Discovery of a Direct Ras Inhibitor by Screening a Combinatorial Library of Cell-Permeable Bicyclic Peptides

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Supporting Information

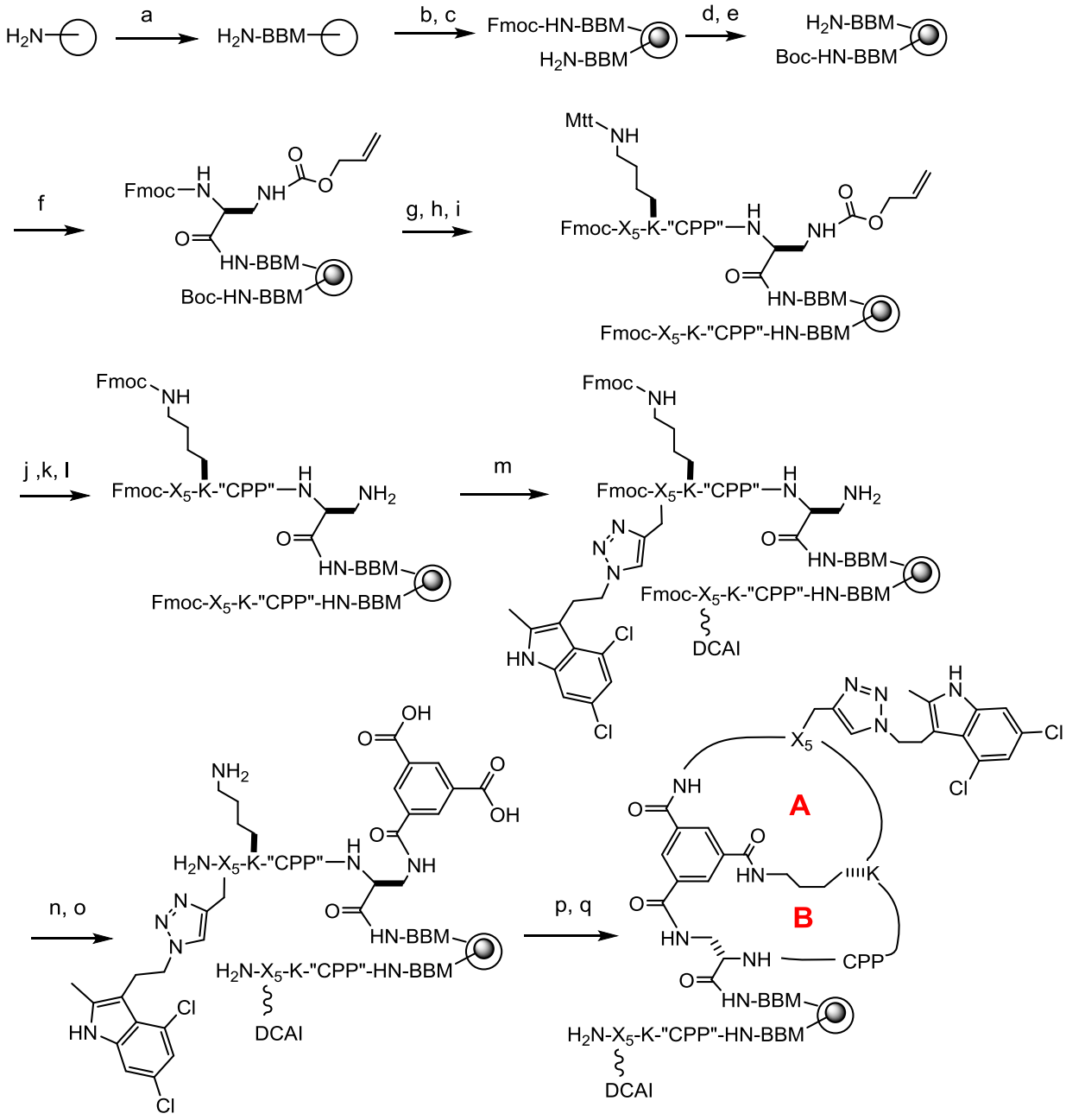
Materials. TentaGel S NH_2 resin (90 μm, 0.26 mmol/g, 2.86×10^6 beads/g) was purchased from Peptides International Inc. (Louisville, KY). All amino acids and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) were purchased from Aapptec (Louisville, KY) or Chem-Impex International (Wood Dale, IL). 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) were purchased from Aapptec. 4,6-Dichloro-2-methyl-3-aminoethylindole (DCAI) was purchased from Princeton BioMolecular Research, Inc. (Princeton, NJ) or Oakwood Chemical (West Columbia, SC). Texas Red-N-hydroxysuccinimide (NHS) was purchased from Life Technologies (Grand Island, NY). Biotin-NHS was from Chem-Impex International. Bio-Rad columns were purchased from Bio-Rad Laboratories (Hercules, CA). 5-Carboxyfluorescein oxysuccinimide ester (FAM-NHS) was purchased from Life Technologies (Grand Island, NY). Fluorescein isothiocyanate (FITC) was purchased from Sigma-Aldrich. All other chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, MO), VWR (West Chester, PA) or Thermo Scientific (Pittsburgh, PA) and used without further purification unless otherwise noted. Peptide libraries were synthesized either manually or on an Aapptec Titan 357 split-and-pool peptide synthesizer. Clean-Blot IP detection reagent (21230) and Supersignal West Femto chemiluminescent substrate kit (1856192) were purchased from Thermo Scientific. Antibodies against p-MEK1/2 (9121S), p-Akt(Thr308) (9275S), MEK1/2 (9122S), and Akt (9272S) were purchased from Cell Signaling Technology (Danvers, MA). Solvents and other chemical reagents were purchased from Sigma-Aldrich or VWR.

