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Review

Macrocyclic peptides as regulators of protein-protein interactions

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ABSTRACT

Protein-protein interactions (PPIs) are recognized as attractive therapeutic targets. However targeting PPIs especially intracellular ones has been proven extremely difficult for conventional drug-like small molecules, and biological drugs such as monoclonal antibodies have difficulty in reaching intracellular targets. Macrocyclic peptides are promising candidates of PPI regulators for their potential in combining high potency and biological stability together. Cell permeability of macrocyclic peptides may also be achieved by structural modifications or conjugation to a cell-penetrating sequence. Significant progress has been made in this research area in recent years. Important technology progress and recent examples of macrocyclic peptide PPI modulators are reviewed.

1. Introduction

Protein-protein interaction (PPI) is the interaction between two proteins, either identical or different, using part of their domains to form interfaces, to regulate the function of the partners. PPIs are involved in most cellular processes in all levels of biological functions. It has been estimated that a total between 130000 and 600000 PPIs exists in the human interactome. Dysregulation of PPIs is associated with broad range of human diseases, many of which we do not have satisfactory therapies currently [1–5].

Both the academia and the pharmaceutical industry have devoted huge efforts in recent decades in this area, and tremendous progress has been made, with success of monoclonal antibodies and some small molecule drugs targeting PPIs. Antibody drugs have been successfully developed against a variety of targets and are approved for various human diseases, such as TNFα in autoimmune diseases, PCSK9 for hypercholestrolemia, PD-1 and PD-L1 in different types of cancer, just to name a few. Venetoclax, a BCL-2 inhibitor with traditional drug-like structure, was approved by FDA in 2016 for chronic lymphocytic leukemia (CLL) associated with 17-p depletion. The drug has a MW of 868 and is orally administered, and is the first FDA approval of a protein-protein interaction inhibitor of BCL-2 [6].

However, compared to the huge number of PPIs and their potential in therapy, the ones already employed in therapeutic use are only a very tiny portion. High-throughput screening against PPI targets using small molecule compound libraries has not been as successful as it worked for other targets like enzymes. This is mainly because of the structural characteristics of PPIs. The interfaces of PPIs are usually flat, with an average area of 800–2000 Å^2, and often without obvious pocket, cleft or groove. Therefore the screening often gives low hit rate [4,5].

On the other hand, antibodies and other protein drugs are suitable for binding to the large, flat interface of PPI targets, and that is the reason for their high specificity, high efficiency, low toxicity and few side effects. However, because antibodies cannot cross the cell membrane, they have only been used against extracellular targets. For the vast number of intracellular PPI targets, new drug modalities are needed. For this purpose, peptides are good candidates. With molecular weight between small molecules and large proteins, the size of peptide is suitable for PPI interface binding. Peptides are in general biologically compatible, with low toxicity, and can achieve high selectivity. Because of these advantages, peptides and peptidomimetics are also widely used as probes in bioanalytical and biomedical research besides pharmaceutical development. However, native peptides often suffer with major drawbacks such as moderate potency, low protease stability, and usually are not permeable to cell membrane. To improve pharmacological properties of peptides, various chemical modifications have been developed, including L to D amino acid substitution, *N*-methylation, incorporation of turn mimetics, helix mimetics or other non-proteinogenic amino acids, using N-cap for α-helix nucleation, and cyclization. Among these methods, macrocyclization may provide peptides

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with improved target affinity through pre-organization, and usually increases the protease stability [7–18].

A diversity of chemistry has been developed for peptide macrocyclization, such as the formation of lactam, lactone, disulfide bond, thioether from Michael addition or nucleophilic substitution, olefin metathesis, and Huisgen 1,3-dipolar cycloaddition. These reactions can be performed on-resin, thus incorporated into the solid-phase peptide synthesis conveniently [3–5,7–10,19]. An approach using pre-prepared orthogonally protected diaminodiacid building blocks, developed by Liu and collaborators, can also be used for the synthesis of macrocyclic peptides. Both all-hydrocarbon and linkers containing heteroatoms or functional groups can be incorporated prior to solid-phase peptide synthesis, enabling straight forward synthesis of macrocyclic peptide with disulfide surrogates among others [20–22]. Reactions that are biocompatible, such as thioether formation and Huisgen 1,3-dipolar cycloaddition, can be applied for peptide macrocyclization in biological systems. Macrocyclization of unprotected peptides can also be carried out under enzyme catalysis, *e.g.*, by sortase A, provided that proper enzyme-recognizing sequence is incorporated [23,24].

