

Inhibition of Ras-Effector Interaction by Cyclic Peptides

Xianghong Wu, Punit Upadhyaya, Miguel A. Villalona-Calero, Roger Briesewitz and Dehua Pei

Supplementary Information

Materials. N-Fmoc amino acids, HBTU, PyBOP, and HOBt were purchased from Advanced ChemTech (Louisville, KY), Peptides International (Louisville, KY), or NovaBiochem (La Jolla, CA). Tetrakis(triphenylphosphine)palladium, TFA, and solvents were purchased from Aldrich. ChemMatrix (100-200 mesh, loading capacity: 0.66 mmol/g) and Rink resins (0.20 mmol/g, 100-200 μm) were purchased from PCAS Biomatrix Inc. and Advanced ChemTech, respectively. Reaction progress in solid phase was monitored by ninhydrin test, whenever possible. MALDI-TOF mass analysis was performed on a Bruker Microflex MALDI-TOF instrument. The data obtained were analyzed by either Moverz software (Proteometrics LLC, Winnipeg, Canada) or Bruker Daltonics flexAnalysis 2.4 (Bruker Daltonic GmbH, Germany). Building block for X¹ was synthesized as previously described.¹

Library Synthesis: The rapalog library was synthesized on 2.0 g of aminomethyl ChemMatrix resin (100-200 mesh, loading capacity: 0.66 mmol/g). All of the manipulations were performed at room temperature unless otherwise noted. The linker sequence (ABBLM) was synthesized with 4 equiv of Fmoc-amino acids, using HBTU/HOBt/DIEA as the coupling reagents. The coupling reaction was typically allowed to proceed for 1.5 h and the beads were washed with DMF (3x) and DCM (3x). The Fmoc group was removed by treatment twice with 20% piperidine in DMF (5 + 15 min) and the beads were exhaustively washed with DMF. To spatially segregate the beads into outer and inner layers, the resin was washed with DMF and water, and soaked in water overnight. The resin was drained and suspended in a solution of Fmoc-OSu (0.40 equiv) in 10 mL of 55:45 (v/v) DCM/diethyl ether. The mixture was incubated on a rotary shaker for 30 min at room temperature. The beads were washed with 1:1 DCM/diethyl ether (3x) and DMF (8x) to remove water from the beads and then treated with excess Boc-Ala-OH/HBTU/HOBt. Next, the Fmoc group on the peptides on the outer layer was removed with 20% piperidine in DMF. To reduce the loading density of rapalogs on the bead surface, a 4:1 mixture of Ac-Val-OH and Fmoc-Glu-OAll (α -allyl ester) was coupled on the peptides on the outer layer of the bead with HBTU/HOBt and DIPEA (4, 4, and 8 equiv). Next,

D-β-homophenylalanine and the building block containing amino acid X¹ (Fig. 1 in main text) were sequentially coupled to the resin by standard Fmoc/HBTU chemistry. After that, the Boc protecting group on the inner layer was removed by 50% TFA in DCM and Boc-X¹-OH was coupled to the inner peptide using HBTU/HOBt, followed by removal of Boc as described above and coupling of Fmoc-Arg-OH on the inner layer. The random region was then synthesized by the split-and-pool method² using 4 equivalents of Fmoc-amino acids and HBTU/HOBt as the coupling agent. To differentiate isobaric amino acids during PED-MS sequencing, 4% (mol/mol) of CD₃CO₂D was added to the coupling reactions of D-Ala, D-Leu, D-Lys, D-Pro and Orn. After the synthesis of random region, the allyl group on the C-terminal Glu residue was removed by treatment with tetrakis(triphenylphosphine)palladium, triphenylphosphine and N-methylaniline (1, 3, 10 equiv, respectively) in anhydrous THF at room temperature for 45 min. Finally, after the removal of the N-terminal Fmoc group (with 20% piperidine), the surface peptides were cyclized by treating the resin with PyBOP/HOBt/ NMM (5, 5, 10 equiv, respectively) in DMF for 3 h. Side-chain deprotection was achieved by treatment with TFA/thioanisole/water/phenol and triisopropylsilane (82.5:5:5:5:2.5 v/v) for 2 h. The resulting library was washed with DCM, DMF, 5%N, N-diisopropylethylamine in DMF, 1:1 (v:v) DCM/diethyl ether, DMF, and DCM extensively and stored at -20 °C.

