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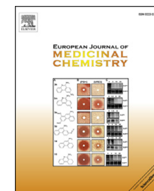

Original article

Peptide therapeutics: Targeting the undruggable space

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A R T I C L E I N F O

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Rapid advancements in genomics have brought a better understanding of molecular mechanisms for various pathologies and identified a number of highly attractive target classes. Some of these targets include intracellular protein–protein interactions (PPIs), which control many essential biological pathways. Their surfaces are part of a diverse and unexplored biological space, where traditional small molecule scaffolds are not always successful. While large biologics can effectively modulate PPIs in the extracellular region, their limitation in crossing the cellular membrane leaves intracellular protein targets outside of their reach. There is a growing need in the pharmaceutical field to push the boundaries of traditional drug design and discover innovative molecules that are able to modulate key biological pathways by inhibiting intracellular PPIs. Peptides are one of the most promising classes of molecules that could deliver such therapeutics in the near future. In this review, we describe technological advancements and emerging chemical approaches for stabilizing active peptide conformations, including stapling, hydrogen bond surrogates, beta-hairpin mimetics, grafting on stable scaffolds, and macrocyclization. These design strategies carry the promise of opening the doors for peptide therapeutics to reach the currently “undruggable” space.

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1. Introduction

Many essential cellular pathways that are implicated in human diseases are controlled by intracellular protein–protein interactions (PPIs) [1]. Such PPIs could be potential drug targets, and thus the ability of molecules to inhibit specific PPIs has remarkable therapeutic value. Small molecule PPI modulators have already reached clinical studies [2], and with many projects in the discovery phase more will do so in the future. Biologics can successfully target PPIs that are accessible outside of the cell [3] and have become the therapeutic of choice for a number of diseases. Nucleic acid therapeutics (e.g. antisense oligonucleotides and RNA interference products) are one of the most important classes of next generation drugs [4], particularly if current delivery limitations can be overcome. Here, I will focus on recent developments that highlight the potential of peptides for intracellular PPI targeting.

1.1. Why peptides?

The historical definition of druggable targets has been continually evolving. It originally referred to any therapeutic protein target that could be modulated with small, orally available molecules [5]. Small organic molecules are well suited for oral administration because of their stability in the digestive tract and their absorption characteristics - they can enter the circulatory system by passively diffusing across the epithelial cells that line the stomach and intestines, a property that also gives them the ability to enter cells and modulate the functions of specific targets within the cell itself.

For decades, the pharmaceutical industry has been dominated by small molecule drugs. In 1997, Lipinski et al. analyzed the existing database of successful small molecule drug candidates in the clinic and developed a guideline, known as the ‘rule of 5’, to predict and reduce the risk of inadequate oral absorption due to poor solubility or poor permeability [6], by favoring molecules with fewer than 5 hydrogen bond donors, fewer than 10 hydrogen bond acceptors, molecular weight less than 500 Da, and an octanol–-water partition coefficient logP no greater than 5. His analysis was

Abbreviations: PPI, protein–protein interaction; HTS, high-throughput screening; PK, pharmacokinetic; mAb, monoclonal antibody; RCM, ring-closing metathesis; aPP, avian pancreatic polypeptide; CCPP, cyclic cell-penetrating peptide; HBD, hydrogen bond donors; CCK, cyclic cysteine knot; HBS, hydrogen-bond surrogate; NRPSs, non-ribosomal peptide synthetases; PEM, protein epitope mimetic; CSA, cyclosporin A; MATCH, macrocyclic template chemistry; DPC, DNA-programmed chemistry; PURE, protein synthesis using recombinant elements; uPA, urokinase-type plasminogen activator; FIT, flexible In vitro translation; RaPID, random nonstandard peptide integrated display; RNAi, RNA interference.

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done partly in response to the development and implementation of combinatorial synthesis and high-throughput screening (HTS), which sometimes led to a bias selection of molecules with increased lipophilicity that were prone to displaying poor pharmacokinetic (PK) parameters. Antibiotics, antifungals, vitamins, and cardiac glycosides, which are often natural products [7], fell outside the 'rule of 5'. Various empirical criteria [8] along with the 'rule of 5' have been applied to the small molecule drug space for more than a decade and have successfully reduced the prevalence of drug candidate attrition due to poor PK. Rational design coupled with novel screening technologies has resulted in small molecule hits for traditional, tractable extra- and intracellular targets such as receptors, ion channels, and enzymes. Yet, the number of new small molecule entities approved per year has remained flat [9,10].

Despite their unique and attractive ability to penetrate cells via passive diffusion, it is a challenge for small of molecules to address novel targets such as intracellular protein–protein interactions [11]. The inherent properties and diversity of PPI interfaces make it extremely difficult to design small molecule inhibitors, even though in recent years we have witnessed a number of successful examples of small synthetic molecules modulating PPI [2,12–16].

The most obvious and unique feature of PPI is the size of the interaction surface. Typical PPI interfaces span the contact area of 1500–3000 A^2, while the interfaces for protein-small molecules are only in the range of 300–1000 A^2 [17]. In addition, most PPI interfaces are relatively featureless, lacking pre-formed and well-defined hydrophobic cavities which can fully accommodate a small molecule ligand. Such single, deep binding pockets that entirely surround the bound ligands are usually found on traditional 'druggable' targets, and on average, they occupy a volume of ~270 A^3 [18] (Fig. 1). In contrast, a PPI interaction surface is a collection of a few smaller binding pockets scattered across the interaction interface, each with a volume of about 100 A^3 [19]. It has been shown that only some of these interactions, called 'hot spots' [20], are essential for affinity as they contribute the majority of the total interaction energy. In order to achieve good affinity and competitive binding, a PPI inhibitor should be large enough to simultaneously interact with multiple 'hot spot' patches and gain a significant part of the distributed free energy [21]. In some cases, when 'hot spots' are localized in close proximity to each other, or when proteins have adaptive binding surfaces [16], they can be effectively modulated by small molecule scaffolds [22,23]. However, in general, molecules with larger contact surfaces such as biologics and peptides are necessary to achieve nanomolar potency at the PPI interface. Not surprisingly, structural analyses of small molecules that have successfully inhibited PPI show that they differ from known small molecule drugs: they tend to have higher molecular weight and more complex topology than typical 'drug-like' molecules adhering to the 'rule of 5' [17].

