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Inhibition of Ras-Effector Interaction by Cyclic Peptides

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Abstract

A combinatorial library of 6×10^6 cyclic peptides was synthesized in the one bead-two compound format, with each bead displaying a unique cyclic peptide on its surface and a linear peptide encoding tag in its interior. Screening of the library against K-Ras identified compounds that bound K-Ras with submicromolar affinity and disrupted its interaction with effector proteins.

Introduction

K-Ras is a member of the small GTP binding protein family which has over 100 members.¹ 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. Approximately 30% of all solid tumors show activating point mutations in K-Ras, rendering it insensitive to down regulation by GAP-mediated hydrolysis of bound GTP. As a result, mutant K-Ras is “frozen” in its activated form which results in constitutive signaling into proliferation and survival pathways. Transgenic mouse models have demonstrated that expression of mutated K-Ras by itself⁴ or in combination with the other oncogenic lesions^{5,6} can promote cancer. Similarly, it was shown that cancer cells undergo apoptosis if oncogenic K-Ras is down regulated by RNA interference.⁷ These data strongly suggest that inhibition of oncogenic K-Ras may have therapeutic benefits in cancer patients.

Despite its great potential for anticancer intervention, K-Ras (or H-Ras and N-Ras) has been a challenging target for small-molecule drug discovery; no effective treatment for Ras mutant tumors is available after decades of efforts by both academic and industrial researchers. Several small-molecule inhibitors have recently been reported, but they are generally weak inhibitors that target the nucleotide exchange function of Ras (with IC₅₀ values in the high μ M range).^{8–12} There have also been efforts to target oncogenic K-Ras proteins by disrupting its subcellular localization with farnesyl transferase inhibitors (FTIs).

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‡Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

However, in clinical trials FTIs have proved largely ineffective in pancreatic and other cancers, possibly because the loss of FT activity is compensated for by geranyl-geranyl transferase.¹³ Ideally, a K-Ras inhibitor should bind directly to K-Ras and disrupt its interaction with effector proteins at the Switch I/II regions. However, the largely flat interfaces between K-Ras and its effector proteins do not allow a small molecule to make sufficient interactions to achieve high affinity and specificity. To our knowledge, only two peptides derived from Raf-1 have been reported to inhibit H-Ras binding to effector proteins with an IC₅₀ values of 7 and 44 μ M.^{14,15} A synthetic peptide was reported to inhibit the SOS1-mediated nucleotide exchange of K-Ras with an IC₅₀ of 25 μ M and was active in a cell-based assay.¹⁶ Clearly, more potent and specific K-Ras inhibitors are necessary for any practical therapeutic value. Herein we report the identification of cyclic peptides, which bind to K-Ras with submicromolar affinity and block the Ras-effector interactions, from a combinatorial library.

