Published in final edited form as:

Tetrahedron. 2014 October 21; 70(42): 7714–7720. doi:10.1016/j.tet.2014.05.113.

Direct Ras Inhibitors Identified from a Structurally Rigidified Bicyclic Peptide Library

Punit Upadhyaya, Ziqing Qian, Nurlaila A. A. Habir, and Dehua Pei*

Department of Chemistry and Biochemistry, The Ohio State University, 100 West 18th Avenue, Columbus, Ohio 43210, USA

Abstract

A one-bead-two-compound (OBTC) library of structurally rigidified bicyclic peptides was chemically synthesized on TentaGel microbeads (90 μ m), with each bead displaying a unique bicyclic peptide on its surface and a linear encoding peptide of the same sequence in its interior. Screening of the library against oncogenic K-Ras G12V mutant identified two classes of Ras ligands. The class I ligands apparently bind to the effector-binding site and inhibit the Ras-Raf interaction, whereas the class II ligand appears to bind to a yet unidentified site different from the effector-binding site. These Ras ligands provide useful research tools and may be further developed into therapeutic agents.

Keywords

Bicyclic peptide; combinatorial library; inhibition; K-Ras; Protein-protein interaction

1. Introduction

The Ras family proteins are small GTP-binding proteins that play critical roles in many signaling pathways and regulate cell proliferation, differentiation, and survival. The three main family members, K-Ras, H-Ras, and N-Ras, are highly homologous in their N-terminal catalytic domains and differ mainly in the C-terminal membrane anchoring sequences.¹ While all three Ras proteins have been shown to drive cancer formation and progression, K-Ras is the most frequently mutated isoform, occurring in ~30% of human cancers. Wild-type K-Ras oscillates between an active, GTP-bound form and an inactive GDP form.^{2,3} The GTP-bound form interacts with multiple effector proteins, such as Raf, PI3K, and Ral-GDS, via its Switch I and Switch II regions. Single point mutations in K-Ras (e.g., G12V) abolish GTPase-activating protein (GAP)-mediated hydrolysis of bound GTP through steric hindrance, rendering the mutant K-Ras constitutively active and causing sustained activation of downstream effector pathways. Ample experimental data suggest that inhibition of oncogenic K-Ras, especially its interaction with effector proteins, should have therapeutic

*Corresponding author. Phone: 614-688-4068; pei.3@osu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

benefits in cancer patients.^{4,5} Unfortunately, K-Ras has been a very challenging target for small-molecule drug discovery, because its binding sites for effector proteins involve flat surfaces without any obvious pockets. As a result, most of the efforts have been focused on inhibition of signaling molecules immediately upstream and downstream of K-Ras or the posttranslational processing/membrane anchoring of K-Ras.^{6,7} Several small-molecule inhibitors have recently been reported to inhibit the nucleotide exchange activity of K-Ras, but they are generally weak inhibitors, with IC₅₀ values in the high μM to low mM range.^{8–12} Covalent inhibitors have recently been developed to selectively target the G12C mutant Ras.^{13,14} Weak peptide ligands against Ras have also been reported.^{15–17} However, despite the intense efforts during the past three decades, no effective treatment for Ras mutant tumors is yet available. In particular, compounds that bind directly to Ras and inhibit the Ras-effector interaction are lacking.

We recently discovered a cyclic peptide inhibitor against K-Ras (K_D of 0.83 μM), which blocks the interaction between K-Ras and its effector proteins Raf, PI3K, and Ral-GDS, demonstrating the feasibility of developing macrocyclic compounds as direct Ras inhibitors.¹⁸ Further development of the cyclic peptide was problematic, however, because the compound was synthetically cumbersome and its lactone moiety was susceptible to hydrolytic degradation. Meanwhile, we devised a general methodology for synthesizing and screening bicyclic peptide libraries displayed on rigid small-molecule scaffolds.¹⁹ Screening of a bicyclic peptide library against tumor necrosis factor- α (TNF α), a protein considered as “undruggable” by the small-molecule approach, identified a potent low-molecular weight TNF α antagonist. This suggests that structurally rigidified bicyclic peptides are effective for binding flat protein surfaces such as the interfaces of protein-protein interactions (PPIs). In this work, we screened the bicyclic peptide library against the K-Ras G12V mutant to identify direct Ras inhibitors as well as assess the generality of bicyclic peptides as PPI inhibitors.

