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Direct Ras inhibitors identified from a structurally rigidified bicyclic peptide library

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ABSTRACT

A one-bead-two-compound (OBTC) library of structurally rigidified bicyclic peptides was chemically synthesized on TentaGel microbeads (90 μm), 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.

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 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 IC_{50} 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-alpha (TNFα), a protein considered as 'undruggable' by the small-molecule approach, identified a potent low-molecular weight TNFα 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

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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], four 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 ~1.4×10^7 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 (~1.5×10^6 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 (0.5 μM) 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 μM)

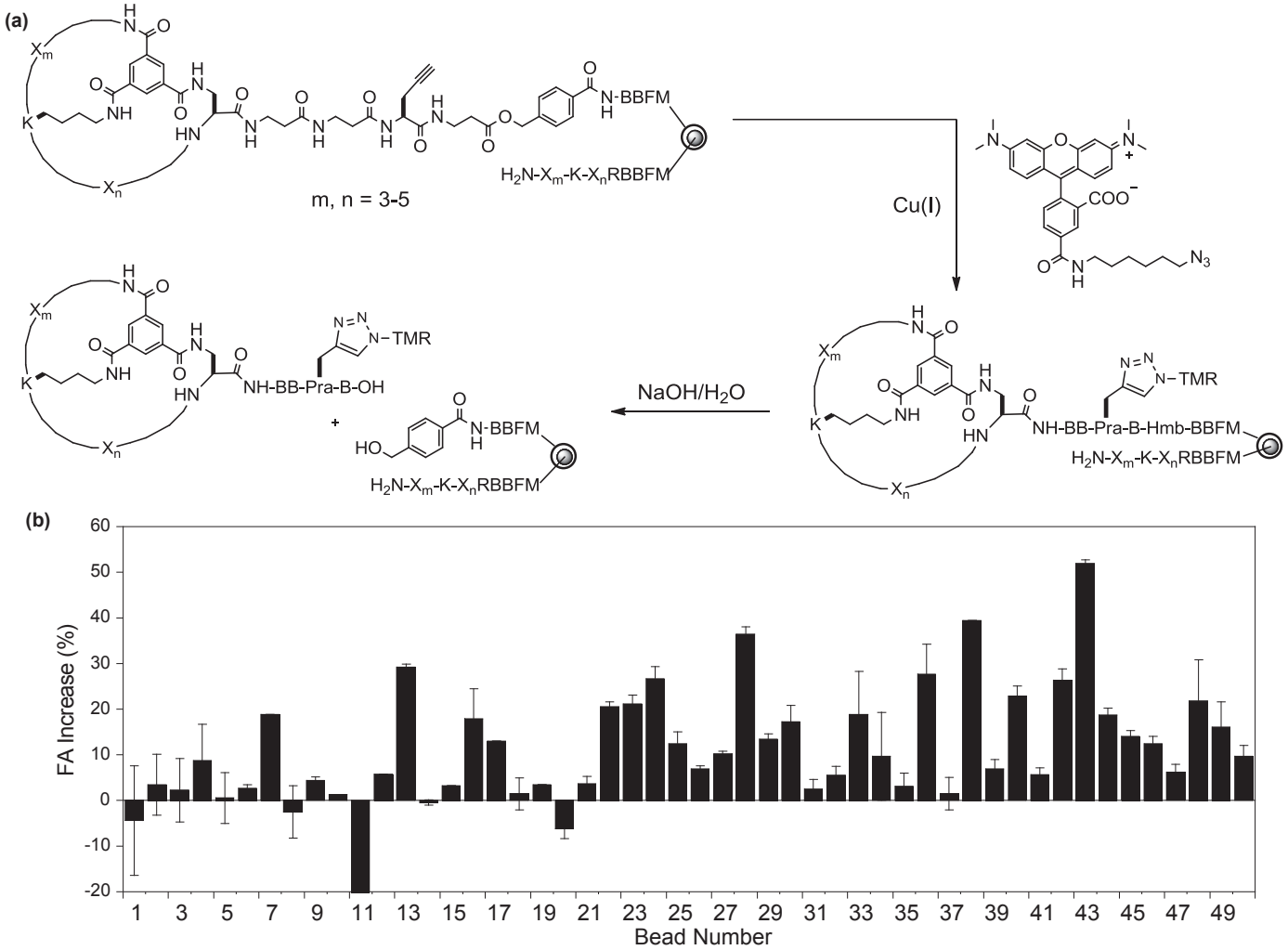

Fig. 1. (a) Structure of the bicyclic peptide library and selective release of bicyclic peptides from resin by base hydrolysis. Hmb, hydroxymethylbenzoyl; Pra, propargylglycine. (b) In-solution analysis of TMR-labeled bicyclic peptides released from single beads (~100 nM) for binding to K-Ras (5 μM)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