The design, structures and therapeutic potential of protein epitope mimetics

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Using a biologically relevant peptide or protein structure as a starting point for lead identification represents one of the most powerful approaches in modern drug discovery. Here, we focus on the protein epitope mimetic (PEM) approach, where folded 3D structures of peptides and proteins are taken as starting points for the design of synthetic molecules that mimic key epitopes involved in protein–protein and protein–nucleic acid interactions. By transferring the epitope from a recombinant to a synthetic scaffold that can be produced by parallel combinatorial methods, it is possible to optimize target affinity and specificity as well as other drug-like ADMET properties. The PEM technology is a powerful tool for target validation, and for the development of novel PEM-based drugs.

Introduction

It is estimated that there are between 5000 and 10,000 potential drug targets encoded within the human genome [1], of which nearly two-thirds could be amenable to traditional small-molecule drugs and nearly one-third to biopharmaceuticals [2]. When discussing druggability and the size of the druggable genome, the focus is generally on proteins having folds that favor interactions with small drug-like molecules [3–5]. However, small-molecule drugs currently on the market are directed against less than 500 different targets [5,6]. A large number of the potential drug targets have, therefore, not yet been addressed, and a substantial proportion of these are proteins that function through protein–protein interactions (PPIs), which are notoriously difficult to hit with small drug-like molecules.

Given the many crucial roles that PPIs play in many biological processes, it is clear that aberrant, inappropriate or poorly regulated interactions have the potential to cause many pathological conditions. There is an urgent need for the discovery of new types of synthetic molecule drugs that can act on these difficult targets. The ability to interfere with specific PPIs would, therefore, provide many attractive opportunities for the treatment of human disease.

But why are PPIs such difficult targets for drug developers? This reflects the experience that high-throughput screening (HTS) of traditional small drug-like molecules usually fails to identify hits on this class of targets, perhaps because typical HTS collections are biased toward G-protein-coupled receptor (GPCR) and enzyme targets. Furthermore, the large surface areas and relatively flat profiles in many protein–protein interfaces, as well as the flexibilities of protein interface sites, conspire to make the design of small-molecule PPI inhibitors difficult [7].

Another factor is that the physicochemical mechanisms of PPIs are still not fully understood [8]. However, some progress has been made in this regard that could lead to the identification of druggable targets in the PPI class. For example, the realization that binding hotspots occur in many protein–protein interfaces was an important conceptual advance. It appears that in many cases, most of the binding energy in PPIs is contributed by only a subset of the many side chains (the 'hot' ones) buried at each interface, as shown in both the barnase–barstar and growth hormone–growth hormone receptor interactions [9,10]. Such hot spot residues often cluster near the center of the interface and are surrounded by energetically less important residues, which may have important

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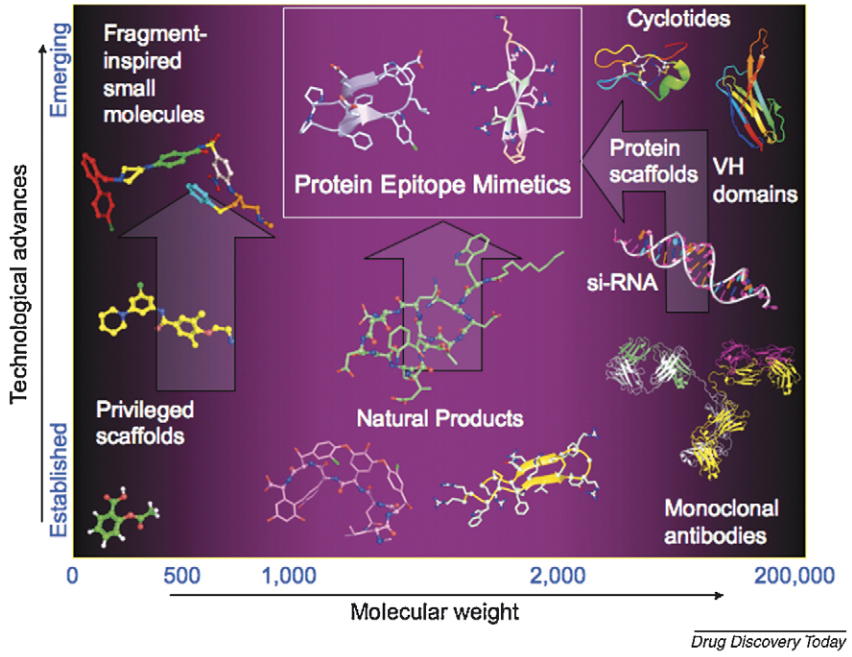