Purification and Labeling of K-Ras. The G12V mutant K-Ras (amino acids 1-185) was recombinantly fused to glutathione S-transferase (GST) at its N-terminus and a hemagglutinin (HA) tag (YPYDVPDYA) at its C-terminus by polymerase chain reaction. The resulting DNA fragment was ligated into prokaryotic expression vector pET-42b. *Escherichia coli* BL21(DE3) CodonPlus cells harboring the above plasmid DNA were grown in LB medium containing kanamycin (50 μg/ml) at 37 ºC until the OD_{600} reached ~0.6. Protein expression was induced by the addition of 0.1 mM of isopropyl-thio-β-D-galactoside (IPTG) for 3 h at 37 ºC. Cells were harvested by centrifugation at 5000 rpm, 4 ºC for 30 min, and the cell pellet was suspended in lysis buffer [50 mM Tris, pH 8, 500 mM NaCl, 5 mM $MgCl_2$, 0.5% Triton X-100, and 10 mM dithiothreitol (DTT)] and lysed by sonication in the presence of protease inhibitors (2 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride, 100 μg/mL soybean trypsin inhibitor). The lysate was centrifuged at 15000 rpm, 4 ºC for 30 min and the clear supernatant was loaded onto a glutathione-Sepharose column. After 30 min incubation at 4 ºC, the column was washed with 150 mL of PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) containing 10 mM DTT. GST-K-Ras was eluted with PBS containing 10 mM reduced glutathione. The protein was concentrated to ~2 mg/mL. To remove the GST tag, GST-K-Ras (~4 mg) in PBS was treated overnight with 20 units of thrombin at 4 ºC. The resulting solution was incubated with 1 mL of glutathione-sepharose resin for 30 min at 4 ºC. The suspension was

centrifuged at 3000 rpm and 4 ºC for 5 min. The supernatant was collected and concentrated by ultracentrifugation, and $MgCl_2$ and tris(2-carboxyethyl)phosphine (TCEP) were added to final concentrations of 5.0 and 1.0 mM, respectively. The protein solution was quickly frozen and stored at -80 °C until use.

Protein labeling with Texas Red or Biotin was achieved by treating the GST-K-Ras protein solution (2 mg/mL) with Texas Red-NHS (3 eq.) or biotin-NHS (2 eq.) for 3 h at 4 ºC. After that, the protein solution was quickly passed through a desalting column to remove any unreacted labeling agent. $MgCl_2$ and TCEP were added to final concentrations of 5.0 and 1.0 mM, respectively, and the protein solution was quickly frozen and stored at -80 °C until use.

