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In vitro selection of a peptide aptamer that potentiates inhibition of cyclin-dependent kinase 2 by purvalanol

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To increase the inhibitory activity of purvalanol against cyclin-dependent kinase 2, we increased the extent of interaction between the inhibitor and the target by coupling a peptide aptamer to purvalanol. The peptide-purvalanol conjugate, selected using ribosome display, had a significantly enhanced inhibitory effect compared with purvalanol alone. The technique is useful as another type of fragment-based drug design tool.

Protein kinases play significant regulatory roles in cellular processes, and their deregulation has been implicated in cancer and a number of neurological, immunological, metabolic, and infectious diseases.[1] Thus, kinase family proteins have become one of the most intensively studied classes of drug targets, and many kinase inhibitors have emerged over the past decades.[2] So far a number of small molecules have been developed as kinase inhibitors. Most small-molecule kinase inhibitors are reversible and ATP competitive.[3] However, because the ATP-binding pocket is highly conserved in all 518 putative human kinases,[4] the discovery and development of selective kinase inhibitors is challenging.

Fragment-based drug design (FBDD) is an important tool for drug discovery and has become an established paradigm for both drug discovery companies and academic institutions.[5] The last decade has witnessed remarkably successful discovery and development of kinase inhibitors using FBDD strategies.[5a,5b,6] Recently, Meyer *et al.* expanded FBDD screening methodology by integrating FBDD with phage display.[7] They tethered a small-molecule inhibitor to the protein Jun without affecting its inhibitory activity and conjugated a phage-displayed cyclic peptide library to the protein Fos. The coiled-coil heterodimers formed by mixing the two conjugates were selected against protein kinase A. The synthesized bivalent inhibitor with the highest frequency among the isolated peptides exhibited a higher inhibitory activity and specificity than either the parent inhibitor molecule or the selected cyclic peptide.

Direct *in vitro* selection of peptides with inhibitory activity has also been reported.[8] In 2003, Li and Roberts introduced penicillin into a mRNA-display peptide library by post-translational modification and isolated peptide-drug conjugates with at least 100-fold higher activity against *Staphylococcus aureus* penicillin-binding protein 2a.[8c] This strategy, however, requires chemical reactions after *in vitro* translation and may produce unwanted by-products. Recently, tRNA carrying an unnatural amino acid has been applied for orthogonal incorporation of such amino acids into peptides.[9]

In the current study, we chose the small molecule purvalanol B (PVB), which inhibits cyclin-dependent kinase 2 (CDK2) with nanomolar IC_{50} values, as our test target because the interaction between PVB and CDK2 has been well studied, and the carboxylic acid of the 6-anilino substituent of PVB can be modified without affecting the inhibitor-kinase interaction.[10] Therefore, the carboxylic acid of PVB was coupled with a non-natural amino acid (aminophenylalanine) and the synthesized amino acid was charged to an amber suppressor tRNA for preparation of a random-sequence peptide library by *in vitro* translation. From the library we isolated a peptide-drug conjugate with higher inhibitory activity due to an increase in interaction sites (Fig. 1).

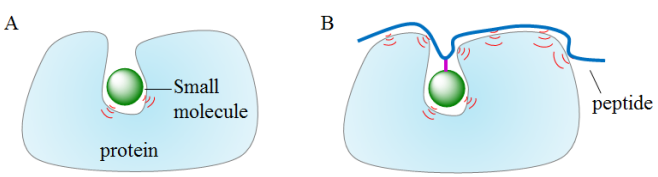

Fig. 1 Principle of enhancement of the inhibitory activity of a small molecule by a peptide. (A) Small molecule interacts with

protein. (B) Peptide-small molecule conjugate interacts with protein.