Another challenge presented by PPI targets is the need for new and diverse scaffold libraries to allow for better sampling of the PPI chemical space. In the absence of small natural substrates or ligands, high-throughput screening (HTS) methods are often used to discover small molecule inhibitors. However, screening is rarely successful. One of the reasons is lack of diversity in the commercial small molecule compound libraries used for screening. The chemical space of the existing libraries is strongly influenced by binding pockets of traditional targets (e.g. enzymes, G-protein-coupled receptors). Consequently, 'drug-like' compound libraries are not diverse enough to contain PPI surface-compatible molecular scaffolds. A number of studies have analyzed known PPI inhibitor molecules and delineated some of the features that differentiate PPI inhibitors from 'drug-like' molecules: more hydrophobic, rigid aromatic scaffolds combined with charged or polar groups, larger in size, macrocyclic, higher number of chiral centers, and three-dimensional in bound conformation [17,18,24–26]. A better understanding of the chemical space of PPI inhibitors will help to create new, focused chemical libraries that could ensure more successful HTS screening in the future [27].

It is evident that novel targets such as intracellular PPIs pose challenges that cannot be answered simply by small, orally available molecules. Large and diverse PPI surfaces, which feature complex topologies of multiple low energy interaction sites, are well-complemented and effectively modulated by protein-based biologic drugs. Due to their greater size and well-defined three dimensional conformations, biologics can bind their protein targets with high affinity and remarkable selectivity. Today, several monoclonal antibodies (mAb) that target extracellular proteins are among the blockbuster therapeutics on the market [28]. Modern developments in drug delivery technologies have allowed non-oral delivery systems, such as injectables, to become acceptable alternative routes of drug administration. Protein therapeutics have expanded the historical definition of "druggable" targets. However, their large size restricts their diffusion across the cell membranes, hindering their ability to reach intracellular targets.

Peptides, which are distinguished from proteins based on their smaller size (50 amino acids or less), mediate various essential biological functions, such as signal transduction, heart rate regulation, food intake, and growth. Natural peptides such as insulin, oxytocin, and cyclosporine are successful drugs [29]. Similar to biologics, peptides can bind large protein targets with high potency and great selectivity, which translates into fewer off-target side effects and less potential for toxicity than small molecule drugs [30]. Unlike small molecules which often trigger side effects by producing toxic metabolites that accumulate in different organs [31], peptides degrade into amino acids, which minimizes the risk

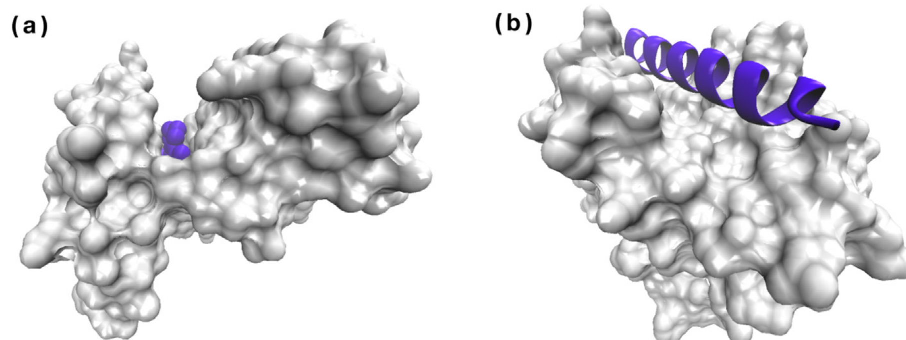

Fig. 1. Examples of two different binding modes: (a) Small molecule inhibitor bound to the EPHA4 receptor tyrosine kinase displays compact binding (PDB: 2XYU), (b) the PPI interface between Bcl-2/BID domains (PDB: 2VOI) is an example of extended binding.

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of toxicity. In comparison to recombinantly produced antibodies and engineered protein drugs [32,33], peptides also pose lower manufacturing costs, are more stable at room temperature and, due to their smaller size, they have a better ability to penetrate tissues [34]. Moreover, non-natural building blocks and various chemical scaffolds can be incorporated into a peptide sequence to create a palette of modified peptides with a wide range of functionalities and chemical diversity.

Therapeutic peptide leads can be derived from various sources: nature (mined directly as peptides or indirectly from the nucleic acids) [30], chemical libraries, and genetic or recombinant libraries [35]. Thus, when considering targeting novel intracellular PPIs, the virtues and versatility of synthetic peptides gives them the capacity to combine beneficial properties of both classes (biologics and small molecules): high specificity and affinity of proteins coupled with less production costs, lower toxicity, and the potential to permeate the cell. However, the challenges that need to be overcome to make these molecules successful pharmaceuticals should not be underestimated. Peptides made of natural amino acids are generally considered to be poor drug candidates mainly because of their pharmacokinetic profiles. Because many proteolytic enzymes recognize common structural features of peptides, unmodified peptides have poor *in vivo* stability against proteases. In addition, they are rapidly cleared from the body by the liver and kidneys, with half-lives often in the range of minutes. Peptides also have poor oral availability and their transport across membranes is limited. Conformationally flexible three dimensional structures, common in unmodified peptides, limit affinity and selectivity.

However, efforts to overcome these hurdles and optimize peptides as successful pharmaceuticals are gradually maturing. During the last three decades there has been an exponential growth in an average number of peptide therapeutics entering clinical studies, as shown in Fig. 2. Peptide drugs are being investigated in a wide variety of therapeutic areas, with the highest number of them entering clinical trials for metabolic disorders and cancer treatments (Fig. 3). However, an overwhelming majority of peptide drugs are directed toward extracellular targets (Fig. 4) with less than 15% binding intracellular proteins, such as enzymes and cytoskeleton proteins [36]. Emergent innovative avenues of research, advanced drug discovery tools, and new platform technologies are promising to engineer next generation peptide-based therapeutics with desired properties that may be able to find a path for unlocking intractable intracellular PPIs.

2. Key design strategies

With the aim to target chemically intractable proteins that represent a vast majority (>80%) of the human proteome [39], a lot of effort is being devoted in industry and academia to the discovery and development of new classes of molecules, such as small “synthetic biologics” [40] that occupy the chemical space between ‘rule of 5’ compounds and large protein-based biologics. This space covers a significant range of molecular weight (500–5000 Da) and many different structural classes with various biophysical properties. Cell-penetrating peptides (CPPs) are a specific class of molecules that can cross the cellular membrane and serve as carriers for various functional groups, as well as incorporate a functional motif [41]. This review, however, addresses emerging chemical approaches for engineering synthetic therapeutics that have the ability to penetrate the cell, bind to the large target protein surface, and successfully modulate the biological pathway, hence combining the advantages of biologics with the benefits of small-molecule drugs. The following sections highlight some of the key design strategies and drug discovery platforms that are progressing toward the delivery of new therapeutic agents that could potentially fill the gap that cannot be addressed by traditional medicinal

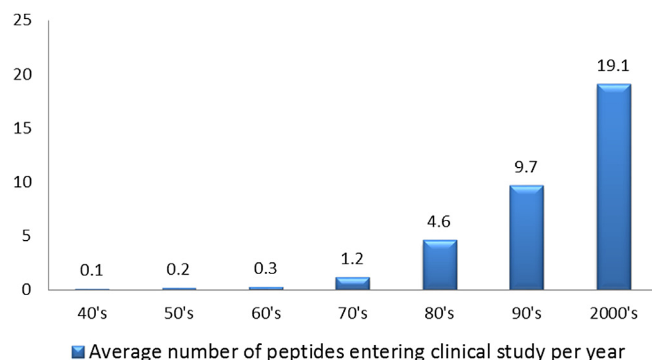

Fig. 2. Average number of peptides entering clinical study per year. Data including the year 2008 was taken from the Development Trends in Peptide Therapeutics Report [36] and was updated through 2010 based on the following publication [37].

