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β-Hairpin Peptidomimetics: Design, Structures and Biological Activities

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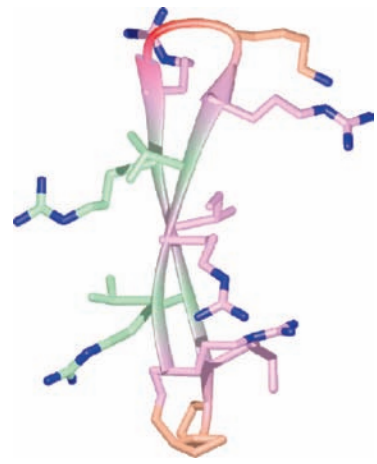

The folded 3D structures of peptides and proteins provide excellent starting points for the design of synthetic molecules that mimic key epitopes (or surface patches) involved in protein−protein and protein−nucleic acid interactions. Protein epitope mimetics (PEMs) may recapitulate not only the structural and conformational properties of the target epitope but also their biological activities. By transferring the epitope from a recombinant to a synthetic scaffold that can be produced by parallel combinatorial methods, it is possible to optimize properties through iterative cycles of library synthesis and screening, and even to evolve new biological activities. One very interesting scaffold is found in β-hairpin motifs, which are used by many proteins to mediate molecular recognition events. This motif is readily amenable to PEM design, for example, by transplanting hairpin loop sequences from folded proteins onto hairpin-stabilizing templates, such as the dipeptide D-Pro-L-Pro. In addition, β-hairpin peptidomimetics can also be exploited to mimic other types of epitopes, such as those based on α-helical secondary structures. The size and shape of β-hairpin PEMs appear well suited for the design of inhibitors of both protein−protein and protein−nucleic acid interactions, endeavors that have so far proven difficult using small "drug-like" molecules. In recent work, it was shown that β-hairpin PEMs can be designed that mimic the canonical conformations of antibody hypervariable loops, suggesting that novel small-molecule antibody mimics may be feasible. Using naturally occurring peptides as starting points, β-hairpin mimetics have been discovered that possess antimicrobial activity, while others are potent inhibitors of the chemokine receptor CXCR4. β-Hairpin PEMs have also been designed and optimized that mimic an α-helical epitope in p53 and so block its interaction with HDM2. A crystal structure of one HDM2−mimetic complex revealed how the surface of the protein had adapted to the shape of the hairpin, thereby enhancing inhibitor affinity. Small folded RNA motifs also make interesting targets for inhibitor design. For example, β-hairpin mimetics have been designed and optimized that bind with high affinity and good selectivity to the TAR and RRE RNA motifs from HIV-1. Solution structures of the mimetics both free and bound to the RNA target provided some surprises, as well as an improved understanding of the mechanisms of binding. These mimetics represent still a relatively new family of RNA-binding molecules, but clearly one with potential for development into novel antiviral agents.

Introduction

This Account describes some recent efforts to design small synthetic molecules that mimic biologically important epitopes on folded peptides and proteins. Epitope mimetic design starts from 3D structural and mutagenesis data, which map the energetically important residues on the protein surface. The challenge is to recapitulate the structural and conformational properties of the chosen target epitope, in a relatively small scaffold that can be produced efficiently by synthetic methods. One motivation for this work is to discover new biologically active molecules. Epitope mimetics have huge therapeutic potential in the design of interaction inhibitors targeting hot-spots at protein−protein and protein−nucleic acid inter-

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faces,[1] as well as in synthetic vaccine design.[2] Interest in these areas has exploded in recent years, with the arrival of functional genomics and proteomics, as well as advances in structural biology and immunology. Such is the enormous complexity of biomolecular interactions, however, that both will remain fertile areas for chemical research in the decades to come.

The most important regular secondary structures found in protein epitopes in Nature are the β-hairpin and the α-helix. The peptide backbones of β-hairpins and α-helices constitute scaffolds upon which energetically important amino acid side chains can be preorganized for binding, as well as themselves providing numerous sites for hydrogen bonding to a target protein or nucleic acid. The β-hairpin is an especially interesting scaffold, since it used by many proteins for biomolecular recognition (e.g., antibodies, T cell receptors), and it is readily amenable to mimetic design.

Although a β-hairpin is composed of two consecutive hydrogen-bonded antiparallel β-strands connected by a turn or loop sequence, this description only poorly conveys the great diversity of conformations that β-hairpins can adopt in folded peptides and proteins. A systematic classification of β-hairpin structures has been introduced,[3] which takes into account polypeptide chain length and hydrogen bonding pattern between two antiparallel β-strands. Analyses of β-hairpin conformations in proteins of known 3D structure have revealed[4,5] that (1) β-hairpins can vary widely in the hydrogen-bonding pattern between the β-strands and in the connecting loop length, although the majority of loops have ≤5 residues and (2) in two residue loops, type-I′ and type-II′ β-turns are strongly favored over type-I and type-II β-turns, which represents a dramatic reversal of the preponderance of the turn-types typically found in proteins.[5] In three residue loops, type-I β-turns are predominant, with residues-1 and -2 most frequently adopting the β-I turn and residue-3 lying in the left-handed α-helical region (α_R-α_R-α_L).[4] Four residue loops also occur frequently, and often contain overlapping type-I/type-III β-turns corresponding to a single turn of a 3_{10}-/α-helical segment. A greater propensity for the occurrence of *cis* peptide bonds is observed in four and five residue loops, involving Xaa-Pro pepide bonds in type-VI β-turns. (3) β-Bulges may also occur within the β-strands. A β-bulge occurs when two residues on one strand lie opposite a single residue on the other strand. β-Bulges affect not only the directionality of the backbone but also and more dramatically the orientation of side chains with respect to the β-hairpin plane. Note also that paired residues on opposite β-strands can exist at hydrogen-bonding (HB) and non-hydrogen-bonding (NHB) positions, and their side chains point to different sides of the hairpin (Figure 1).