2. Results and discussion

The bicyclic peptide library consisted of a random peptide sequence of 6–10 residues “wrapped” around a trimesoyl group (Fig. 1a).¹⁹ Peptide cyclization was mediated by the formation of three amide bonds between trimesic acid and the N-terminal amine, the side chain of a C-terminal L-2,3-diaminopropionic acid (Dap), and the side chain of a fixed lysine within the random region. The resulting bicyclic peptides contained 3–5 random residues in each ring and 24 different amino acids at each random position. The 24-amino acid set included 10 proteinogenic amino acids [Ala, Arg, Asp, Gln, Gly, His, Ile, Ser, Trp, and Tyr], 4 nonproteinogenic α -L-amino acids [L-4-fluorophenylalanine (Fpa), L-norleucine (Nle), L-ornithine (Orn), and L-phenylglycine (Phg)], and 10 α -D-amino acids [D-2-naphthylalanine (D-Nal), D-Ala, D-Asn, D-Glu, D-Leu, D-Lys, D-Phe, D-Pro, D-Thr, and D-Val]. The library was synthesized in the one bead-two compound (OBTC) format on spatially segregated TentaGel microbeads (90 μm ; ~ 100 pmol peptide/bead; 2.86×10^6 beads/g).^{20,21} Each bead displayed ~ 50 pmol of a unique bicyclic peptide on its surface and ~ 50 pmol of a linear peptide of the same sequence in its interior as an encoding tag (Fig. 1a). In addition, the library was synthesized in such a manner that the bicyclic peptides were attached to the solid support through a linker sequence containing a propargylglycine (Pra)

residue (for later labeling of the bicyclic peptide) and an ester bond between a β -alanine and a hydroxymethylbenzoyl (Hmb) moiety (for selective release of cyclic peptides by base hydrolysis), whereas the linear encoding peptides were attached to the support via stable amide bonds. The library has a theoretical diversity of 6.6×10^{13} ; however, the actual library size is limited to $\sim 1.4 \times 10^7$ different compounds by the amount of resin that can be conveniently handled in a research laboratory (5 g in this case).

Approximately 0.5 g of the library ($\sim 1.5 \times 10^6$ beads/compounds) was subjected to four rounds of screening against recombinant K-Ras G12V.¹⁹ To facilitate the screening experiments, the K-Ras mutant was produced as a fusion protein with glutathione-S-transferase at the N-terminus (GST-Ras) and labeled at a surface lysine residue(s) with a biotin or fluorescent dye molecule (Texas red). During the first round, the bicyclic peptide library was incubated with biotinylated GST-Ras (1.0 μ M) and streptavidin-coated magnetic particles, and the resulting magnetic beads (~ 1500 beads) were isolated from the library by magnetic sorting.^{22,23} The 1500 beads were washed and subjected to a second round of screening against the biotinylated GST-Ras (0.25 μ M) using an on-bead enzyme-linked assay involving a streptavidin-alkaline phosphatase (SA-AP) conjugate.^{24,25} Binding of GST-Ras to a positive bead recruited SA-AP to the bead surface. Subsequent incubation with the phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) produced a turquoise colored precipitate on the bead. This procedure resulted in 450 turquoise beads, which were manually isolated with a micropipette under a dissecting microscope. During the third round of screening, the turquoise beads were washed exhaustively to remove the bound proteins and dye molecules and incubated with Texas-red labeled GST-Ras (0.25 μ M). After reaching the binding equilibrium, the 200 most fluorescent beads were isolated with a micropipette with the aid of a fluorescence microscope.

Next, the 200 beads were treated with tetramethylrhodamine (TMR) azide in the presence of Cu(I), resulting in selective labeling of the bicyclic peptides at the Pra residue installed in the linker sequence (Fig. 1a). The beads were separated into individual microcentrifuge tubes (1 bead/tube) and the TMR-labeled bicyclic peptide was released from each bead by base hydrolysis of the ester linkage. After neutralization, the bicyclic peptide from each bead was tested for binding to K-Ras G12V (no GST tag) in solution by fluorescence anisotropy (FA).²⁶ Each bicyclic peptide (~ 100 nM) was incubated with 5 μ M K-Ras and the FA increase (relative to the control without K-Ras) was measured. Eight of the peptides that showed 25% FA increase (Fig. 1b) were further assayed against varying concentrations of K-Ras (0–20 μ M) to determine their dissociation constants (K_D). Six of the peptides had K_D values of 0.05 to 5 μ M (Fig. S1 in Supplementary data). Finally, beads corresponding to these 6 binding peptides were retrieved from the microcentrifuge tubes and the linear encoding peptides inside the beads were sequenced by partial Edman degradation-mass spectrometry (PED-MS).^{27,28} Unambiguous sequences were obtained for five of the beads (Table 1, No. 13, 28, 38, 82, and 105; Fig. S2). The sequence of the sixth bead/peptide (No. 123) could not be reliably determined due to poor MS spectral quality.

Bicyclic peptides 13, 28, 38, 82, and 105 were resynthesized, labeled with fluorescein isothiocyanate (FITC) at an added C-terminal Lys (Fig. 2a and S3), and analyzed for binding to K-Ras by FA. Peptides 28, 38, 82 and 105 bound to recombinant K-Ras with K_D values of

6.3, 0.49, 2.1, and 2.6 μM (Fig. 2b and Table 1) and were named as “cyclorasin B1-4” (for *cyclic ras inhibitor bicyclic*), respectively. Peptide 13 did not show significant binding to K-Ras. The discrepancy between the binding affinities derived from single-bead analysis and those determined with resynthesized and purified peptides may be caused by impurities present in the peptide samples released from the single beads (e.g., truncated peptides), which may interfere with the binding of bicyclic peptide with K-Ras. Cyclorasin B2-4 were selected for further characterization because of their relatively high potencies.

