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Targeting intracellular protein-protein interactions with cell-permeable cyclic peptides

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Abstract

Intracellular protein-protein interactions (PPIs) are challenging targets for conventional drug modalities, because small molecules generally do not bind to their large, flat binding sites with high affinity, whereas monoclonal antibodies cannot cross the cell membrane to reach the targets. Cyclic peptides in the 700–2000 molecular-weight range have the sufficient size and a balanced conformational flexibility/rigidity for binding to flat PPI interfaces with antibody-like affinity and specificity. Several powerful cyclic peptide library technologies were developed over the past decade to rapidly discover potent, specific cyclic peptide ligands against proteins of interest including those involved in PPIs. Methods are also being developed to enhance the membrane permeability of cyclic peptides through both passive diffusion and active transport mechanisms. Integration of the permeability-enhancing elements into cyclic peptide design has led to an increasing number of cell-permeable and biologically active cyclic peptides against intracellular PPIs. In this account, we review the recent developments in the design and synthesis of cell-permeable cyclic peptides.

Graphical abstract

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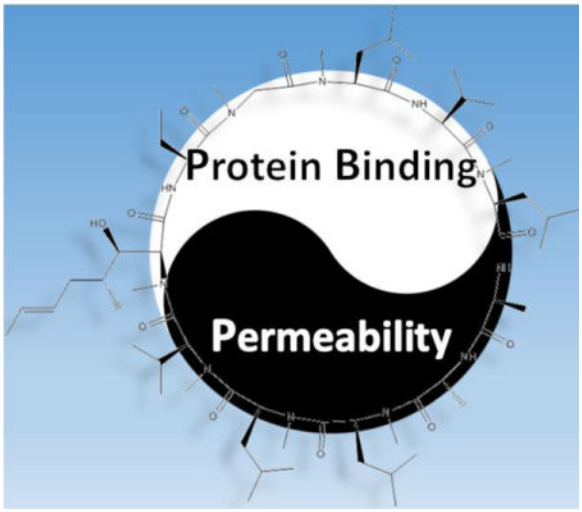
Conflict of Interest

The authors declare no conflict of interest.



Introduction

Protein-protein interactions (PPIs) serve as the foundation of essentially all cellular processes by enabling and regulating the function of individual proteins. Molecules that are capable of specifically modulating PPIs are in high demand as molecular probes and therapeutic agents. Unfortunately, PPIs, especially those that occur intracellularly, have proven challenging targets for conventional drug modalities, namely small molecules and biologics. While small molecules excel in targeting proteins containing deep binding pockets, PPIs typically involve large, flat binding sites which are devoid of any major binding pocket. Biologics such as monoclonal antibodies are effective for recognizing PPI interfaces, but cannot cross the cell membrane to reach the intracellular PPIs. Clearly, a general solution to the problem of inhibiting intracellular PPIs requires exploring chemical spaces beyond the conventional drug modalities. Therefore, in recent years, many investigators have turned their attention to macrocycles, particularly cyclic peptides, as potential PPI inhibitors [1–3]. With sizes that are typically 3–5 times larger than conventional small-molecule drugs and a balanced conformational flexibility/rigidity, macrocycles in the 700–2000 molecular-weight range are capable of binding to the flat PPI interfaces with antibody-like affinity and specificity. At the meantime, macrocycles retain many of the drug-like properties of small molecules such as metabolic stability [4,5]. Cyclic peptides are also synthetically accessible and generally less toxic than small molecules. Nevertheless, developing cyclic peptide inhibitors against intracellular PPI targets still faces two significant challenges: 1) how to engineer a macrocyclic structure to engage a target of interest with high affinity and specificity, often in the absence of any structural information; and 2) how to achieve cell permeability for the macrocycle. In this perspective, we will provide an overview of the recent developments toward overcoming both challenges, followed by selected examples of cell-permeable macrocyclic PPI inhibitors.