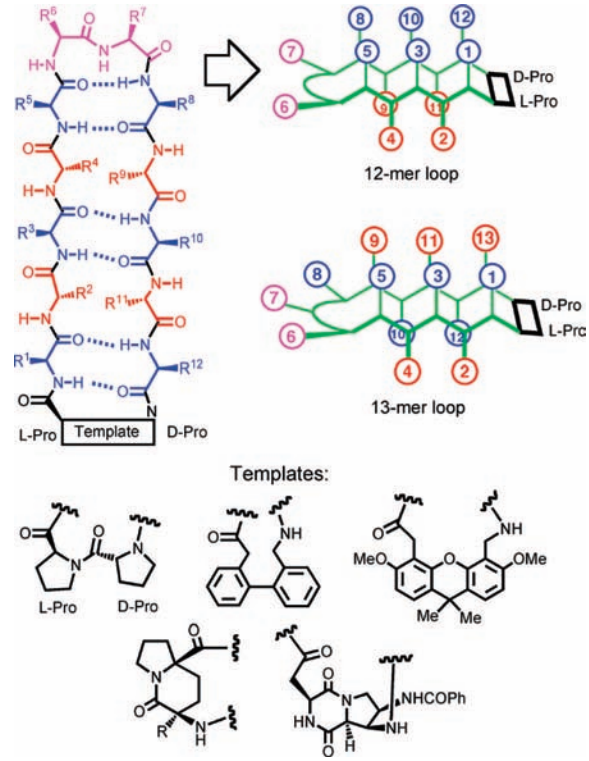
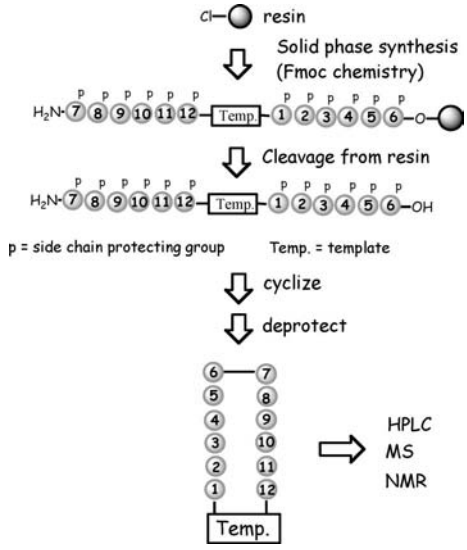


FIGURE 1. A prototypical template-bound 12-mer β-hairpin loop, and some of the templates studied to date.

The most straightforward approach to hairpin mimetic design involves transplanting a hairpin loop from a protein of known structure onto a semirigid hairpin stabilizing template, to afford macrocyclic, conformationally defined, template-bound β-hairpin protein epitope mimetics (PEMs) (Figure 1). As we will see, however, β-hairpin PEMs are versatile scaffolds that can also be used to mimic other epitopes, such as those based on natural α-helical secondary structures.

The template assumes an important role in macrocyclic β-hairpin mimetic design. The overall effects of backbone cyclization, the conformational bias imposed by the constrained template, and the influence of the hairpin loop length and sequence should act cooperatively to stabilize β-hairpin structures. For example, the relative position and orientation of the bond vectors at the N- and C-terminal attachment sites in the template must be carefully chosen to favor stable β-hairpin loop geometries. As templates, a variety of cyclic systems have already been investigated,[6] but certainly the most convenient to use is the dipeptide D-Pro-L-Pro (Figure 1). This dipeptide is known to adopt a stable type-II′ β-turn,[7] and so is ideal to nucleate β-hairpin conformations possessing the preferred right-handed twist typically observed between adja-

SCHEME 1. Synthesis of a Typical Template Bound β-Hairpin Mimetic



cent antiparallel β-strands in proteins. Indeed, Kopple and co-workers had shown earlier that D-Pro-L-Pro can be used to fix β-turn positions in cyclic hexapeptides,[8] and Marshall and co-workers used conformational search methods to show that a type-II′ β-turn is strongly favored by D-Pro-L-Pro.[9]

When transplanting a hairpin loop from a protein of known structure onto D-Pro-L-Pro, this template must be inserted at a NHB position. The N- and C-terminal loop residues will then be forced into a HB position and the ensuing hydrogen-bonding pattern should be maintained along the hairpin (Figure 1). Lengthening a given loop, by inserting one residue at the C-terminus, causes all the residues in HB positions along this strand to move into NHB positions, and vice versa, and so the distribution of side chains on the two sides of the hairpin is completely altered. The template, therefore, exerts an important influence over the preferred hairpin conformation and geometry.

An attractive feature of such β-hairpin mimetics is the modular approach that can be taken for their synthesis. Typically, a linear precursor can be assembled on a solid phase using peptide chemistry, and then macrocyclized and deprotected in solution (Scheme 1). This assembly process is amenable to parallel combinatorial methods.[10] Proteinogenic and nonproteinogenic amino acids, as well as an array of related building blocks (e.g., peptoids), are available for mimetic design. In terms of size (1−2 kDa), the peptidomimetics populate a relatively underexplored area of molecular space that lies between traditional small drug-like molecules (<500 Da) and the world of biopharmaceuticals (e.g., monoclonal antibodies, among others).

There are many potential applications of peptidomimetics, not least for the very challenging goals of designing protein−protein and protein−nucleic acid interaction (PPI, PNI) inhibitors. Thus, high throughput screening of small drug-like molecule libraries usually fails to identify hits on these targets. Protein−protein interfaces tend to be relatively large (approximately 650−2000 Å^2), and generally do not possess the relatively deep ligand binding pockets typical of enzymes. However, binding hotspots do occur in many protein−protein interfaces. Thus, in the barnase−barstar and growth hormone−growth hormone receptor interactions it was first shown that most of the binding energy is contributed by only a subset of the many residues (the energetically most important or "hot" ones) buried at each interface.[11−13] Protein−protein binding interfaces often have a modular architecture, in which energetically important interactions can be grouped into independent clusters, and the networks of interactions within a cluster contribute cooperatively to the stability of the complex. The contributions of distinct independent hot clusters may then be additive.[14] Conformational flexibility and adaptivity also play important roles in protein−protein interactions, by optimizing the complementarity of protein binding surfaces. For example, in some cases, protein−protein binding reactions are associated with a transition from a largely unfolded state (free) to a folded state (bound) in at least one protein partner (e.g., p53-HDM2 and Tat-TAR).

