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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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Materials and Methods

Preparation of Purvalanol B-C6-AF-tRNA (PVBaa-tRNA)

Purvalanol B-C6-AF-tRNA was synthesized as shown in scheme 1. Purvalanol B (16 μmol), NHS (1.5 equiv, 24 μmol) and EDC·HCl (1.5 equiv, 24 μmol) were dissolved in DMF (640 μL) on ice. After stirring for 22 h on ice, Inhibitor-succinimide ester was purified by reverse-phase HPLC (Waters XTerra C18; 2.5 μm, 4.6 mm x 20 mm) at a flow rate of 1.5 mL/min, with a linear gradient of 0%-100% acetonitrile in 0.1% trifluoroacetic acid for 10 min. Aqueous $NaHCO_3$ (1M, 30 μL) was added to a mixture of DMSO solution of the resulting purvalanol B-succinimide ester (44 mM, 40 μL), *p*-(aminocaproyl)-aminophenylalanine-pdCpA (C6-AF-pdCpA) (44 mM, 20 μL) and DMSO (510 μL). After incubation at 37 ˚C for 30 min, Purvalanol B-C6-AF-pdCpA was purified by reverse-phase HPLC (Waters XTerra C18; 2.5 μm, 4.6 mm x 20 mm) at a flow rate of 1.5 mL/min, with a linear gradient of 0%-100% acetonitrile in 0.1% trifluoroacetic acid for 10 min. The product was confirmed by MALDI-TOFMS (calculated for $[N-H]^-$ 1324.42, found 1323.66).

The aminoacylated pdCpAs were ligated to an amber suppressor tRNA derived from *Mycoplasma capricolum* Trp_1 tRNA without the 3′ dinucleotide by chemical ligation method as described.[1, 2] These aminoacyl-tRNAs can be obtained as commercially available reagents (CoverDirect tRNA reagents for site-directed protein labeling, ProteinExpress, Japan).

Construction of DNA library for ribosome display

A DNA pool was constructed to incorporate with purvalanol B into the translated peptide as reported previously.[3-5] Single-stranded DNA (ssDNA) was purchased from Operon (Japan); 5′-ATATGGCCATGCAGGCC(NNB)$_3$TAG(NNB)$_7$GGCCAGCTAGGCCAGTT-3′ where the N and B represent G, C, T, or A, and T, G or C, respectively. Double strand DNA (dsDNA) was prepared by PCR using dsDNA and Ex Taq (Takara, Japan) in the presence of a reverse primer rp-2 (5′-AACTGGCCTAGCTGGCC-3′) primer. The resulting dsDNA and 13Trx plasmid [5] were digested with *SfiI* enzyme (New England Biolabs, USA; *Sfi*I sites are underlined) and fused (DNA Ligation Kit, Takara, Japan). Finally, a double-stranded DNA library was prepared by PCR using primerfp-1 (5′-TTAATACGACTCACTATAGAACATGAGGATCACCCATGTAAAAGTCGACAA TAATTTTGTTTAACTT-3′) and primer rp-1 (5′-AAACAGCTATGACCATGATTA-3′).

Selection of peptide inhibitors against CDK2/Cyclin A

The DNA library was transcribed into mRNA using RiboMAX (Promega, USA) and purified with RNeasy® Mini kit (Qiagen, Germany). *In vitro* translation was performed using Puresystem Δ1 RF1 (Wako, Japan) in the presence of 80 pmol of PVBaa-tRNA at a final volume of 25 μL. 93.5μL of the selection buffer with Tween 20 (54 mM Tris-acetate, pH 7.5; 160 mM KCl; 54 mM magnesium acetate; 0.11% Tween 20; BSA 1.1 mg/ml) was added to 6.5 μL of translation solution. The diluted translate solution was incubated CDK2/Cyclin A (Invitrogen, USA) which was immobilized by on Glutathione agrose according to manufacturer's protocol at 4°C for 1 h. The agarose beads were collected by centrifugation and washed three times with washing buffer 1 (50 mM Tris-acetate, pH 7.5; 150 mM KCLl; 0.1 % Tween 20; 50 mM $Mg(AcO)_2$1.1mg/ml BSA) and three times with washing buffer 2 (50 mM Tris-acetate pH 7.5, 150 mM KCl, 50 mM magnesium acetate; 100 μM Purvalanol B; 0.05 % Tween 20; 1.0 mg/ml BSA) at 4°C. Then the agarose beads were incubated with elution buffer