Results and discussion

Library design and synthesis

In order to enhance the likelihood of identifying a Ras inhibitor, we chose to apply the rapamycin analogue (“rapalog”) concept for inhibition of protein-protein interactions (PPIs).¹⁷ In this approach, a bifunctional, macrocyclic molecule first binds to endogenous FK506-binding protein 12 (FKBP) to form a binary complex, which in turn binds to the target protein. The large size of the rapalog-FKBP complex establishes a steric block which may prevent PPIs at sites that are distal to the drug binding site. To generate a rapalog inhibitor against K-Ras, we synthesized a one bead-two compound (OBTC) library of rapamycin analogues on aminomethyl-ChemMatrix resin (100–200 mesh). In the library, each bead was spatially segregated into two layers^{18,19} and carried a unique pair of compounds: a macrocyclic compound (rapalog) in the surface layer and an encoding tag (a linear peptide) in the interior. The design and synthesis of the library is illustrated in Fig. 1. All library compounds contained a common Dkb-Pip-D-homoPhe moiety [where Dkb = 3,3-dimethyl-2-ketobutyryl; Pip = L-pipecolate; D-homoPhe = (*R*)-3-amino-5-phenylpentanoic acid], which provides a minimal structural unit for binding to FKBP.¹⁷ To generate an effector moiety that permits potent and specific binding to K-Ras, a random peptide sequence of 4–6 amino acid residues (X^1 – X^6 , with side chains R^1 – R^6 , respectively) was fused to the FKBP-binding moiety. In addition, an invariant glutamate was added at the C-terminus for N- to C- cyclization and to provide a handle for attachment of the macrocycle to the solid support (via its side chain). The X^1 position had 5 different building blocks (D-Ala, Leu, Val, Phe, and D-Nle), whereas the X^2 – X^6 positions were randomized with a 25-amino acid set. This set included 11 proteinogenic L-amino acids (Ala, Gly, Ser, Pro, Ile, Gln, Glu, His, Arg, Tyr, Trp), 9 D-amino acids (D-Ala, D-Val, D-Pro, D-5 Thr, D-Leu, D-Asn, D-Lys, D-Phe, D-Asp), and 5 unnatural amino acids [L-ornithine (L-Orn), L-phenylglycine (L-Phg), L-para-fluorophenylalanine (L-Fpa), D-2-naphthylalanine (D-Nal), D-norleucine (D-Nle)]. The 25-aa set was chosen to give maximum structural diversity and protease resistance while minimizing the costs (all commercially available). Since we did not know what ring size would be optimal for binding to K-Ras, we varied the ring size by cyclizing an aliquot of the library peptides after the addition of X^4 , X^5 and X^6 . The theoretical diversity of the library is >10 million compounds. The actual diversity was, however, limited by the number of beads used for the library, which was ~6 million (2 g). The inner layer of each bead also contained a linear peptide of the same sequence (X^1 – X^6) as the macrocycle on the surface. The loading of cyclic peptide in the surface layer was reduced by 5-fold to increase the stringency of library screening and minimize nonspecific binding due to avidity effects.^{20,21} The details for library synthesis are provided in Electronic Supplementary Information.

Library screening and hit identification

We chose to screen the library against the G12V K-Ras, one of the most frequently observed K-Ras mutations. Recombinant K-Ras(G12V) as a fusion protein with glutathione-S-transferase (GST) was chemically labeled with the fluorescent dye Texas Red on a lysine residue. Since GST contains many more Lys than K-Ras, the dye should be mostly on GST and thus does not affect K-Ras function. The library beads were incubated with the fluorescently labeled GST-K-Ras and recombinant FKBP fused to EGFP (enhanced green fluorescent protein) (~500 nM each). Under a fluorescent microscope, a small number of beads showed both green and red fluorescence on their surfaces, indicating that they had bound both GST-K-Ras G12V and FKBP-EGFP (Fig. 2). Note that the ChemMatrix resin is impermeable to macromolecules such as proteins, K-Ras and FKBP only have access to the macrocyclic compounds on the bead surface (no interference from the encoding peptides inside the bead). Screening of ~1 g of the library (~3 million compounds) produced 24 “hits”, which were isolated and individually sequenced by the partial Edman degradation-mass spectrometry (PED-MS) method²² to give 22 unambiguous sequences, of which 20 sequences were unique (two sequences each appeared twice, likely due to breaking of originally one bead into two smaller beads during library screening) (Table 1). Interestingly, the selected compounds were dominated by larger rings (i.e., having all six residues in the random region) and rich in aromatic (e.g., D-Nal, Trp, Tyr, Fpa, and Phg) and basic residues (e.g., Arg).

In vitro inhibition of K-Ras function

Compounds **1–15** (Table 1) were resynthesized on a larger scale (~10 mg each) individually and tested for their ability to bind to K-Ras G12V and FKBP simultaneously using a bead binding assay. In this assay, GST-FKBP was immobilized to glutathione beads. Recombinant K-Ras G12V, which was fluorescently labeled with Texas Red, was added to the beads. By itself, K-Ras G12V did not bind to the FKBP beads. However, K-Ras G12V was recruited to the bead surface in the presence of the compounds (except for compounds **3** and **9**) as detected by fluorescence microscopy (Figure S1 in Supplementary Data). The specificity of the observed binding was supported by the complementary experiment in which GST-K-Ras G12V was immobilized on beads and FKBP-EGFP in solution was recruited to the beads when compounds were added. These results suggest that most of the identified compounds can simultaneously bind K-Ras G12V and FKBP to form a trimeric complex.