Library Screening: The library resin was first swollen in DCM, washed extensively with DMF, doubly distilled H₂O, and HBST blocking buffer (30 mM HEPES, pH 7.4, 150 mM NaCl, 0.05% Tween 20, 3% BSA), and incubated overnight at 4 °C in the blocking buffer. The library was then incubated in the blocking buffer containing 3 μM FKBP-EGFP and 640 nM of Texas Red-labeled GST-K-Ras for 2 h at 4 °C. The library beads were viewed under an Olympus SZX12 microscope equipped with a fluorescence illuminator (Olympus America, Center Valley, PA). The most intensely colored beads under both the RFP2 and GFP filters were manually isolated as positive hits. The positive beads were washed exhaustively and the screening procedure was repeated to ensure the validity of the hits and remove any false positives. The positive beads were sequenced by PED-MS as previously described.³

Synthesis of Individual Peptides. Each peptide was synthesized on 200 mg of Rink Resin LS (0.2 mmol g⁻¹) in a manner similar to that employed for the library synthesis except the

synthesis was started with a lysine residue followed by the coupling of Fmoc-Glu-OAll for the ring cyclization. Peptide cyclization was monitored by a ninhydrin test to detect any remaining amines. The peptides were released from the resin by reagent K, evaporated to a minimum volume under a nitrogen atmosphere, and triturated three times with cold diethyl ether. The resulting crude peptides were purified by reversed-phase HPLC on a C₁₈ column and the authenticity of the peptides was confirmed by MALDI-TOF MS analysis. Fluorescein-labeled and biotinylated peptides were prepared by treating the peptides with 3 equiv of 5-carboxyfluorescein or biotin succinimidyl ester in 100 mM sodium bicarbonate buffer (pH 8.5) and dimethyl sulfoxide (DMSO) 1:1 (v/v) mixture with a total reaction volume of 100 μL. The reaction was allowed to proceed at room temperature for 20 min and any unreacted dye or biotin ester was quenched by treatment with 5 μL of 1 M Tris-HCl buffer (pH 8.5) for 5 min. All labeled peptides were purified and characterized by MS as described above.

Fluorescence Polarization: FA titration experiment was performed by incubating 100 nM Fluorescein-labeled cyclic peptide with varying concentration (0-20 μM) of K-Ras G12V in PBS for 2 h. FA values were measured on a Molecular Devices Spectramax M5 spectrofluorimeter, with excitation and emission wavelengths at 494 and 520 nm, respectively. Equilibrium dissociation constants (K_d) were determined by plotting the fluorescence anisotropy of Fluorescein-labeled cyclic peptide as a function K-Ras concentration. The titration curves were fitted to the following equation, which assumes a 1:1 binding stoichiometry:

$$Y = (A_{\min} + (A_{\max} * Qb/Qf - A_{\min}) * ((L + x + K_d) - \sqrt{(L + x + K_d)^2 - 4 * L * x}) / 2L) / (1 + (Qb/Qf - 1) * ((L + x + K_d) - \sqrt{(L + x + K_d)^2 - 4 * L * x}) / 2L)$$

where Y is the measured anisotropy at a given concentration x of K-Ras; L is the cyclic peptide concentration; Qb/Qf is the correction factor for dye-protein interaction; A_{\max} is the maximum anisotropy when all the peptidomimetic is bound to K-Ras, while A_{\min} is the minimum when all the peptide is free; and K_d is the dissociation constant.

HTRF Assay: Recombinant HA-tagged K-Ras G12V and GST-Raf-RBD (100 nM each) were incubated with a monoclonal anti-HA labeled with d2 (2 μg/mL) and a monoclonal anti-

GST labeled with Tb (0.25 µg/mL) (Cisbio). Increasing concentrations of rapalog compounds were added in a total volume of 20 µL using a 384-well plate. The plate was incubated overnight at 4 °C and the HTRF signal was detected using a Molecular Devices Spectramax M5 plate reader.

On-Bead Assay for Trimeric Complex Formation: In a total volume of 50 µL, compounds 1-15 (100 µM) were incubated for one hour at room temperature with: A. Glutathione beads coated with GST-FKBP plus K-Ras G12V (500 nM) (Fig. S1A). B. Glutathione beads coated with GST-K-Ras G12V plus EGFP-FKBP (Fig. S1B).

On-Bead Assay for Inhibition of Ras-Effector Interaction. GST-Ras G12V and GST-Raf-RBD were immobilized on glutathione beads. Myc-tagged Ral-GDS and Tiam1 (Origene) were immobilized on Protein G beads that were coated with a monoclonal anti-Myc (Origene). Fluorescein labeled Raf-RBD (150 nM) was added to the GST-Ras G12V beads. Similarly, Texas Red labeled K-Ras G12V (150 nM) was added to the Raf-RBD, Ral-GDS and Tiam1 beads. The bead slurries were incubated in the presence or absence of compound **12** (10 µM) for one hour at room temperature, washed with buffer, and then examined under a fluorescence microscope.