The ability of cyclorasin B2-4 to block Ras-effector interactions was first evaluated by a qualitative bead-binding assay.¹⁸ Briefly, GST-Raf was immobilized on glutathione beads and incubated with Texas red-labeled Ras protein; binding of the Ras protein to the immobilized Raf rendered the beads intensely red (Fig. 3a). However, the Ras-Raf interaction was completely abolished in the presence of 10 μM cyclorasin B3 or B4 (Fig. 3b), but not cyclorasin B2. The potency for inhibition of the Ras-Raf interaction was next determined by a homogeneous time resolved fluorescence (HTRF) assay,²⁹ giving an IC_{50} value of $\sim 1.4 \mu\text{M}$ for cyclorasin B3 (Fig. 3c). Cyclorasin B2 again showed no inhibition. The HTRF assay failed for cyclorasin B4 due to aggregation and precipitation of B4 at higher concentrations. We also examined the ability of cyclorasin B2-4 to compete with the Ras-binding domain (RBD) of Raf (GST-Raf RBD) and compound 12, the monocyclic K-Ras inhibitor we previously reported,¹⁸ for binding to K-Ras using an FA-based competition assay. Addition of GST-Raf RBD (Fig. 3d) or compound 12 (Fig. 3e) inhibited the binding of FITC-labeled cyclorasin B3 to K-Ras in a concentration-dependent manner. Compound 12 also abolished the binding of cyclorasin B4 but not B2 to K-Ras (Fig. S4). These results suggest that like compound 12, cyclorasin B3 and B4 bind to a site(s) at or near the Ras-Raf interface, whereas cyclorasin B2 binds to a site different from the Ras-Raf interface.

To assess the specificity of cyclorasin B2-4 for K-Ras, we examined the ability of cyclorasin B2-4 to self-compete for binding to K-Ras and to bind other proteins. Addition of unlabeled cyclorasin B2 inhibited the binding of FITC-labeled B2 to K-Ras in a concentration-dependent manner (Fig. S4). Likewise, unlabeled cyclorasin B4 inhibited the binding of FITC-labeled B4 to K-Ras (Fig. S4). These results further support the notion that cyclorasin B2-4 each bind to a specific site on K-Ras. To determine whether the bicyclic structure is important for binding to K-Ras, we synthesized the monocyclic and linear counterparts of cyclorasin B2 (Fig. S3) and measured their binding affinity for K-Ras. The monocyclic and linear peptides bound to K-Ras with K_D values of 7.4 and 58 μM , or 15- and 120-fold lower affinity than cyclorasin B2, respectively (Fig. 2b). Therefore, both the bicyclic structure and the amino acid sequence are required for high-affinity binding to K-Ras. To test whether the cyclic peptides have any selectivity for the signaling-active form of Ras, we prepared K-Ras specifically loaded with GTP, GDP, or GPPNP (a GTP analog). Cyclorasin B2 bound to all three Ras forms with essentially the same affinity ($K_D = 0.49, 0.64,$ and $0.76 \mu\text{M}$, respectively) (Fig. 2d). In contrast, cyclorasin B3 bound to Ras-GTP and Ras-GPPNP with approximately 8-fold higher affinity than Ras-GDP (K_D values of 1.2, 1.6, and 9.3 μM , respectively) (Fig. 2e). Finally, we tested cyclorasin B2 and B3 for binding to five arbitrarily selected proteins, including bovine serum albumin (BSA), protein-tyrosine phosphatase 1B (PTP1B), GST-SHD SH2 domain fusion protein, maltose-binding protein-XIAP BIR3

domain fusion (MBP-BIR3), and GST-FKBP12 fusion protein. Cyclorasin B2 is a selective K-Ras ligand, showing only weak binding to BSA, GST-SHD SH2, and MBP-BIR3 proteins ($K_D = 23\text{--}57\text{ }\mu\text{M}$, which are 47-120-fold higher than that of K-Ras) but no binding to PTP1B or GST-FKBP12 (Fig. S5). Cyclorasin B3 is somewhat less selective than cyclorasin B2 and bound to MBP-BIR3 and BSA with K_D values of 16 and 17 μM , respectively, and very weakly to PTP1B, GST-SHD SH2, and GST-FKBP12 (Fig. S5).

Cyclorasin B2-B4 were tested for inhibition of cell proliferation by the MTT assay.³⁰ None of the compounds showed significant effect on the proliferation of cultured cancer cells up to 50 μM concentration, due to poor membrane permeability of the cyclic peptides (as determined by confocal microscopy of cells treated with FITC-labeled peptides). When attached to an oleic acid group to improve its membrane permeability (Fig. S6),³¹ cyclorasin B3 exhibited modest anti-proliferative activity against H358 lung cancer cells (Fig. 3f). Further study with more potent compounds is needed to ascertain that the observed cell growth inhibition is a specific outcome of Ras inhibition. Conjugation of cyclorasin B2 to a fatty acid or cell-penetrating peptide (Arg₁₁) failed to confer any cellular activity.

