Exploring the Chemistry-Biology Interface: A Novel Discovery Platform of Pseudo-natural Products

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Molecular modality for drug development is evolving from traditional small molecules to macromolecules. The successes in therapeutic antibodies represent such a transition, but current interest in the drug discovery field is also extending to mid-sized molecules, such as peptides and oligonucleotides. In this article we shall discuss another modality, referred to as pseudo-natural peptides and products with macrocyclic scaffolds.

Section 1: Background

PTM enzymes in RiPPs biosynthesis

Ribosomally synthesized and post-translationally modified peptides (RiPPs) are a diverse group of secondary metabolites present in all life forms. They begin as linear precursor peptides that acquire chemical and structural variety through enzymatic post-translational modifications (PTM). A RiPP precursor peptide typically composed of: (i) a leader peptide (LP) or follower peptide, which serves as a key recognition element for PTM enzymes and is removed at some point during biosynthesis, and (ii) a core peptide (CP), which undergoes post-translational modifications by PTM enzymes and is proteolytically released to form the final natural product.

The PTM enzymes involved in RiPP biosynthesis are highly specific in their reactions on peptide substrates but are tolerant of mutations in the core region of the precursor peptide. By "mixing-and-matching" the enzymes responsible for PTMs, the same RiPP peptide precursors can be used to produce a wide range of natural products. Due to their versatility, the PTM enzymes are potentially applicable to create macrocyclic peptide libraries.

Thiopeptides with unique cyclization motifs

In nature, many ribosomally produced post-translationally modified peptides (RiPPs) share macrocyclization as a common structural feature. Macrocyclization reduces conformational flexibility and reorganizes functional groups to enhance the binding to a biological target. Additionally, macrocyclic peptides demonstrate increased resistance to proteolysis and enhanced cell-penetrating activity. Natural peptides adopt various cyclization strategies to enhance their interaction and selectivity with the target proteins.

Thiopeptides are a class of RiPPs produced by bacteria. They are characterized by three main components – a central six-membered heterocycle, a core macrocyclic ring and a tail. The core macrocyclic ring is decorated with natural amino acids as well as multiple oxazole, thiazole or thiazoline substituents and dehydroamino acids. Some thiopeptides feature a secondary side ring with distinct chemical modifications such as glycosylation, methylation, and hydroxylation. These compounds are commonly classified based on the oxidation state of the central six-membered heterocycle (**Figure 1**).

Figure 1 | Classification of thiopeptides according to the oxidation state of the central heterocycle. (a) Series *a*–*e* were shown based on the oxidation state of the central six-membered heterocycle. (c) Representative thiopeptides in series *a*–*e*. The number of bonds in the ring is shown in the middle.

Thiopeptides are characterized by their six-membered nitrogen-containing heterocycles along with thiazoles and thiazolines. Recently, pyritides, a group of pyridine-based macrocyclic peptides, lacked the sulfur-containing heterocycles, were also discovered. Generally, pyridine synthases are hypothesized to involve a formal [4 + 2]-cycloaddition between two dehydroalanine (Dha) moieties. In the proposed mechanism, the C-terminal Dha undergoes tautomerization to an iminol, which would act as a pseudo-diene. The N-terminal Dha then functions as the complementary dienophile. Subsequent cycloaddition, either stepwise or concerted, results in formation of a cyclic hemiaminal intermediate, known as the Bycroft–Gowland intermediate (III).[1] These intermediate serves as a common mechanistic step in the biosynthesis of pyridine- and (dehydro)piperidine-containing thiopeptides. Consequently, it remains unclear how the active site residues engage the substrate and catalyze [4 + 2]-cycloaddition, dehydration, and leader peptide elimination (see **Figure 2f**). Nevertheless, gene disruption experiments and in vitro studies of thiopeptides such as thiocillin and thiomuracin support an enzyme-catalyzed [4+2] cyclization process with strong evidence suggesting that a conserved Tyr

Figure 2 | Biosynthesis of lactazole A (LazA). (a) LazA (precursor peptide) and PTM enzymes encoded in the genome. (b) Precursor peptide sequence. (c) Chemical structure of LazA. (d) Azole synthesis pathway catalyzed by LazDE and LazF. (e) Dehydroalanine synthesis pathway catalyzed by LazB and LazF. (f) Pyridine synthesis along with N-terminal leader peptide cleavage catalyzed by LazC.

(Tyr319 in the case for TbtD) plays an important role in reaction coordinate monitoring, chemical rescue, and proximity-mediated substrate cross-linking.

Section 2: My recent research contributions to Chemistry-Biology Interface of mid-sized drug discovery

mRNA display platform for the discovery of bioactive *de novo* macrocyclic peptides

Macrocyclic peptides are highly promising therapeutic candidates. To identify potential drug molecules through high-throughput screening, it is essential to generate a diverse library of cyclic peptide sequences. The mRNA display, where the linear peptide is attached to its encoding mRNA sequence via a puromycin linker at the C-terminus.