1. Wu, X., Wang, L., Han, Y., Regan, N., Li, P.-K., Villalona-Calero, M., Hu, X., Briesewitz, R., and Pei, D. (2011) Creating Diverse Target-Binding Surfaces on FKBP12: Synthesis and Evaluation of a Rapamycin Analogue Library. *ACS Comb. Sci.* 13, 486-495.
2. Thakkar, A., Wavreille, A.-S., and Pei, D. (2006) Traceless capping agent for peptide sequencing by partial Edman degradation and mass spectrometry. *Anal. Chem.* 78, 5935–5939.
3. (a) Lam, K. S., Salmon, S. E., Hersh, E. M., Hruby, V. J., Kazmierski, W. M., & Knapp, R. J. (1991) A New Type of Synthetic Peptide Library for Identifying Ligand-Binding Activity. *Nature* 354, 82-84. (b) Houghten, R. A., Pinilla, C., Blondelle, S. E., Appel, J. R., Dooley, C. T., & Cuervo, J. H. (1991) Generation and use of synthetic peptide combinatorial libraries for basic research and drug discovery. *Nature* 354, 84–86. (c) Furka, A., Sebestyen, F., Asgedom, M., & Dibo, G. (1991) General method for rapid synthesis of multicomponent peptide mixtures. *Int. J. Pep. Prot. Res.* 37, 487-493.

Fig. S1. Ability of compounds **1-15** to promote a trimeric complex between K-Ras G12V to FKBP beads. Compounds **1-15** were incubated with: A. FKBP beads plus K-Ras G12V labeled with Texas Red. B. K-Ras G12V beads plus EGFP-FKBP. Binding of K-Ras G12V-Texas Red (A) and FKBP-EGFP (B) to the beads was assessed by fluorescence microscopy.

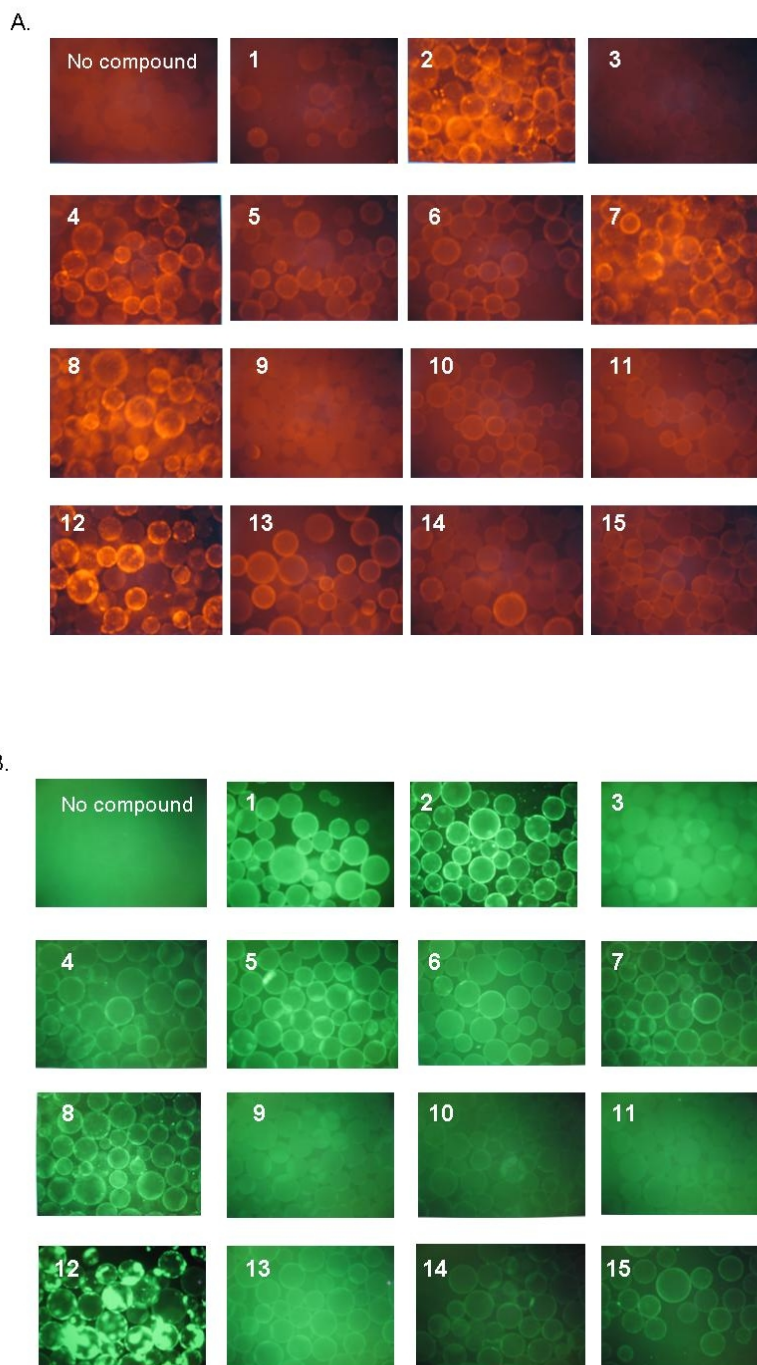


Fig. S2. Tests of compound **12** for binding to PTP1B, TC-PTP, GST-TSAD SH2, BSA, and MBP-VP35 by fluorescent anisotropy. Compound **12** showed no significant binding to any of the proteins up to 20 μM .