3. Conclusion

We discovered several relatively potent ligands against K-Ras, a previously “undruggable” protein target, by screening a naïve bicyclic peptide library. Together with our earlier success against TNF α ,¹⁸ our study demonstrates that structurally rigidified bicyclic peptides are effective (and perhaps even privileged) for recognizing flat protein surfaces such as the interfaces of protein-protein interactions. Moreover, out of the five bicyclic peptides with confirmed binding to their target proteins, four (anticachexin C1 and C2 for TNF α and cyclorasin B3 and B4 for K-Ras) bind specifically to the intended PPI interfaces. This suggests that the PPI interfaces possess unique features that distinguish them from typical surfaces of globular proteins, making them “hot spots” for ligand binding during library screening. Although the current bicyclic K-Ras inhibitors have modest potency and limited membrane permeability, both properties may be greatly improved by exploring the vast structural space untapped in this work (only $\sim 1.5 \times 10^6$ out of 6.6×10^{13} possible structures were screened in this study). This may be achieved by synthesizing and screening second-generation libraries and/or conventional medicinal chemistry efforts. Cyclic peptides have been rendered membrane permeable by either N^α-methylation of their peptide bonds^{32–34} or incorporation of a minimal number of arginine residues.³⁵ Such efforts are already underway in our laboratory.

4. Experimental section

4.1. Materials

Reagents for peptide synthesis were purchased from Peptides International (Louisville, KY), NovaBiochem (La Jolla, CA), Anaspec (San Jose, CA), Chem-Impex International Inc. (Wood Dale, IL), or Aapptec (Louisville, KY). N-Hydroxysuccinimidyl biotin was purchased from Chem-Impex International (Wood Dale, IL) and N-(9-fluorenylmethoxycarbonyloxy) succinimide (Fmoc-OSu) was from Advanced ChemTech. Phenyl isothiocyanate (PITC) was purchased in 1-mL sealed ampoules from Sigma-Aldrich,

and a freshly opened ampoule was used in each experiment. Isopropyl β -D-1-thiogalactopyranoside (IPTG), ampicillin and kanamycin sulfate were also purchased from Sigma Aldrich. Dynabeads M-280 streptavidin, Texas red N-hydroxysuccinimide ester, and TMR azide were purchased from Invitrogen (Calsbad, CA). Cell proliferation kit (MTT) was purchased from Roche (Indianapolis, IN). Cell culture media, fetal bovine serum, penicillin-streptomycin, 0.25% trypsin-EDTA, DPBS (2.67 mM potassium chloride, 1.47 mM potassium phosphate monobasic, 137 mM sodium chloride, 8.06 mM sodium phosphate dibasic) were purchased from Invitrogen (Carlsbad, CA). Anti-GST-Tb and Anti-HA d2 antibodies for HTRF assay were purchased from Cisbio (Bedford, MA). Solvents and other chemical reagents were purchased from Sigma-Aldrich or VWR (West Chester, PA).

4.2. Expression and purification of K-Ras and GST-Raf RBD

The G12V mutant K-Ras (amino acids 1–185) was recombinantly fused to glutathione S-transferase at its N-terminus and an HA tag (YPYDVPDYA) at its C-terminus and expressed in *Escherichia coli* BL21 cells. The cells were grown at 37 °C in Luria broth supplemented with 0.05 mg/mL kanamycin to an OD₆₀₀ of 0.6 when protein expression was induced by addition of 0.1 M isopropyl β -D-1-thiogalactopyranoside (IPTG). After 5 h of incubation at 30 °C, the cells were harvested by centrifugation. The cell pellets were lysed in lysis buffer (40 mM Tris-HCl, 150 mM NaCl, 0.5% Triton X-100, 5 mM β -mercaptoethanol, pH 8.0) containing a protease inhibitor cocktail (1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 0.1 mM phenylmethylsulfonyl fluoride and 1 μ g/ml pepstatin A). The crude cell lysate was loaded onto a glutathione-Sepharose 4B column (GE Healthcare) and the bound GST-K-Ras was eluted with 50 mM Tris-HCl, 10 mM glutathione, pH 8.0. After buffer exchange into PBS (10 mM phosphate, 137 mM NaCl, pH 7.4), the protein was quickly frozen and stored at –80 °C. To generate K-Ras without the GST tag, the GST-K-Ras protein was treated with thrombin (GE Healthcare) for 16 h at 4 °C in PBS and purified by affinity chromatography on a glutathione-Sepharose 4B column. The Ras binding domain (RBD) of Raf was expressed as N-terminal GST fusion in *E. coli* BL21 cells. The cells were grown at 37 °C in Luria broth supplemented with 0.05 mg/mL ampicillin to an OD₆₀₀ of 0.6 when protein expression was induced by addition of 0.1 mM IPTG. GST-RBD was purified as described above for GST-K-Ras.