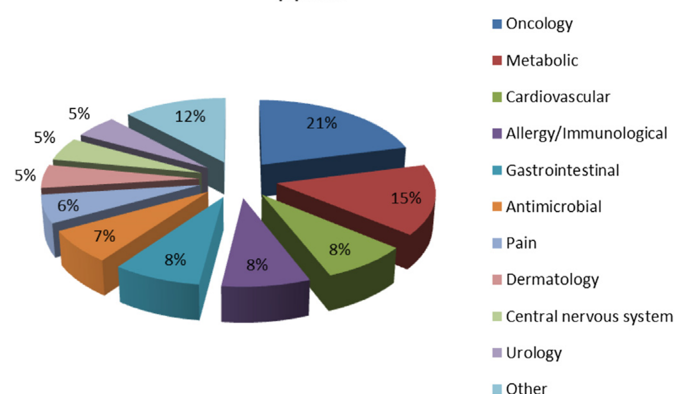

Fig. 3. The distribution of peptide pipeline drugs by therapeutic areas. Other indications that are not specified on the chart include: Endocrinology, Respiratory, Bones, Hematology, Ophthalmology, OB-GYN (obstetrics and gynecology). The highest number of peptides has entered clinical study for cancer treatments and metabolic disorders. Data was taken from a 2012 report provided by the Peptide Therapeutic Foundation [37]. The detailed analysis of the data by Phase I/II/III is published in the following review [38].

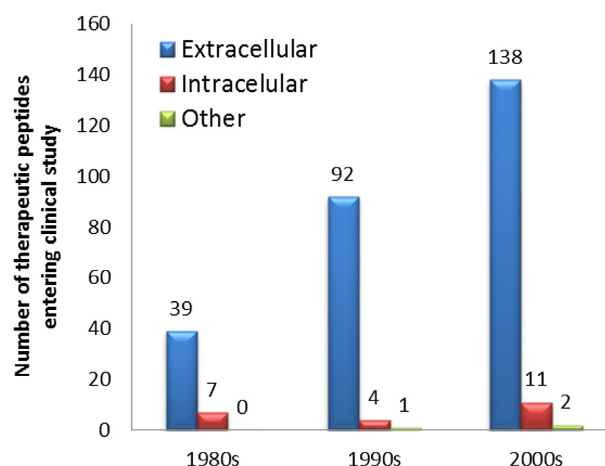

Fig. 4. The location of targets (intracellular vs extracellular) for therapeutic peptides that have entered clinical studies in three time-periods. Data was taken from the Development Trends in Peptide Therapeutics Report [36].

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chemistry. Namely, we focus on constrained secondary structures, grafting bioactive peptides on stable miniprotein scaffolds, macrocycles, and some evolutionary selection technologies.

2.1. Constrained secondary structures

Extensive analysis of the available structural data of protein–-protein interaction complexes shows that PPIs are usually mediated via various folded subdomains, and α-helices are the largest structural class that facilitate this interaction [42,43]. For peptidic ligands, conformation is believed to be the primary determinant of bioactivity and bioavailability [44]. Hence, it is intuitive to start designing bioactive peptides by carefully mimicking structural elements such as α-helices or β-hairpins at the PPI recognition interface. To identify and rationally design protein domain mimics, a host of computational strategies have been established, as described in a recent review [45]. However, when peptide fragments are removed from their parent protein, they generally lose their well-defined, biologically active conformation and their proteolytic stability,. a number of synthetic approaches can be used to stabilize the α-helical structure of a peptide, as reviewed recently [46–50], including side chain crosslinking with a lactam bridge [51,52], a "click" triazole linker [53,54], and an *m*-Xylene thioether linker [55]. In the following section some of the stabilization strategies are highlighted as they were utilized in designing cell-permeable peptides to target intracellular interactions.

2.1.1. Hydrocarbon stapled peptides

In 2000, the Verdine group proposed a unique combination of strategies, known as hydrocarbon stapling, to create a novel class of therapeutics with the ability to modulate both intracellular and extracellular targets [56]. The stapled stabilization method utilizes α,α-disubstituted non-natural amino acids linked with a membrane-compatible, chemically and metabolically stable all-hydrocarbon bridge (in i,i+4 or i,i+7 positions) with the intention to facilitate cell penetration (Fig. 5) [57]. For ring-closing, the olefin metathesis reaction was chosen for its compatibility with solid-phase peptide synthesis under mild conditions [58]. Today, the variety of staples that have been developed include multiple bridges in a single peptide and a 6-carbon cross-link in i,i+3 positions [59]. The effect of the stapling on peptide fragments is threefold: it promotes the structural stability of an α-helix, improves its resistance to proteolytic degradation and, when combined with the appropriate biophysical parameters of a peptide [60], it can facilitate cell-penetration by energy dependent endocytosis pathway [61,62]. Accumulated structural data has also revealed that a staple can interact with its target via multiple mechanisms [63]. In 2004, Walensky et al. demonstrated that the all-hydrocarbon stapled α-helical BID BH3 segment was protease resistant, cell-permeable, and showed *in vitro* and *in vivo* biological activity [61]. During the following decade, stapled peptide technology has been applied to a number of extra- and intracellular targets, including most intractable transcription factors and other promoters of cancer proliferation [61,64–67]. A comprehensive list of all biological systems that are targeted using stapled peptide technology has been recently compiled in a review by Bird *at al* [68]. Based on accumulated data from a series of stapled peptide studies, the ability of a stapled peptide to penetrate the cell is a result of a combination of factors such as charge, hydrophobicity, and structural rigidity, which in turn depends on peptide sequence and the position of the staple. A recent report indicates that cell permeation ability in stapled peptides is strongly related to staple type and formal charge, while other parameters do not show a significant effect [62].

In addition to their remarkable ability to modulate intracellular PPIs, the proteolytic stability of all-hydrocarbon stapled peptides could transform the therapeutic potential of natural bioactive peptides. As shown by Bird et al. [67], the insertion of two independent staples in an HIV-1 fusion inhibitor peptide results in improved pharmacokinetic properties. In addition, low but measurable concentrations of the full-length peptide were detected in plasma samples from all treated animals after oral administration.