Generating Cyclic Peptides as PPI Inhibitors

Over the past decade, a variety of structure-based design and combinatorial library approaches have been developed to discover cyclic peptide inhibitors against PPIs (Figure 1). Since these methodologies have been the subject of several recent, exhaustive reviews [6–10], we will provide here only a brief overview of them. The proper method of choice depends on the nature of the PPI in question and the expertise available within a research laboratory. If the PPI is predominantly mediated by a contiguous structural epitope (e.g., an α-helix, a β-turn, or a peptide loop) on one of the binding partners and structural information is available, rational design has been a productive approach for generating potent cyclic peptide PPI inhibitors. One special type of cyclic peptides that have been extensively explored are “stapled peptides”, which are stabilized α-helices by covalently crosslinking their side chains at *i* and *i+4* (or *i+7*) positions [11–15]. Compared to their linear counterparts, stapled peptides often have greatly improved binding affinity to the intended target, better metabolic stability, and in some cases increased cell permeability. Another common practice is to mimic the β-turn motifs found at PPI interfaces with cyclic peptides. For example, recognizing that the Grb2 SH2 domain binds to a phosphotyrosine-containing peptide ligand in a β-turn conformation, Burke and co-workers rationally designed highly potent cyclic peptide inhibitors against the Grb2 SH2 domain (K_D = 75 pM; Figure 2) [16]. Zhou et al. designed a cyclic peptidomimetic inhibitor against menin-mixed lineage leukemia-1 (MLL1), starting from a conserved menin-binding motif (RWRFPARP) [17]. Further SAR studies yielded a highly-potent, cell-permeable cyclic inhibitor with a *K*i of 4.7 nM against the MLL1-menin interaction (Figure 2). More recently, the stapling strategy has been applied to generate structurally constrained hairpin loops as PPI inhibitors [18]. These examples demonstrate that naturally occurring epitope sequences at PPI interfaces, when available, can serve as good starting points for rational design of potent cyclic peptide PPI inhibitors.

When structural information is not available or the PPI is mediated by multiple epitopes that are distant in the protein sequence (which is true for most PPIs), rational design of cyclic peptide ligands is very challenging. A more productive approach involves synthesizing and screening large combinatorial libraries of mono- and bicyclic peptides. Several complementary platform technologies have been developed over the past decade to synthesize cyclic peptide libraries of enormous structural diversity (up to 10^{14} different compounds), either biologically or chemically (Figure 1). The biological methods include phage display [7,19], mRNA display [8,9,20], and peptide splicing mediated by split inteins [21,22], all of which involve ribosomal peptide synthesis. In the case of phage and mRNA display libraries, the peptides are physically linked to their encoding DNA/RNA, which can be readily sequenced to reveal the identity of a peptide ligand. The displayed peptides are converted into mono- or bicyclic peptides posttranslationally by reacting the N-terminal amine and/or side chain nucleophiles (usually Cys and Lys) with a bifunctional (e.g., disuccinimidyl glutarate) [23] or trifunctional crosslinking agent [e.g., *tris*(bromomethyl)benzene] [24]. In intein-mediated libraries, peptides are produced within living cells and N-to-C cyclized through a protein splicing mechanism, with each cell harbouring a different macrocycle and the corresponding encoding DNA [21]. The most

widely practiced chemical methods include one bead-two compound (OBTC) libraries [25,26] and macrocycle libraries derived from DNA-templated synthesis (DTS) [27,28] or diversity-oriented synthesis [29,30]. In OBTC libraries, each library bead displays a unique cyclic or bicyclic peptide on its surface and contains a linear peptide of the same sequence in the interior as an encoding tag, which can be sequenced by Edman degradation or mass spectrometric methods. In DTS libraries, each macrocycle is covalently linked to the DNA template that serves as the "blueprint" for its construction, analogous to that of mRNA display. The advantages of biological libraries include greater diversity (up to 10^{14} different compounds), compatibility with library amplification (via the polymerase chain reaction) and iterative selection, and straightforward hit identification (by sequencing the encoding DNA/mRNA). However, ribosomal synthesis limits the library building blocks to the 20 proteinogenic amino acids and certain modified amino acids (e.g., N^{α}-methylated amino acids) [31]. In contrast, both natural and unnatural building blocks (e.g. D-amino acids) can be readily incorporated into chemically synthesized libraries to generate more structurally diverse cyclic peptides. The rational design and combinatorial library approached have also been combined to generate stapled peptide inhibitors of exceptionally high potencies [32,33].