4.3. Protein labeling

To label GST-K-Ras with biotin, a freshly thawed Ras protein solution (50 μ M, 1 mL) was adjusted to pH 8.0 by the addition of 1 M NaHCO₃ and treated with two equivalents of N-hydroxysuccinimidyl biotin dissolved in DMSO. The reaction was allowed to proceed for 2 h at 4 °C and quenched by the addition of 500 μ L of 1 M Tris buffer (pH 8.0). The mixture was passed through a Sephadex G-25 column (which was eluted with 10 mM PBS, 150 mM NaCl, pH 7.4) to remove any free biotin. Labeling with Texas red was carried out in a similar manner.

4.4 Preparation of Ras-GDP, Ras-GTP and Ras-GPPNP

GST-K-Ras (100 μ L at 100 μ M) was loaded on to ~100 μ L of glutathione-Sepharose 4B resin and incubated for 40 min for each exchange. To prepare Ras-GTP and Ras-GDP, the

resin-bound GST-K-Ras was incubated with 20 mM EDTA plus 2 mM GTP (or GDP) at room temperature for 2 h. After that, 8 μ L of 1M MgCl_2 was added and the solution was incubated for 1 h. The resin was washed with PBS 3 times and eluted with 50 mM Tris-HCl and 10 mM glutathione (pH 8.0). The eluted protein was exchanged into PBS using a Slide-A-Lyzer Mini dialysis unit (Thermo). To prepare Ras-GPPNP, the glutathione bead-bound GST-K-Ras was incubated with 20 mM EDTA for 1 h and washed extensively with EDTA-free PBS. The resin was suspended in 100 μ L of 50 mM Tris, 0.1 mM ZnCl_2 , pH 8.0 containing 2 mM GPPNP (final concentration) and 3 units of calf intestinal alkaline phosphatase (New England Biolabs) and incubated at 4 °C overnight. After that, 8 μ L of 1 M MgCl_2 was added and following incubation for 1 h, and the resin was washed three times with PBS and eluted with 50 mM Tris-HCl and 10 mM glutathione (pH 8.0). The eluted protein was exchanged into PBS as described above. The nucleotide loading was monitored by reversed-phase HPLC under ion pairing conditions as previously described.³⁶

4.5. On-bead library screening

The peptide library (500 mg) was swollen in DCM, washed exhaustively with DMF, doubly distilled H_2O , and buffer A (30 mM sodium phosphate, pH 7.4, 150 mM NaCl, 0.05% Tween 20, and 0.1% gelatin), and incubated overnight at 4 °C in a blocking buffer (buffer A plus 3% BSA). The resin was drained and incubated in the blocking buffer containing 500 nM biotinylated GST-K-Ras for 3 h at 4 °C. The unbound protein was removed by washing with buffer A. The resin was suspended in the blocking buffer (10 mL) and 10 μ L of M280 streptavidin-coated Dynabeads was added. The mixture was incubated for 1 h at 4 °C with gentle rotary mixing and the magnetic beads were collected using a TA Dynal MPC-1 magnetic particle concentrator (Invitrogen). The positive beads were transferred into a Bio-Spin column (0.8 mL, BioRad) and incubated in 0.8 mL of the blocking buffer containing the SA-AP conjugate (1 μ g/mL final concentration) at 4 °C for 10 min. The beads were quickly washed with the blocking buffer (3×1 mL) and a staining buffer (30 mM Tris, pH 8.5, 100 mM NaCl, 5 mM MgCl_2 , 20 μ M ZnCl_2) (3×1 mL). The beads were suspended in 1 mL of the staining buffer and 100 μ L of a BCIP stock solution (5 mg/mL) was added. The mixture was incubated at room temperature with rotary mixing and intense turquoise color developed on positive beads in 25 min. The staining reaction was quenched by the addition of 1M HCl and the turquoise colored beads were manually removed with a micropipette under a dissecting microscope. After exhaustive washing with buffer A, dd H_2O , and 8 M guanidine hydrochloride to remove the bound proteins, the beads were incubated overnight at 4 °C with 200 nM Texas red-labeled GST-K-Ras in the blocking buffer in a petri dish. The beads were viewed under an Olympus SZX12 microscope equipped with a fluorescence illuminator (Olympus America, Center Valley, PA) and the most intensely colored beads were manually collected.

4.6. On-bead peptide labeling and release

Positive beads (200 beads) derived from on-bead screening were pooled, washed with water and DMF, and soaked in 60 μ L of 1:1 (v/v) water/DMF mixture. The labeling reaction was initiated by the addition of 20 μ L of freshly prepared ascorbic acid and copper sulfate solutions (each at 5 mg/mL in water) and 5 μ L of TMR azide in anhydrous DMSO (10 mM). The reaction was allowed to proceed overnight at room temperature in the dark and

terminated by extensive washing of the beads with water/DMF. The beads were transferred into individual microcentrifuge tubes (one bead/tube) and the cyclic peptide was released from each bead by treatment with 5 μL of 0.1 M NaOH solution for 4 h at room temperature in the dark. The solution was neutralized by the addition of 5.5 μL of 0.1 M HCl, transferred to a new tube, evaporated to dryness in a vacuum concentrator. The crude bicyclic peptide was dissolved in 26 μL of ddH₂O to generate a stock solution of $\sim 1 \mu\text{M}$. The beads containing linear encoding peptides were kept in the original tubes and stored at 4 °C for later PED/MS sequencing.

