Biosynthetic Strategies for Macrocyclic Peptides

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Macrocyclic peptides are predominantly peptide structures bearing one or more rings and spanning multiple amino acid residues. Macrocyclization has become a common approach for improving the pharmacological properties and bioactivity of peptides. A variety of ribosomal-derived and non-ribosomal synthesized cyclization approaches have been established.

macrocyclic peptides | bicyclic peptides | biosynthesis | ribosomal synthesis | chemoenzymatic strategy | library screening | in vitro display

1. Introduction

Macrocyclic peptides, including monocyclic and bicyclic peptides, are privileged molecular modalities which can be used for diagnosis (e.g., biosensors, glucose sensors), disease treatment (e.g., antimicrobial, cancer therapy), and drug delivery [1][2]. Although peptide therapeutics have greater potency compared to the small-molecule therapies, they often suffer reduced bioavailability due to their limited permeability and metabolic stability, which in turn decreases their clinical efficacy [3]. Macrocyclization, as one feature shared by structurally diverse molecules, generally improves several pharmacological features of a peptide, revealing the potential as a novel method for improving bioactivity [4]. The design of peptide macrocyclization methods has become an important part of strategies.

Various macrocyclization reactions have been designed over the years, utilizing different mechanisms such as backbone cyclization and sidechain to sidechain cyclization [5][6][7], where backbone cyclization presents the most conformational constraint. Commonly, macrocyclic peptides are produced by non-ribosomal peptide synthetases [8][9][10][11] or ribosome-derived peptides by enzymatic posttranslational modifications [12][13][14]. Considering the chemical variety and stereochemical complexity, special emphasis on the selective transformation of polycyclic compounds arises for methods using enzymes and microbial whole cells. The use of microorganisms allows for obtaining enantiomerically pure compounds via one-stage synthesis [15].

In particular, the use of genetically encoded peptide libraries constitutes an attractive strategy to generate large collections of these molecules, which have been amenable to screening through display platforms to accelerate the discovery of cyclopeptide binders [16][17][18][19]. For instance, in vitro translation of cyclic peptides [20][21][22] or cyclization of mRNA- or phage-displayed peptides via chemical [23][24][25][26] or enzymatic processes [15][27], have

been successfully applied for this purpose, resulting in the identification of cyclic peptides capable of interacting with a variety of targets. The respective methods disclosed in this review (Figure 1) will provide new opportunities to prepare collections of macrocyclic peptides and bicyclic peptides, as well as composed libraries with a variety sequence levels and chemical diversity, with a focus on biosynthesis. More importantly, features, advantages as well as insights into peptide cyclization platforms and perspectives for future development will be discussed.

2. Biosynthetic Strategies

The flexible in vitro translation (FIT) system comprises the integration of a reconstituted E. coli translation system and NAA-tRNAs prepared by flexizymes (artificially evolved ribozymes able to catalyze the aminoacylation of tRNA with a variety of non-proteinogenic amino acids (NAAs). FIT facilitates expression of various peptides containing NAAs from designed mRNA templates according to the newly designated genetic table by means of genetic code reprogramming (Figure 2A) Therefore, the advantage of the FIT system as a cyclization method is the incorporation of more NAAs as the backbone macrocyclic peptides

mRNA display is a reliable methodology for mass peptide library screening (~1013 members) and has been used for peptide drug discovery (Figure 2B) [16][17][28]. It is superior to other screening methodologies in terms of rapidness and peptide selection based on affinity potencies against the proteins of interest. In the RaPID system, in vitro translation is modified to use reprogrammed genetic codes to enable spontaneous peptide macrocyclization, including NAAs such as α-hydroxy acids, N-methyl-, D-, β-amino acids, and amino acids bearing nonstandard sidechains [22][29][30][31][32]. Furthermore, the advantages of RaPID include the use of diverse molecular topologies of macrocyclic peptides to generate a trillion unique members and enrichment or enhancement for low-abundance, high-affinity ligands [33].