FIGURE 1

Protein epitope mimetics (central, top) occupy a region of molecular space with a size and structural complexity between that of typical small drug-like molecules (M.Wt. $\leq$ 500, left) and large recombinant macromolecules (biopharmaceuticals 10–200 kDa, right); a region that is shared also with some natural products (middle; vancomycin, polyphemusin and daptomycin are shown). Owing to their size and properties, molecules in this central region may be particularly well suited as inhibitors of protein–protein interactions.

functions, such as acting as an 'O-ring' to occlude bulk water from the hot interactions [11,12]. However, in one interesting example, hot spot mimicry of a cytokine receptor (IL-2Rα) was demonstrated by a small synthetic molecule that binds with high affinity to IL-2, and targets the same hot spot residues that the receptor uses to bind IL-2 [13]. Thus, here at least, the 'O-ring' concept does not apply, because the small-molecule mimic cannot provide such a large solvent-excluded zone.

When protein–protein interfaces are examined for size (typically in the range 800–2500 Å^2) and chemical character of the entire interface, solvent accessibility, packing density of atoms and presence of polar versus hydrophobic interactions, binding epitopes are on average only weakly differentiated from the rest of the protein surface [14–18]. Despite this, many studies have highlighted the frequent occurrence of Trp, Tyr and Arg in hot spot and core regions, which perhaps relates to the ability of their side chains to participate in hydrophobic interactions, van der Waals interactions, hydrogen bonding and polar π-interactions [11,19,20].

Recent studies suggest that protein–protein interfaces often have a modular architecture, in which energetically important interactions can be grouped into independent clusters [21,22]. It is also apparent that the contributions of distinct independent hot clusters may be additive. Perhaps most important is the realization that the hot spot patches at protein interfaces have surface areas close to those of synthetic macrocyclic molecules, and many natural products of 1–2 kDa. Such synthetic molecules are, therefore, very interesting candidates for inhibitor design.

Approaches to protein–protein interaction (PPI) inhibitors in drug discovery

An illustration of some current approaches to the targeting of PPIs is given in Fig. 1 [23–27]. Among these, fragment-based approaches have received the most attention and have produced some promising initial results. According to a recent review [28], about 50 projects using a fragment-based approach are currently at the discovery, preclinical and clinical stages in small biotech or large pharmaceutical companies. ABT-737, targeting the Bcl-2 family of proteins, serves as one of the highlights in this field, and is now in clinical trials as an anticancer agent [29]. Natural products, such as cyclic peptides, depsipeptides and macrolactones are also the source for several important clinical candidates and marketed products. Macrocyclic natural products, in particular, hit a wide variety of different targets, including PPIs. This may be because natural products cover a larger chemical space than typical small drug-like molecules. Natural products, however, can sometimes be difficult to synthesize, optimize and produce on a large scale.

Protein epitope mimetic (PEM) technology

An important new approach to drug discovery, especially to target PPIs, involves the application of protein epitope mimetic (PEM) technology. This approach is based upon the design of synthetic molecules that mimic the functionally important epitopes in biologically relevant peptides and proteins. The epitope is transferred from a recombinant to a synthetic scaffold that can be produced by parallel combinatorial methods. Activity, selectivity