4.7. Fluorescence anisotropy

For FA experiments, K-Ras protein (non-GST fusion, 0–20 μM) was incubated with TMR-labeled peptide (50 or 100 nM) in 20 μL of the blocking buffer for 2.5 h at 24 °C. The FA values were measured on a Molecular Devices Spectramax M5 spectrofluorimeter, with excitation and emission wavelengths at 545 and 585 nm, respectively. Equilibrium dissociation constants (K_D) were determined by plotting the FA values as a function of K-Ras concentration and fitting the data to the equation

$$Y = \frac{\left(A_{min} + \left(A_{max} \times \frac{Q_b}{Q_f} - A_{min} \right) \left(\frac{(L+x+K_d) - \sqrt{((L+x+K_d)^2 - 4Lx)}}{2L} \right) \right)}{\left(1 + \left(\frac{Q_b}{Q_f} - 1 \right) \left(\frac{(L+x+K_d) - \sqrt{((L+x+K_d)^2 - 4Lx)}}{2L} \right) \right)}$$

where Y is the measured anisotropy at a given K-Ras concentration x; L is the peptide concentration; Q_b/Q_f is the correction fact for dye-protein interaction; A_{max} is the maximum anisotropy at saturating K-Ras concentration; and A_{min} is the minimum anisotropy.

4.8. Peptide sequencing by PED-MS

Beads containing linear encoding peptides were placed into individual wells of an AcroPrep 96-well filter plate (Pall Corporation, PN5030) with one bead per well. A solution of Fmoc-OSu (2 μmol) and PITC (100 μmol) dissolved in 25 μL of anhydrous pyridine was quickly mixed with 25 μL of 2:1 (v/v) pyridine/water containing 0.1% triethylamine and the mixture was immediately added into each well. The reaction was allowed to proceed for 6 min at room temperature and drained on a universal vacuum manifold system designed for 96-well plates (United Chemical Technologies, Inc.). The bead was washed with DCM (5 $\times$ 300 μL) and TFA (300 μL) and incubated in 100 μL of TFA (2 $\times$ 6 min). The bead was washed with DCM and pyridine (3 $\times$ 300 μL each) and PED cycle was repeated 11 times. After the final PED cycle, the N-terminal Fmoc group was removed by treatment with 300 μL of 20% piperidine in DMF for 15 min. Prior to MS analysis, the bead was treated with 100 μL of TFA containing ammonium iodide (1.0 mg) and dimethylsulfide (10 μL) for 20 min to reduce any oxidized Met. The bead was washed with water, transferred into a microcentrifuge tube, and incubated overnight in 20 μL of CNBr in 70% TFA (40 mg/mL) in the dark. The solvent was evaporated under vacuum and the released peptide was dissolved in 5 μL of 0.1% TFA in water. One μL of the peptide solution was mixed with 2 μL of saturated 4-hydroxy- α -cyanocinnamic acid in 1:1 (v/v) acetonitrile/0.1% TFA and 1

μL of the mixture was immediately spotted onto a MALDI sample plate. MS analysis was performed on a Bruker Microflex MALDI-TOF instrument and the data were analyzed by Bruker Baltonics flexAnalysis 3.3 (Bruker Daltonic GmbH, Germany).

4.9. Individual peptide synthesis

Each peptide was synthesized on Rink resin LS (0.28 mmol/g) in a manner similar to that employed for the library.¹⁸ Briefly, Fmoc-lysine(Boc)-OH was coupled onto the Rink resin to provide a side chain amine for later labeling with a fluorescent probe. Fmoc-Dap(Alloc)-OH was then added to provide a cyclization point. Coupling of the remaining residues followed standard Fmoc/HBTU chemistry, except that a Fmoc-Lys(Alloc)-OH residue was added to the sequence in between the sequences of the two rings. After removing the N-terminal Fmoc group by piperidine, the exposed N-terminal amine was acylated by treatment with an excess of trimesic acid, HATU and DIPEA (10, 3, and 20 equivalents) for 1 h. The Alloc groups on Dap and lysine side chains were removed by treatment with $\text{Pd}(\text{PPh}_3)_4$, PPh_3 and N-methylaniline (0.5, 5, and 5 equivalents) overnight. The peptide was then cyclized using PyBOP, HOBT and DIPEA (10, 10, and 20 equivalents) for 3 h. The peptide was released from the resin and deprotected by treatment with 95% TFA, 2.5% triisopropylsilane and 2.5% H_2O and purified to near homogeneity by reversed-phase HPLC. Their identity was confirmed by MALDI-TOF mass spectrometric analysis. For fluorescent labeling, peptides were dissolved in 20 μL of DMSO, 30 μL of H_2O and 5 μL of 1 M NaHCO_3 and treated with 2 equiv. of fluorescein isothiocyanate (Sigma) for 2 h and purified by reversed-phase HPLC.