I consider below first some examples of β-hairpin mimetic design, starting from peptides and proteins of known 3D structure, containing β-hairpins that are important for biologically activity. I then go on to illustrate two examples of how β-hairpin PEMs can be used to mimic epitopes based on α-helical structures. It should become apparent that great scope exists for further applications of this approach in drug and vaccine design.

β-Hairpin Mimetics Based on Antibody and Cytokine Receptor Loops

Template-bound β-hairpin mimetics have been designed to structurally mimic canonical conformations found in antibody hypervariable loops. Of the six complementarity-determining regions (CDRs) in IgG antibodies, four adopt β-hairpin structures. Although considerable sequence diversity can be accommodated in the six loops, analysis of crystal structures has shown that this is achieved within a restricted set of "allowed" or canonical backbone conformations.[15] Mimetics of selected canonical conformations were made by transplanting loops from the immunoglobulin framework onto a D-Pro-L-Pro template.[16] The mimetic **1**, for example, contains 8

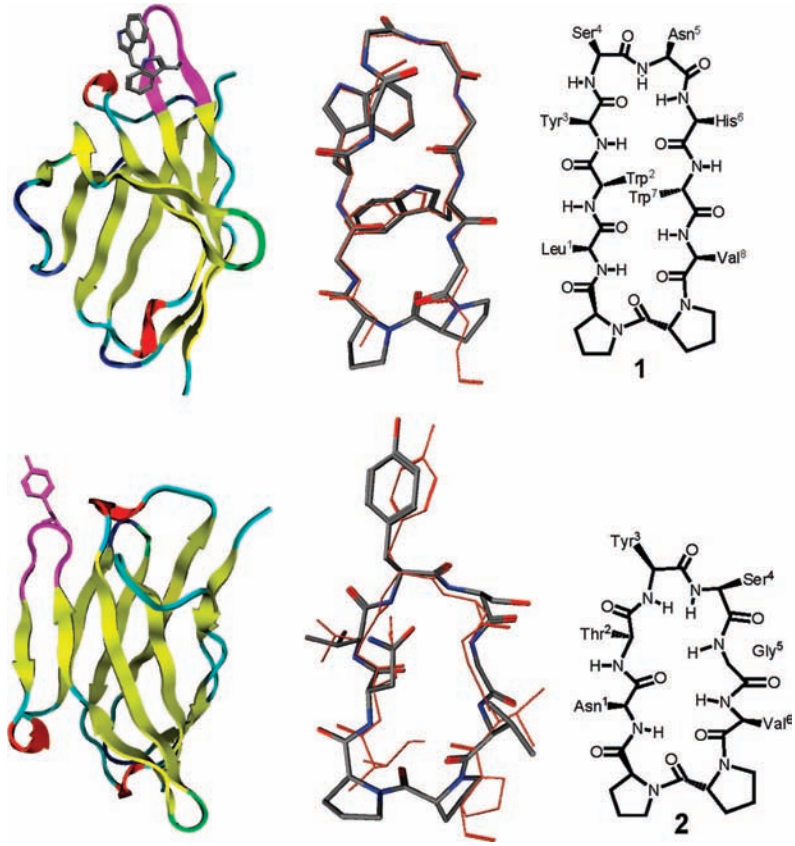


FIGURE 2. Hairpin mimetics (**1** and **2**) based on antibody CDR loops. The antibody domains are shown left with the CDR loops purple, and the solution structures of the mimetic (**1** and **2**) shown in gray/blue/red (blue = N atoms, red = O atoms) superimposed upon the corresponding CDR loops from the crystal structures (1GIG, 1TET) in red.

residues from the light chain L3 loop of antibody HC19, which binds to influenza hemagglutinin (Figure 2). NMR studies showed that this mimetic adopts a backbone 2:2 hairpin conformation and cross-strand side-chain aromatic stacking interactions between tryptophan side chains that are essentially identical to those seen in the antibody crystal structure. It is now known that such stacking interactions help to stabilize β-hairpin conformations also in linear peptides.[17]

In another example, a six-residue H2 loop was transplanted from the anticholera toxin antibody TE33 to the same template, to give mimetic **2** (Figure 2). NMR again showed that the average solution structures of **2** were very similar to that seen in the crystal structure of the antibody fragment. These studies showed that accurate structural mimetics of these L3 and H2 canonical conformations were indeed possible. More generally, the approach may have practical value in the design of small molecule antibody mimetics, although in the cases mentioned above, corresponding tests for antigen-binding activity were not carried out.

Similar approaches have also been reported to prepare a hairpin mimic of a 12-residue loop present in the extracellular interferon γ receptor,[18] as well as one based on a protruding hairpin loop in platelet-derived growth factor-B (PDGF-B).[10]

β-Hairpin Mimetics of Antimicrobial and Antiviral Peptides

The large family of naturally occurring cationic antimicrobial peptides represent interesting targets for peptidomimetic research. In vertebrates, these peptides are part of the innate immune system, providing a first line of defense against bacterial and viral infection.[19] One group of cationic antimicrobial peptides possesses β-hairpin structures stabilized by disulfide bridges, including the protegrins, polyphemusins, and tachyplesin, among others (Figure 3). These peptides show broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria. The main mechanism of action involves lysis of the bacterial cell membrane.[20] These cationic peptides are attracted electrostatically to the outer bacterial cell surface, due to the presence of excess negatively charged phospholipids and glycolipids. They then invade and disrupt the membrane bilayer, causing cell lysis.[20] However, the peptides also lyse (at a higher concentration) typical mammalian