Aileron Therapeutics Inc. was founded in 2005 and has been developing first-in-class drugs based on a stapled peptide platform [69]. Its novel, long-acting, growth-hormone-releasing factor (GRF) agonist (ALRN-5281) is the first stapled peptide that entered Phase I clinical trial in 2013 for orphan endocrine disorders [38]. Aileron is also developing a stapled peptide drug (ATSP-7041) that targets intracellular PPI. ATSP-7041 is a highly potent molecule that restores the activity of human transcription factor p53 by disrupting its interaction with two inhibitory proteins, MDM2 and MDMX. The p53 is a tumor suppressor protein that has been named as "the guardian of the genome", as it protects cells from malignant transformation [70]. The MDM2 and MDMX proteins are overexpressed in cancers to deactivate the p53 function. Although a number of small-molecule MDM2 antagonists are currently in clinical trials [2], developing small molecule drugs that could effectively bind both inhibitory proteins has proved challenging [70]. Interestingly, a recent study showed that resistance mutations in HDM2 protein selectively reduce affinity for Nutlin, while the mutated HDM2 target is still responsive to stapled p53-peptide analogs [71]. The data suggests that the larger interaction footprint of stapled peptides, compared to small molecules, might be responsible for higher resilience to resistance mutations.

The ATSP-7041 peptide is the first highly potent and specific dual inhibitor of MDM2/MDMX with drug-like properties that demonstrated *in vitro* and *in vivo* activity [70], hence proving the concept that stapled peptides have the potential to be the next generation peptide therapeutics that can modulate undruggable

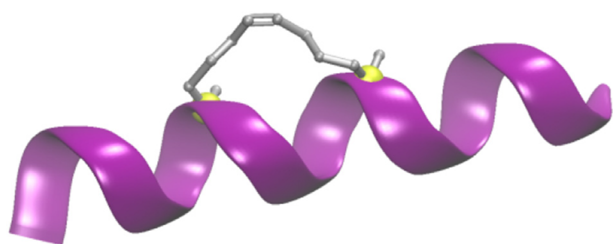

Fig. 5. A single all-hydrocarbon staple (gray) stabilizing a helical conformation. The alpha carbons of i, i + 4 position are depicted with yellow spheres. The α,α-disubstituted non-natural amino acids have S, S stereochemistry at the α-carbon.

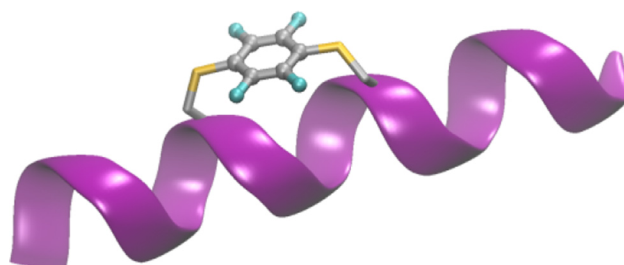

Fig. 6. The perfluoroaryl cross-linked peptide in i, i + 4 positions, stabilizing a helical conformation. Fluorine atoms are shown in cyan, sulfur in yellow.

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pathways in cancer biology. A potent and selective molecule of this class is planned to enter phase I clinical trial.

2.1.2. Perfluoroaromatic staple

Recently, the Pentelute group [72] discovered a nucleophilic aromatic substitution reaction between cysteine and perfluoroaryl substrate that takes place at room temperature with remarkable selectivity and functional group tolerance. This prompted them to apply this chemistry toward stapling unprotected peptides in order to generate a new class of molecules (Fig. 6). As a starting point for the model system, an α-helical peptide, CAI was chosen, which binds the C-terminal domain of HIV-1 capsid *in vitro* but cannot penetrate the cell [73]. The model peptide was stapled with the perfluoroaromatic group and was evaluated for structural stability, protease resistance, and cell permeability. When compared to its unstapled analog, the perfluoroaryl cross-linked peptide was more helical, less prone to amide-bond cleavage, and showed significant cellular uptake with subsequent localization in the endosomes and cytosol. While further research is in progress, the reported approach of helix stabilization opens up new avenues for altering the biophysical properties of peptides to generate drug-like synthetic biomolecules.

2.1.3. Hydrogen bond surrogate approach

An alternative approach for stabilizing the α-helical conformation is to introduce a covalent mimic of a main-chain hydrogen bond between the i and i+4 residues of a peptide. Initially, using solid-phase synthesis, Satterthwait [74] replaced the backbone hydrogen bond (C=O ··· H–N) with a hydrazine linkage to create a nucleation site and overcome the energy barrier for α-helix formation. Later, the Arora group [75] applied a ring-closing metathesis (RCM) reaction to the N-terminal residues of a short peptide (Fig. 7). This generates a stable and irreversible linkage that preorganizes the α-turn, which initiates and stabilizes an α-helical structure [76]. This approach is called the hydrogen-bond surrogate (HBS) strategy and has been reviewed recently [77]. HBS helices have been shown to modulate protein targets with high affinity and specificity [78]. One of the main advantages of using an HBS linker is that, while stabilizing helical conformation, peptide side-chains are not altered so the ligand retains its entire functional surface for molecular recognition. In 2011, the design of the first direct inhibitor of Ras–Sos interaction was reported, a cell-permeable, HBS-stabilized α-helix which was shown to downregulate Ras signaling in cultured cells [79]. In the most recent study, an HBS-stabilized protein domain mimetic was rationally designed to target the interaction of the p300/CBP coactivator with the transcription factor hypoxia-inducible factor-1α [80]. The results showed the remarkable efficacy of the HBS-stabilized peptide in suppressing tumor growth in a mouse tumor xenograft model. These findings demonstrate the effectiveness of the strategy and its potential for future inhibitor design.