(50 mM Tris-acetate pH7.5, 150 mM KCl, 50 mM EGTA) at 4°C for 30 min. The supernatant was collected after centrifugation and the mRNA was recovered and purified using the RNeasy® Mini kit (QIAGEN, Germany) and reverse transcribed by PrimeScript (Takara, Japan). Preparative PCR was performed to amplify the reverse transcription products using primers T7-fp-rec-1 (5′-TTAATACGACTCACTATAGAAAAGTCGACAATAATTT TGTTTAACTT-3′) and rp-fp-M13-NS (5′-AAACAGCTATGACCATGATTA-3′). The DNA product was purified by PCR Purification kit (QIAGEN, Germany) and the quality and concentration were verified by 6% PAGE and UV absorbance. The purified dsDNA was used as a template for the next round of selection.

Peptide synthesis

Synthesis of Fmoc-Purvalanol B labeled amino acid (Fmoc-PVBaa)

The preparation of Purvalanol B labeled amino acid is shown in Scheme 2. Purvalanol B (216.1 mg, 0.5 mmol, 1.0 equiv.), ***N***-Hydroxysuccinimide (NHS) (69.0 mg, 0.6 mmol, 1.2 equiv.) and 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl) (134.2 mg, 0.7 mmol, 1.4 equiv.) were dissolved in DMF (50 mL) and stirred at r.t. for 5 h. Fmoc-*p*-(aminocaproyl)-aminophenylalanine (296.0 mg, 0.57 mmol, 1.14 equiv.) was added to the above mixture and stirred at r.t. for another 3 h. The solvent was evaporated under reduce pressure and the crude residues was purified by flash chromatography on silica gel (CH_2Cl_2:CH_3OH:CH_3COOH, 50:2:1, v/v) to afford Fmoc-PVBaa (213.4 mg, 0.23 mmol, yield 46%) as white solid. The product was confirmed by MALDI-TOF mass analysis. Calculated $[M+H]^+$ 930.399, found 930.354; $[M+Na]^+$ 952.389, found 952.329, $[M+K]^+$ 968.497, found 968.637.

The peptide aptamer C5 was synthesized using the Fmoc chemical method in the RIKEN Brain Science Institute. The synthesized peptide was confirmed by MALDI-TOF mass analysis. Calculated $[M+H]^+$ 1790.8, found 1791.1.

Surface Plasmon Resonance (SPR) AnalysisSPR measurements were performed on a

Biacore T100 instrument with a CM5 sensor chip (Biacore, Sweden). All experiments were performed at 25°C with a constant flow rate of 30 μL/min. To determine the binding events, the A5 and CP peptides were immobilized on the chip surface using 10 mM sodium acetate buffer, pH 5.0. CDK2/cyclin A was injected in a running buffer (50 mM Tris-HOAc, pH 7.5, 50 mM $Mg(OAc)_2$, 150 mM KCl, 0.05% surfactant P20) over the sensor chip surface for 1.5 or 2 min; 300 μL of running buffer was then injected over the surface of the sensor chip to disassociate CDK2/cyclin A from the chip surface. The sensor chip was regenerated by injecting 10 μL of 10 mM NaOH and 10 μL of 10 mM HCl, followed by flushing with the running buffer for 5 min to stabilize the baseline. The sensorgrams were analyzed using Biacore T100 Evaluation Software (version 2.0.3, Biacore) and the curves were fitted to a 1:1 binding model.