4.10. HTRF assay

Recombinant HA-tagged K-Ras (no GST) and GST-Raf-RBD (50 nM each) were incubated with a monoclonal anti-HA antibody labeled with a small-molecule fluorescence acceptor d2 (2 $\mu\text{g}/\text{mL}$) and a monoclonal anti-GST antibody labeled with fluorescence donor Tb (0.25 $\mu\text{g}/\text{mL}$) (Cisbio). Increasing concentrations of cyclic peptide (0–20 μM) were added to the solutions (total volume of 20 μL) in a 384-well plate. The plate was incubated overnight at 4 $^\circ\text{C}$ and the HTRF signal was measured on a Molecular Devices Spectramax M5 plate reader. The data were fit to a dose response inhibition curve using Graphpad Prism 6.0 (Graphpad Software Inc., La Jolla, CA).

4.11. MTT Assay

MTT assays were performed with H358 lung cancer cells. One hundred μL of H1299 cells (0.5×10^5 cells/mL) were placed in each well of a 96-well culture plate and allowed to grow overnight. Varying concentrations of cyclic peptide (0–25 μM) were added to the each well and the cells were incubated at 37 $^\circ\text{C}$ with 5% CO_2 for 72 h. Ten μL of a MTT stock solution (5 mg/ml) was added into each well. The plate was incubated at 37 $^\circ\text{C}$ for 4 h. Then 100 μL of SDS-HCl solubilizing buffer was added into each well, and the resulting solution was mixed thoroughly. The plate was incubated at 37 $^\circ\text{C}$ overnight. The absorbance of the formazan product was measured at 570 nm using a Molecular Devices Spectramax M5 plate reader. Each experiment was performed in triplicates and the cells without any peptide added were treated as control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health (GM062820, CA132855, and GM062820).

References and notes

1. Rajalingam K, Schreck R, Rapp UR, Albert S. *Biochim Biophys Acta-Mol Cell Res.* 2007; 1773:1177–1195.
2. Giehl K. *Biol Chem.* 2005; 386:193–205. [PubMed: 15843165]
3. Walker K, Olson MF. *Curr Opin Genet Dev.* 2005; 15:62–68. [PubMed: 15661535]
4. Singh A, et al. *Cancer Cell.* 2009; 15:489–500. [PubMed: 19477428]
5. Gysin S, Salt M, Young A, McCormick F. *Genes Cancer.* 2011; 2:359–372. [PubMed: 21779505]
6. Wang W, Fang G, Rudolph J. *Bioorg Med Chem Lett.* 2012; 22:5766–5776.
7. Wang Y, Kaiser CE, Frett B, Li H. *J Med Chem.* 2013; 56:5219–5230. [PubMed: 23566315]
8. Taveras AG, et al. *Bioorg Med Chem.* 1997; 5:125–133. [PubMed: 9043664]
9. Maurer T, et al. *Proc Natl Acad Sci U S A.* 2012; 109:5299–5304. [PubMed: 22431598]
10. Sun Q, et al. *Angew Chem Int Ed.* 2012; 51:6140–6143.
11. Waldmann H, et al. *Angew Chem Int Ed.* 2004; 43:454–458.
12. Shima F, et al. *Proc Natl Acad Sci U S A.* 2013; 110:8182–8187. [PubMed: 23630290]
13. Ostrem JM, Peters U, Sos ML, Wells JA, Shokat KM. *Nature.* 2013; 503:548–551. [PubMed: 24256730]
14. Lim SM, Westover KD, Ficarro SB, Harrison RA, Choi HG, Pacold ME. *Angew Chem Int Ed.* 2014; 53:199–204.
15. Clark GL, et al. *Proc Natl Acad Sci U S A.* 1996; 93:1577–1581. [PubMed: 8643674]
16. Barnard D, Sun HY, Baker L, Marshall MS. *Biochem Biophys Res Commun.* 1998; 247:176–180. [PubMed: 9636675]
17. Patgiri A, Yadav KK, Arora PS, Bar-Sagi D. *Nat Chem Biol.* 2011; 7:585. [PubMed: 21765406]
18. Wu X, Upadhyaya P, Villalona-Calero MA, Briesewitz R, Pei D. *Med Chem Commun.* 2013; 4:378–382.
19. Lian W, Upadhyaya P, Rhodes CA, Liu Y, Pei D. *J Am Chem Soc.* 2013; 135:11990–11995. [PubMed: 23865589]
20. Liu R, Marik J, Lam KS. *J Am Chem Soc.* 2002; 124:7678–7680. [PubMed: 12083920]
21. Joo SH, Xiao Q, Ling Y, Gopishetty B, Pei D. *J Am Chem Soc.* 2006; 128:13000–13009. [PubMed: 17002397]
22. Kodadek T, Bachhawat-Sikder K. *Mol Biosyst.* 2006; 2:25–35. [PubMed: 16880920]
23. Hu BH, Jones MR, Messersmith PB. *Anal Chem.* 2007; 79:7275–7285. [PubMed: 17713965]
24. Lam KS, Salmon SE, Hersh EM, Hruby VJ, Kazmierski WM, Knapp RJ. *Nature.* 1991; 354:82–84. [PubMed: 1944576]
25. Sweeney MC, Wavreille AS, Park J, Butchar J, Tridandapani S, Pei D. *Biochemistry.* 2005; 44:14932–14947. [PubMed: 16274240]
26. Hintersteiner M, et al. *Chem Biol.* 2009; 16:724–735. [PubMed: 19635409]
27. Thakkar A, Wavreille AS, Pei D. *Anal Chem.* 2006; 78:5935–5939. [PubMed: 16906744]
28. Liu T, Qian Z, Xiao Q, Pei D. *ACS Comb Sci.* 2011; 13:537–546. [PubMed: 21848276]
29. Leyris JP, et al. *Anal Biochem.* 2011; 408:253–262. [PubMed: 20937574]
30. Mosmann TJ. *Immunol Methods.* 1983; 65:55–63.
31. Nelson AR, Borland L, Allbritton AL, Sims CE. *Biochemistry.* 2007; 46:14771–14781. [PubMed: 18044965]

