Ribosome-Mediated Synthesis of Natural Product-Like Peptides Via Cell-Free Translation

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Abstract

Peptide natural products (PNPs) represent a unique class of compounds known for their fascinating structural motifs with important biological activities. Lately, PNPs have garnered a lot of interest for their application in drug discovery. Nevertheless, lack of diversity oriented synthetic/biosynthetic platforms to generate large natural product-like libraries has limited their development as peptide therapeutics. The promiscuity of cell-free translation has allowed for the synthesis of artificial PNPs having complex structural features. Modified cell-free translation systems coupled with the display technologies have generated diverse natural product-like peptide libraries and led to the discovery of several biologically active molecules. Such technologies have drastically decreased the time to obtain peptide drug leads and therefore, revolutionized the field of peptide drug discovery. In this account, we review recent developments in the synthesis of natural product-like peptides via cell-free translation.

Introduction

Peptide natural products (PNPs) possess interesting structural features, which make them great therapeutic agents and allow them to modulate difficult ‘undruggable’ targets not easily accessible by small molecules. Some common structural designs observed in PNPs are ring topologies, non-standard amino acids (nsAAs) including *N*-methyl amino acids, D-amino acids, amino acids with non-canonical side chains, β -/ γ -amino acids and backbone heterocyclic motifs such as oxazolines and thiazolines (Figure 1). In the past decade, substantial research efforts were dedicated to generate diverse and large set of peptide libraries having either natural product-like motifs or novel architectures [1]. Genetic code manipulation approaches and chemical post-translational modifications allowed phage and mRNA display technologies to revolutionize the field of peptide therapeutics [1,2]. Cell-free translation system integrated with mRNA display technology [3] has provided several potent bioactive natural product-like peptides with high target-binding affinities [1,2,4]. Moreover, a recently described modified translation/mRNA display format [5] has significantly decreased the time and efforts spent for the search of bioactive peptide sequences. Moving beyond the drug discovery application, coupled translation-selection systems have generated peptides, which were also useful as co-crystallization ligands [6], fluorogenic peptide aptamers [7,8] and fluorescent probes [9]. Clearly, such discoveries were made possible by the ability of cell-free translation to incorporate diverse structural motifs observed in PNPs into peptides. In this review, we will focus on different strategies employed for the ribosomal synthesis of natural product-like peptides using cell-free translation systems and their applications.

Macrocyclization

A prevalent structural motif among PNPs is a ring topology (macrocylic structures), which decreases the number of conformations available for a peptide. Such a structural rigidity decreases the entropic cost to adopt the bioactive conformation and thus, improves target-binding affinity [10,11]. Moreover, macrocyclization distorts the alignment of residues around an amide bond, which in turn decreases the chance to be recognized by peptidases and therefore, potentially increases resistance against proteolysis. In addition, some studies have shown that cyclization could improve passive membrane permeability by promoting intra-

molecular interactions [12,13]. Due to these advantages, methodologies for peptide cyclization have been extensively explored [4,14,15].

