De novo discovery and structural analysis of thiopeptide pseudo-natural products acting as TNIK kinase inhibitors

Despite the decades of impressive progress in drug discovery, natural products remain a major source of new therapeutics. Natural products have evolved to modulate protein functions in the biological milieu, and their structures are optimized for high metabolic stability, cell permeability, and other pharmacological properties. Therefore, harnessing natural product-like scaffolds in drug discovery is a promising strategy to generate high-quality lead compounds for medicinal chemistry campaigns.

To this end, we have previously *in vitro* reconstituted the biosynthesis of lactazole A, a thiopeptide natural product produced by *Streptomyces lactacystinaeus* (Fig. 1(a)) [1]. As with other ribosomally synthesized and post-translationally modified peptides [2], lactazole biosynthesis commences with the transcription and translation of the structural gene, which produces a precursor peptide. The five biosynthetic enzymes then catalyze multiple post-translational modifications, which include the formation of dehydroamino acids, azole heterocycles and peptide macrocyclization, in the precursor to furnish the mature natural product. We replicated this biosynthetic logic by using an *in vitro* translation system to express the precursor peptide and converted it to lactazole A by adding the biosynthetic enzymes to the translation mixture. We further discovered that the enzymes can accept structurally diverse precursor peptides and convert them to natural product analogs [1,3], which enabled us to construct a selection platform for *de novo* discovery of pseudo-natural products with biological activities of interest [4]. The platform combines *in vitro* lactazole biosynthesis with the mRNA display technology (Fig. 1(b)). To produce a library of thiopeptides, mRNA-barcoded LazA analogs are translated and converted into pseudo-natural lactazole analogs as described above. The resulting thiopeptide library can be subjected to a pulldown against an immobilized protein target.

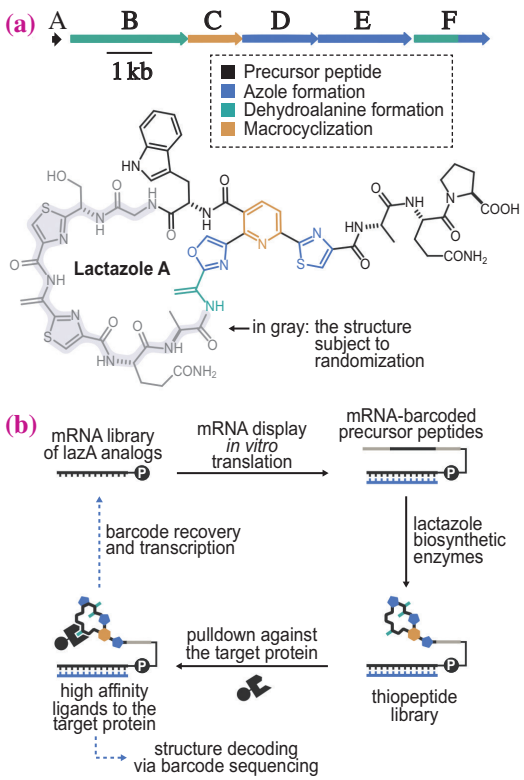

Fig. 1. **(a)** The biosynthetic gene cluster for lactazole A, the parent natural product, and its chemical structure. **(b)** An overview of the platform for the discovery of pseudo-natural product peptides with bioactivities against protein targets of choice.

In the proof-of-concept study [4], we utilized Traf2- and NCK-interacting kinase, TNIK, as the protein target. TNIK is involved in colorectal cancers and lung squamous cell carcinomas, and its inhibition is a confirmed therapeutic strategy, which makes this kinase an attractive drug target [5]. Here, we utilized the established selection platform to identify a series of thiopeptide inhibitors of TNIK, which resulted in the identification of 11 ligands with high affinities to the target protein (K_D ~ 1–60 nM). The compounds also acted as strong enzyme inhibitors, with two thiopeptides, TP1 and TP15 (Fig. 2(a)), demonstrating sub-μM IC_{50} values in a kinase inhibition assay. TP15 also underwent cell uptake, and inhibited TNIK in cell assays.

To understand the molecular basis of target engagement by the discovered compounds, we sought to conduct a structural analysis of the interactions. TNIK·TP1 and TNIK·AMPPNP·TP15 complexes were crystallized, and the X-ray diffraction data was collected at SPring-8 **BL32XU**, allowing us to solve the crystal structures to 2.1 and 2.3 Å resolution, respectively (Fig. 2(b)).