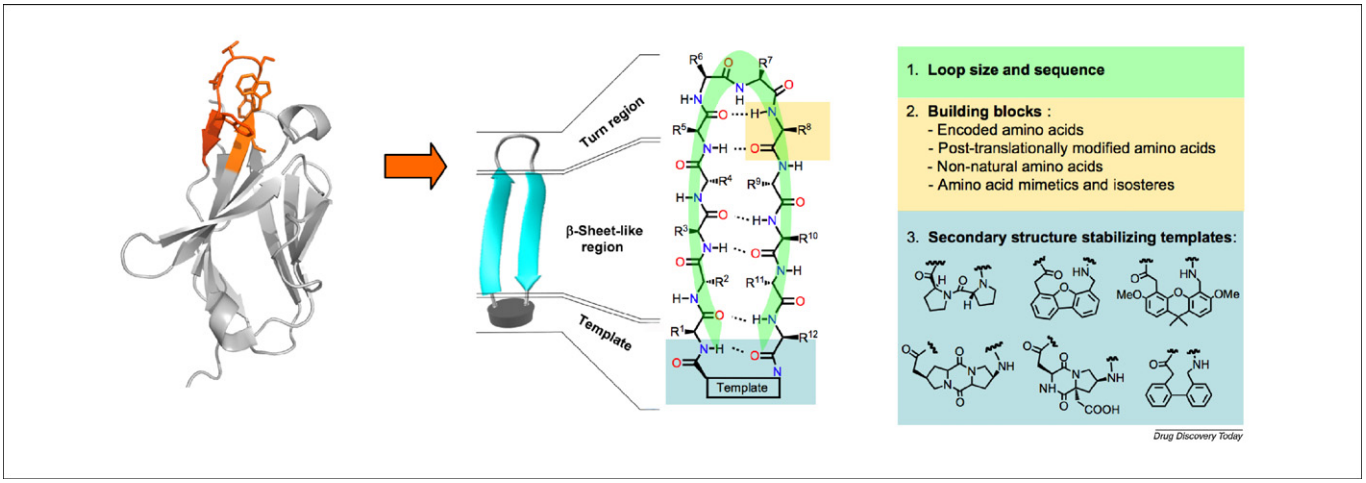

FIGURE 2

Protein epitope mimetics based on constrained template-bound β-hairpin loops, illustrating the three main variables (loop size, building blocks and template) that can be used to generate structural diversity.

and ADMET properties of an initial hit can then be efficiently optimized through iterative rounds of library synthesis and screening.

The β-hairpin is an especially interesting naturally occurring scaffold used by many proteins for biomolecular recognition, and thus is an attractive tool for mimetic design. For example, hairpin loop sequences may be transferred from folded proteins onto semirigid hairpin-stabilizing templates to afford macrocyclic, conformationally restrained β-hairpin PEM molecules (Fig. 2). Such template-fixed β-hairpin PEM molecules are versatile scaffolds that can also be used to mimic epitopes based on other types of secondary structures, including α-helices. This versatility represents one important difference to traditional peptidomimetic design, which typically involves efforts to mimic unique secondary structure motifs, such as β-turns. The template assumes an important role in the design of macrocyclic β-hairpin PEM molecules. The overall effects of backbone cyclization, the conformational bias imposed by the template and the influence of the hairpin loop size and sequence can act cooperatively to stabilize β-hairpin conformations. A variety of bi- and tri-cyclic systems can be envisaged as hairpin templates, but one of the most convenient systems to use is the dipeptide D-Pro-L-Pro, which adopts a very stable type-II′ β-turn [30] and is ideal to nucleate β-hairpin conformations.

PEM molecules can be rapidly and efficiently produced using a mixed solid- and solution-phase parallel synthesis process [31]. As shown in Fig. 2, the PEM technology is highly versatile; variation of the hairpin loop size, the sequence, the template and the nature of the amino acid building blocks (natural, non-natural and isosteres), all offer a virtually unlimited repertoire of permutations. This modular approach to PEM synthesis provides a powerful tool for optimizing biological and drug-like properties, which represents a further difference to traditional peptidomimetic design. Owing to their macrocyclic, conformationally constrained nature, β-hairpin PEM molecules can be endowed with excellent small-molecule drug-like pharmacokinetic properties, significantly more favorable than those of linear peptides. The 3D structures of PEM molecules both free in solution and in complexes with the target receptor can be investigated by NMR and X-ray crystallography, and the generation of structure–activity relationships may provide feedback into the library design and structure-guided optimization processes. The NMR structures of several PEM molecules have in fact been described already [32–40], including two in complexes with their target receptors [33,36].

There are various possible starting points for applying the PEM technology in drug discovery projects. These vary in the level of prior structural and functional information on the target biomacromolecules and have been described in a number of recent studies.

Mimics of natural β-hairpin loops

For example, mimetics of several 'canonical conformations' observed in antibody hypervariable loops were studied [41,42]. β-Hairpin PEM molecules were designed starting from crystal structures of antibody Fab fragments [38]. In one case, eight residues at the tip of the light chain loop-3 in the antibody HC19 were transferred from the immunoglobulin fold onto a D-Pro-L-Pro template (Fig. 3a). NMR studies on the resulting mimetic in solution revealed a stable hairpin conformation, which was an accurate structural mimetic of the L3 canonical loop in the antibody. This example suggests that the design of structural and functional mimetics of antibody combining sites based on PEM-like molecules is possible, although in the examples studied biological assays were not pursued.

