertheless, the practical importance of being able to design protein ligands and PPI inhibitors is enormous. Much of biology and medical research today is concerned with reducing complex biological processes to sets of protein–protein, or more generally, protein–ligand interactions. Unfortunately, the experience of many pharmaceutical companies is that PPIs are a very difficult family of targets to hit by using traditional small organic drug-like molecules.[4–7]

The report by Chong Li et al.,[8] is therefore of great interest because it illustrates one successful approach to PPI inhibitors based on the design of protein epitope mimetics. Protein epitope mimetics can be viewed as relatively small synthetic molecules (about 1–2 kDa) that mimic the surface patches on folded proteins involved in receptor binding. The mimetics should provide a well-defined (conformationally constrained) structural scaffold upon which the groups important for molecular recognition can be optimally displayed for interaction with the receptor. The synthetic origin of the mimetics is important, because then a myriad of synthetic methods can be used to ring the changes needed to optimize chemical, biological, and perhaps also other drug-like properties. Apart from high affinity and specificity for the target receptor, a very important property required to inhibit intracellular PPIs is cell-membrane permeability. This issue was also addressed successfully in the work reported by Li et al.

In a field as challenging as this, it is a good idea not to start your design efforts with the structurally most complex PPIs. The example studied by Li et al. involves the interaction of a single short helical segment of the tumor-suppressor protein p53 with two regulators of p53 activity, called "mouse double minute 2" (MDM2) and MDMX.[9,10] The oncoproteins MDM2 and MDMX both negatively regulate the activity of p53 by binding to the same short helical epitope, with the sequence **F19**S20D21L22**W23**K24-L25**L26**, which is located near the N terminus of p53.[11,12] Inhibitors of these PPIs have attracted great interest recently as anticancer agents.[13,14] The N-terminal segment of p53 is at best loosely folded in solution and only becomes fully helical upon binding to MDM2 or MDMX.[15–17] In a helical conformation, however, the amino acids every 3/4 residues along the sequence align on the same face of the helix. In this case, it is the residues shown above in bold (**F19**, **W23**, **L26**) whose side chains can then dock into relatively hydrophobic pockets on the surface of MDM2 and MDMX (see Figure 1). These side chains are also energetically important for the affinity and selectivity of the interactions between p53 and MDM2 and MDMX.

By way of digression, it is interesting to note here that many PPIs are known in which short helical epitopes dock with complementary binding sites on a receptor protein. For example, the transactivation domains of many transcription factors contain short helical segments (so-called LXXLL motifs) that interact with other components of the transcriptional machinery.[18] So targeting this type of PPI with selective inhibitors could be used to inhibit a variety of biological processes, some with interesting applications in drug discovery.

Returning to the p53-MDM2/X story, the target for epitope mimetic design in this case was the helical segment of p53, which encompasses residues 19–26. The aim was to transfer the energetically important groups (the side chains of **F19**, **L22**, **W23** and **L26** in p53) onto a relatively rigid synthetic scaffold in a way that preserves binding affinity to the protein targets. Having a relatively rigid synthetic scaffold is a major advantage as it allows inhibitor optimization (affinity, specificity, stability, cell permeability, toxicity) by using clear structure–activity relationships, and in a way that is often

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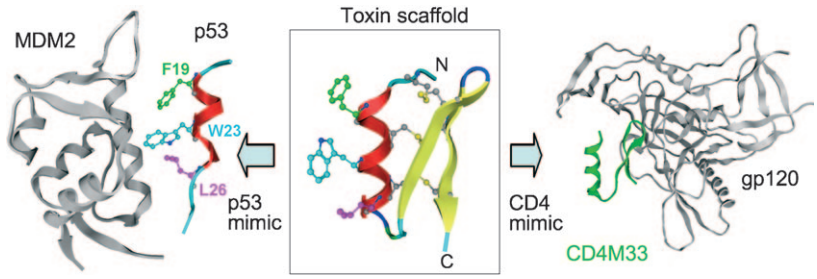

Figure 1. The scorpion toxin scaffold (center; based on the PDB crystal structure 1R1G),[21] with Ser6, Val10 and Val13 changed to Phe, Trp and Leu, respectively, to mimic the helical epitope in p53 (left; based on the PDB crystal structure 1YCR).[11] The same toxin scaffold has also been used to mimic a β-hairpin epitope in CD4. Right: the mimetic (called CD4M33) bound to gp120 (PDB crystal structure: 1YYL).[22]

not possible in a linear flexible peptide molecule. The choice of scaffold then becomes a key parameter in epitope mimetic design.

Li et al. chose for mimetic design a small, naturally occurring, highly crosslinked peptide scaffold that belongs to the family of short-chain K^+-channel toxins isolated from scorpion venom, called BmBKTx1.[19] Both X-ray and NMR solution structures are available (PDB ID: 1Q2K and 1R1G) for this 31-residue peptide,[20,21] which can be efficiently synthesized and oxidatively folded in vitro. This scaffold comprises a three-turn N-terminal α-helix, crosslinked by three disulfide bridges to a 14-residue β-hairpin (Figure 1). Both the α-helical segment and the β-hairpin in BmBKTx1 provide conformationally stable segments onto which foreign epitopes can be grafted.

