Selection-based discovery of macrocyclic peptides for the next generation therapeutics

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Naturally occurring macrocyclic peptides represent a unique class of compounds that exhibit various biological activities ranging from antibiotics to immunosuppressant. Although the discovery of such macrocyclic peptides had relied on their isolation from living organisms, recent advances in ribosomal peptide synthesis and in display techniques made it possible to use artificially generated macrocyclic peptide libraries for selection of ligands for biologically relevant proteins. In this review, we discuss the technologies and their applications for the discovery of peptide ligands.

Introduction

As an alternative to testing compounds at random, chemical libraries (or compound libraries) were first introduced about 25 years ago to enable systematic discovery of compounds with drug-like properties (*i.e.* lead-compounds) [1]. Two different approaches were established in the field of compound-libraries design: target-oriented and diversity-oriented [2]. The target-oriented libraries generally rely on the structural information on the target available *a priori* and are often comprised of compounds of particular class [3,4]. On the other hand, functionally diverse libraries often cover wider chemical space, thus being less biased. Such libraries are preferred when little structural information on a target is available [5].

Irrespective of library design, it is often a major priority to establish a library with high diversity, in order to provide a representative sample of the total chemical space or a part of chemical space. Chemical libraries comprised of tens of thousands compounds have recently become commercially available (*e.g.* ChemBridge library 5×10^4 compounds). However, there has been major progress in combinatorial synthesis of small-compound libraries, allowing one to synthesized libraries with diversity over 10^6 [6]. Combinatorial synthesis enables the production of highly diverse chemical libraries in a single process. The diversity of combinatorial library is dependent upon the number of degenerate positions available or monomers combined (*i.e.* diversity points) to produce a final compound as well as the diversity of such monomers. Peptide library can be also constructed in a similar combinatorial manner, in which the number of degenerate positions in a peptide chain defines the number of diversity points (X), with diversity (N) equal the number of different amino acids available for each of the degenerate positions. Thus the diversity of a peptide library can be calculated as N^X ; the diversity of such libraries can be controlled by varying the number of degenerate positions in a peptide chain or the diversity of amino acids used. For the peptide libraries, in which all 20 proteinogenic amino acids are used, the diversity will be 20^X . Introducing non-proteinogenic amino acids, effectively increasing the value of N, can further expand the diversity of a peptide library.

Effective screening of the highly diverse peptide libraries and deconvolution of active species would be key to the successful discovery of potent peptide ligands against drug targets. Screening of such libraries can be performed using *in vitro* display techniques such as phage display suitable for the libraries up to 10^{12} species [7], and mRNA display — up to 10^{14} species [8]. Even though such selection methods were developed quite some time ago, remarkable success in the discovery of potent inhibitors has been achieved in recent years by the integration of the aforementioned chemical approaches of macrocyclization of the displayed peptides. We here review these technologies for the design and the deconvolution of ribosomally synthesized macrocyclic peptide libraries, highlighting thus discovered bioactive ligands with their structural properties.

Peptide Cyclization Methods Using Proteinogenic Amino Acids

Naturally occurring macrocyclic peptides play a wide variety of roles, such as hormones (somatostatin [9], vasopressin [10]) and neurotransmitters (oxytocin [10]). Such macrocyclic peptides were shown to be not only potent antibiotics such as micrococcin [11], daptomycin [12] and pristinamycin [13], but also an immunosuppressant known as cyclosporine [14,15]. The function of these macrocyclic peptides imply that macrocyclization of peptides via a covalent bond enables stable-complex formation with the target-protein presumably through constraining the conformational freedom of peptides. Moreover, cyclized peptides were found to be more resistant to degradation in the presence of a peptidase. For instance, substitution of a labile S-S bond of oxytocin with C-S bond produced a more stable analogue — Carbetocin — thus increasing the bioavailability of the compound [16].

Classical peptide libraries are mostly composed of linear peptides due to the facility for chemical and ribosomal syntheses. Recent trends in library design incorporate well-defined secondary structures, such as α -helix [17,18], β -sheet [19–22], or β -turn [23–26] based on the previously discovered in natural proteins or peptide ligands. Even though, thus developed structurally focused peptide libraries are more likely to provide biologically active peptides, imposed structural constraints often limit the application of such libraries to certain target proteins. Alternatively, to constrain the conformation of individual peptides in a library while maintaining the structural diversity of the library as a whole, macrocyclic peptide backbone structures composed of random sequences could be produced. For their ribosomal synthesis, two conceptually distinct technologies are currently available for post-translational peptide cyclization; one relies on the expression of peptides consisting of only proteinogenic amino acids, but the sidechain group of defined residues, such as the sulfhydryl group of cysteine, are selectively crosslinked by a chemical reagent(s); and the other method relies on incorporation(s) of a non-proteinogenic amino acid(s) into peptide chain using a genetic code manipulation technique [27] and expressed peptides are self-crosslinked between the reactive group on the non-proteinogenic amino acid and a reactive group of a different proteinogenic or non-proteinogenic residue.