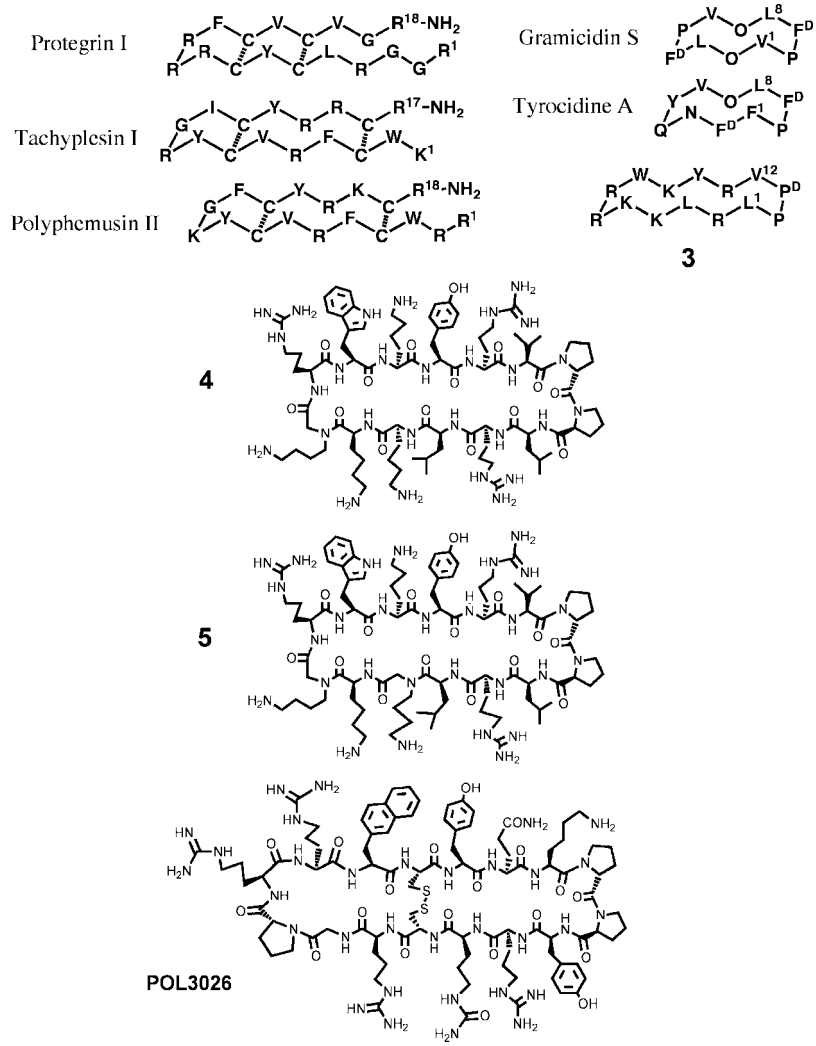


FIGURE 3. *β*-Hairpin cationic antimicrobial peptides, selected mimetics, and other microbial natural products.

cell membranes (e.g., red blood cells), so toxicity is a serious problem that has so far prevented clinical applications.

It is interesting to note that Nature has also evolved cyclic *β*-hairpin peptides with potent antimicrobial activity. Examples include gramicidin S and tyrocidine (Figure 3). Tyrocidine and gramicidin are constituents of tyrothricin which was first isolated in 1939. Both gramicidin S and tyrocidine are highly toxic to erythrocytes, liver and kidney, and have only found use as mixtures in topical applications.

The *β*-hairpin mimetic approach was taken to discover potential new antimicrobials related to protegrin I. Peptide loops with sequences related to protegrin I were mounted on the D-Pro-L-Pro template, and the disulfide bridges were eliminated by replacing the cysteines with a variety of other residues. In this way, a family of template-bound protegrin mimetics was discovered, exemplified by the mimetic **3** (Figure 3), which possess potent broad spectrum antimicrobial activity.[21] By screening small libraries of mimetics it was possible to identify analogues with considerably reduced hemolytic activity on red blood cells.[22] However, the principal mechanism of antibacterial action still involves lysis of the bacterial cell membrane. A stable hairpin structure was observed by NMR only when the mimetic was dissolved in a micelle (membrane-like) environment.[21] Nevertheless, efforts have been made to understand the observed antimicrobial and hemolytic structure−activity relationships in terms of a QSAR model.[23] The best model suggested that the antimicrobial potency correlates with the peptide charge and amphipathicity, while the hemolytic effects correlate with the lipophilicity of residues forming the nonpolar face of the *β*-hairpin.

Some *β*-hairpin mimetics have also been made with a mixed peptide-peptoid backbone (**4** and **5**, Figure 3),[24] while others contain templates based on a xanthene[21] or a biaryl derivative.[25] It is noteworthy that the mixed peptide−peptoid derivative (**4**) now showed stable *β*-hairpin structures by NMR

even in free aqueous solution, perhaps due to the enhanced stability of a type-II′ turn at the hairpin tip.[24] All these derivatives retained a significant broad spectrum antimicrobial activity.

More recently, a new structure−activity trail has been followed starting from **3**, which ultimately provided access to new derivatives with a much higher high potency against *Pseudomonas aeruginosa*. These new derivatives do not cause cell lysis, and only one enantiomer retains significant potency, suggesting a different mechanism of action (to be published).

A different cationic antimicrobial peptide called polyphemusin II (Figure 3), isolated from the American horseshoe crab, has also been a source of inspiration in β-hairpin mimetic design. Polyphemusin and related synthetic derivatives, such as T22, TC14011,[26] have been shown to possess potent inhibitory activity against the chemokine receptor CXCR4, where they block binding of the natural ligand stromal-derived factor (SDF)-1α (or CXCL12). Interest in CXCR4, a G-protein coupled receptor, has blossomed in recent years with the realization that the SDF-1α/CXCR4 interaction guides trafficking and homing of many different cell types in the human body.[27] The SDF-1/CXCR4 axis is also important in several pathophysiological processes, including cancer cell proliferation, migration and angiogensis, as well as artherosclerosis, and HIV infection. To date, only a few small-molecule CXCR4 antagonists are known, including AMD3100[28] and KRH-1636.[29]

Recently, new β-hairpin mimetics of polyphemusin II were designed and tested as CXCR4 antagonists.[30] Through successive rounds of parallel synthesis, both the CXCR4 inhibitory activity and drug-like properties were optimized. These studies resulted in highly potent CXCR4 inhibitors, such as POL3026 (Figure 3), with excellent plasma stability, high selectivity for CXCR4, and favorable pharmacokinetic properties in animal models. These compounds may be useful as HIV-1 entry inhibitors, for treatment of cancer and inflammation, and for hematopoietic stem cell transplant therapy.