Enhancing the Membrane Permeability of Cyclic Peptides

Cyclic peptides violate Lipinski's Rule of 5 (molecular weight ≤500, ≤5 hydrogen bond donors, ≤10 hydrogen bond acceptors, and cLog P≤ 5) [34] and are generally impermeable to the cell membrane. However, some naturally-occurring cyclic peptides (e.g., cyclosporine A, Figure 2) possess the unusual ability of crossing the cell membrane by passive diffusion and are orally bioavailable. Investigating the physiochemical and molecular properties of these natural products, with the ultimate goal of designing cell-permeable and orally bioavailable synthetic cyclic peptides, has been an active area of research [4,5,35–39]. In silico modelling of naturally occurring cell-permeable cyclic peptides identified some shared structural features, such as N^{α}-methylation of the peptide backbone and formation of extensive intramolecular hydrogen bonds [39]. Using model systems, researchers have demonstrated that N^{α}-methylation, formation of intramolecular hydrogen bonds, and strategic placement of sterically occluding hydrophobic groups are effective for improving the membrane permeability of synthetic cyclic peptides [4,35–38]. These methods decrease the solvent-accessible polar surface area, thus reducing the desolvation energy associated with membrane crossing. Using Sanguinamide A (Figure 2) as a model system, Bockus et al. noted the importance of conformational flexibility when designing cyclic peptides with favourable balance between solubility and permeability [40]. Kihlberg and co-workers performed NMR- and computer-based conformational analysis of orally administered macrocycle drugs (e.g. cyclosporine A) and clinical candidates (e.g. cyclic HCV protease inhibitors) and again demonstrated the importance of conformational dynamics in achieving the proper balance between membrane permeability and aqueous solubility [5]. When in an aqueous environment, the cyclic peptides exist in open conformations, allowing the hydrogen bond donors and acceptors to interact with water or a protein target; whereas in the hydrophobic region of a lipid bilayer, they adopt closed conformations by forming intramolecular hydrogen bonds. The practical challenge inherent in developing passively

diffusible cyclic peptides is how to properly balance membrane permeability against tolerating varieties of hydrophilic functional groups that may be required to engage diverse protein targets. So far, essentially all of the designed cell-permeable cyclic peptides have been small model systems consisting of almost exclusively hydrophobic amino acids. None of these hydrophobic cyclic peptides have demonstrated intracellular PPI inhibition or any other biological activity, although they may be useful for targeting hydrophobic PPI interfaces.

A second and likely more general strategy to engineer cell-permeable cyclic peptides is to incorporate cell-penetrating motifs into the cyclic peptide design. Earlier efforts involved covalent attachment of cyclic peptide ligands to short cell-penetrating peptides (CPPs) such as HIV Tat peptide (YGRKKRRQRRR) and nonaarginine (R_9) [41–43]. This approach had only limited success, because the linear CPPs have poor cytosolic delivery efficiencies (<5% [44]) and undergo rapid proteolytic degradation *in vivo*. A major breakthrough in the field was the recent discovery of a family of small amphipathic cyclic peptides as exceptionally active and metabolically stable CPPs [e.g. cyclo(D-Phe-Nal-Arg-D-Arg-Arg-D-Arg-Gln) or CPP9, where Nal is L-2-naphthylalanine], which have cytosolic delivery efficiencies of up to 120% (or 60-fold higher than that of the Tat peptide) [45,46]. The cyclic CPPs (and the CPP-cargo conjugates) bind directly to the plasma membrane phospholipids and enter cells by endocytosis [46]. They then efficiently escape from the early endosome into the cytosol, apparently through budding and subsequent rupture of small vesicles [47]. The cyclic CPPs have provided a general, effective system for intracellular delivery of a wide variety of cargos including peptides and proteins (see below).