The bacterial phage display strategy has brought notable success in isolating active ligand peptides, including those cyclized by a disulfide bond(s), against various targets. Although the potency of thus selected peptides varies from sub nM to high μ M, the intrinsic instability of a disulfide bond under intracellular reducing conditions or the susceptibility of peptide bonds of a linear peptide to proteolysis makes it difficult to develop bioactive peptide drugs. Unfortunately, since this display system relies on ribosomal peptide synthesis for the production of the bacterial phage particles, the incorporation of non-proteinogenic amino acids into the nascent peptide chain is rather technically demanding and complicated. In fact, there is little success in isolating bioactive peptides containing a non-proteinogenic amino acid by means of genetic code expansion [28].

To overcome this difficulty, post-translational cyclization methods based on proteinogenic amino acids were developed. 1,3,5-tris-(bromomethyl)benzene (TBMB) was used to produce a bicyclic-peptide library (Figure 1a). This library was used to select for a very potent inhibitor ($K_i = 1.5$ nM) against plasma kallikrein, PK15 [29]. To design bicyclic-peptide library, the positions of three cysteines were

fixed and the peptide-chain length between cysteines was varied 3–6 amino acid residues (Cys-X_n-Cys-X_m-Cys; 3 ≤ n, m ≤ 6). This approach was utilized to discover bicyclic-peptide ligands for a range of disease-associated secreted serine proteases such as urokinase type plasminogen activator (uPA) [30,31] and coagulation factor XII [32]. Other related methodologies for post-translational cyclization have been also recently reported using *N,N'*-[1,2-ethanediyloxy-2,1-ethanediy]bis(2-bromoacetamide) (EBB) (Figure 1b) [33●], light-responsive 3,3'-bis(sulfonato)-4,4'-bis(chloroacetamido)-azobenzene (BSBCA linker) (Figure 1c) [34], disuccinimidyl glutarate (DSG) (Figure 1d) [35], and α,α' -dibromo-*m*-xylene (DBX) (Figure 1e) [36].

Peptide Cyclization Methods Using Non-Proteinogenic Amino Acids

The RaPID (Random non-standard Peptides Integrated Discovery) system [37,38] combines the advantages of a custom-made reconstituted *E. coli* cell-free translation system [39] coupled with the flexizyme technology [40–42●], referred to as Flexible In vitro Translation (FIT) system [43], and the peptide-puromycin-mRNA fusion, so-called mRNA display. The FIT system largely facilitates the expression of peptides not only containing various non-proteinogenic amino acids, such as N-methyl-amino acids, into the nascent peptide chain by means of the genetic code reprogramming [44–46] but also a unique reactive N-terminal group, thus readily expanding the repertoire of chemical properties exhibited by the peptides (Figure 2a). The FIT expression of thioether-macrocyclic peptides is achieved through an intramolecular nucleophilic substitution reaction between the chloroacetyl group (ClAc) of the N-terminal amino acid designated by the initiator-codon reassignment (ClAc-aa-tRNA^{Met}_{CAU}; aa denotes an amino acid of choice) and the sulfhydryl group of a cysteine sidechain at a downstream position (Figure 2b) [47●–49].

The above macrocyclization strategy could be adapted to an mRNA display method, *e.g.* the RaPID system, enabling for rapid selection of potent macrocyclic ligands against various protein targets [47●–51]. For instance, the RaPID selection using a macrocyclic peptide library containing a K^{Tfa} (ϵ -N-trifluoroacetyl lysine) warhead yielded potent (IC₅₀ = 3.2 nM) inhibitors of SIRT2 (or Sirtuin 2; adenine dinucleotide-dependent deacetylase). The selected inhibitors were shown to be 200-fold selective over other isoforms of sirtuin (*vide infra*) [49]. Moreover, the RaPID selection from a library consisting of natural product-like macrocyclic N-methyl-peptides produced very potent ligands (K_D = 0.6–1.3 nM) to E6AP (E3 ubiquitin ligase, HECT domain) and also exhibited inhibitory activity of E6AP-catalyzed ubiquitination on p53 [47●]. Importantly, this study showed the combination of the backbone N-methylation and macrocyclic scaffold was critical to exhibit the observed potency of the above peptides.