As the first showcase of this system, Yamagishi et al. utilized it for selection of anti-E6AP macrocyclic N-methyl-peptides, where one of the abundant classes of selected peptides exhibited an inhibitory activity against E6AP-catalyzed polyubiquitination of target proteins such as p53 and peroxiredoxin 1 [20]. This work demonstrated the potential of the RaPID system for the discovery of a novel class of nonstandard peptides against previously non-druggable targets [20]. integrated both the chemical synthesis of proteins and screening against trillion-member macrocyclic peptide libraries using RaPID, making post-translationally modified targets accessible for drug discovery [22]. Overall, RaPID screening has been proven to be a powerful system for the discovery of bioactive macrocyclic peptides, and has been used to generate a number of high-affinity, highly selective binders.

To fully exploit the potential of RiPPs as drug candidates, Urban et al. reported a phage display-based tool for systematic engineering with examples of lanthipeptides, a subclass of RiPPs characterized by multiple thioether cycles that are enzymatically introduced in a regio- and stereospecific manner (Figure 2C) [15][27]. The phage display system was found to be suitable for generating lanthipeptide ligands for protein targets and could be adapted to other library designs [34]. Furthermore, the C-terminal display on pIII could be applicable for the engineering of other RiPP classes, and acts as a valuable source to identify therapeutic peptide alternatives [27].

The lanthipeptide biosynthetic enzyme systems were demonstrated to be amenable for the display of lanthipeptides by N- or C-terminal phage display and yeast display, suggesting strong applications [15].

Another novel discovery approach relying on the in cellulo production of macrocyclic peptides is the split-intein circular ligation of peptides and proteins (SICLOPPS) method [4]. SICLOPPS utilizes the natural process of intein spicing to generate macrocyclic peptides (Figure 2D) [35][36]. The general method to produce macrocyclic peptides using intein chemistry involves a process in which an intein domain is arranged at the C-terminal region of the objective precursor peptide and an N-terminal Cys bearing Compared with other discovery platforms, limitations include the constraint of the tolerance of its host cell, challenges in system modifications and evolution, and limited accessibility of a single type of cyclic peptide topology [37].

Benefiting from high versatility for the multimerization and conjugation of the modular platform, bicyclic peptides could be implemented for assembling multimers including tandems, trimers, tetramers and drug conjugates, which could be applied as standalone therapeutics [38]. The unique bicyclic peptides screening platform based on phage display could also be used for selection of bicyclic peptides with amenable links to other molecular payloads such as cytotoxins or other bicyclic peptides, to create complex molecules with combinatorial pharmacology [38]. Taking drug conjugate as an example, a tripartite complex is formed via bicyclic peptide conjugates through: (1) bicyclic peptides binding to a specific tumor antigen; (2) a designed, selectively cleavable linker (only by enzymes within specific microenvironment); and (3) a payload (small molecule) [38][39]. More importantly, bicyclic peptides are promising therapeutics that present a novel and flexible platform of building blocks to address intractable challenges for oncology medicines and other therapeutic practice [38].

Furthermore, enzyme-catalyzed peptide cyclization has been exploited due to the cost-effectiveness and high chemo-selectivity of biocatalysts [40]. Non-ribosomal machinery has been discovered for peptide synthesis using multienzyme complexes as an assembly line to catalyze stepwise peptide cyclization. For instance, hydrolytic release is observed for vancomycin with a backbone constrained by post-synthetic oxidative cross-linking reactions [41]. As another example, an isolated TE demonstrated the capability of catalyzing the cyclization of linear peptides via a phosphopantetheine linker and building a cyclic peptide library derived from the antibiotic tyrocidine [9][10][40].