β-Hairpin Mimetic Protease Inhibitors

The Bowman−Birk (BB) family of serine protease inhibitors represents another interesting starting point for β-hairpin mimetic design. One of the smallest members of the BB family is a cyclic peptide isolated from sunflower seeds. A crystal structure of this peptide bound to the active site of trypsin was taken as a starting point for mimetic design.[31] β-Hairpin mimetics were designed by transplanting either 11 or 7 residues from the BB reactive loop onto a D-Pro-L-Pro template.[32] NMR studies on the resulting mimetics **6** and **7** (Figure 4) revealed well-defined β-hairpin conformations in aqueous

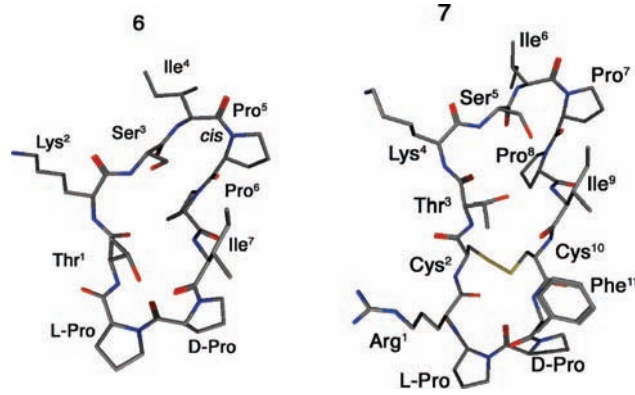


FIGURE 4. Solution structures of trypsin inhibitor mimetics based on the reactive Bowman−Birk loop.

solution. The conformation of the hairpin in both mimetics was essentially identical to that seen in the reactive loops of BB protease inhibitors. The turn region contains a characteristic 5-residue loop, including a *cis*-Ile-Pro peptide bond in a type-VI β-turn. Enzymic assays showed that both mimetics inhibit bovine trypsin with mid to low nanomolar K_i values, and an alanine scan confirmed the energetically important role of a Lys side chain at the P1 position.

β-Hairpin Mimetics Targeting Protein−Protein Interactions

Many PPIs are mediated by α-helices, so small molecule mimics of α-helical epitopes are of great interest. One example is the interaction of p53 with the human version of mouse double minute 2 protein (HDM2). One domain of HDM2 acts as an inhibitor of the tumor suppressor protein p53, which is activated in response to oncogenic stress to prevent the emergence of cancer cells. p53 is a transcription factor that modulates the expression of numerous target genes.[33] Recent studies have shown that HDM2 acts both as an antagonist of p53, and also downstream of p53, helping to adjust the biological outcome (growth arrest when the oncogenic stress is mild, or apoptosis when the stress is severe) to the nature of the triggering signal.[34] HDM2 can block activation by p53 both by binding to it and by targeting p53 for degradation by ubiquitinylation. However, in tumors HDM2 can become overexpressed, and levels of free p53 are then no longer sufficient for tumor suppression. Hence, inhibitors of the p53-HDM2 interaction are of interest as potential anticancer agents.

A crystal structure showed that a p53-derived peptide in complex with the inhibitory domain of HDM2 adopts an amphipathic α-helical backbone conformation (Figure 5).[35] When free in solution, however, the N-terminal region of p53 is unfolded.[36] In the complex, the side chains of Phe19, Trp23

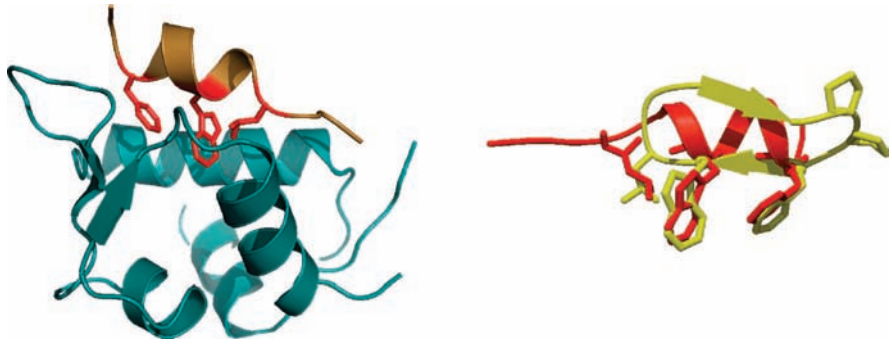


FIGURE 5. The p53−HDM2 crystal structure (PDB 1YCR) (p53 brown/red, HDM2 blue). A model template-bound β-hairpin (yellow) is shown superimposed on the p53 helical peptide (red).

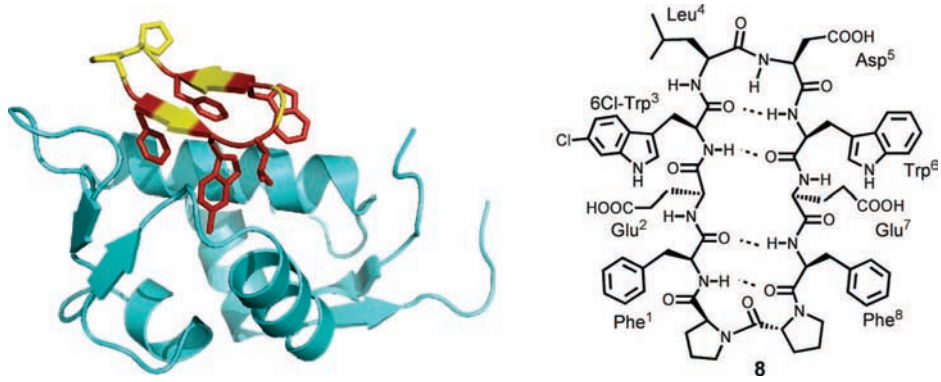


FIGURE 6. An optimized p53−HDM2 inhibitor (**8**), and crystal structure (PDB 2AXI) of the mimetic (**8**) bound to HDM2.