2.1.4. β-Hairpin mimetics

Another interesting and often functionally important epitope used for molecular recognition by PPI interfaces is the β-hairpin. In the past, a number of β-turn and β-turn-mimetic scaffolds have been proposed that are capable of nucleating and facilitating β-hairpin formation, thus minimizing the conformational entropy of a peptide [81–87]. An example of this approach, known as the protein epitope mimetic (PEM) technology, is being developed by Polyphor. The PEM approach incorporates an active peptide loop into a semi-rigid, synthetic, hairpin-stabilizing template yielding conformationally restrained, macrocyclic β-hairpin molecules (Fig. 8) [88]. Due to their modular nature, PEM structures can be varied and linked together rapidly by parallel synthesis methods. The diversity of PEM compound libraries comes from variations in the template, the hairpin loop size, and an array of possible building blocks including non-proteinogenic amino acids offering a powerful tool for sampling relatively unexplored molecular space [89]. Iterations of library synthesis and screening are used to optimize the biological activity and drug-like pharmacokinetic properties of an initial hit. In addition to the rational approach that mimics the natural β-hairpin loop of the target protein, Polyphor also applies phage display for hit identification. Phage-selected peptide sequences can be translated into synthetic PEM molecules for further optimization. In one of their studies, stabilized β-hairpin PEM was used to mimic some features of an α-helical epitope of p53 and antagonize the interaction between p53 and HDM2 [90]. In 2010, Polyphor discovered a new class of antibiotics that kill bacteria by blocking the function of a protein that is vital for outer cell membrane formation [91]. The developed antibiotic drug, the β-hairpin mimetic POL7080, is in human clinical trials, demonstrating the

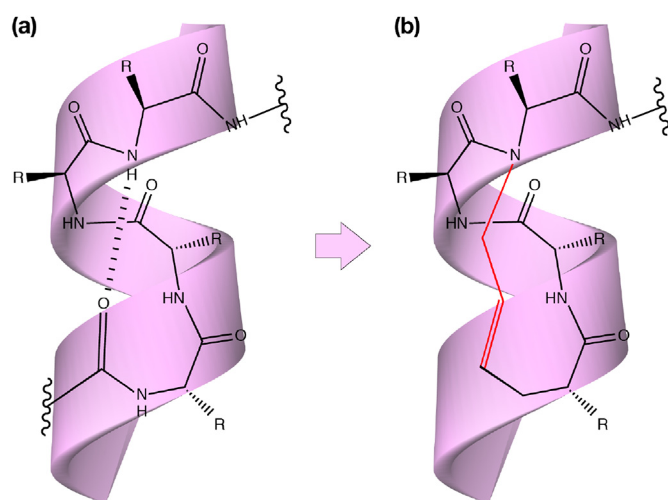

Fig. 7. Schematic representation of α-helix stabilization strategy. (a) A canonical α-helix with (i + 4, i) backbone hydrogen bond, (b) Olefin HBS linkage nucleating α-helix (depicted in red).

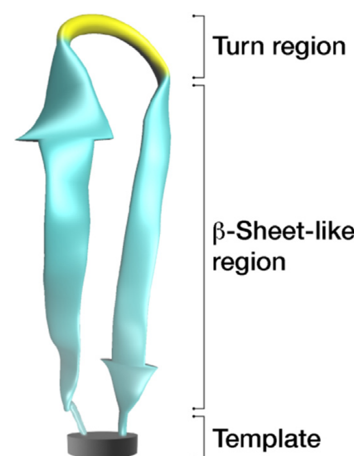

Fig. 8. Schematic representation of PEM, conformationally restrained macrocyclic β-hairpin. Three regions depict the modular nature of the PEM, where variation comes from the template (gray), loop size, and building blocks in the β-sheet-like (cyan) and the turn (yellow) regions.

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ability of PEM technology to address challenging membrane protein targets. However, to modulate intracellular PPI, new approaches need to be developed to engineer the necessary cell permeability properties [92].

2.2. Grafting bioactive peptides on mini-protein scaffolds

The grafting approach usually involves incorporating an active peptide epitope into a small stable protein scaffold to lock its bioactive conformation, increase stability, and improve cell permeability. This strategy has been widely used by different groups utilizing various scaffolding moieties such as miniature proteins [93] or disulfide-rich peptides (e.g. knottins, cyclotides) [94] and animal toxins [95–98]. One of the major limitations of most of these scaffolds in targeting intracellular proteins is their inefficient cellular uptake. A study by Daniels and Schepartz [99] has demonstrated that embedding a cell-permeable cationic motif into the primary sequence of a small, thermostable avian pancreatic polypeptide (aPP) confers cell permeability without destroying the structure. In 2007, Greenwood et al. reported the first cyclotide (MCoTI-II) to penetrate the cell [100]. Subsequent studies with live cell imaging showed that disulfide-rich cyclic peptides MCoTI, kB1, and SFTI-1 readily cross mammalian cell membranes by various mechanisms, hence defining a new class of cyclic cell-penetrating peptides (CCPPs) [101,102]. Due to their cyclic cysteine knot (CCK) motif, cyclotides are exceptionally stable, and therefore extremely useful as pharmaceutical scaffolds [95]. In a recent study, it was reported for the first time that an engineered MCoTI-I cyclotide could efficiently modulate intracellular PPI; namely, it could activate the p53 tumor suppressor pathway both *in vitro* and *in vivo* [103]. To design an active construct, Ji et al. [103] grafted the phage-selected α-helical peptide PMI [104] with low nanomolar affinity for both MDM2 and MDMX, into loop 6 of the MCoTI-I cyclotide scaffold. To ensure the grafted PMI secondary structure was in the correct, biologically active conformation, a small linker insert was designed based on a bee-venom neurotoxin (apamin) sequence, which has been previously shown to correctly display grafted p53 helical analogs [105]. The resulting engineered cyclotide (Fig. 9), MCo-PMI, stabilized the PMI α-helical domain, retained binding to both MDM2 and MDMX, demonstrated stability in human serum, and inhibited tumor growth in a murine xenograft model by disrupting intracellular protein–protein interaction.

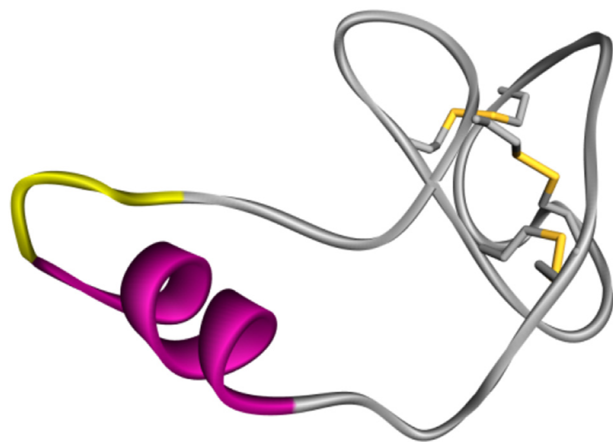

Fig. 9. An engineered cyclotide MCo-PMI. Coordinates are taken from the solution structure of the MCo-PMI complexed with HDM2 (PDB code: 2M86). The purple ribbon represents the PMI helix grafted into loop 6 of MCoTI-I cyclotide scaffold (gray). The apamin-derived linker is shown in yellow.