Structural features of macrocyclic peptides

In recent years, we are fortunate to witness co-crystal structures of artificial macrocyclic peptides in complex with their protein targets of interest, revealing at molecular levels how the macrocyclic scaffolds

contribute to not only potent binding to proteins but also the stability of own tertiary structure. To date, four examples of the co-crystal structures of protein with bioactive thioether-macrocyclic peptide ligands isolated by the RaPID system are available in literatures [52●●,53●,54●●] Here we discuss three distinct macrocyclic peptides among them, and provide insights into their individual conformation. It should be noted that the selected macrocyclic peptides could act as not only inhibitors but also co-crystallization chaperones for transmembrane proteins.

Multidrug and toxic compound extrusion (MATE) family transporters are involved in the export of secondary metabolites, including alkaloids [55]. As a result of selection using macrocyclic peptide libraries against a MATE family transporter from *Pyrococcus furiosus* (PfMATE), several macrocyclic-peptide ligands were discovered [51●,52●●,56]. In its outward-open conformation, PfMATE possesses a cleft formed between the N-terminal lobe and the C-terminal lobe (Figure 3a). From the X-ray crystal structures of the peptide-protein complexes, it was observed that partial insertion of MaL6 into the cleft of PfMATE stabilizes the outward-open conformation. MaL6 has a relatively compact conformation for having a ring size composed of 17 amino acids (Figure 3b). MaD3S, another selected macrocyclic-peptide ligand of PfMATE, is composed of a smaller 7-mer ring and a 9-mer spoke (Figure 3c, d). These peptides are both able to fit into the cleft of PfMATE regardless of their differing ring sizes. Formation of an antiparallel β -strand, stabilized by 7 intramolecular hydrogen bonds (H-bonds), allowed the larger ring of MaL6 to partially fit into the cleft of PfMATE. Interestingly, the complex structure of MaL6 forms and projects a cleft-binding β -turn motif similar to those found in nanobodies [57–59]. Although the 5 H-bonds between MaL6 and PfMATE appear to help stabilize the complex, the majority of the interactions appear to be hydrophobic (Figure 3a). MaD3S is able to bind PfMATE's substrate binding pocket due to its smaller ring size and high shape complementarity with its binding surface. It is evident from this study that macrocyclic peptide libraries provide peptide ligands with various cycle sizes to cater to a given protein structure. More recently, another co-crystal structure of a macrocyclic peptide with a eukaryotic ABC-drug transporter was solved, revealing that its sidechains interact with two α -helices of the transmembrane portion of the protein. This suggests that the RaPID system could be used to identify macrocyclic peptides that bind to shallow epitopes [53●].

SIRT2 regulates cell cycle progression [60] by deacetylating ε -N-acetyl group from specific lysine residues in α -tubulin [61] and histone H4K16 [62]. A 14-mer peptide, S2iL5, was one of an inhibitor of SIRT2 derived from the macrocyclic peptide library including a K^{Tfa} (ε -N-trifluoroacetyl lysine) as a 'warhead' [49] described above and was successfully cocrystallized with SIRT2 [54●●] (Figure 3e). Direct and water-mediated H-bonds cause the guanidinium group of R8 to be flipped within the cyclic scaffold while other intramolecular H-bonds in the peptide orient K^{Tfa}7 to extend outside of the cyclic scaffold and occupy the acetyllysine-binding site of SIRT2, where the overall conformation of S2iL5 was referred to as "power button icon"-like macrocycle (Figure 3f). Importantly, S2iL5 induces an open-to-closed domain movement of SIRT2 and an unanticipated helix-to-loop transition in a SIRT2-specific region (residues 289–304). As a result of the rigid and unique conformation of S2iL5 and drastic conformational changes in SIRT2, the backbone amide groups of K^{Tfa}7 and R8 take part in

H-bonds with SIRT2 (Figure 3e), respectively, creating an intermolecular β -sheet-like interaction between S2iL5 and SIRT2. Additional H-bonds between Y4, R9 and Y12 and SIRT2 and a number of hydrophilic and hydrophobic interactions with SIRT2 burying 42% of the total surface area of S2iL5 can be also observed. The SPR (surface plasmon resonance) analysis of S2iL5 against immobilized SIRT2 on a sensor chip revealed remarkably tight binding ($K_D = 1.0$ nM). While single mutations of S2iL5 in which one of amino acids responsible for the H-bond formation was replaced with alanine, did not result in a large impact on the affinity towards SIRT2 (*e.g.*, K_D of Y4A = 1.9 nM, R9A = 7.9 nM and Y12A = 0.42 nM), a triple mutant Y4A/R9A/Y12A showed further reduction in complex stability ($K_D = 29$ nM). Although a 14-mer linear peptide analog of S2iL5 showed only a modest decrease in K_D (= 6.8 nM), a 5-mer linear peptide that retains the 5 residues adjacent to K^{Tfa}7 (H5–R9) showed a drastic reduction in K_D with 2 orders of magnitude (= 145 nM). These mutational analyses indicated the importance of the synergistic contributions of the multiple interactions and unique conformation of S2iL5 acquired from macrocyclization.