Phage display in PEM design

Rather than starting PEM design with a natural peptide or protein, phage display offers an alternative means for selecting peptides and proteins with novel binding functions from large combinatorial libraries [43]. In some cases, peptides isolated from phage libraries have been shown to adopt β-hairpin structures, either free in solution or when bound to their target receptor [43]. Several spectacular examples of the successful application of phage technology to derive hairpin-shaped agonists and antagonists of cell surface receptors have been reported, including for example, the discovery of a peptide that mimics erythropoietin (EPO) and binds

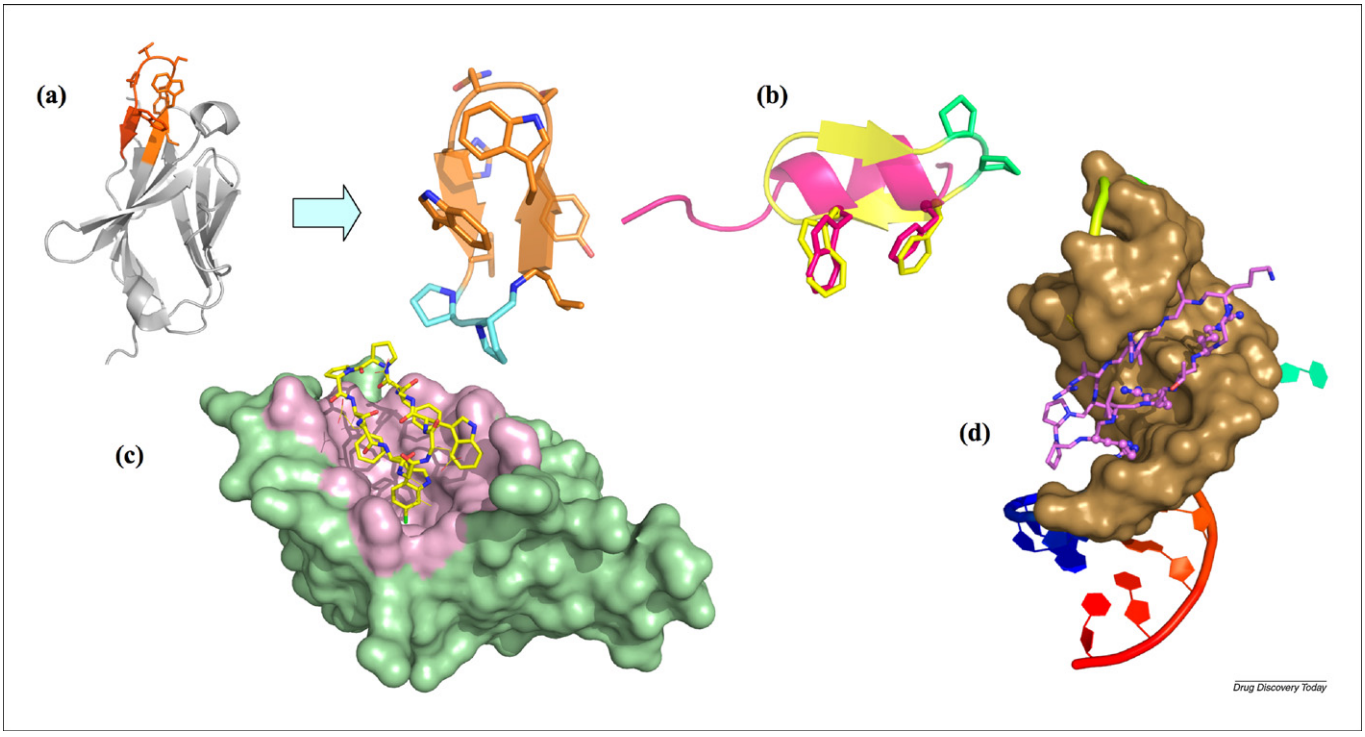

FIGURE 3

(a) A β-hairpin mimetic designed by transplanting a hairpin loop from the immunoglobulin fold (left) onto a D-Pro-L-Pro template (right). **(b)** Using a template-bound β-hairpin to mimic an α-helix, the overlay illustrates how the hairpin scaffold (yellow, D-Pro-L-Pro template green) can present side chains in 3D space at positions equivalent to those aligned along one face of a helix (pink). **(c)** Crystal structure (pdb 2AXI) of a β-hairpin mimetic (yellow) bound to a paratope in HDM2 that normally interacts with a helical epitope in p53. **(d)** NMR structure of a β-hairpin mimetic (shown as stick model) bound in the major groove of the transactivation-response region TAR of BIV mRNA (shown colored) (pdb 2A9X).