2.3. Macrocyclic peptides

Naturally occurring cyclic peptides are the smallest biomolecules that have the complexity to effectively recognize large surfaces of challenging target proteins while demonstrating drug-like metabolic stability and membrane permeability [106]. Various classes of cyclic peptide natural products have been recently reviewed in detail [107]. Many of them are biosynthesized via non-ribosomal peptide synthetases (NRPSs), which introduce remarkable chemical diversity by incorporating non-proteinogenic amino acids, epimerization, N-methylation, and backbone cyclization. Due to these features tailored by natural selection, many cyclic peptide natural products penetrate biological membranes [107]. This class of compounds could be a potential source for diverse chemical scaffolds and structural information to guide the design of drug-like properties. However, the challenging synthesis of natural product-like non-standard cyclic peptides has hindered a systematic exploration of the macrocyclic chemical space for drug discovery. The most prominent example of a natural cyclic peptide is cyclosporin A (CSA), which has been an orally administered immunosuppressant drug for 30 years. CSA is a non-polar cyclic peptide with highly N-methylated backbone, and it penetrates the cell by passive diffusion. Backbone N-methylation introduces proteolytic stability and reduces the number of hydrogen bond donors (HBD). Macrocyclization also increases enzymatic stability by eliminating charged N- and C- termini and introducing conformational distortion of endopeptidase recognition sites [108,109]. In addition, cyclization pre-organizes peptides close to their biologically relevant three dimensional structures by significantly reducing their conformational space, which can translate into improved target affinity and specificity. However, cyclic peptides have sufficient flexibility to undergo conformational change upon target binding; e.g. CSA adopts a “locked” conformation in low-dielectric environments by forming intramolecular hydrogen bonds, but it exposes its polar groups during interaction with polar solvents or a protein partner [110–113]. Such conformational flexibility might be one of the key facilitating factors for passive membrane permeability. Using simple model systems of non-N-methylated synthetic peptides, Lokey and Jacobson [114,115] showed that the ability to form conformations with internal hydrogen bonds in non-polar media correlates with passive diffusion. They also demonstrated that the N-methylation pattern that helps in the formation of transannular hydrogen bonds also enhances passive permeability and oral bioavailability [116]. Kessler and colleagues [117,118] studied the effect of N-methylation on model cyclic peptides, their pharmacokinetic properties, and bioactivity. They demonstrated that specific N-methylation drastically improves metabolic stability and the intestinal permeability of the designed somatostatin analog [119,120]. Yet cyclization, epimerization to D-configuration, N-methylation, and transannular hydrogen bond formation are only general strategies for achieving proteolytic and metabolic stability as well as cell penetration, since the design rules and structural features that govern and fine-tune cell permeability are not well understood.

Today, 68 macrocyclic drugs on the market are almost equally distributed between two classes of compounds - cyclic peptides and macrolides; all peptides are delivered parenterally, except for CSA [121]. However, in clinical trials, cyclic peptides represent the largest group of macrocycles with three orally administered candidates: SCY365, voclosporin, and NXL-103 [121]. Recent advances in synthetic strategies [122–124] grant access to structurally diverse cyclic peptide libraries and allow for rapid screening. The latest review by Bhat *at al.* [125] offers a comprehensive analysis of lead discovery and optimization strategies for peptide macrocycles, emphasizing their potential and key advantages as compared to

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natural products and synthetic macrocycles. Systematic studies of known binding modes of macrocycles, as demonstrated by Villar et al. [126], provide clues on prevalent features of protein targets that are suitable for macrocyclic ligands. A new computational approach developed in Kritzer's group [127] identifies hot loops at loop-mediated PPI interfaces and provides clever starting points for the rational design of macrocycles as PPI inhibitors. Continued exploration of the structure-transport properties of macrocyclic compounds may improve our understanding of the structural and physicochemical characteristics that influence cellular uptake. Such knowledge will aid in the discovery of new drug-like chemical scaffolds that are able to modulate intracellular targets.

2.4. Discovering macrocyclic peptides

There are a number of ways to create macrocyclic libraries to allow for the fast and extensive search of chemical space for hit identification [128,129]. Libraries can be generated with synthetic platforms such as utilizing amphoteric reagents for small peptidomimetic macrocycles [130,131], Macrocyclic Template Chemistry (MATCH) [132], Chemical LInkage of Peptides onto Scaffolds (CLIPS) [133], DNA-Programmed Chemistry (DPC) [134], and side-chain macrocyclization of an unprotected peptide library utilizing cysteine perfluoroarylation via S_NAr transformation [135]. Another successful technique in the discovery of bioactive macrocycles is on-bead combinatorial library synthesis and screening [136]. In this approach, a cyclic peptide is displayed on the microbead surface and is accessible to the target, while a linear peptide resides in the bead interior and serves as an encoding tag [137]. Chemical synthesis allows for the incorporation of unnatural building blocks, increasing the structural diversity of the library.

Biological selection methods are a powerful alternative to synthetic platforms and rational design strategies. The selection techniques (e.g. phage-, ribosome-, yeast-, mRNA-display) allow for fast and cost effective generation and screening of very large libraries (10^9–10^{13}) with the amplification of target-bound peptide sequences during iterative selection rounds. In this section, two main technologies are highlighted: mRNA-display and phage selection of bicyclic peptides.

2.4.1. mRNA display

mRNA-display is a peptide *in vitro* selection technique that generates large libraries (10^{13}) of peptides covalently linked with their corresponding mRNAs [138]. Initially, the 3′ end of the mRNA pool is modified with puromycin and subjected to *in vitro* translation (Fig. 10). When ribosomes translate each mRNA into a peptide, they recognize the 3′ puromycin due to its structural similarity to the 3′ end of aminoacyl-tRNA, and the peptide is linked to puromycin. A covalent mRNA-peptide fusion is generated where mRNA encodes the attached peptide sequence. The mRNA portion of the conjugate is reverse transcribed to cDNA for later PCR amplification, while the peptide portion is cyclized. Subsequently, cyclic peptide-mRNA-cDNA fusions are screened for affinity against immobilized protein targets, and cDNAs of the selected peptide hits are amplified by PCR. Notably, in the mRNA-display approach, peptide cyclization takes place before the selection step against the target protein, which allows macrocyclic ring size optimization [109].