Cysteine-mediated macrocyclization strategies, where the nucleophilic reactivity of sulfhydryl group is exploited, are frequently applied. Inspired by the posttranslational modifications and cyclization in lantipeptide biosynthesis [16], Szostak and co-workers developed a cyclization method to produce lantipeptide analogues (Figure 2a) [17]. Using in vitro translation, 4-selenolysine (**1**, Figure 3a) and 4-selenoisoleucine amino acids were incorporated into a ribosomal peptide and subsequently oxidized by hydrogen peroxide to generate dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively. Upon deprotection of a cysteine, a selective nucleophilic attack on a more reactive Dha over Dhb residue afforded desired macrocyclization (lanthioine formation). Afterwards, this strategy was combined with mRNA display to generate a library of functional lantipeptides (10^{11} membered library) and a low micromolar ($3\ \mu\text{M}$) binder of Sortase A was obtained from the selection [18]. Eventually, Suga and co-workers devised a more versatile strategy involving a reaction between cysteine and the electrophilic carbon of chloroacetyl moiety on the N-terminus of a peptide (figure 2b) [19]. In this work, a custom-made reconstituted cell-free translation system called Flexible In Vitro Translation (FIT) system [3] was used. Central to the FIT system is a ribozyme-mediated aminoacylation of tRNAs (called flexizyme technology) [20,21] and genetic code reprogramming [22,23] for the incorporation of non-canonical amino acids [24]. Methionine residue was removed from the FIT system and initiation event was reprogrammed to accept N^{α} -(2-chloroacetyl)-Trp (**2**, Figure 3a). After translation, spontaneous cyclization occurred between C-terminal cysteine side-chain and N-terminal head of the tryptophan residue (Figure 2b). Later on, this strategy was integrated into mRNA display format and was called the Random non-standard Peptide Integrated Discovery (RaPID) system [3]. The FIT/RaPID system have been successfully utilized for the construction and screening of large macrocyclic peptide libraries (10^{13}) to afford active natural product-like molecules, which gave remarkable results as enzymes inhibitors [25,26], modulators of protein-protein interactions [27,28] stable hormone analog [29], protein co-crystallization ligands [30] and fluorescent imaging probe [9].

Bicyclic structures are not common among PNPs. However, larger size bicyclic peptides may give rise to more extensive interactions with their protein

targets as compared to monocyclic counterparts. In this direction, Suga *et al.* reported strategies to obtain bicyclic [31] and recently, tricyclic [32] peptides using cell-free translation system. For bicyclization, two orthogonal (intramolecular) reactions were carried out after the ribosomal synthesis of peptide. Using genetic code reprogramming and a modified cell-free translation system (which is now known as the FIT system), 4-(2-chloroacetyl)aminobutyric acid (Cab, **3**, Figure 3a) having a cysteine reactive functionality and a cycloaddition reaction pair azidohomoalanine (Aha, **4**, Figure 3a) and propargylglycine (Pgl, **5**, Figure 3a) were incorporated. Cu (I) mediated click reaction between azide and alkyne side chains led to the first cyclization event, whereas thioether formation between cysteine and Cab residue afforded fully bicyclized peptides [31]. Recently, chemically synthesized bicyclic peptide libraries were employed to select inhibitors of protein-protein interaction [33]. On the other hand, utilizing 1,3,5-tris(bromomethyl)-benzene (TBMB) mediated cyclization of phage displayed peptide libraries (Figure 2c), Heinis and Winter reported a potent bicyclic peptide inhibitor of human urokinase-type plasminogen activator ($K_d = 53$ nM) [34] having improved biological activity over previously reported monocyclic inhibitors. [35]

Iwasaki *et al.* demonstrated that the N^α -(2-chloroacetyl) group on initiator amino acid (position 1) in a peptide cyclizes with the nearest cysteine irrespective of the presence of downstream cysteine residues, however it does not react with cysteine present right next (position 2) to the initiator amino acid [36]. Bashiruddin *et al.* benefitted from this selective cyclization and reported a strategy to obtain tricyclic peptides (Figure 2d) [36]. In this account, a peptide containing N -chloroacetyl- D Trp at the N-terminus and four cysteine residues was translated. After ribosome-mediated translation of the desired peptide, first cyclization event occurred between the N -chloroacetyl group and a downstream cysteine, leaving cysteine at position 2 of the peptide unreacted. Subsequently, addition of 1,3,5-tris(bromomethyl)-benzene (TBMB) led to two cyclization events involving three cysteine residues, which afforded a fused tricyclic peptide (Figure 2d).