The utility of this scorpion α/ββ toxin fold for epitope mimetic design has been recognized for some time.[19] For example, the β-hairpin motif in the toxin has been exploited to generate mimics of an epitope on the cellular receptor CD4,[22–24] which binds to the HIV-1 glycoprotein gp120. HIV-1 viral entry is initiated by the binding of gp120 to CD4 on host cells. Crystallographic studies have shown that the key epitope on CD4, used for binding to gp120, is based largely on a surface β-hairpin loop.[25] Transplanting this hairpin epitope from CD4 onto the scorpion toxin scaffold afforded, after optimization, mimetics that bind tightly to gp120 and inhibit HIV-1 entry to cells (Figure 1).[22–24]

In the work of Li et al., the focus is on the α-helical segment of the scorpion toxin scaffold. The residues S6, R9, V10 and V13 in BmBKTx1 were replaced with the topologically equivalent residues from p53 (**F19**, **L22**, **W23** and **L26**), and the four terminal residues (A1, A2, and Y30, K31) were deleted, thus giving a 27-residue peptide called stoppin-1. In this way, the key hydrophobic side chains required for interaction with both MDM2 and MDMX should be displayed in a helical array on the surface of the mimetic (Figure 1). In addition to these hydrophobic contacts, two key hydrogen bonds should be formed between the mimetic and the protein, namely, those equivalent to the ones seen between the **F19** (p53 numbering) backbone amide NH and the carbonyl side chain of Q72 in MDM2 (Q71 in MDMX) and the indole NH of **W23** and the L54 backbone carbonyl in MDM2 (M53 in MDMX). Stoppin-1 was studied in solution by CD spectroscopy, which supported the presumed structure. Then, in direct binding assays, it was shown that stoppin-1 binds to both MDM2 and MDMX, with K_d values of 790 and 994 nM, respectively. For the linear p53 peptide $p53^{res.15-29}$ the K_d values were 123 and 279 nM, respectively. By contrast, the native wild-type toxin BmBKTx1 showed no affinity for either MDM2 or MDMX.

A further challenge was to engineer the mimetic to allow its uptake by cancer cells. At present there is growing interest in understanding how to design cell-permeable peptide-like molecules, for example, through the N-methylation of peptide bonds in backbone cyclic peptides.[26–28] The N-methylation of peptide NH groups appears to aid transfer of such molecules through a lipophilic membrane environment, and so to facilitate their cell uptake, when these NHs are normally solvent exposed and able to hydrogen bond to bulk-solvent water molecules. The approach taken by Li et al., however, was different. They exploited the observation that some naturally occurring peptides containing multiple, closely spaced, cationic residues (called protein transduction domains, or simply, cell-penetrating peptides)[29] are efficiently taken up across cell membranes. The mechanism(s) of cell uptake of cationic peptides is/are still the subject of much scrutiny. The stoppin-1 mimetic was re-engineered, by replacing five residues near the C terminus (which are nonessential for binding to MDM2/X) with arginines, to create a cluster of eight cationic residues projecting from the C-terminal β-hairpin motif. The resulting mimetic, stoppin-2, was shown to still bind to MDM2 ($K_d = 493$ nM). Next the ability of stoppin-2 to kill cancer cells was studied. Stoppin-2 was shown to reduce the viability of a $p53^+$ cancer cell line, and, when added daily, was able to kill the cells quantitatively. A variety of control experiments were performed to strengthen the conclusion that stoppin-2 kills tumor cells in a p53-dependent manner.

Given the widespread occurrence of α-helical epitopes in many PPIs, it is not surprising that many groups have reported efforts to design α-helix mimics.[30,31] For example, another relatively small folded peptide that has been used for engineering experiments is the avian pancreatic polypeptide (aPP).[32] aPP contains five turns of α-helix in its C-terminal half, linked to a ten-residue extended N-terminal segment, which is backfolded onto the α-helix. The p53 epitope has also been successfully grafted onto this aPP scaffold to create miniprotein inhibitors of the p53–MDM2 interaction.[33] The cyclotides and conotoxins represent two other families of macrocyclic crosslinked peptides, each with great potential as scaffolds in protein ligand design.[34–36] Another approach to stabilize α-helical conformations in linear peptides makes use of so-called "stapled helices". This involves covalently linking the side chains of residues lying on the same face of the helix (*i* and *i*+4) through amide, disulfide, or double (or

similar) bonds.[37,38] Stapled helical peptide mimetics of p53 have been reported that bind to MDM2 and reactivate the p53 tumor-suppressor pathway.[39] Another strategy for helix mimicry reported recently involves the design of bi- and tri-aryl systems.[30,40,41] Due to restricted rotation in biaryl systems, substituents can be appended to the aromatic groups in a way that allows close mimicry of side-chain residues *i*, *i*+4 and *i*+7 in α-helices. In this way, for example, terphenyls were designed to mimic the key helical region of p53, and also inhibit the p53–MDM2 interaction.[41] Finally, there have been several examples reported of the use of β-hairpin templates to mimic α-helical epitopes.[42–44] These exploit the close positional relationship between side chains at positions *i* and i+2 in a β-strand with those at positions *i* and *i*+4 in an α-helix. Hairpin mimetics were designed in this way to mimic the helical epitope in p53, and so inhibit the p53–HDM2 interaction.[42,43] Additional examples of p53–MDM2 inhibitors, including others based on helix-forming β-peptides, have been described recently in an excellent review.[45]

Protein epitope mimetic design is clearly a very promising strategy in the search for novel PPI inhibitors. The great importance and potential for exploitation in this area of molecular design are certainly very clear.

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