and activates the EPO-receptor [44]. In principle, phage display can be used to identify ligands for any target protein, so it is an attractive idea to harness the advantages of phage technology for the design of novel synthetic β-hairpin PEM molecules. One example to illustrate this point is a phage peptide (DCAWHL-GELVWCT) that binds the Fc fragment of a human IgG antibody. In this example, a crystal structure of the phage peptide bound to an Fc fragment revealed a β-hairpin structure in which side chains displayed on one side of the hairpin make intimate contact with the surface of the Fc protein, burying a surface of about 650 $Å^2$ [45]. Inserting a D-Pro-L-Pro template into this phage peptide produces a backbone macrocyclic PEM that adopts a stable β-hairpin conformation and binds to the Fc domain with significantly higher affinity than does the phage peptide [35]. There is clearly great potential in the marriage of these recombinant and synthetic technologies, and further efforts are underway to use phage peptides as starting points for PEM design.

α-Helical epitopes in PEM design

α-Helical epitopes are also attracting great interest in the design of PPI inhibitors [46–48]. In a recent study, a β-hairpin PEM was used to mimic an α-helical epitope in p53, and inhibit p53 binding to the human double minute 2 protein (HDM2) [49]. A crystal structure showed that a p53-derived peptide in complex with the inhibitory domain of HDM2 adopts an amphipathic α-helical backbone conformation [50]. In the complex, the side chains of Phe19, Trp23 and Leu26 align along one face of the helix, and insert into deep hydrophobic pockets on the surface of HDM2. To mimic this α-helical epitope, a short eight-residue β-hairpin was designed by transferring the three hot residues aligned along one side of the p53 helix onto one strand of the hairpin (Fig. 3b) [37]. The affinity for HDM2 of the first designed mimetic, although weak ($IC_{50} \approx 125$ μM), was optimized in an iterative process of library synthesis and screening. The optimized β-hairpin mimetic binds to HDM2 with $K_D \approx 25$ 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 [51].

More recently, a crystal structure of the hairpin mimetic bound to HDM2 confirmed that the residues Phe1, 6-ClTrp3 and Leu4 in the first β-strand fill the hydrophobic pockets on the surface of HDM2 (Fig. 3c) [36]. However, aromatic groups in the second β-strand, Trp6 and Phe8, are also important and they 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. In this way, the binding site on HDM2 has adapted to optimize structural complementarity with the mimetic.

PEM inhibition of protein–RNA interactions

PEM molecules can also inhibit specifically protein–RNA interactions of therapeutic relevance. For example, there are PEM molecules that can potently and selectively inhibit the interaction between the bovine immunodeficiency virus (BIV) Tat protein and its target transactivation-response region (TAR) RNA [52], an interaction that is essential for viral replication. Optimization

of initial hits based upon an NMR structure of the mimetic-TAR complex (Fig. 3d) provided new inhibitors with nanomolar affinity to BIV-TAR. Several of the mimetics synthesized in this study were also shown to be potent inhibitors of the HIV Tat–TAR interaction [33].

A second case study highlights the discovery of β-hairpin PEM inhibitors of the HIV-1 Rev–Rev response element (RRE) interaction [53]. This interaction plays an important role in the temporal control of HIV-1 mRNA splicing in the nucleus. A small segment of the Rev protein binds to a stem loop region of the HIV-1 mRNA, called the RRE, in an α-helical conformation [54]. These PEM molecules mimic the helical epitope in Rev and bind tightly to the RRE RNA. This is still a relatively new class of RNA-binding molecules, but clearly one with great potential for development into anti-infective agents, and further studies are aimed at optimizing their biological and pharmacokinetic properties.

Case studies: from β-hairpin-shaped natural products to PEM drug leads

Disulfide-bridged, β-hairpin-shaped natural peptides such as the sunflower seed trypsin inhibitor, the antimicrobial peptide protegrin I and the antiviral polyphemusin II are excellent starting points for PEM-based drug design projects (Fig. 4a). In each case, the natural product was used to design initial PEM molecules. The potency, selectivity and ADMET properties (absorption, distribution, metabolism, excretion and toxicity) of these molecules were subsequently optimized efficiently in iterative cycles of focused PEM library design and screening.