D-amino acids

D-amino acids are often found in PNPs and known to increase serum stability of a peptide due to decrease in proteolytic degradation [37] and induce turn

conformations in peptides [38]. Ribosome-mediated incorporation of D-amino acids into peptides was carried out by taking advantage of promiscuous substrate specificity of translation initiation [39,40]. Different *N*-formyl and *N*-acyl-D-aminoacyl-tRNA^{fMet} were prepared via aminoacylation carried out by flexizymes. In the absence of methionine, ribosome utilized reprogrammed tRNA^{fMet}s for translation initiation to synthesize peptides bearing D-amino acids in the FIT system [39]. In a very recent application of this approach, a 14-mer macrocyclic peptide having *N*-chloroacetyl-D³Phe (**6**, Figure 3b) residue was selected from a large peptide library, to bind the extracellular domain of EpCAM with a K_d of 1.7 nM and a fluorescent tag enabled the visualization of EpCAM-expressing MCF7 cells [9]. This peptide based fluorescent probe was under 3000 Da and stained every live cell under high cell-density conditions.

Fujino *et al.* reexamined the elongation efficiency of 19 D-amino acid residues by reassigning UCC codon to D-aminoacyl-tRNA^{Asn}_{GGU} in the FIT system. Twelve D-residues were individually translated in 10% or higher yields and peptides containing either two D-Ala or D-phe residues (**7** or **8**, Figure 3 b) were also efficiently synthesized [41]. Lately, using in vitro reconstituted dehydrogenases, posttranslational conversion of dehydroalanine into D-alanine was reported [42]. Enzymes NpnM and maltose-binding protein (MBP)-fused NpnA3 were co-expressed in *E. coli* along with the substrate peptides. NpnM catalyzed the formation of dehydroalanine and dehydrobutyrine residues, whereas NpnA3 selectively reduced dehydroalanine to D-alanine in presence of NADPH.

N-Alkyl Amino Acids

Another frequently observed structural feature among PNPs is *N*-methylation, where nitrogen atom of an amide bond is methylated [43]. Similar to macrocyclization, it increases conformational rigidity as well as resistance to peptidases. Due to *N*-methylation, amide bond is unable to participate in hydrogen bonding with near by water molecules, which is believed to promote passive membrane permeability [44-46]. Cyclosporin A (Figure 1), an 11-residue cyclic peptide having seven *N*-methylated amide bonds, is the most studied natural cyclic peptide for its good pharmacological properties including cell permeability [12,47]. In nature, *N*-methylated PNPs are not synthesized by the ribosome or derived from

ribosomally synthesized peptides, rather a large set of multifunctional enzymes working in tandem, called non-ribosomal peptide synthetases (NRPSs), carry out the biosynthesis of *N*-methylated peptides [48,49]. Nevertheless, ribosomal acceptance of *N*-methylated amino acids is known for a long time [50]. In addition, ribosomal tolerance of different *N*-methylated amino acids has been evaluated by various research groups [51-53].

Subtelny *et al.* demonstrated the ribosome-mediated incorporation multiple *N*-methylated amino acids into peptides by chemical methylation of amine on the canonical aminoacyl-tRNAs followed by their use in a cell-free translation system [52]. Although, they produced *N*-methylated peptides with reasonable efficiencies, this methodology suffered with misincorporations and truncated peptide products. In the first example of its kind, Kawakami *et al.* reported the incorporation of up to eight consecutive *N*-methyl amino acids (including **9-12**, Figure 3c) into peptides using the FIT system [53]. Moreover, macrocyclization of *N*-methylated peptides was also demonstrated, which enabled the preparation and application of natural-product like macrocyclic peptide libraries to search for biologically active molecules with interesting pharmacological properties. So far, two examples of *N*-methylated peptide inhibitors are reported. First, five *N*-methyl amino acids were included during the preparation of peptide library, thereby screening this library against a ubiquitin ligase E6AP to yield several multiple *N*-methylated macrocyclic peptides (Figure 4a) with low to sub-nanomolar binding affinities (as low as $K_d = 0.6$ nM) [26]. Second, using a slightly different display system called transcription-translation coupled with association of puromycin linker (TRAP) [5], Ishizawa *et al.* reported a potent *N*-methyl-macrocyclic peptide inhibitor against VEGFR2, a human membrane protein growth factor receptor, with a K_d of 33 nM [54]. To produce more complex *N*-alkylated peptides (known as peptoids) via ribosome synthesis, Kawakami *et al.* utilized the FIT system to synthesize peptoid-peptide hybrids (Figure 4b) by using non-branched, branched and functionalized alkyl chain substituted *N*-alkyl amino acid substrates (**13-28**, figure 3c) [55].