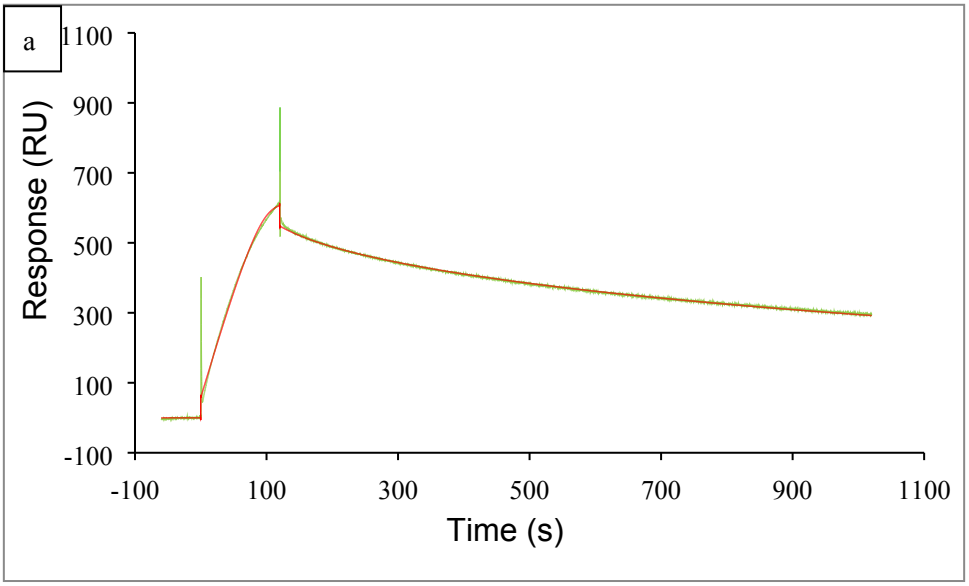
Kinase Assays

CDK IC_{50} determinations were performed using the FRET-based Z′-LYTE Ser/Thr 12 kit (Invitrogen, USA). A mixture of CDK2/cyclin A (0.68 ng/μL) and Ser/Thr 12 peptide substrate (4 μM) in Kinase Buffer A (5 μL) was introduced into a solution of test inhibitor in Kinase Buffer A with 4% DMSO (2.5 μL) in a black 384-well plate (Corning, USA). The kinase reaction was initiated by adding 2.5 μL of ATP (142 μM) in kinase buffer A; the final 10 μL kinase reaction consisted of 3.4 ng of CDK2/cyclin A, 31 μM ATP, and 2 μM Ser/Thr 12 in 50 mM HEPES, pH 7.5, 1 mM EGTA, 10 mM $MgCl_2$, 0.01% Brij 35, and 1% DMSO. A 0% phosphorylation control, 100% phosphorylation control, and 0% inhibition control were performed at the same time according to the manufacturer's protocol. After 1 h incubation at room temperature, 5 μL of a 1:2048 dilution of Development Reagent A was added and the reaction mixture was incubated at room temperature for another hour. The reaction was stopped by addition of 5 μL of Stop Reagent and the fluorescence emission signals were measured

on a Mithras LB 940 microplate reader (Berthold, Germany; excitation 405 nm, emission 460 and 535 nm). Blanks consisting of test inhibitor were subtracted from the respective emission signals. Each final percent inhibition value is the average of four independent runs. The data were plotted using GraphPad Prism 5 (GraphPad Software) and the curve was fitted to a sigmoidal dose-response (variable slope) model (R^2 = 0.9896).