Designing libraries of natural product-like, backbone-modified peptide scaffolds could be a powerful tool for the selection of cell-penetrable, orally available bioactive compounds that target PPI. The incorporation of proteolytically stable D-amino acids allows for the sampling of unexplored conformational space in peptides, while backbone N-methylation could introduce desirable pharmacological properties. To allow for the configuration of mRNA-display with non-natural amino acids, a reconstituted *in vitro*

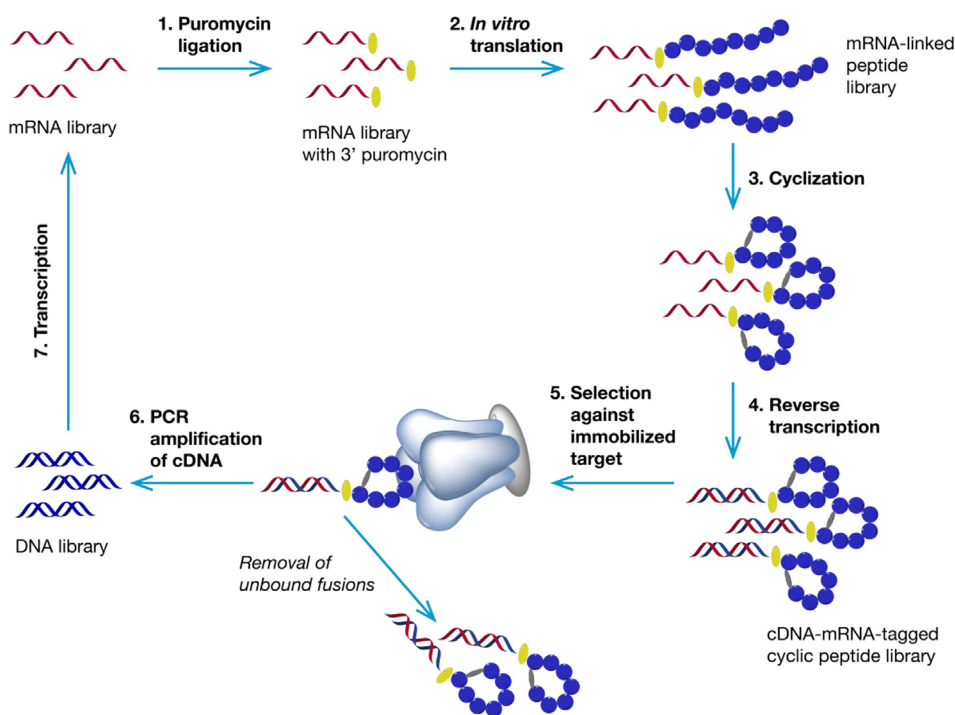

Fig. 10. Schematic representation of mRNA-display: a single round of *in vitro* selection. An initial mRNA library (top left) is shown in red. The puromycin added to the 3′ end is depicted in yellow (step1). In vitro translation and cyclization steps result in a cyclic peptide library, where peptides (shown in blue) are covalently attached to the encoding RNAs (red). The mRNA portion is reverse transcribed to cDNA. Peptides that bind the target protein are selected, while unbound fusion molecules are washed away (step 5). The cDNAs are amplified by PCR (step 6).

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translation system (PURE, Protein synthesis Using Recombinant Elements) has been developed [139–141], where natural amino acids can be replaced with chemically similar and tRNA-synthetase compatible non-natural ones. Utilizing the PURE system, Szostak and colleagues showed that over 50 non-natural amino acids could be incorporated into peptides, generating a library of highly modified cyclic compounds [140,142]. While simple and straightforward, this approach limits the choice of non-proteinogenic building blocks. An alternative method, developed by the Suga group [143] and termed FIT (Flexible *In vitro* Translation) [144], relies on 'flexizyme', an engineered flexible ribozyme that does not recognize a side chain or an amino group of the activated amino acid, and therefore is able to charge a wide variety of non-proteinogenic building blocks [145]. The merging of mRNA-display with the FIT system was named Random nonstandard Peptide Integrated Display (RaPID) [146]. The FIT system has validated the successful translation of D-amino acids, N-methylation, N-substituted glycines, and side chain modified lysines [146]. To achieve post-translational peptide macrocyclization in mRNA-display library, where the C-terminus of a peptide is blocked by puromycin-mRNA, a number of synthetic approaches have been developed for head-to-side chain cyclization [142,147–149]. Most commonly, the N-terminal chloroacetyl group is reacted with a cysteine residue, forming a non-reducible thioether bond and effectively producing macrocyclic rings of up to 25 amino acids [150,151].

mRNA-display technology coupled with the customized *in vitro* translation systems that are capable of incorporating non-natural building blocks in macrocyclic peptides is able to generate libraries of great diversity (>10^{12}). Iterative rounds of affinity-based selection can deliver leads with extremely high binding affinities, which could be further optimized for cell permeability. The Suga group has shown that the RaPID platform discovered selective macrocyclic peptide inhibitors for previously undruggable protein targets, thus demonstrating proof-of-technology [152–154]. Macrocycles show the ability to bind various protein surfaces, such as α-helices, β-sheets, and random coils. Importantly, even though peptide leads were selected by mRNA-display for binding against immobilized protein targets, they displayed isoform-selective inhibitory potency against the active protein [153].

Different versions of mRNA-display technology are being developed by PeptiDream and Ra Pharmaceuticals.

2.4.2. Phage selection of bicyclic peptides

PPI interfaces often have complex architectures. When an interaction surface involves several binding subdomains held together in close proximity by the tertiary fold of a protein, the side chains that take part in the interaction belong to the non-contiguous polypeptide chain [155]. Such discontinuous PPI recognition interfaces are challenging to mimic with a single peptide segment. An elegant approach to generate complex peptide loop topologies is offered by the tethering to the synthetic scaffold tris-(bromomethyl)benzene (TBMB) (Fig. 11a). Linear peptides are linked to the organic TBMB core through reactive cysteine side chains, generating multiple-loop peptide constructs [133]. Utilizing the cyclization with TBMB, a phage display approach was developed for generating bicyclic peptide libraries [156]. Linear peptides containing three cysteine residues were displayed on phage and subsequently cyclized with the TBMB scaffold, generating two macrocyclic rings prior to affinity selection. Screening bicyclic peptide libraries has yielded a number of potent antagonists with nanomolar or picomolar affinities to several serine proteases, such as plasma kallikrein, cathepsin G, and urokinase-type plasminogen activator (uPA) [157].