Cell-Permeable Cyclic Peptide PPI Inhibitors

Most of the cell-permeable cyclic peptide PPI inhibitors reported so far enter cells by active transport mechanisms. Cyclic peptides can be rendered cell-permeable by integrating short CPP motifs into their structures, through either rational design or combinatorial approaches (Figure 1). Lian et al. fused membrane impermeable cyclic peptide inhibitors against intracellular enzymes PTP1B and Pin1, which had previously been discovered by screening OBTC libraries, with a cyclic CPP [48]. The resulting bicyclic peptides were readily cell-permeable while retaining the enzyme inhibitory activities, and potently inhibited the signalling pathways downstream of the enzymes in cell culture at nM concentrations (e.g., a PTP1B inhibitor with IC_{50} = 30 nM; Figure 2). The generality of the bicyclic delivery strategy was later demonstrated by the synthesis of a combinatorial library of 5.7 million cell-permeable bicyclic peptides [49]. Screening of the library against the K-Ras protein identified a bicyclic peptide inhibitor (Figure 2) which blocked the Ras-Raf interaction with an IC_{50} value of 3.4 μM, inhibited MEK and AKT phosphorylation, and induced apoptosis of lung cancer cells at low μM concentrations. Upadhyaya et al. designed an OBTC cyclic peptide library by integrating a CPP-like pentapeptide motif into 1.5 million different target-binding sequences [50]. Screening of the library against G12V K-Ras followed by medicinal chemistry efforts resulted in a cycloundecapeptide (cyclorasin 9A5; Figure 2) which is cell-permeable and potently blocked the interaction of K-Ras with its downstream effectors (e.g., Raf) with an IC_{50} value of 120 nM, inhibited signalling pathways downstream of Ras, and induced apoptosis of lung cancer cells (LD_{50} ~3 μM).

Some PPIs require one of the binding partners in the extended conformation for binding (e.g., PDZ domains); in such cases, cyclization of the binding peptide reduces or abolishes the binding affinity. To render the linear peptide ligands cell-permeable and proteolytically stable, Qian et al. devised reversible cyclization strategies, in which the linear peptide ligands were fused with a short CPP motif (e.g., Arg-Arg-Arg-Arg-Nal-Phe) and cyclized into mono- or bicyclic structures by disulphide bond(s) [51,52]. When outside the cell (e.g., in serum), the cyclic peptides have dramatically improved cellular uptake efficiency and metabolic stability relative to their linear precursors. Upon entering the cytosol, the disulphide bond(s) is reduced by intracellular glutathione to release the biologically active linear peptide for binding to the intended target. As a proof-of-concept, the investigators designed a cyclic peptide inhibitor against the PDZ domain of CFTR-associated ligand (CAL-PDZ; Figure 2), which inhibited the CAL-CFTR interaction and reduced lysosomal degradation of CFTR, thereby increasing CFTR chloride channel activity in lung epithelial cells that harbour a defective CFTR mutant (ΔF508) [51]. More recently, the same group applied the reversible bicyclization strategy to design a peptidyl inhibitor against the PPI between nuclear factor-κB (NF-κB) essential modulator (NEMO) and IKKs. The bicyclic peptide (Figure 2) demonstrated greatly improved serum half-life (10 h vs 15 min for the linear counterpart) and cell permeability, and was ~10-fold more potent for inhibition of NF-κB activation in a cell-based assay relative to the linear peptide (NBD) [52].

It is worth noting that other types of cell-permeable peptidyl PPI inhibitors have also been found to enter cells by endocytosis. Energy-dependent endocytosis was demonstrated to be the major mechanism of uptake of a stapled peptide inhibitor ($SAHB_A$; Figure 2) against the Bcl-2/BH3 interaction, which effectively inhibited the proliferation of a human leukemia xenograft *in vivo* [53,54]. Likewise, cyclotides, which are a family of highly-stable, disulphide-rich cyclic peptides, were also shown to enter mammalian cells by energy-dependent mechanisms [55,56]. By grafting a known α-helical peptidyl ligand of Hdm2 protein into *Momordica cochinchinensis* trypsin inhibitor (MCoTI), Camarero and co-workers developed a cell-permeable cyclotide that effectively inhibited the p53/Hdm2 interaction *in vitro* and *in vivo* [57].

Conclusion

Intracellular PPIs were once considered as undruggable targets. It is now generally accepted that cyclic peptides and other types of macrocycles may provide a general modality for targeting this PPI class. Over the past decade, tremendous progress has been made toward overcoming the two major challenges associated with developing cyclic peptide drugs: target engagement and membrane permeability. The advent of several powerful cyclic peptide library technologies has now made it a relatively routine exercise to discover cyclic peptide ligands against most proteins including those involved in PPIs. Our improved understanding of membrane permeation of cyclic peptides via both passive diffusion and active transport mechanisms has begun to allow rational design of cell-permeable cyclic peptides that specifically target intracellular PPIs. Some of these cyclic peptides have already provided useful molecular probes for chemical biology applications. We are confident that during the next decade, some of these cyclic peptides will advance into clinical applications.