and Leu26, in particular, align along one face of the helix, and insert into deep hydrophobic pockets on the surface of HDM2. It was shown that a short 8-residue β-hairpin could be designed to mimic this α-helical epitope, by transferring the three hot residues aligned along one side of the p53 helix onto one strand of the hairpin, as shown in Figure 5.[37] The affinity of the first designed mimetic for HDM2, although weak ($IC_{50} \approx 125\ \mu M$), was optimized in an iterative process involving library synthesis and screening. The optimized β-hairpin mimetic **8** binds to HDM2 with $K_D \approx 40$ nM, and includes a 6-chlorotryptophan (6-ClTrp) at position-3, which had been used earlier by a group at Novartis to improve the affinity of a phage-derived peptide to HDM2.[38]

A crystal structure of **8** bound to HDM2 confirmed that the residues Phe, 6-ClTrp and Leu in the first β-strand fill the hydrophobic pockets on the surface of HDM2 (Figure 6).[39] However, unexpected were the interactions that aromatic groups in the second β-strand make with the protein. In particular, Trp6 and Phe8 participate in stacking interactions with the side chain of Phe55 in HDM2. In the p53−HDM2 complex, the Phe55 side chain is rotated away and makes no contact with the p53 peptide. Thus, the plasticity of the binding site on HDM2 adapts to optimize structural complementarity with the mimetic. Recently, the structure of ligand-free HDM2 (apo-HDM2) solved by NMR spectroscopy[40] revealed even more dramatic structural differences from the ligand bound form. In apo-HDM2, the hydrophobic p53 binding pockets become largely occluded by the inward displacement of two helices comprising the walls of the p53-binding cleft, and the N-terminal segment of HDM2 folds back and occludes the shallow end of the p53-binding cleft.

It should be noted that a large number of small drug-like molecules have now been discovered that inhibit the p53−HDM2 interaction.[33,41] These inhibitors all target the deep hydrophobic pockets in the p53-bound form of HDM2, features that are fairly atypical for most PPI interfaces. An important goal of future work, therefore, is to target other interesting PPIs with β-hairpin mimetics, to determine how general this approach is to PPI inhibitor design.

Phage display is another well-established technology for selecting peptides and proteins with novel binding functions.[42] An important feature of phage display is that very large libraries ($>10^9$) can be produced and screened. It might, therefore, be useful to harness phage technology for use in β-hairpin mimetic design. To examine this point, a phage peptide that binds the Fc fragment of human IgG was taken as a starting point for PEM design.[43] This phage peptide binds the Fc fragment in a β-hairpin conformation, with side chains on one side of the hairpin contacting the surface of the protein and burying a surface of about 650 Å^2.[44] The backbone cyclic mimetic

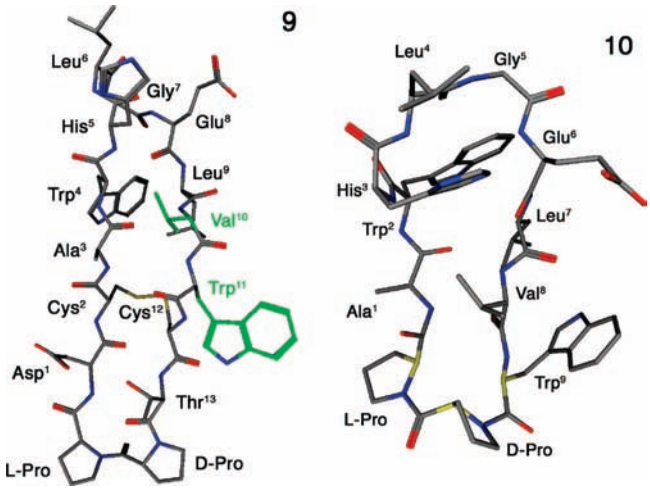


FIGURE 7. β-Hairpin mimetics that bind the Fc domain of an IgG. The mimetic **9** contains a β-bulge at Val[10]; mimetic **10** contains two *cis* peptide bonds at Trp[9]-D-Pro and D-Pro-L-Pro (C(α) atoms yellow).

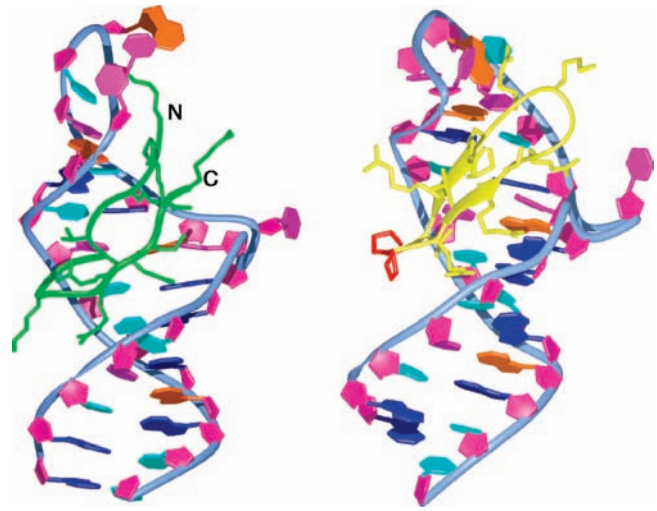


FIGURE 8. Solution structures of BIV Tat–TAR RNA complex (PDB 1MNB) and the BIV-2–TAR RNA complex (PDB 2A9X). Note how the N- and C-termini of Tat (green) are oriented to the tip of the RNA, while the template (red) in BIV-2 (yellow) is oriented to the base.

9 was shown to adopt a stable β-hairpin structure in solution (Figure 7), essentially identical to that seen in the crystal structure of the Fc-bound phage peptide. The peptide contains a β-bulge in the second strand, with the side chains of two consecutive residues (Val[10] and Trp[11]) on the same side of the hairpin interacting with the protein surface. The mimetic (**9**) was shown to bind to the Fc domain with 80-fold higher affinity than the phage peptide.