Chemo-enzymatic strategies have been developed to combine chemical linear peptide synthesis with enzymatically catalyzed cyclization to reprogram existing non-ribosome-derived produced peptides, utilizing nature-developed stereo- and regioselective peptide cyclization enzymes (Figure 2G) [8]. Furthermore, based on the diversity of natural cyclization strategies, chemoenzymatic approaches for the cross talk between biology and chemistry present a new source of diversative cyclic peptides with altered features and diversification. Future research efforts could include the generation of custom-made catalysts for cyclization of a specific sequence, the use of excised TE-domains [42], and investigation of the efficiency of combinatorial cyclization using these enzymes [8][9][10]. The advantages of chemoenzymatic approaches include broad substrate tolerance, the feasibility of producing glycosylated cyclopeptides and lipopeptides, and the extension of carbohydrate complexity into peptides as a useful toolkit for a large cyclic library search [8].

3. Discussions and Perspectives

Macrocyclic peptides and bicyclic peptides represent a golden middle ground as they have a size between that of small molecules and biological ligands, have functional attributes including unique selectivity, versatility, and structural stability, and are promising alternatives to small-molecule and macromolecule scaffolds [1]. The biosynthesis of cyclic and bicyclic peptides has attracted particular attention, bearing promising advantages over traditional methods. Each of reviewed platforms boasts its own strengths and has its own features, as reviewed above. The highlighted insights were summarized with regard to their perspectives.

Genetically encoded libraries of cyclic peptides generated through various approaches such as phage display, mRNA display, and split-intein circular litigation are increasingly being applied for macrocyclic compounds, with their own advantages and disadvantages [43]. These chemistries can also be used to generate bicyclic peptide libraries considering that bicyclic peptide phage libraries can further be generated by chemical cross-linking. Additionally, another emerging library via yeast surface display has also been used for efficient isolation and characterization of cyclic peptides produced from combinatorial libraries, suggesting that the yeast surface display enables selectivity and affinity screening [44]. Compared to in vitro screening, in cellulo screening may be limited by library sizes; nevertheless, this approach has other benefits for successful candidates; for instance, peptides which are soluble and more resistant to proteolytic degradation, are more likely to be screened by the cell host [45][46].

An interesting strategy is the combination of in vitro and in cellulo approaches in tandem to benefit from the advantages of large library size and desirable properties through in-cell selection [45]. In addition, combinational insights applied for various libraries will assist in the future trend of cyclic peptide screening. The yeast surface display in combination with phage display has been shown to be an approach for the quick identification of protein binders and the filtering of non-functional peptides [47]. For example, the yeast surface display was implemented to select peptides binding to wild-type IL-23, helping to envisage the phage display as a future strategy for drug leads [47].

The intracellular environment provides the unique advantage of activity-based selection [48]. Reverse two-hybrid screening could be coupled with a high-throughput platform for candidates in vivo [1]. Only SICLOPPS is compatible with activity-based two-hybrid screening, which makes it more reliable for discovering bioactive hits, whereas the other methods are more prone to discovering high-affinity binders that lack biological activity.

Non-ribosome-derived peptides exhibit structural diversity in terms of D-configured residues, oxidation, methylation, halogenation, lipidation, heterocyclization, and macrocyclization [8]. This feature is reflected in a broad spectrum of biological activities of non-ribosome-derived peptides, including antibacterial, immunosuppressive, and antitumor properties. Acidic lipopeptide antibiotics have demonstrated the structure versatility of non-ribosomal derived peptides such as calcium-dependent antibiotics (CDAs), daptomycin, A54145, friulimicins, and amphomycins [49][50][51][52][53][54]. These attributes will allow for the design of novel and diversified classes of bioactive peptides.

The chemical space of available peptides is expanding via the synthesis of peptides containing NAAs with the aim of adding more functionalities such as stability, or to form specific tertiary structures to discover peptide therapeutics [55][56]. Nowadays, macrocyclic peptide-compatible screening technologies can also be tolerated with the introduction of peptides containing NAAs. For instance, the RaPID approach benefits from the possibility of incorporating unnatural amino acids through the FIT [43]. Thus, a larger chemical space will be available for the bioactive peptide using synthetic and screening methods together, for example RaPID, RiPPs, and even chemoenzymatic synthesis [1].