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Highlights

- Cyclic peptides are an emerging class of drug modality for PPI inhibition
- Rational design and library screening are established to generate lead peptides
- Membrane permeability can be enhanced by various techniques
- Integration of membrane permeability and binding affinity is essential

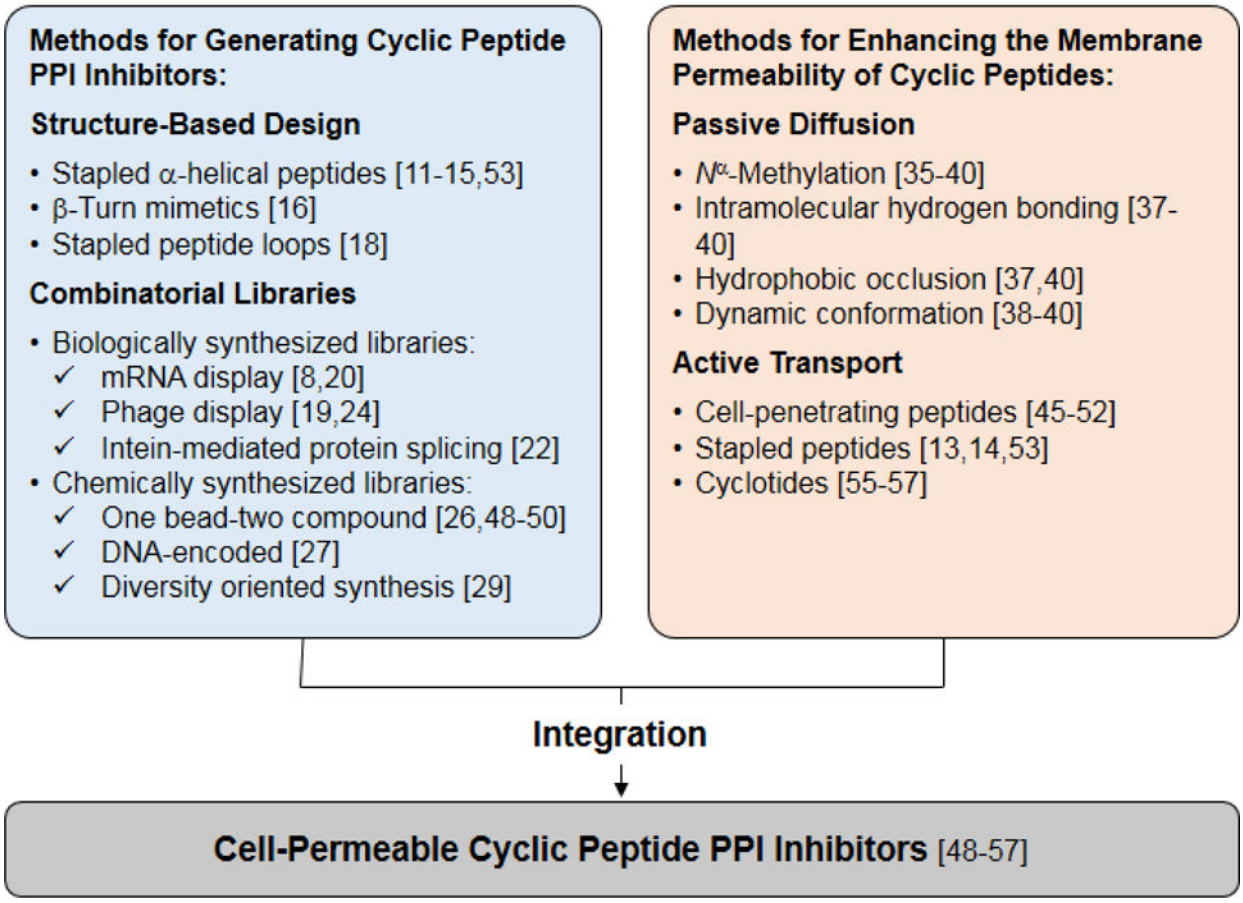

Figure 1.
Summary of methods that have been developed to generate cyclic peptide ligands for inhibition of PPI targets and to enhance their membrane permeability.