An attempt was also made to reduce the size of the mimetic, by transplanting just nine residues at the tip of the loop onto the template, to give **10**. However, for reasons that are still uncertain, the backbone underwent a profound rearrangement, adopting a stable conformation with two *cis* peptide bonds between Trp[9]-D-Pro[10] and between D-Pro-L-Pro in the template.[43] As a result, the Trp[9] side chain (equivalent to Trp[11] in the phage peptide) now appeared on the wrong face of the hairpin, and not surprisingly, the mimetic bound only weakly to the Fc domain.

β-Hairpin Mimetics Targeting Protein–RNA Interactions

RNA exhibits a rich variety of folded structures, and many RNA motifs have now become interesting targets in drug discovery.[45] However, the design of small molecules that target protein–RNA interactions with high specificity and affinity remains a major challenge. Many RNA binding molecules are based on natural products, such as the aminoglycosides,[46] and interact relatively unselectively with RNA.

An opportunity to apply β-hairpin peptidomimetic design to an RNA target was afforded by the solution structure of a peptide derived from bovine immunodeficiency virus (BIV) Tat protein bound to its target transactivation response (TAR) region RNA (Figure 8).[47] The N-terminal region of Tat is unfolded when free, but a short stretch adopts a β-hairpin conformation when bound to TAR. The 3D structure of the Tat-TAR complex from HIV-1 has not yet been reported. But the two systems show many sequence similarities, and in both, the binding of Tat to TAR is essential for viral replication.

β-Hairpin mimetic BIV TAR–Tat inhibitors were first designed by using the available NMR structure of the complex.[48] Simply transplanting the Tat hairpin loop onto a D-Pro-L-Pro template did not afford a useful inhibitor, although the 11-residue loop included all the residues in Tat that are energetically important for binding to TAR. This poor mimicry seemed to be due to a distortion of the hairpin, and a resulting large energetic penalty associated with the adoption of the structure required for binding to TAR. However, good Tat–TAR inhibitors were found by extending the loop to 12-residues, which allows the population of regular 2:2 β-hairpin structures. One of these, called BIV-2, binds to TAR with a $K_D \approx$ 150 nM in electrophoretic mobility shift assays, in the presence of a large excess of tRNA as a control for nonspecific binding. Moreoever, BIV-2 adopts a stable 2:2 β-hairpin conformation free in aqueous solution (Figure 9).

Insights into how this mimetic is bound by the RNA came from an NMR structure of the BIV-2–TAR RNA complex (Figure 8).[49] The structure revealed a regular β-hairpin conformation in the bound mimetic, which occupies the major groove binding site used by Tat. However, it was a surprise to discover that the mimetic had bound in an orientation that is flipped 180° compared to that expected, with the template ori-

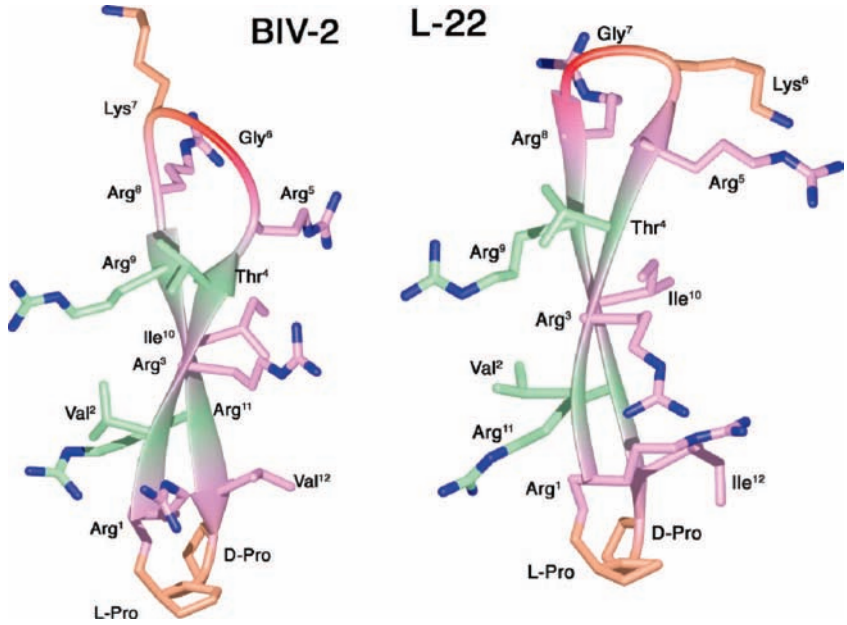


FIGURE 9. Average solution NMR structures of the Tat mimetics BIV-2 and L-22 (PDB 2NS4).

ented toward the base rather than the tip of the RNA-loop. Structure–activity studies provided insights into the reasons for this flip and the origins of the binding affinity, as well as providing access to even more potent BIV Tat–TAR inhibitors, and to potent inhibitors of HIV Tat–TAR.[50]

The β-hairpin in BIV-2 binds to the RNA with side chains on one face contacting the RNA, and those on the opposite face exposed to solvent. Three Arg side chains are buried at the interface in similar positions in both the BIV-2–TAR and Tat–TAR complexes. These are Arg70, Arg73 and Arg77 in Tat, and correspondingly, Arg3, Arg1 and Arg5 in BIV-2 (Figure 10). In Tat these three Arg residues are located on both β-strands, whereas the three in BIV-2 are located along one β-strand. Nevertheless, they superimpose remarkably well in the two complexes. The Arg3, Arg1 and Arg5 in BIV-2 are energetically important, since the substitution of any one, even by Lys or Orn, leads to large losses in binding affinity. It seems that the electrostatic interactions these side chains make with the RNA play an important role energetically in driving complex formation.

Among the many other derivatives of BIV-2 studied, several with nanomolar affinity to BIV–TAR were identified. One of the tightest binders, called L-22, adopts a very stable β-hairpin conformation in solution (Figure 9). The hairpin stability in this case seems to derive from a stable type-I′ β-turn at the hairpin tip (Lys^6-Gly^7), and possibly also from cross-strand van der Waals side chain interactions along the two strands.