In general, there are two ways to discover macrocyclic peptides for PPI modulation, high-throughput screening including combinatorial chemistry and biological display technologies, and structure-based rational design. There are plenty of examples of hit identification of cyclic peptides for both approaches, and a combination of these two methods in the iterative optimization process following the hit identification is often employed [5,8,11–13].

2. Hit identification by high-throughput screening

Combinatorial peptide library synthesis and screening methods are now more often used in peptide lead optimization. Various display methods using biological systems like phage, bacteria, yeast, and *in vitro* protein expression, are used in initial peptide hit generation instead. A combination of chemistry and biology methods has been used to adapt these technologies for macrocyclic peptide library generation and screening. Following are some prominent examples.

2.1. Phage display

Bacteriophage is engineered to uniformly display a library of peptides on the surface. The library is applied to the immobilized target protein, and those phages having peptide sequence with affinity to the target may stay on the target and those without affinity are washed away. After several rounds of panning, the sequences with highest affinity may be isolated [25].

To increase conformational constraint to the displayed peptide, cysteines are often added to both the N- and C-terminus of the displayed sequence, and disulfide bridges are formed before selection. Inspired by natural peptides with multiple disulfide bridges such as cyclotides and conotoxins, researchers designed and synthesized bicyclic phage library by using four cysteines in the peptide library design. Because of the increased constraint on the peptide, bicyclic peptide libraries may facilitate identification of high affinity ligands [26]. However, formation of multiple isomers by different pairing pattern and isomerization by disulfide exchange in systems containing free thiol became an issue in this case. Heinis *et al.* developed another method to construct bicyclic phage library by using biocompatible thioether formation. The peptide sequences encoded by a phage library contain three invariable cysteines, which react with 1,3,5-tris-(bromomethyl) benzene, or other analogs with the same symmetry, to give bicyclic peptides [27,28]. This method has been applied in discovery of PPI inhibitors for targets such as Notch1 and β-catenin, as well as proteases and others. As an example, bicyclic peptide **1** (Fig. 1) binds to the negative regulation region of Notch1 with the K_d value of 150 nmol/L [29].

2.2. Ribosomal display

Cell-free methods like mRNA and ribosomal display enable several orders larger library size than phage display (10^{14} *vs.* 10^{9}). However with the 20 proteinogenic amino acids, cyclization chemistry is basically limited to cysteines. To increase the structural diversity of the library, non-proteinogenic amino acids need to be incorporated into the expression system with technical feasibility. Suga *et al.* has reported the use of a ribosomal display method, termed Random non-standard peptides integrated discovery (RaPID), to circumvent this issue [30]. Instead of using aminoacyl t-RNA synthases, they created promiscuous aminoacylating ribozymes, so-called flexizymes, which essentially recognize activated carboxylates, to charge tRNA with amino acids. Flexizymes thus allow extensive genetic code reprogramming with non-canonical amino acids, which can be used for peptide cyclization on the mRNA. This method enables the use of cyclization method that are compatible to the peptide and expression system, for example, 1,3-dipolar cycloaddition and thioether formation.

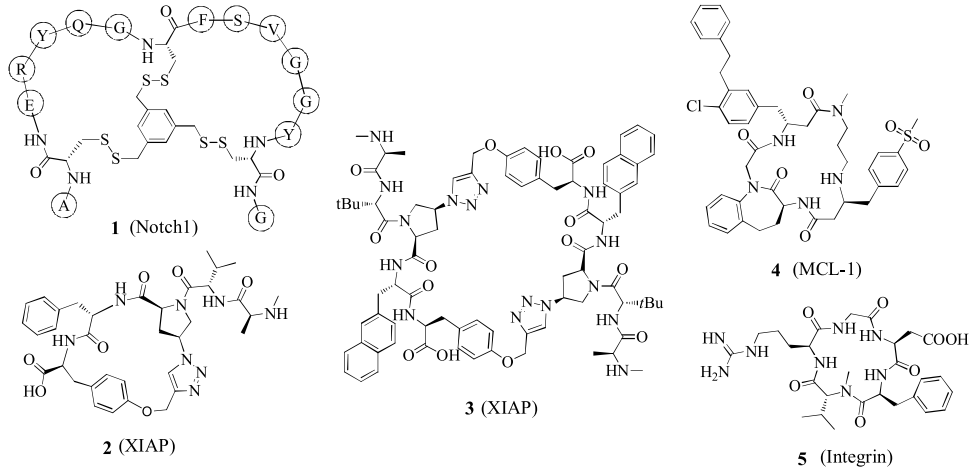

Fig. 1. Structures of macrocyclic peptides **1**–**5**.

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