Molecular Dynamic Simulation

To visualize the binding interface between CDK2 and ligand or peptide, we carried out 50ns MD simulations of CDK2-ligand (PVB) or –A5 peptide complex. The system was constructed by replacing a naturally existing CDK2-ligand complex (PDB entry 1CKP)[6] with A5 peptide. All MD simulations were carried out using a constant number of molecules, a pressure of 1 atom, and a temperature of 310 K, according to Berendsen's algorithm with a coupling time of 0.2 ps after each system had been heated to 310 K over the first 100 ps. The size of the initial system was ~110 × 90 × 110 $Å^3$, and it contained about 90,000 atoms. The time step was set at 1 fs. The bond lengths involving the hydrogen atoms were constrained to equilibrium lengths using the SHAKE method. Parm99 and gaff parameters were used for the amino acid residues and PVBaa (PVB) molecule. The electrostatic potential (ESP) of PVB residue was calculated by MP2/6-31G** level using Gaussian03[7] and the restrained electrostatic potential (RESP) charges were assigned using the ANTECHAMBER module from amber 12.[8] The particle mesh Ewald method was used, and the direct space cutoff distance was set to 12 Å. The program package that we used for the MD simulations was amber 12.



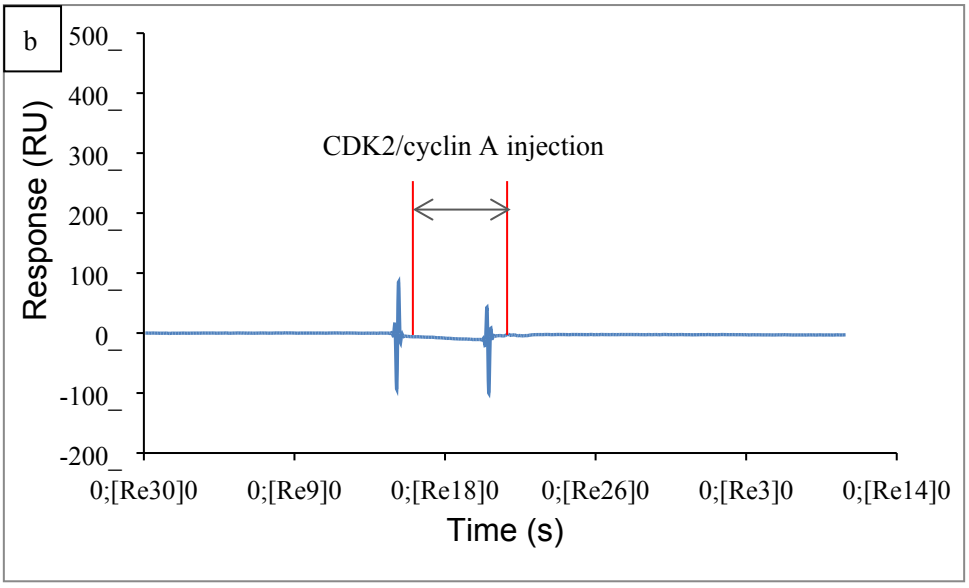


Fig. S1 SPR sensorgram of immobilized A5 (a) and CP (b) against CDK2/cyclin A. The CDK2/cyclin A was injected at a concentration of 175 nM. (a) The green line represents the observed binding and the red line represents the fitted curve using a 1:1 binding model. The association rate constant, dissociation rate constant, and equilibrium dissociation constant are k_a = 7.10 × 10^5 M^{-1} s^{-1}, k_d = 2.72 × 10^{-3} s^{-1}, and K_d = 3.83 × 10^{-9} M, respectively. (b) The results show that CP does not bind to CDK2/cyclin A.