Scheme S1. Synthesis of peptide library



Reagents and Conditions: (a) Standard Fmoc/HBTU chemistry; (b) soak in water; (c) 0.4 eq. of Fmoc-OSu; (d) Boc anhydride, DMF; (e) 20% piperidine; (f) Fmoc-Dap(Alloc)-OH, HATU; (g) 95:5 TFA/H_2O; (h) 20% piperidine; (i) split-and-pool synthesis by standard Fmoc/HATU chemistry; CPP = FΦR_4 or R_4ΦF; (j) 2% TFA in DCM (10x); (k) Fmoc-OSu, NMM, DMF; (l) $Pd(PPh_3)_4$; (m) click chemistry; (n) trimesic acid, HATU, NMM; (o) 2% DBU in DMF; (p) PyBOP, HOBt, NMM; (q) modified reagent K.

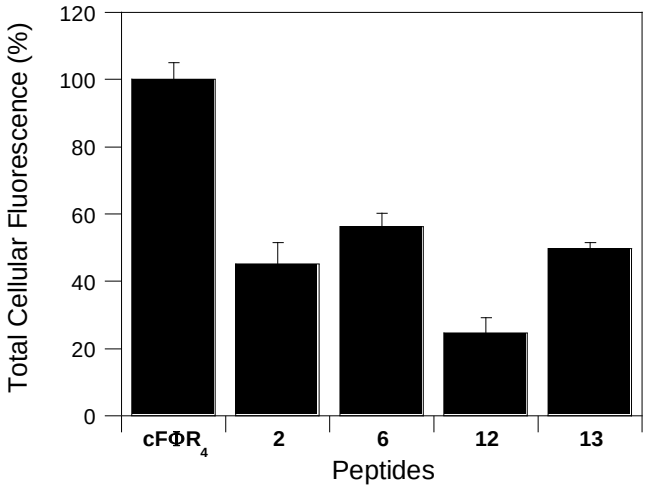


Figure S1. Comparison of the cellular uptake of bicyclic peptides **2**, **6**, **12**, and **13** derived from library screening. H1299 cells were incubated with 5 μM FITC-labeled peptides for 2 h and the total cellular uptake was determined by flow cytometry analysis. Data reported are relative to that of $cF\Phi R_4$ (100%) and represent the mean ± SD from 3 independent experiments.

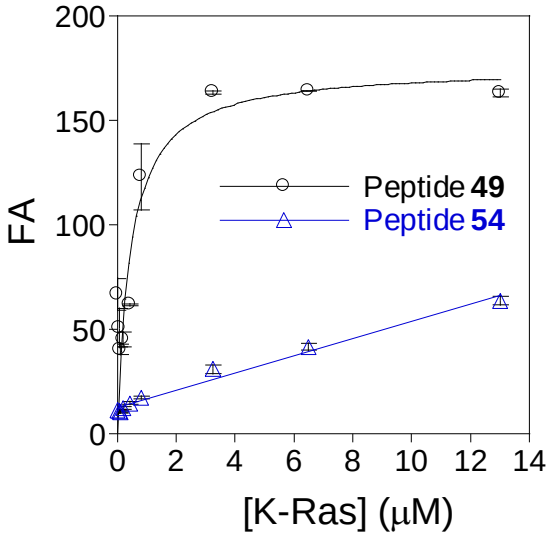


Figure S3. Binding of FITC-labeled peptides **49** and **54** (100 nM each) to recombinant K-Ras G12V as purified from *E. coli*. The K-Ras protein contained a mixture of Ras-GDP and Ras-GTP. Peptides **49** and **54** bound to the protein with apparent K_D value of 0.21 ± 0.10 and 17 ± 11 μM, respectively.