The potential advantages of peptide macrocyclization, such as increased proteolytic stability and higher affinity due to smaller entropic penalty, are further enhanced for bicyclic peptide scaffolds. The two macrocyclic rings protect each other from enzymatic degradation, therefore bicyclic peptides remained intact, while corresponding monocyclic peptides are rapidly degraded in blood plasma *ex vivo* [158]. In addition, bicyclic peptides have a unique ability to interact with large surfaces by engaging both loops, which makes them suitable to address different types of binding sites. As illustrated by the crystal structure of a bicyclic inhibitor of uPA, such peptides form a large recognition interface (>700 Å) with the target protein by using both loops for creating multiple interactions. The

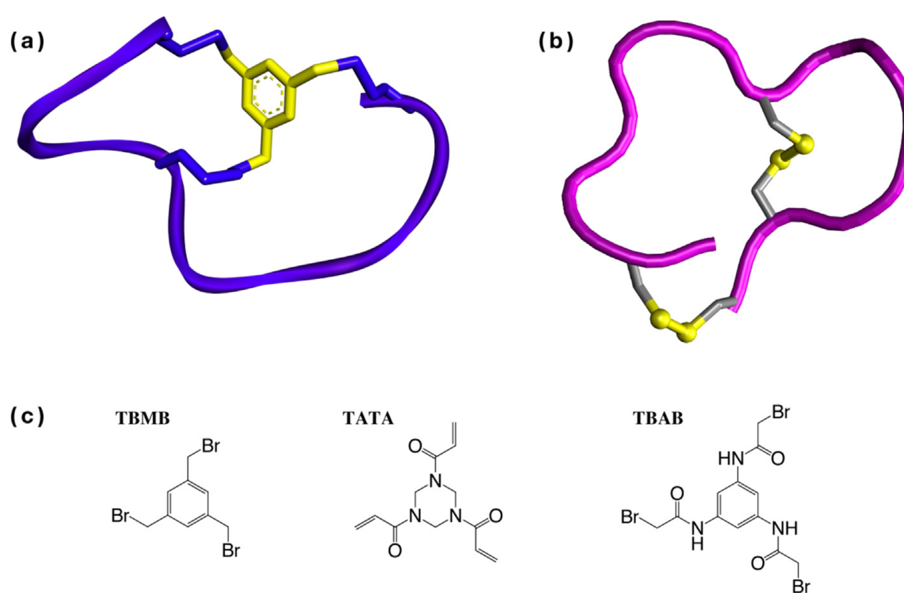

Fig. 11. Examples of bicyclic peptides and organic cores. (a) Structure of bicyclic inhibitor (UK18) of uPA with TBMB core shown in yellow; coordinates are taken from the X-ray structure of protein/peptide complex (PDB: 3QN7). (b) Structure of bicyclic peptide inhibitor UK504 (PDB: 4GLY) with two disulfide bridges shown in yellow. (c) The various thiol-reactive chemical cores for the cyclization of cysteine-rich peptides.

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bicyclic antagonist showed 200-fold higher potency than the best monocyclic inhibitor of uPA [159].

Recently, Heinis and colleagues [160] reported a simple and robust method to screen topologically complex bicyclic peptide libraries without an organic scaffold (Fig. 11b). By design, the peptide library has cysteines in three constant positions while allowing for a forth cysteine to occur in the randomized peptide region. Since four cysteines can pair in three different ways to form a disulfide linkage, variable loop sizes are formed within one library display. As a result, disulfide-linked peptides are structurally more diverse than the peptides from the TBMB-based approach that uses a single bicyclic format per library.

Another limitation of bicyclic peptides is their flexible structure in solution. To stabilize their unbound conformation, various novel hydrophilic scaffolds (Fig. 11c) have been developed to replace the hydrophobic TBMB core [161]. Small polar synthetic molecules at the center of bicyclic peptides can form non-covalent intramolecular interactions with the loops, and thus further pre-organize bicyclic peptide structures. As revealed by X-ray results, these non-covalent interactions contribute to the high affinity binding.

Phage-selected bicyclic peptides are limited to only proteinogenic amino acids. Unnatural building blocks can be introduced synthetically during further optimization steps. In a recent study, a glycine residue with a positive ϕ angle was replaced with a small D-amino acid, which increased the inhibitory activity and proteolytic stability of a bicyclic antagonist [162]. Since cyclic peptides adopt conformations complementary to that of the target protein surface, the necessary macrocyclic ring flexibility might be provided by glycine residues. Analysis of X-ray structures showed that many protein-bound cyclic peptides have glycines with positive ϕ angles, therefore, it was suggested that the mutation of glycine to D-amino acid could be a broad strategy applied to cyclic peptide drug design [162].

Bicyclic peptides have the potential to penetrate cell membrane due to their small size (1–3 kDa). A recently published study [163] elucidated their structure-transport properties by introducing arginine residues, either into the cycles or attached to the termini. Uptake efficiency positively correlated with the number of arginines, and uptake was more effective for bicyclic peptides than for their linear counterparts. The demonstrated cell-penetrating capacity and uptake-promoting structural principles could guide future design of bicyclic peptides for intracellular targets.

3. Concluding remarks

Facing the complex nature of PPI interfaces and the urgent need to modulate biological pathways via intracellular protein interaction networks, peptide-based synthetic molecules are well positioned to become the next generation therapeutics. Peptides have the unique ability to mimic, complement, and modulate large PPI interfaces with multiple shallow pockets, while they can also penetrate the cells if appropriate peptide motifs are selected.

In the absence of a natural ligand or hormone, finding successful PPI modulators is a challenging task. Having detailed structural knowledge of the PPI interaction surfaces enables us to identify 'hot spots' and the key binding motifs essential for creating protein epitope mimetic peptides as a starting point for a rational, iterative design to achieve the desired potency. Often, the conformational flexibility of unconstrained peptides compromises their affinity and drug-like properties. In this paper, a number of elegant synthetic approaches were reviewed for stabilizing various secondary structures of peptides, such as stapling, hydrogen bond surrogates, β-hairpin mimetics, grafting on stable scaffolds, and macrocyclization. Many of these strategies have successfully demonstrated their ability to generate potent, selective, and proteolytically stable inhibitors that modulate previously undruggable or challenging targets. However, tuning pharmacokinetic properties and achieving cell permeability remains a hurdle. The identification of common structural features and physicochemical properties of peptides that correlate with the ability to penetrate the cell is one of the biggest challenges. The detailed mechanisms of passive diffusion or active transport via endocytosis, by which various peptides enter the cell, are not well understood. In addition, peptides can adopt a range of conformations, and the structure-transport activity relationships vary for different three dimensional structural moieties.

Biological selection methods, such as phage selection of bicyclic peptides and mRNA-display, have different strengths. They do not rely on prior structural knowledge of a PPI interface, because peptides are able to bind to the target proteins during the selection cycles. Phage-display based bicyclic peptides can generate structurally constrained peptide libraries with complex loop architectures and enhanced proteolytic stability. In addition, their cell-penetrating capacity has been recently explored. However, the successful design of cell permeable bioactive bicyclic molecules for intracellular targets has not been demonstrated yet. The mRNA-display technology with customized *in vitro* translation systems allows for the generation of naturally occurring cyclic peptide-like libraries that incorporate features that govern drug-like properties, e.g. D-amino acids and N-methylation. As library design improves through continuous discovery, so does the potential of mRNA-display for direct selection of functionally active peptides with cell permeability and oral bioavailability. With these technologies maturing, we anticipate seeing peptide-based therapeutics successfully unlocking intractable intracellular PPI networks.

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