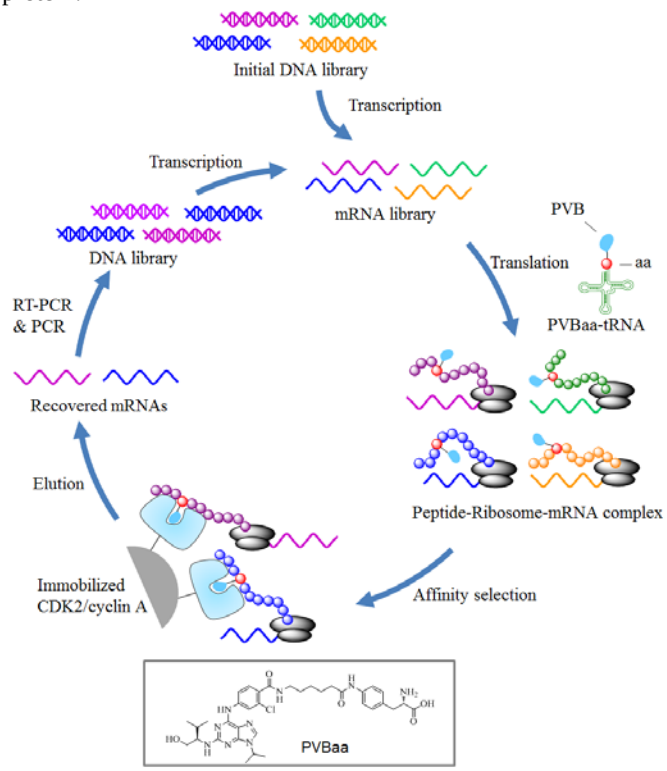

Fig 2. Schematic representation of the selection of peptide-small molecule conjugates using ribosome display. The initial DNA library encoding random sequences with an amber stop codon was transcribed into a mRNA library and subsequently translated into a peptide library using PVBaa-tRNA. The peptide-ribosome-mRNA complexes (PRMs) were formed after ribosomes were halted at ribosome arrest sequences. The PRMs were incubated with immobilized CDK2/cyclin A and the bound complexes were collected. The mRNAs of interest were recovered, reverse transcribed into DNA, and amplified to repeat the selection cycle. The chemical structure of PVBaa is shown in the gray box.

An overview of the selection system used in this study is shown in Fig. 2. We prepared a mRNA library consisting of $(NNB)_3UAG(NNB)_7$, where UAG (amber stop codon) incorporated aminophenylalanine coupled with PVB (PVBaa). *In vitro* translation was performed using PURESYSTEM Δ1 RF1 in the presence of amber suppressor tRNA carrying PVBaa. The target molecule CDK2/cyclin A was immobilized to glutathione-agarose beads and then incubated with the translated complexes (ribosome display library). The unbound complexes were washed away and EDTA was added to dissociate the peptide-ribosome-mRNA complexes and elute mRNAs. The recovered mRNAs were then reverse transcribed and amplified by PCR. The amplified DNA was used for the next round of the selection process. After six iterative rounds of selection and amplification, the enriched DNA pool was cloned and 56 clones were sequenced. Among the analyzed clones, five peptides were found at least twice (Table 1). Peptide A5 appeared most frequently (31 of 56 clones) and was chosen for further study.

Table 1 Sequences of selected and analyzed peptides

Name	Sequences[a]	Clones
A4	THNXSMVWFMC	3
A5	SKLXRFTGCSC	31
B11	SGLXRARSIVF	2
C4	SVHXRHIPILL	2
C8	FRFXSLSKSLY	2

[a] X represents the non-canonical amino acid PVBaa.

We first examined the binding of the chemically synthesized peptide A5 to the CDK2/cyclin A complex using a surface plasmon resonance (SPR) method. The CDK2/cyclin A complex bound tightly to immobilized A5 (K_d of 4 nM) (Fig. S1a) but did not bind to an immobilized control peptide (CP) in which PVBaa was replaced with phenylalanine (Fig. S1b). These SPR results indicate that PVBaa is required for binding.

Next we examined the inhibitory activity of A5 and PVB as well as CP using the fluorescence resonance energy transfer-based Z′-LYTE assay kit. A5 inhibited CDK2/cyclin A with an IC_{50} of 34 nM, while PVB was a less potent inhibitor of CDK2/cyclin A, with an IC_{50} value of 263 nM. As we expected, CP did not exhibit inhibitory activity (Fig. 3). Because PVBaa exibited nearly the same inhibitory activity as PVB (Fig. S2), the higher inhibitory activity of A5 compared with PVB arises from the peptide.

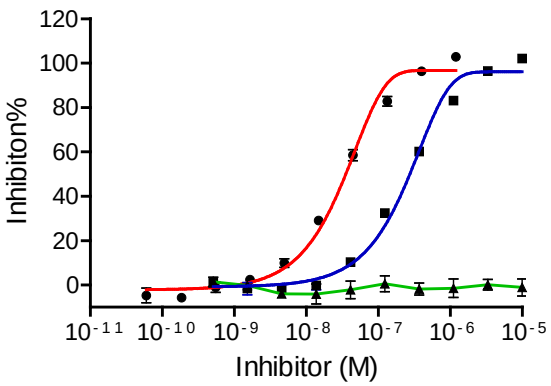

Fig. 3 Inhibitory activities of A5 (circles), PVB (squares), and CP (triangles) against CDK2/cyclin A. The results indicate that the peptide enhances the inhibitory effect of PVB against CDK2/cyclin A.