The analysis revealed that both TP1 and TP15 engage with the substrate-binding site of TNIK, and the substrate mimicry is particularly apparent for TP15. The Thr3–Ile4–Arg5 motif in TP15 resembles a prototypical TNIK substrate, and accordingly, Thr3 is positioned at the entrance to the P-site, Ile4 occupies a hydrophobic pocket that constitutes the P+1 site,

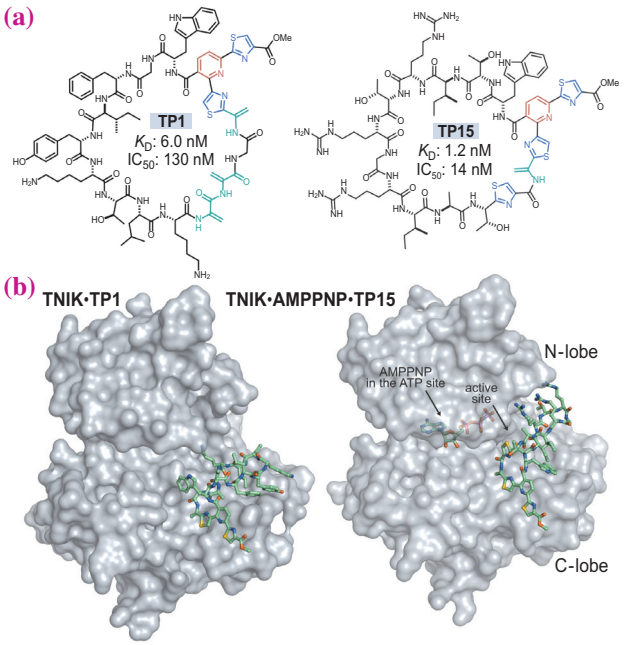

Fig. 2. Chemical structures of discovered thiopeptides **(a)** TP1 and TP15 and **(b)** their interaction with TNIK (pdb 7xzr and 7xzq).

and Arg5 makes ionic contacts with the αC-helix of TNIK (Fig. 3(a)). The phosphate transfer to the side-chain of TP15's Thr3 appears to be obstructed by the partially closed Gly-rich loop of the kinase, which dislocates the γ-phosphate of ATP and sterically occludes it from the substrate. The unique mechanism of enzyme inhibition seen in the TNIK·TP15 complex may serve as the basis for the development of a new type of peptidic kinase inhibitors.

The structures also revealed how multiple non-proteinogenic elements in TP1 and TP15 aid the thiopeptide folding. Both compounds adopt β-hairpin secondary structures featuring a β-turn facing the heteroaromatic scaffold, and the enzymatically installed post-translational modifications (azoles, dehydroalanines, and pyridines) appear to rigidify the fold. Despite similar secondary structures, TP1 and TP15 fold and interact with TNIK in distinct ways. TP15 adopts a "pyridine carbonyl out" conformation that, in combination with a nearby thiazole, helps with the antiparallel positioning of β-strands, whereas the "pyridine carbonyl in" in TP1 leads to a twisting of two adjacent dehydroalanines. As a result, although the aromatic scaffold is positioned similarly in the complexes, the relative strand orientations are inverted. In TP15, the amino acid occupying the P+1 pocket (Ile4) is located on the N-terminal strand, while in TP1, the analogous residue (Leu9) is on the C-terminal side.

Overall, the structures of TNIK·TP1 and TNIK·AMPPNP·TP15 complexes revealed how enzymatically installed non-proteinogenic elements facilitate diverse conformational landscapes of pseudo-natural thiopeptides. The obtained insights will be used to devise the next-generation selection platforms for rapid and efficient discovery of natural product-like structures for drug discovery applications.

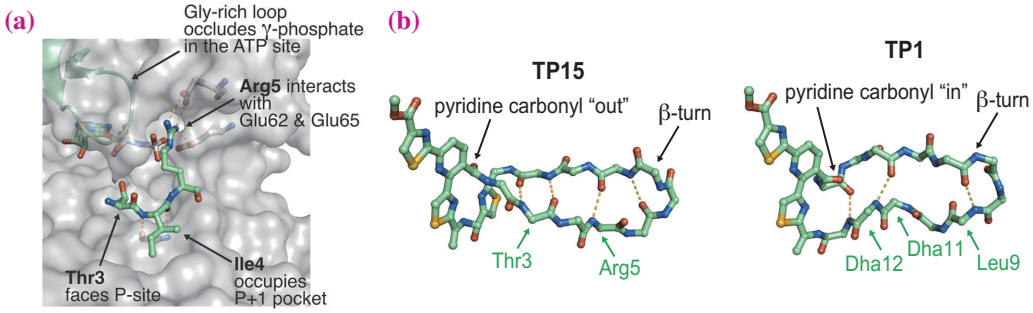

Fig. 3. **(a)** Important molecular interactions between TP15 and TNIK. Only the Thr3–Ile4–Arg5 motif of TP15 is shown. **(b)** Folding of TNIK-bound TP1 and TP15. The protein and amino acid side-chains of the thiopeptides are omitted for clarity. Dha: dehydroalanine.

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