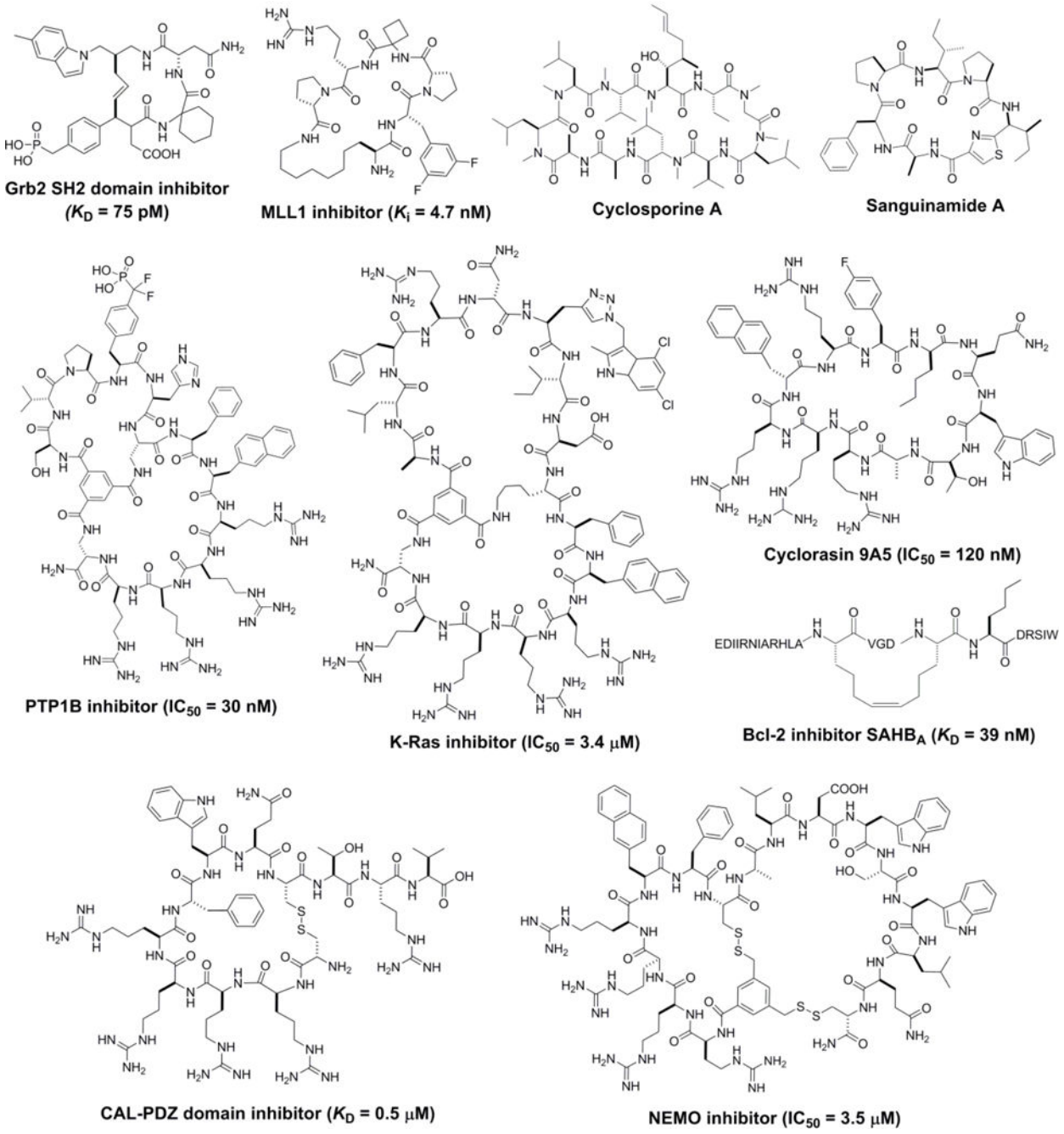

Figure 2.

Examples of cell-permeable and biologically active cyclic peptides which were derived from natural sources or rational design and/or combinatorial library screening.