The combinatorial method could provide large libraries of macrocyclic peptides created with both natural and unnatural amino acids as well as building blocks, which can subsequently be screened for novel or improved bioactivity. Furthermore, alterations in the substrate specificity of TE-domains by directed protein evolution will increase the utility of these macrocyclization catalysts [8]. However, little is known about the chemoenzymatic potential of tailoring enzymes, which can contribute to the structural diversity and rigidity of non-ribosomal derived peptides, including their tolerance in vitro for their specified reactions. Furthermore, it could be questioned as to whether exercised TE-domain swapping is an applicable tool for the production of novel peptides in vivo [8].

The overall philosophy of new methodologies needs to expand the “toolbox” in order to expand opportunities in many ways with: (1) the creation of structurally and functionally diverse libraries of peptide macrocycles; (2) compatibility with functional assays; (3) integration with high-throughput display platforms; (4) genetic build-in structural features; (5) efficiency to produce cyclics with various ring size and amino acid sequences; (6) predictability of regioselectivity; and (7) amenability to coupling with well-established display platforms or intracellular selections Iannuzelli et al. developed and characterized an expanded toolbox of unnatural amino acids suitable for directing the biosynthesis of thioether-linked macrocyclic peptides via a cysteine cross-linking reaction by means of electrophilic non-canonical amino acids in bacterial cells [57]. These new cyclization strategies benefited from several features: the functionality and efficiency of electrophilic unnatural amino acid-mediated cyclic peptide formation; the variability of inter-sidechain linkages; enabling 21 amino acid residues in cellulo; various ranges of cyclization from short to long; accessibility with respect to broader scaffolds; and variations in the unnatural amino acid modules for function modulation. Bionda et al. developed methodologies to guide the production of “natural product-like” macrocyclic peptides constrained by an intramolecular thioether bridge in bacterial cells via the combination of a chemo-selective reaction (between encoded cysteine and a cysteine reactive unnatural amino acid) via intein-catalyzed protein splicing, inspired by the biosynthetic logic of natural products [37].

The breakthrough application of phage displays in bicyclic peptides opens the door for bicyclic peptides, multimers and drug conjugates. Although many aspects (such as antitumor efficacy, toxic effects, and PK) need to be addressed as bicyclic peptides classes move into clinical phases, library-based bicyclic peptide platforms enable a new path for a wide range of therapeutics. The biosynthetic properties of bicyclic peptides critically enhance the versatility and functionality of macrocyclic peptides in drug discovery. Furthermore, future investigation could advance biosynthetic approaches and perspectives of bicyclic peptides to other drug modalities.

In particular, integration with in vitro and in cellulo libraries will provide high-throughput applications for cyclic peptides screening. Specifically, these in vitro and in vivo screening libraries enable ultra-high throughput screening accessible to laboratory settings without special resources. Inspired by biosynthetic logic for future directions, expanding the toolkit of current methodologies will broaden the opportunities for structural diversification and enable library integration and functional screening at a high-throughput level. Furthermore, progress in the cyclization of peptides and their screening platforms will enable broader future applications in a variety of fields, including structure diversity and space exploration, therapeutics, drugging of undruggable targets, and building blocks for macromolecules.

4. Concluding Remarks

In summary, the biosynthesis of macrocyclic peptides has substantially developed to provide a competitive platform technology in the field of drug discovery, accompanying in vitro display and DNA-encoded library technologies. In contrast to organic synthesis, biosynthesis provides a unique and robust process to identify early-hit cyclic peptides that can be further optimized. The integration of cyclic peptide libraries was emphasized based on different categories such as mRNA display and phage display. The biosynthetic characteristics of in vitro display such as phage display enable structural diversity for natural product-like bicyclic peptides.

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