To investigate the origin of the better inhibitory activity of A5 compared with PVB, we performed a 50 ns molecular dynamics (MD) simulation of the interaction between CDK2 and A5 and between CDK2 and PVB (Fig. 4). We found interactions of Ile10, Glu81, Leu83, Asp86, and Gln131 with

PVB in both complexes (Fig. 5). A lysine residue also interacted with PVB, but the residue number was different in CDK2-PVB (Lys9) and CDK2-A5 (Lys89) (Fig. 5). Considering the CDK2-PVB (Lys89) was reported by co-crystal[10a], there is a certain limitation of MD simulation. However, as we expected (Fig. 1), additional interactions of Val7, Glu8, and Lys20 with peptide A5 were apparently observed in the CDK2-A5 complex (Fig. 5). In addition to these intermolecular interactions, we observed intramolecular interactions that stabilized the bound form of A5. These intermolecular and intramolecular interactions could account for the stronger inhibitory activity of A5 compared with PVB.

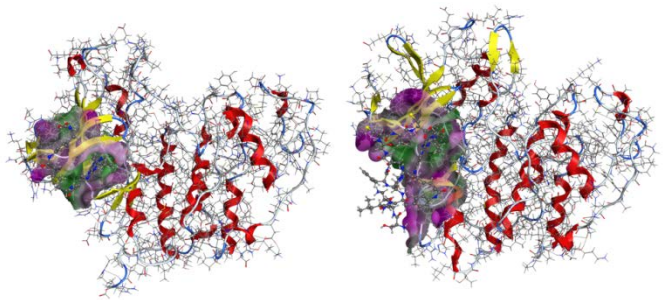

Fig. 4 Differences of binding sites in CDK2-PVB (left) and CDK2-A5 (right) complexes. The area of binding sites is shown in molecular surface mode. The area in CDK2-A5 is wider than that in CDK2-PVB, indicating that interactions in CDK2-A5 were different from those in CDK2-PVB (interactions are shown in Fig. 5).

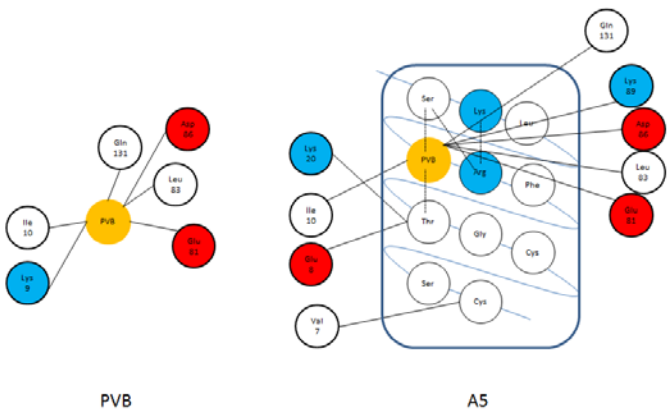

Fig. 5 Diagrammatic illustration of interactions in CDK2-PVB (left) and CDK2-A5 (right) complexes. Interactions in CDK2-A5 were slightly greater than those in CDK2-PVB. MD simulations suggested that these additional interactions induced an increase of binding affinity between the A5 peptide conjugate and CDK2/cyclin A. Red, blue, and white circles indicate acidic, basic, and other residues, respectively.

Previously, Li and Roberts considered that compounds that strongly inhibit translation, such as kinase inhibitors or GTP analogs, or side chains that might be too large to fit the exit tunnel of the ribosome may not be efficiently inserted via tRNAs carrying modified amino acids.[8c] However, we found that polyethylene glycol can be translated through the ribosome.[12] We therefore tried to incorporate PVB in the *in vitro* selection. The ribosome display used in this investigation offers a significant advantage because the ribosomally bound mRNA can be recovered by the addition of chelating agents, which destabilize the ribosomal complex.[13] This advantage and the high steric allowance of translation contribute to the usefulness of this technology.

We have used *in vitro* selection to isolate a peptide-inhibitor conjugate with improved inhibitory activity, and we assume that the specificity would also be improved if appropriate negative selections were performed. In addition, the peptide-inhibitor conjugate could be introduced into mammalian cells and delivered to its target protein using nanoparticles or conjugation with a cell-permeable peptide that does not interfere with the inhibitory effect. Unlike other fragment-based screening methods, *in vitro* selection does not depend on detailed structural information to guide optimization. The current study validates this strategy as another type of FBDD tool useful for development of peptide-drug conjugates.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [Preparation of PVBaa-tRNA; *in vitro* selection; peptide synthesis; SPR analysis, inhibitory assay and molecular dynamics simulation]. See DOI: 10.1039/c000000x/

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