Finally, several of the mimetics made in this study were also shown to be potent inhibitors of the HIV Tat–TAR interaction.[50] Some interesting SAR differences were apparent, sug-

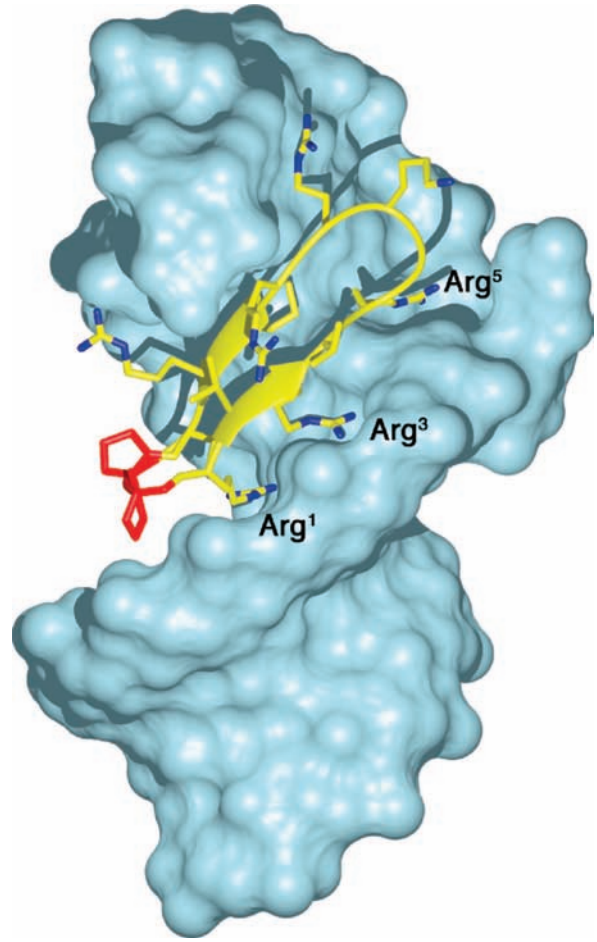


FIGURE 10. Structure of BIV-2 bound to BIV–TAR (PDB 2A9X) showing the buried Arg^1, Arg^3 and Arg^5 side chains.

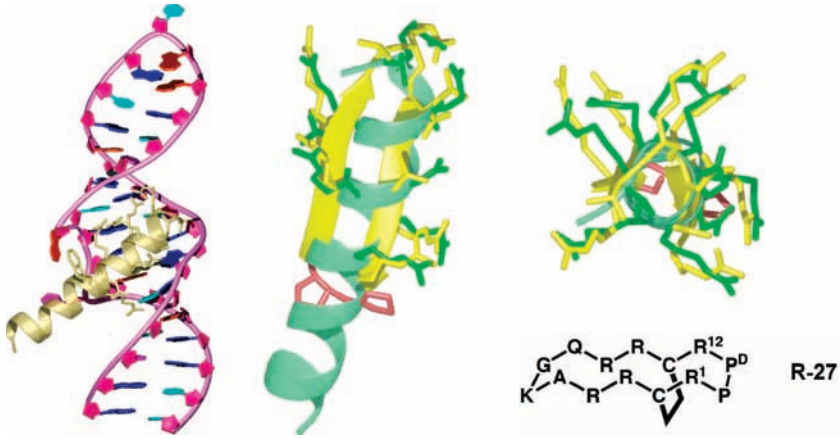


FIGURE 11. Structure of Rev bound to HIV-RRE (PDB 1ETF); mimicry of the helical Rev peptide by a model β-hairpin. The superimposition shows how side chains attached to the helix (green) can be mimicked by side chains attached to the hairpin (yellow). Mimetic **R-27** is shown.

gesting that while the binding modes of the mimetics to BIV and HIV TAR are similar, they are not identical. Studies are now underway into the antiviral activities of selected mimetics on whole cells, and into the 3D structures of HIV TAR–mimetic complexes.

Another very interesting target for hairpin mimetic design is the HIV-1 Rev–RRE interaction, which plays an important role in the temporal control of HIV-1 mRNA splicing. Rev binds to a region of the HIV-1 mRNA called the Rev response element (RRE). The solution structure of a stem loop RRE RNA bound to a Rev-derived peptide showed that the RNA contains a deep binding pocket that binds Rev in an α-helical conformation (Figure 11).[51] Encouraged by the success in using a β-hairpin to mimic an α-helical epitope in p53 and inhibit the p53–HDM2 interaction,[37] a similar approach was explored to β-hairpin mimetic inhibitors of the Rev–RRE interaction.[52]

The Rev-RRE structure shows that key RNA-interacting side chains are displayed around almost the entire circumference of the Rev α-helix. Modeling suggested that the relative positions of all the key side chains in Rev could be mimicked in a 12-residue model 2:2 β-hairpin mimetic (Figure 11). This led to a first mimetic (called **R-01**), that was shown to bind to RRE RNA with a $K_D \approx 1$ μM in the presence of excess tRNA. Screening of a small library or related mimetics gave the inhibitor **R-27**, which in a gel shift assay (in the absence of excess tRNA) showed a $K_D \approx 2$ nM. It was notable that by NMR all the mimetics tested did not adopt stable β-hairpin conformations but instead appeared in free solution to be disordered. One exception was **R-27**, in which the hairpin structure is stabilized by introduction of a disulfide bridge. An interesting and critical test of the RNA binding specificity involved a comparison of binding affinities to RRE and TAR RNA. Here only the constrained **R-27** showed a good level of discrimination between these two similar receptors, suggesting that the conformational properties of the mimetic have a crucial influence on binding specificity.

These cyclic template β-hairpin peptidomimetics represent still a relatively new class of RNA-binding molecules. The promising results obtained so far suggest that further studies are warranted to explore the mechanisms of binding, and to further optimize their biological activities.

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BIOGRAPHICAL INFORMATION

John Robinson studied chemistry (BSc) at University College London, and completed a PhD at Cambridge University. He subsequently carried out postdoctoral work in the University of Karlsruhe, before joining the Chemistry Department of Southampton University in 1979 as a lecturer. He moved to Zurich as Full Professor of Organic Chemistry in 1989.

FOOTNOTES

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