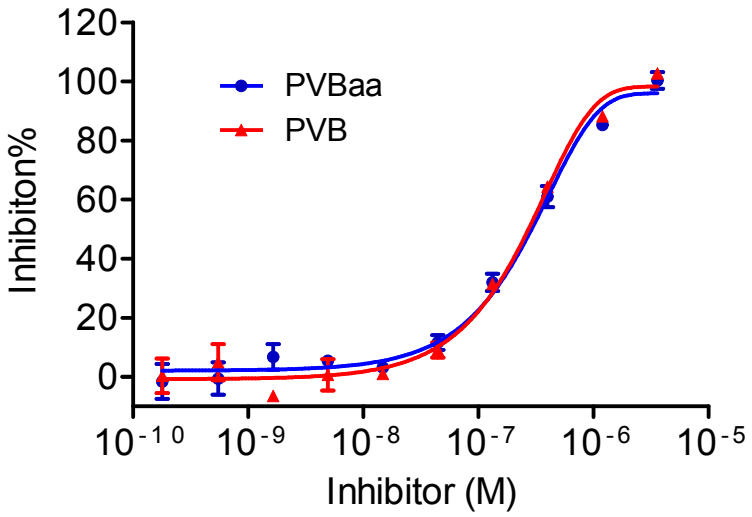
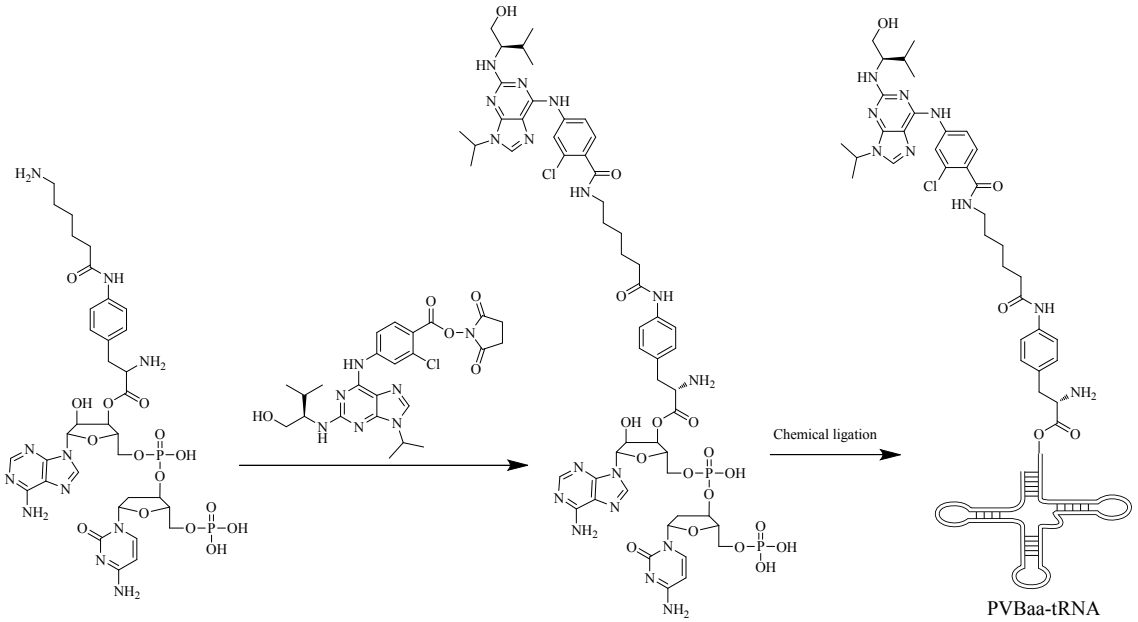
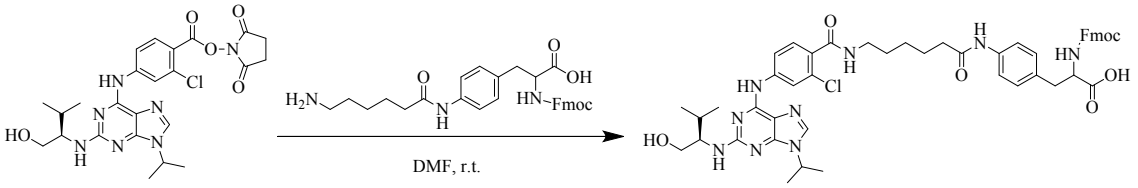


Fig. S2 Inhibitory activities of PVBaa (circles, blue line) and PVB (triangles, red line) against CDK2/cyclin A. The results indicate that PVBaa and PVB exhibit nearly the same inhibitory activity against CDK2/cyclin A.



Scheme 1 Preparation of PVBaa-tRNA



Scheme 2 Preparation of Fmoc-PVBaa.

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