Serine protease inhibitors based on the Bowman–Birk motif

The reactive site loop of the Bowman–Birk (BB) family of serine protease inhibitors comprises a β-hairpin loop, and represents an interesting starting point for the design of PEM-based serine protease inhibitors. One of the smallest members of the BB family was isolated from sunflower seeds, and a crystal structure of the inhibitor bound to the active site of trypsin was taken as a starting point for PEM design [55]. Initially, PEM molecules were designed by transferring 11 or 7 residues from the BB reactive loop onto a D-Pro-L-Pro template [34]. NMR studies on the resulting mimetics revealed a well-defined β-hairpin conformation in aqueous solution, essentially identical to that seen in the natural peptide (Fig. 4b). Enzymatic assays showed that both mimetics inhibit bovine trypsin with low to mid nanomolar K_i values, and an alanine scan confirmed the energetically important role of a Lys side chain, which occupies the P1 position [34].

This PEM scaffold was recently used to engineer new inhibitors targeting pharmaceutically important serine proteases [56], among them cathepsin G [57], neutrophil elastase [58] and tryptase [59], which are implicated in chronic obstructive pulmonary disease (COPD) and asthma. New PEM libraries were produced,

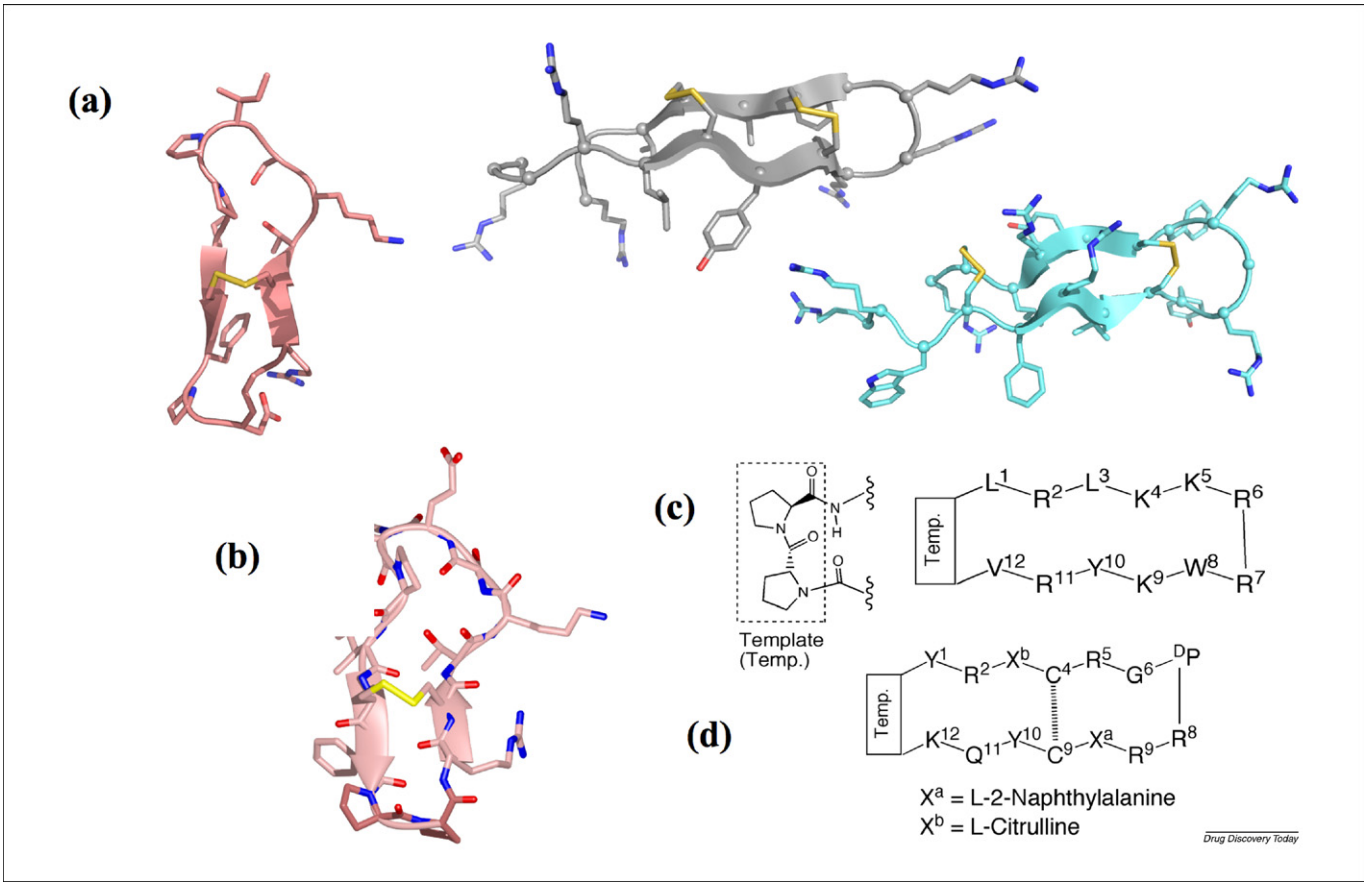

FIGURE 4

PEM drug leads from β-hairpin-shaped natural products. **(a)** three natural products, the sunflower seed trypsin inhibitor (pink), protegrin I (gray) and polyphemusin I (blue). **(b)** A PEM-based serine protease inhibitor. **(c)** PEM-based protegrin mimetic with broad-spectrum antimicrobial activity. **(d)** A PEM-based CXCR4 antagonist (POL3026).

and initial hits were optimized in an iterative process of library synthesis and screening to improve inhibitory potency, selectivity and *in vitro* ADMET properties (e.g. plasma stability, microsomal stability and protein binding). In this way, potent and selective inhibitors of cathepsin G (POL5196; K_i = 25 nM), neutrophil elastase (POL5975; K_i = 1.1 nM) and tryptase (POL9428; K_i = 8.0 nM) were discovered. These inhibitors are very selective (selectivity index >5000) with respect to other serine proteases, such as trypsin, chymotrypsin, chymase, thrombin and factor VIIa, and they exhibit excellent *in vitro* ADMET properties (unpublished data).

Approximately 1000 PEM-based cathepsin G, elastase and tryptase inhibitors have now been fully characterized. The PEM inhibitors are fully reversible competitive inhibitors. A detailed SAR analysis revealed that the potency and selectivity of the inhibitors could be subtly modulated by variations at positions within the hairpin loop that are remote (>10 Å) from the active site P1 residue (to be published).