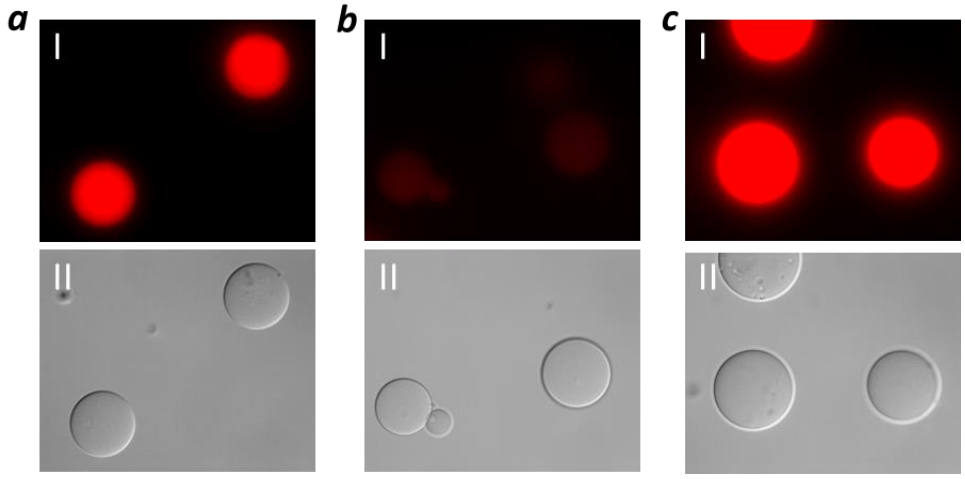


Figure S3. Effect of peptides **49** and **54** on Ras-Raf interaction. *Experimental Procedure*: Streptavidin-coated agarose beads (Sigma-Aldrich; ~1 mg) were incubated with biotinylated K-Ras G12V (500 nM) for 30 min, washed to remove unbound protein, and then incubated with Texas red-labeled GST-Raf RBD (500 nM) in the absence (***a***) or presence of 8 μM peptide **49** (***b***) or **54** (***c***) for 30 min. The bead suspension was diluted into 10 volumes of PBS (pH 7.4) and imaged in the mcherry channel (I) or under DIC (II). *Result*: Binding of GST-Raf RBD to the immobilized K-Ras rendered the beads red (***a***). The presence of 8 μM peptide **49** (but not peptide **54**) almost completely abolished the Ras-Raf interaction (***b*** and ***c***).

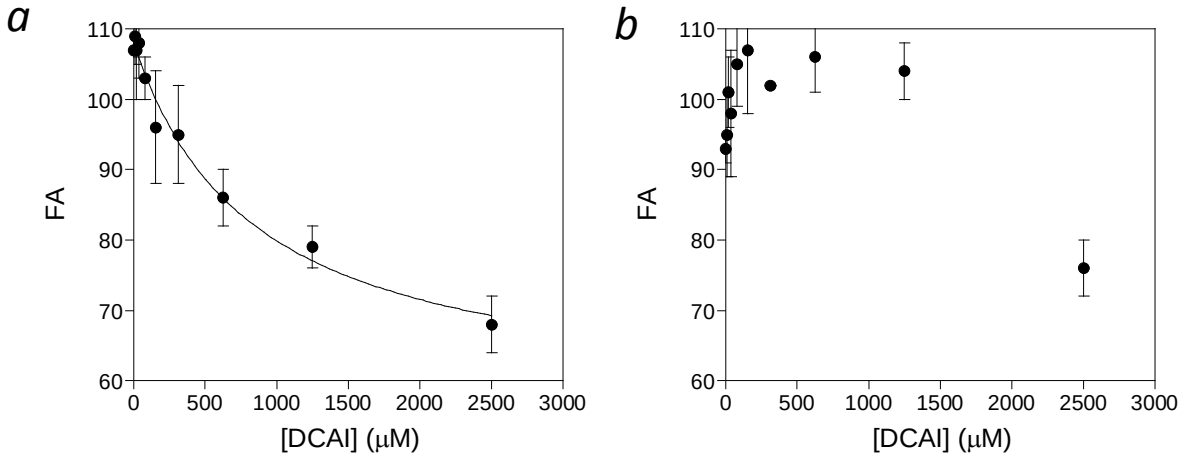


Figure S4. (***a***) Competition between peptide **49** and DCAI for binding to K-Ras G12V. K-Ras (0.2 μM) was incubated with 100 nM FITC-labeled peptide **49** in the presence of indicated concentrations of DCAI (0-2.5 mM) for 1 h at 24 °C and the FA values were recorded and plotted against the DCAI concentration. (***b***) Same as (***a***) except that FITC-labeled peptide **54** (100 nM) and 10 μM K-Ras G12V were employed.