In a latest account, Schwachert *et al.* chemically synthesized different peptoids and peptoid-peptide hybrids and explored their cell permeable properties [56]. They observed that a strategic peptide to peptoid substitution contributed towards enhanced cell permeability. This report and the remarkable ability of the FIT

system to incorporated *N*-alkylated amino acids will attract more interest in peptoid–peptide hybrids.

Other Non-Proteinogenic α -Amino Acids

Incorporation of α -amino acids with non-canonical side chains into polypeptides has been periodically explored and applied in drug discovery. Szostak group prepared an mRNA displayed highly reprogrammed cyclic peptide library (10^{13}) to include 12 non-canonical amino acids (**29–40**, Figure 3d) and screened against the protease thrombin [57]. Two macrocyclic peptides with low nanomolar binding affinities were isolated and the non-canonical amino acids were shown to be essential for the biological activity of these peptides.

To search for strong inhibitors against a histone deacetylase SIRT2, Suga lab prepared peptide libraries having a non-canonical amino acid K^{Tfa} (ϵ -*N*-trifluoroacetyl lysine, **41**, Figure 3d) in the randomized region [25]. The selection yielded several peptide inhibitors having a conserved sequence around the ‘warhead’ amino acid K^{Tfa} and bound SIRT2 with 10–100 fold selectivity over SIRT1 and SIRT3. The peptide S2iL5 bound with a K_d value of 1.0 nM and was used as a co-crystallization ligand to successfully obtain the structure of S2iL5 bound SIRT2 [58]. Different peptide binders selected from FIT/RaPID system were also used to explore the structure and functions of various therapeutically relevant proteins [30,59,60]. Environment sensitive fluorescent amino acids have been used to study protein structure and functions [61].

In a latest report, Manandhar *et al.* incorporated an environment sensitive fluorescent NBD (7-nitro-2,1,3-benzoxadiazole) group containing amino acid (**42**, Figure 3d) into a random peptide library and, by using mRNA display, selected aptamers that bind to verotoxin [7]. One of the peptide (VT4) showed decrease in fluorescence produced by NBD upon binding to verotoxin possibly due to change in peptide conformation. This fluorescent peptide probe can now be used for the detection of verotoxin.

Non- α -Amino Acids

Other important structural motifs that are known to contribute towards the biological activity of PNPs include non- α -amino acids such as β -amino acids and

backbone heterocycles (Figure 3e) [62-64]. In general, ribosome-mediated incorporation of β -amino acids into peptides and proteins has been challenging. However, Suga and Murakami labs successfully demonstrated the ribosome-mediated synthesis of peptides having multiple β -amino acids (**43-58**, Figure 3e) by genetic code reassignment coupled with the FIT system [65]. Authors successfully demonstrated the incorporation of up to three different β -amino acids into peptides (Figure 4c).

Earlier, Hecht group reported ribosomes with mutations in the peptidyltransferase center (PTC) that enabled the incorporation of both aliphatic and aromatic β -amino acids into a single pre-determined site via suppression of amber stop codon by chemically aminoacylated β -aminoacyl-tRNA [66-68]. The acceptance and thus incorporation of backbone heterocyclic amino acid analogues including thiazol(in)es and oxazol(in)es into polypeptides by the wild-type ribosomes has not been established yet. However, Maini *et al.* designed and incorporated a highly fluorescent oxazole amino acid derivative (**59**, Figure 3e) into green fluorescent protein (GFP) and *E. coli*. dihydrofolate reductase (DHFR) using mutant ribosomes via amber suppression technology [69]. Moreover, a dipeptide residue having a thioamide backbone (**60**, Figure 3e) was also incorporated into DHFR.