Mimetics of protegrin I as novel antibacterials

Interesting starting points for PEM design are represented by the large family of naturally occurring cationic antimicrobial peptides [60], including the protegrins, polyphemusins and tachyplesins, which adopt β-hairpin structures stabilized by multiple disulfide bridges. The first target was protegrin I, isolated from porcine leucocytes, whose mechanism of action involves lysis of the bacterial cell membrane [61]. A synthetic analog of protegrin I, IB367 (Iseganan) has already been in clinical trials for the treatment of oral mucositis [62]. Peptide loops with sequences related to protegrin I were synthesized, mounted on a D-Pro-L-Pro template, in which disulfide bridges were replaced by a variety of other residues. In this way, several families of PEM-based protegrin mimetics were discovered (Fig. 4c) possessing broad-spectrum antimicrobial activity and a considerably reduced hemolytic activity on red blood cells [39,63–65].

More recently, a new structure–activity trail has provided access to novel analogs with a much higher antimicrobial potency and a remarkable selectivity toward *Pseudomonas aeruginosa*. These new PEM molecules do not cause cell lysis and only one enantiomer retains significant activity, suggesting a different (nonlytic) mechanism of action (to be published). Further optimization of the ADMET properties produced analogs active against multidrug resistant *P. aeruginosa in vivo*, one of which (POL7080) is now in preclinical development.

Inhibitors of CXCR4: from design to the clinic

CXCR4 belongs to a subfamily of the seven transmembrane GPCRs, known as chemokine receptors. The natural ligand of CXCR4 is the 67 residue stromal cell-derived factor-1α (SDF-1α or CXCL12). CXCR4 is used as a coreceptor for CD4-dependent HIV infection of human T-cells and plays a major role in bone marrow (BM) where it is expressed on the surface of the majority of hematopoietic stem and early progenitor cells (HSCs and HPCs). The SDF-1/CXCR4 axis is important for the retention of these cells in the BM stromal compartment and for proper hematopoiesis [66]. Disruption of the SDF-1/CXCR4 axis results in mobilization of hematopoietic stem and precursor cells from the BM to peripheral blood. Finding new protocols for improving mobilization and harvesting efficiencies of HSCs for transplantation is clinically important for the treatment of many hematological malignancies, including leukemia and lymphoma. CXCR4 inhibitors, therefore, hold great promise as future therapeutics for the efficient mobilization and harvesting of peripheral blood HSCs, in cancer therapy as antimetastatic agents, as well as in inflammation and tissue repair.