We subsequently tested the compounds for their ability to inhibit the Ras-Raf interaction using an assay based on homogeneous time-resolved fluorescence (HTRF).²³ Raf is a key K-Ras effector protein that activates the MAPK pathway. The Ras binding domain of Raf (GST-RBD) was incubated with HA-tagged G12V K-Ras. Fluorescently labeled anti-GST and anti-HA antibodies were added to the solution. The binding between GST-RBD and K-Ras brings the anti-GST and anti-HA antibodies into proximity, which can be detected by the HTRF measurement. Surprisingly, the addition of increasing concentrations of many of the rapalogs resulted in concentration-dependent decrease in the HTRF signal, even in the absence of FKBP (Fig. 3). This finding suggests that the active rapalogs can bind to the Ras-Raf interface and disrupt the Ras-Raf interaction independent of FKBP. A plot of the HTRF signal against rapalog concentration produced a titration curve, from which the IC₅₀ value was estimated. Compounds **5** and **12** were most potent in this assay, with IC₅₀ values of 0.20 and 0.70 μ M, respectively.

Compounds **5** and **12** were further characterized by labeling with fluorescein at the side chain of the invariant Glu/Gln residue (Fig. 4a) and determining their binding affinity to K-Ras by fluorescence polarization. Compound **12** bound to K-Ras with a K_D value of 0.83

μM (Fig. 4b). Binding of K-Ras to the immobilized compound **12** by surface plasmon resonance gave a similar K_D value. In addition, unlabeled compound **12** competed with the dye-labeled **12** for binding to K-Ras in a concentration-dependent manner (by fluorescence polarization), suggesting that compound **12** binds to a specific site on K-Ras. When tested against five arbitrarily chosen control proteins (bovine serum albumin, protein tyrosine phosphatase 1B and TCPTP, the GST fusion protein of TSAD SH2 domain, and the maltose-binding protein fused with a viral coat protein VP35), compound **12** showed no significant binding to any of the proteins up to $20 \mu\text{M}$ concentration (Figure S2 in Supplementary Data), demonstrating that compound **12** is a selective K-Ras ligand. To our surprise, compound **5** failed to bind to K-Ras in this assay, the reason of which is not yet clear, although it is possible that labeling of the compound with a large, hydrophobic dye molecule may have altered the conformation of compound **5**.

Finally, compound **12** was tested for inhibition of K-Ras G12V-binding to effector proteins, Raf, Ral-GDS, and Tiam1 using a bead-binding assay. GST-RBD of Raf was immobilized to glutathione beads, whereas Myc-tagged Ral-GDS or Tiam1 were bound to protein G beads that had been coated with an anti-Myc antibody. The protein-loaded beads were incubated with fluorescently labeled K-Ras G12V ($0.15 \mu\text{M}$) in the absence and presence of compound **12** for 60 min, washed with buffer, and examined under a fluorescence microscope. As shown in Fig. 5, K-Ras G12V bound to the beads with immobilized Raf, Ral-GDS, or Tiam1 in the absence of compound **12** (DMSO control). However, addition of $10 \mu\text{M}$ compound **12** completely abolished the interaction between K-Ras G12V and the effector proteins. Analysis of the Ras-Raf interaction in the reverse order (i.e., fluorescein-labeled Raf binding to beads immobilized with K-Ras G12V) confirmed the inhibitory activity of compound **12**.

Cellular activity

We have also carried out some preliminary cellular assays. Singh et al. used RNA interference to down regulate mutant K-RAS in a large number of human cancer cell lines.⁷ They reported that the H358 cell line is sensitive to losing mutant K-Ras whereas the A549 cell line is not (both are lung cancer cell lines). In these and other cell lines with mutant K-Ras, we tested the effect of compound **12** on the phosphorylation of Mek and Erk, two downstream effectors of Raf which are expected to be dephosphorylated if the Ras-Raf interaction is disrupted. Incubating cells with compound **12** for up to 16 h did not lead to a decrease in Mek or Erk phosphorylation. The lack of cellular activity is likely due to poor cell penetration of compound **12**.