Various cyclization approaches, such as side chain-to-side chain or side chain-to-N-terminus cyclization, are integrated with mRNA display to screen cyclic peptide libraries. Disulfide bond formation between two cysteine residues is the simplest strategy, but using bridging reagents and incorporating non-native amino acids can create a wide range of cyclization patterns, especially with multiple reactive residues present.

The Suga group has developed the flexible in vitro translation (FIT) system, which includes pre-charged nonproteinogenic amino acid-tRNAs prepared using flexizymes for in vitro genetic code reprogramming.[2] The FIT system enables the ribosomal synthesis of random peptide libraries incorporating various nonproteinogenic amino acids (npAAs), containing over a trillion ($>10^{12}$) of unique members. The most convenient and reliable cyclization method involves translating peptides with an N-chloroacetyl-amino acid initiator that can react with a downstream cysteine residue. This approach takes advantage of the spontaneous and selective formation of a thioether bond between the N-terminal chloroacetyl group and the sulfhydryl group of the nearest cysteine residue. By integrating the FIT system with mRNA display, referred to as RaPID (Random nonstandard Peptides Integrated Discovery) system,[3–5] Suga group has constructed various libraries of macrocyclic peptides and efficiently screen potent macrocycle ligands with highly specific and potent activities against more than 40 protein targets.[4-6]

Figure 3 | Laz-RaPID selection of bioactive pseudo-natural products against a protein of interest (TNIK). (a) Minimal scaffold of LazA and its map of PTM enzymes. (b) Design of a library of pseudo-natural LazA. (c) RaPID display of the library of pseudo-natural LazA against a protein of interest, TNIK, which is a kinase involving the Wnt signaling pathway.

Integration of mRNA display with thiopeptide biosynthesis enzymes

The Suga group recently succeeded in integrating the FIT system with the PTM enzymes of thiopeptides, more specifically lactazole (Laz) A, referred to as LazA-FIT system (**Figure 2**).[7] After optimization of conditions, the one-pot synthesis of LazA was achieved in the FIT system with Laz enzymes within 24 hours. This allowed for construction of a library of pseudo-natural LazA by placing random sequences sandwiched by the essential residues of the precursor peptides at the N-terminal and C-terminal regions. Furthermore, this LazA-FIT was integrated with mRNA display, referred to as Laz-RaPID system,[8,9] enables for selection of potent ligands against proteins of therapeutic interest, *e.g.* TNIK (**Figure 3**). TNIK is a protein kinase downstream of Wnt signaling, whose inhibition leads to prohibition of tumor growth. This selection campaign yielded potent inhibitors, one of which, TP15, exhibits 1.2 nM K_D and 14 nM IC_{50} with serum stability of 2 hours $t_{1/2}$.[10] The X-ray structure of the complex of TP15 and TNIK was solved, reveling that specific hydrophilic and hydrophobic interactions with many residues between TP15 and TNIK (**Figure 4**). Thus far, three different proteins were targeted by means of the Laz-RaPID system, and in all cases desired potent binders were successfully obtained in a wide variety of sequences and tertiary structures.[11,12] This work has established a novel discovery platform of pseudo-natural products for therapeutic innovation. Some improvements remain to be needed for further development. For instance, some LazA analogs are cell membrane permeable and intracellularly functional, but they are still modest in the level of a few

μM concentration range. Their cell membrane permeability is indeed comparable to Tat and poly-arginine, so-called membrane permeable peptides. However, this is not enough to maximize the inhibitory potential of pseudo-natural products. If higher membrane permeable molecules are discoverable by the Laz-RaPID system, the technology becomes more powerful for the drug development. We demonstrated that the metabolic stability can be improved when the molecules are more modified containing non-peptidic structures.[12] However, certain charged amino acid residues, such as lysine and arginine, play critical role in interaction with the target protein. Thus, it is required to alter these residues into non-proteinogenic amino acids to gain more resistance against peptidase/proteases.

Figure 4 | X-ray structure of TP15 complexed with TNIK. (a) Overall structure. (b) Specific interactions of the pyridine-thiazole region to the His-cluster of TNIK. (c) Specific interactions of arginine residues of TP15 with the acidic cluster of TNIK.