The starting point for PEM design in this case was polyphemusin II (Fig. 4a), an 18-amino acid peptide isolated from the American horseshoe crab (*Limulus polyphemus*) and a close analog, T22 ([Tyr5, 12, Lys7]-polyphemusin II) [67,68]. T22 is a potent inhibitor of CXCR4, and prevents T-tropic HIV-1 cell fusion and entry. NMR analysis showed that T22 adopts a β-hairpin structure in solution. On the basis of this structure, several PEM libraries were designed, synthesized and evaluated in biological assays. Several rounds of optimization led to early leads such as POL3026 (Fig. 4d). POL3026 is a highly potent and selective CXCR4 antagonist in Ca^{2+} flux and ligand displacement assays (IC_{50} = 1–3 nM for both) and is a potent inhibitor of T-tropic HIV-1 entry [69]. POL3026 does not inhibit other chemokine receptors *in vitro* at concentrations up to 10 μM and does not block infection by M-tropic (CCR5 dependent) HIV-1. Further studies showed that POL3026 possesses small-molecule-like PK properties in dogs (half-life 3.4 h at a dose of 1.5 mg/kg administered s.c.) [69].

Additional libraries were synthesized from POL3026 to optimize ADMET properties while maintaining or improving potency and selectivity. This optimization resulted in compounds POL5551 and POL6326, which were selected for further development as mobilizing agents for hematopoietic stem cells. The *in vivo* efficacy of these compounds was confirmed in a murine colony forming cell (CFC) assay, which enumerates circulating HPCs before and after the application of the compounds. In contrast to the current standard G-CSF treatment, which requires multiple injections over several days to achieve a significant number of circulating HSCs, a single injection of POL3026 or POL6326 gives a 11–12-fold increase in circulating progenitor cells with a peak at 2–4 hours postdosing. The mobilization potential of POL6326 was also assessed in *Cynomolgus* monkeys, where very similar mobilization kinetics was observed. If confirmed in humans, this rapid, transient increase in PB $CD34^+$ cells (a marker for HSCs) would be a distinct advantage over current protocols, and would allow precise timing in the harvesting of HSCs from peripheral blood, with a more convenient harvesting procedure, and most probably a single administration of the mobilizing agent. Recently, POL6326 successfully completed phase I clinical trials for HSC mobilization.

Summary and outlook

Despite ever-increasing efforts by the pharmaceutical and biopharmaceutical industries to optimize the drug discovery process, the number of novel and innovative drugs approved by the US FDA and European EMEA has decreased over recent years. An analysis of the potential druggable targets has revealed that only around 30–50% of these will be amenable to traditional small-molecule approaches. In this review, we have highlighted the PEM technology, which uses biologically relevant peptides and proteins as starting points for ligand design. In terms of size, β-hairpin PEM molecules represent a bridge between the molecular space occupied on one side by biopharmaceuticals and on the other by

traditional small-molecule drugs (Fig. 1). PEM molecules can be produced by an efficient parallel synthesis process, which is a prerequisite for rapid iterative lead optimization of biological activity and ADMET properties. PEM molecules are also chemically stable, usually soluble in aqueous media and hence are amenable to establish drug formulation technologies. Up-scaling of PEM molecule production to the quantities required for preclinical and clinical development seems so far not to require extensive process development.

The applications of PEM technology highlighted in this review hint at its broad potential in identifying potent and selective lead compounds in drug discovery. Extracellular proteins have already been successfully targeted, however, ongoing work suggests that intracellular targets will also be accessible. This requires knowledge about the structural modifications needed (e.g. amide *N*-methylation, as seen in many natural products) to make PEM molecules cell permeable and orally bioavailable, by design. Novel formulation strategies will also broaden the spectrum of applications amenable to the PEM technology. The successful development of the CXCR4 inhibitor POL6326, the first PEM molecule to enter the clinic where it has recently completed successfully phase I trials in the field of hematopoietic stem cell mobilization, is an important milestone in the further validation and development of this approach to drug discovery.

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