32. Rezai T, Yu B, Millhauser GL, Jacobson MP, Lokey RS. J Am Chem Soc. 2006; 128:2510–2511. [PubMed: 16492015]
33. White TR, et al. Nat Chem Biol. 2011; 7:810–817. [PubMed: 21946276]
34. Chatterjee J, Gilon C, Hoffman A, Kessler H. Acc Chem Res. 2008; 41:1331–1342. [PubMed: 18636716]
35. Qian Z, Liu T, Liu YY, Briesewitz R, Barrios AM, Jhiang SM, Pei D. ACS Chem Biol. 2013; 8:423–431. [PubMed: 23130658]
36. John J, Sohmen R, Feuerstein J, Linke R, Wittinghofer A, Goody RS. Biochemistry. 1990; 29:6058–6065. [PubMed: 2200519]

Fig. 1.

(a) Structure of the bicyclic peptide library and selective release of bicyclic peptides from resin by base hydrolysis. Hmb, hydroxymethylbenzoyl; Pra, propargylglycine. (b) In-solution analysis of TMR-labeled bicyclic peptides released from single beads (~100 nM) for binding to K-Ras (5 μM) by fluorescence anisotropy (FA). Representative data derived from positive beads 1–50 (after the third round of screening) are shown.

Fig. 2.

(a) Structures of cyclorasin B2 and B3. (b) FA analysis of K-Ras (mixture of Ras-GTP and Ras-GDP) binding by FITC-labeled cyclorasin B2 and its monocyclic and linear counterparts. (c) FA analysis of K-Ras binding by cyclorasin B3. (d) Comparison of FITC-labeled cyclorasin B2 binding to Ras-GTP, Ras-GDP, and Ras-GPPNP. (e) Comparison of FITC-labeled cyclorasin B3 binding to Ras-GTP, Ras-GDP, and Ras-GPPNP.

Fig. 3.

Biological characterization of cyclorasin B3. (*a, b*) On-bead assay of inhibition of Ras-Raf interaction by cyclorasin B3. In the absence of inhibitor (*a*), binding of Texas red-labeled K-Ras (500 nM) to immobilized GST-Raf RBD rendered the beads red, whereas the addition of 10 μM cyclorasin B3 abolished Ras-Raf interaction (*b*). (*c*) Determination of cyclorasin B3 potency by HTRF assay. (*d*) Effect of GST-Raf RBD on FITC-cyclorasin B3 (100 nM) binding to K-Ras (3 μM). (*e*) Effect of compound 12 on FITC-cyclorasin B3 (100 nM) binding to K-Ras (3 μM). (*f*) Effect of oleiylated cyclorasin B3 on the growth rate of H358 lung cancer cells as measured by MTT assay.

Table 1

Sequences and Binding Affinities of Ras Ligands Selected from the Peptide Library

Bead No.	Peptide sequence ^a	K_D from single-bead analysis (μM)	K_D of resynthesized peptide (μM)
13	Gln-Gln-val-Asp-Lys-Fpa-phe-nal-ala-Gly-Dap	5.1 ± 1.8	NA
28	Tyr-nal-leu-Lys-ala-Gln-Ala-Gly-Ser-Dap	3.2 ± 1.6	6.8 ± 4.5
38	Trp-phe-Asp-Lys-phe-asn-His-Dap	2.6 ± 0.6	0.49 ± 0.08
82	nal-Ser-Gln-nal-Phg-Lys-phe-Arg-val-Arg-Dap	3.3 ± 1.1	2.1 ± 0.9
105	Orn-Arg-nal-Arg-Fpa-Lys-phe-glu-Gly-Dap	0.052 ± 0.020	2.6 ± 1.3

^aThe three-letter codes for L-amino acids have the first letter capitalized, whereas those of D-amino acids have all lower-case letters. NA, no significant binding activity.