Section 3: Outlook to future developments of research on Chemistry-Biology Interface of mid-sized drug discovery

Mid-sized molecules, such as macrocyclic peptides, are yet under development for pre-clinical and clinical study stages. However, we expect tremendous potentials of the mid-sized molecules in the future development as follows:[13] (1) specificity and potency against a protein target of interest; (2) manufactural cost; and (3) bioavailability. The antibody-like high specificity and potency of (1) has been well documented when the molecules were discovered by display formats, such as the RaPID system. Thus, this potential has been well recognized by the community. The current manufactural cost of (2) relying on chemical synthesis is more expensive than small molecules, but less expensive than antibody productions. We expect that their cost of synthesis will be lower when more industrial mass productions are initiated.[14] Despite a huge expectation of bioavailability of (3), the present knowledge is limited. However, they are small enough to become orally available. In fact, recent clinical practice of peptides, GLP-1 analogs, a PCSK9 inhibitor, and KRAS inhibitor (all of which were developed by pharmaceutical companies), showed orally availability with appropriate formulations including permeation enhancers. We expect that more accumulation of knowledge for bioavailability (and biodistribution) of mid-sized molecules will lead us a right direction for the improvement of their drug properties. From a view of academic challenges, it is critical to build library designs and selection strategies to directly discover mid-sized molecules with higher bioavailability, which accelerates the development of mid-sized molecule for therapeutic innovation.[2]

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References and citations

1. D.T. Nguyen, T.T. Le, A.J. Rice, G.A. Hudson, W.A. van der Donk, D.A. Mitchell: Accessing Diverse Pyridine-Based Macrocyclic Peptides by a Two-Site Recognition Pathway. **J. Am. Chem. Soc.**, 144, 25, 11263-11269 (2022)
2. H. Murakami, A. Ohta, H. Ashigai, H. Suga: A highly flexible tRNA acylation method for nonnatural polypeptide synthesis. **Nature Methods** 3, 357-359 (2006)
3. Y. Yamagishi, I. Shoji, S. Miyagawa, T. Kawakami, T. Katoh, Y. Goto, H. Suga: Natural product-like macrocyclic N-methyl-peptide inhibitors against a ubiquitin ligase uncovered from a ribosome-expressed de novo library. **Chemistry & Biology** 18, 1562-1570 (2011)
4. T. Passioura, T. Katoh, Y. Goto, H. Suga: Selection-Based Discovery of Druglike Macrocyclic Peptides. **Annual Review of Biochemistry** 83, 727-752 (2014)
5. Y. Huang, M.M. Wiedmann, H. Suga: RNA Display Methods for the Discovery of Bioactive Macrocycles. **Chemical Review** 119, 10360-10391 (2018)
6. Y. Goto; H. Suga: The RaPID Platform for the Discovery of Pseudo-Natural Macrocyclic Peptides. **Acc. Chem. Res.**, 54, 3604-3617 (2021)
7. A.A. Vinogradov; M. Shimomura; Y. Goto; T. Ozaki; S. Asamizu; Y. Sugai; H. Suga; H. Onaka: Minimal lactazole scaffold for in vitro thiopeptide bioengineering. **Nature Communications,** 11, 2272 (2020)
8. Y. Goto; H. Suga: Artificial In Vitro Biosynthesis Systems for the Development of Pseudo-Natural Products. **Bull Chem Soc Japan** 91, 410-419 (2018)
9. A.A. Vinogradov; H. Suga: Introduction to Thiopeptides: Biological Activity, Biosynthesis, and Strategies for Functional Reprogramming. **Cell Chemical Biology,** 27, 1032-1051 (2020)
10. A.A. Vinogradov, Y. Zhang, K. Hamada, J.S. Chang, C. Okada, H. Nishimura, N. Terasaka, Y. Goto, K. Ogata, T. Sengoku, H. Onaka, H. Suga. De Novo Discovery of Thiopeptide Pseudo-natural Products Acting as Potent and Selective TNIK Kinase Inhibitors. *J. Am. Chem. Soc.*, **144**, 44, 20332−20341, (2022).
11. J.S. Chang, Alexander A. Vinogradov, Y. Zhang, H. Onaka, Y. Goto, H. Suga. Deep learning-enabled library design for de novo discovery of bioactive thiopeptides. *ACS Cent. Sci*. **2023**, 9, 11, 2150–2160.
12. A.A. Vinogradov; Y. Zhang; K. Hamada; S. Kobayashi; K. Ogata; T. Sengoku; Y. Goto; H. Suga: A Compact Reprogrammed Genetic Code for De Novo Discovery of Proteolytically Stable Thiopeptides. **J Am Chem Soc**, 146, 8058-8070 (2024)
13. A.A. Vinogradov, Y. Yin, H. Suga. Macrocyclic Peptides as Drug Candidates: Recent Progress and Remaining Challenges. *J. Am. Chem. Soc*., **141**, 10, 4167-4181, (2019).
14. L.J. Walport; R. Obexer; H. Suga: Strategies for transitioning macrocyclic peptides to cell-permeable drug leads. **Current Opinion in Biotechnology** 48, 242-250 (2017)