Biosynthetic routes to various ribosomally synthesized and post-translationally modified peptides (RiPPs) including thiazol(in)es and oxazol(in)es containing peptides have been studied extensively. Recently, several labs have reported successful *in vitro* reconstitution of different enzymes involved in the synthesis of RiPPs and their use for post-translational modification of ribosomally synthesized peptides [70]. While, other labs utilized *E. coli* for the co-expression of the enzyme of interest and their substrate peptides to synthesize azol(in)e (**61** and **62**, Figure 3e) based peptides *in vitro* [70], Goto *et al.* developed a cell-free translation system called FIT-PatD for the synthesis of PNPs [71]. In this study, translation by the FIT system was coupled with post-translational modifications by recombinant PatD enzyme to synthesize multiple azoline containing peptides (Figure 4d). Adding to the growing list of backbone amino acid incorporated into peptides using the FIT system, Torikai and Suga reported a ribosome-mediated synthesis of macrocyclic peptide (Figure 4e) containing a 12-carbon long polyunsaturated chain, which is a peptide analogue of amphotericin B (an amphiphilic membrane-interacting antifungal natural product) [72]. D-cysteine, but not the L-derivative, having the hydrophobic terpene moiety

linked to the S-atom was accepted by the ribosome during the translation initiation event. The authors speculated that the long and bulky side chain in the L-cysteine derivative is not accommodated in the P-site, whereas in case of D-cysteine derivative, the side chain may sit in the exit tunnel assisting in a more stable initiation complex.

Conclusion

Here, we have reviewed the contribution of cell-free translation system to produce large natural product-like peptide libraries and its integration with high throughput screening systems to identify interesting peptide drug leads. Several bioactive molecules against difficult therapeutic targets including kinases, and membrane receptors were discovered by the coupled peptide translation-molecular display/selection systems (for example FIT/RaPID system). Moreover, we also discussed that such systems are not limited to drug discovery, rather are also being applied in structural biology as well as fluorescence imaging. As illustrated by examples, cell-free translation systems such as the FIT system have exhibited remarkable ability to incorporate several prominent structural features found among PNPs into ribosomally synthesized peptides. However, the strategies discuss in this review have only been able to access a small portion of vast chemical space available. Further expansions in peptide translational approaches can potentially yield molecules that may go beyond the scope of nature. More research efforts are required to develop current translation-display systems to directly identify orally bioavailable and cell permeable bioactive peptide molecules.

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Figure captions

Figure 1. Representative structures of peptide-natural products (PNPs).

Figure 2. Posttranslation cyclization methods. **(a)** Cyclization between cysteine and dehydroalanine to yield a lanthionine derivative. **(b)** Macrocyclization via a spontaneous reaction between *N*-chloroacetyl group and cysteine. **(c)** Tris(bromomethyl)-benzene-mediated bicyclization on phage displayed peptides. **(d)** Topologically controlled tricyclization by combining strategies in illustrated **(b)** and **(c)**.

Figure 3. PNP structural motifs and novel architectures incorporated into ribosomally synthesized peptides via cell free translation.

Figure 4. Natural product-like peptides synthesized via cell free translation. **(a)** Macrocyclic peptide having four *N*-methylated residues. **(b)** Peptoid-peptide hybrid. **(c)** Peptide having different β -amino acids. **(d)** Peptide having different backbone heterocyclic motifs. **(e)** Peptide analogue of Amphotericin-B. Flag = DYKDDDDK.

Figure 1

Cyclosporine A

Aurilide

Patellamide A

α -Amanitin

Gramicidin S

Figure 2

Figure 3

Figure 4