Conclusions

We were able to identify cyclic peptides that disrupt the interaction between K-Ras and its effector proteins. This study thus demonstrates that it is possible to develop potent small-molecule inhibitors (at least cyclic peptides) of Raf-Ras interaction. Since compound **12** inhibits K-Ras in an FKBP-independent manner, it should be possible to replace the FKBP-binding moiety with random sequences and screen the resulting library against K-Ras to discover more potent K-Ras inhibitors. It should also be possible to identify compounds that bind to K-Ras in the FKBP-dependent manner. Both types of studies are currently underway in our laboratories.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Synthesis of the rapalog library. Reagents and conditions: a) standard Fmoc/HBTU chemistry; b) soak in water; c) 0.5 equiv Fmoc-OSu in Et₂O/DCM; d) Boc-Ala-OH, HBTU; e) 20% piperidine in DMF; f) 1:4 Fmoc-Glu-OAlI/Ac-Val-OH, HBTU; g) Fmoc-D-homoPhe-OH, HBTU; h) building block for X¹, HBTU; i) 50% TFA in DCM; j) Boc-X¹-OH, HBTU; k) Fmoc-Arg(Pbf)-OH, HBTU; l) split-and-pool synthesis by Fmoc/HBTU chemistry; m) Pd(PPh₃)₄; n) PyBOP, HOBT; and (o) TFA, TIPS.

Fig. 2. Representative positive beads, viewed under a GFP (left) or a red filter (right) using a confocal microscope. Because the protein molecules were too large to diffuse into the beads, they formed a fluorescent “shell” around each positive bead.

Fig. 3. Inhibition of the interaction between HA-tagged K-Ras (G12V) and GST-RBD using HTRF assay. A) A representative titration curve with compound **12**. B) IC₅₀ values for compounds **1–15** as determined by the HTRF assay.

Fig. 4. (a) Structure of compound **12**. (b) Binding of compound **12** to K-Ras as determined by fluorescence anisotropy (FA) assay.

Fig. 5.

Inhibition of K-Ras G12V interaction with Raf, Ral-GDS, and Tiam1 by compound **12**. Top panel, binding of fluorescein-labelled Raf or GST-Ras (0.15 μ M) to beads immobilized with GST-Ras, GST-Raf, or Myc-tagged Ral-GDS and Tiam1. Bottom panel, same as above but the dye-labelled proteins were incubated with the beads in the presence of 10 μ M compound **12**.

Table 1

Sequences of K-Ras-binding peptides

#	X ¹	X ²	X ³	X ⁴	X ⁵	X ⁶
1	Val	D-Asn	Arg	Arg	Fpa	Tyr
2	D-Nle	D-Nal	D-Nal	D-Lys	Arg	Fpa
3	Phe	Trp	D-Lys			
4	Phe	Arg	Gly	Tyr	D-Nal	Om
5	Leu	Arg	Arg	Tyr	Fpa	Arg
6	Phe	Arg	Tyr	Pro	Tyr	D-Phe
7	Phe	Phg	Fpa	Arg	Ala	
8	Thr	D-Nal	D-Ala	Arg	Tyr	Arg
9	Val	Phg	D-Thr	Pro	Glu	Ile
10	Phe	Arg	Ser	Tyr	D-Lys	
11	Leu	Glu	Phg	D-Phe	Om	Pro
12	D-Nle	Fpa	Arg	D-Nal	Arg	Arg
13	Phe	Arg	D-Nal	Arg	D-Phe	Arg
14	Leu	D-Nal	Arg	D-Val	Arg	Ala
15	Phe	Ala	Tyr	Arg	D-Thr	Gly
16	D-Ala	Om	D-Val	Gly	D-Nal	Arg
17	Phe	Arg	Tyr	Arg	D-Nal	Arg
18	D-Ala	Arg	Fpa	Trp	Phg	Ser
19	D-Ala	D-Nal	D-Nal	Arg	H	Tyr
20	Phe	D-Nal	Arg	Phg	Ser	Gly