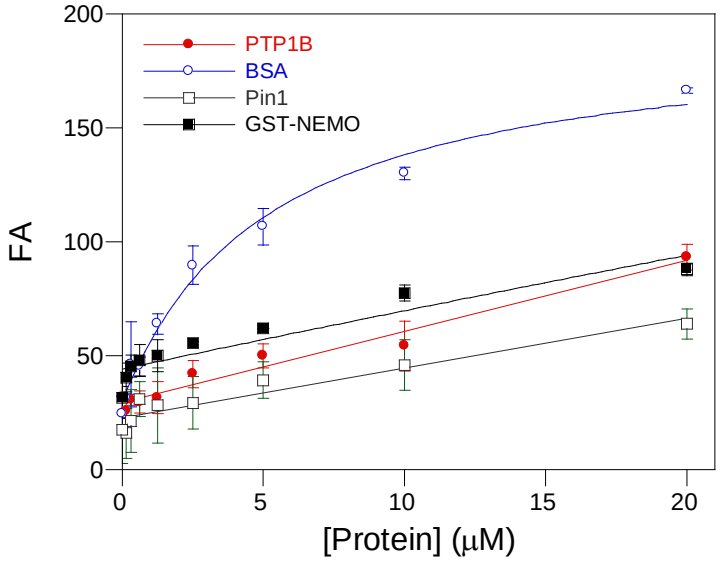


Figure S5. Binding of peptide **49** to arbitrarily selected proteins as analyzed by fluorescence anisotropy (FA). Each reaction contained 100 nM FITC-labeled peptide **49** and the indicated concentrations of protein-tyrosine phosphatase 1B (PTP1B), BSA, peptidyl-prolyl cis-trans isomerase Pin1, and glutathione-S-transferase-NFκB essential modulator (GST-NEMO) in PBS (pH 7.4) and incubated for 1 h at 24 °C prior to FA measurement.

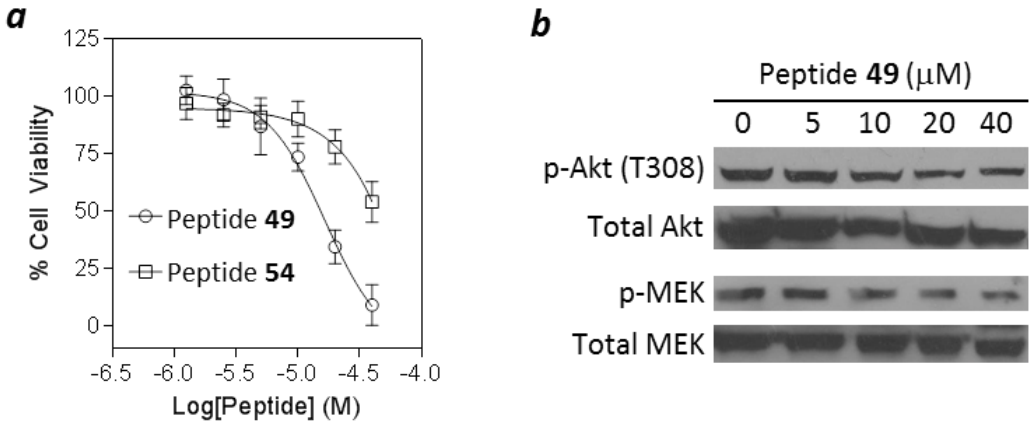


Figure S6. Effect of peptide **49** on H441 lung cancer cells. (a) MTT assay of cell viability as a function of Ras inhibitor concentration. (b) Western blots showing the dose-dependent inhibition of MEK and Akt phosphorylation in H441 cells by peptide **49**. The cells were treated with peptide **49** for 5 h and stimulated with 50 ng/mL epidermal growth factor for 10 min prior to lysis.