Macrocyclic peptide ligands obtained by the RaPID system adopt constrained but diverse conformations, such as lariat-, β -hairpin-, and power button icon-like structures as described above. These observations prove the outstanding structural diversity of macrocyclic peptides and the advantage of ligand development using combinatorial macrocyclic peptide libraries with large sequence diversities.

Conclusion and perspective

In this review, we discussed newly emerged technologies for the selection of bioactive non-standard macrocyclic peptides from combinatorial libraries, which are ribosomally expressed and displayed using bacterial phage or mRNA-puromycin fusion. The advantage of using non-standard macrocyclic peptide libraries is their high sequence diversity combined with constrained 3D-structural diversity of individual peptides. The integration of such macrocyclic peptide libraries with one of the aforementioned selection methods creates a powerful platform technology for the discovery of functional ligands against a wide range of target-proteins. Novel cyclization strategies and incorporation of N-methyl-amino acids or an artificial amino acid with a chemical warhead assisted by genetic code reprogramming enable the introduction of new chemical groups into the macrocyclic peptides, thereby, making it possible to access the ‘fine-tuned’ chemical properties of macrocyclic ligands of protein targets of interest rapidly and effectively.

On going development of peptide cyclization techniques and introduction of novel functional groups into peptides make macrocyclic peptides a very attractive class of compounds for the discovery of ligands for the broad spectrum of proteins. We have already seen remarkable *in vitro* and cell/*vivo* bioactivities for macrocyclic peptides to some extracellular targets with specificities. The macrocyclic peptides against intracellular targets have also exhibited remarkable *in vitro* activities in terms of potency and specificities, but yet met little success in cell/*vivo* activities due to their insufficient cell-membrane permeability. The next generation of technologies for the discovery of macrocyclic peptides with not only potency but also

cell-membrane permeability would be important to accelerate the drug development for intracellular non-druggable targets.

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Figure legends

Figure 1

Peptide cyclization methods using proteinogenic amino acids. (a) Thioether-closed bicyclic peptides. Three Cys residues were post-translationally modified by TBMB (tris-(bromomethyl)benzene), yielding a bicyclic peptide library displayed on M13 phages. (b) Crown ether-like macrocyclic peptides. A peptide library displayed on T7 phages was treated with EBB (*N,N'*-[1,2-ethanediyl-oxy-2,1-ethanediyl]bis(2-bromoacetamide)) to construct a crown ether-like macrocyclic peptide library. (c) Macrocyclic peptides closed via an azobenzene linker. Conjugation of two Cys residues with BSBCA (3,3'-bis(sulfonato)-4,4'-bis(chloroacetamido)-azobenzene) provided macrocyclic peptides whose conformation can be controlled by photoirradiation. (d) Macrocyclic peptides closed via glutaramide linker. The N-terminal amino group and a side chain amino group of C-terminal Lys residue were connected by DSG (disuccinimidyl glutarate) to construct a mRNA-displayed macrocyclic peptide library. (e) Thioether-closed macrocyclic peptides. Three Cys residues were post-translationally modified by DBX (α,α' -dibromo-*m*-xylene), yielding a macrocyclic peptide library displayed on mRNAs.

Figure 2

Peptide cyclization methods using non-proteinogenic amino acids. (a) Genetic code reprogramming using the FIT system. Non-proteinogenic amino acids were charged onto tRNA by artificial tRNA acylating ribozymes, flexizymes. Addition of the resulting non-proteinogenic aminoacyl-tRNA into *in vitro* reconstituted translation system enabled us to express peptides containing non-proteinogenic amino acids. (b) Thioether-closed macrocyclic peptide libraries displayed on mRNA molecules. The N-terminal chloroacetyl group incorporated via the genetic code reprogramming technology undergoes spontaneous macrocyclization with the sulfhydryl group of a downstream Cys residue.

Figure 3

Crystal structures of macrocyclic peptide ligands-protein complexes. The peptide ligands are shown with the following element color code; carbon in cyan, nitrogen in blue, oxygen in red, and sulfur in yellow. Intramolecular and intermolecular hydrogen bonds are shown as blue and red dashed lines, respectively. (a) Crystal structure of PfMATE in complex with MaL6 peptide (PDB ID: 3WBN). The N-terminal and C-terminal lobes of PfMATE are shown in light green and dark green, respectively. (b) β -hairpin-like

conformation of MaL6. (c) Crystal structure of PfMATE in complex with MaD3S peptide (PDB ID: 3VVS). (d) Lariat-shaped conformation of MaD3S. (e) Crystal structure of SIRT2 in complex with S2iL5 peptide (PDB ID: 4L3O). (f) “Power button icon”-like conformation of S2iL5.

Figure 1.

Figure 2.

Figure 3.