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K-Ras(G12D)-selective inhibitory peptides generated by random peptide T7 phage display technology

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ABSTRACT

Amino-acid mutations of Gly^{12} (*e.g.* G12D, G12V, G12C) of V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (K-Ras), the most promising drug target in cancer therapy, are major growth drivers in various cancers. Although over 30 years have passed since the discovery of these mutations in most cancer patients, effective mutated K-Ras inhibitors have not been marketed. Here, we report novel and selective inhibitory peptides to K-Ras(G12D). We screened random peptide libraries displayed on T7 phage against purified recombinant K-Ras(G12D), with thorough subtraction of phages bound to wild-type K-Ras, and obtained KRpep-2 (Ac-RRCPLYISYDPVCRR-NH_2) as a consensus sequence. KRpep-2 showed more than 10-fold binding- and inhibition-selectivity to K-Ras(G12D), both in SPR analysis and GDP/GTP exchange enzyme assay. K_D and IC_{50} values were 51 and 8.9 nM, respectively. After subsequent sequence optimization, we successfully generated KRpep-2d (Ac-RRRRCPLYISYDPVCRRRR-NH_2) that inhibited enzyme activity of K-Ras(G12D) with IC_{50} = 1.6 nM and significantly suppressed ERK-phosphorylation, downstream of K-Ras(G12D), along with A427 cancer cell proliferation at 30 μM peptide concentration. To our knowledge, this is the first report of a K-Ras(G12D)-selective inhibitor, contributing to the development and study of K-Ras(G12D)-targeting drugs.

1. Introduction

Somatic mutations in small GTPase Ras drive neoplasia in various cancers. The K-Ras isoform is most frequently mutated in 86% of Ras-driven cancers [1], with 83% of K-Ras amino-acid mutations at residue Gly^{12} where G12D is the major substitution [2]. Therefore, development of anti-cancer drugs targeting mutated K-Ras will benefit several patients.

Despite being a promising drug target for cancer therapeutics, effective drugs targeting mutated K-Ras have not been marketed [3]. K-Ras remains a challenging target, and the generation of direct inhibitors remains difficult, because the K-Ras molecular surface is round and has less druggable pockets for conventional small molecules; furthermore, no allosteric regulatory sites have been reported to date [4]. Moreover, K-Ras changes its structure in the presence/absence of GDP or GTP, and binding affinities between K-Ras and GDP/GTP are too strong (picomolar affinity) to be inhibited by small molecules [5].

In this context, some direct K-Ras inhibitors, based on novel approaches, were reported, such as covalent inhibitors or peptide inhibitors [6]. The former strategy involves an irreversible binding to Cys^{12} of K-Ras(G12C). For example, Ostrem et al. screened 480 disulfide-fragment compounds by protein mass spectrometry and identified several fragments that react with the G12C mutant but not with the wild-type (WT) K-Ras, in the presence of GDP [7]. The latter strategy involves using a peptide alternative to small molecule compounds. Patgiri et al. extracted a K-Ras-binding sequence from son of sevenless 1 (SOS1), which catalyzes the transition of K-Ras/GDP (inactive-form) to K-Ras/GTP (active-form), and stabilizes its alpha-helix structure through a hydrocarbo-staple method to inhibit protein-protein interactions (PPIs) between K-Ras and SOS proteins [8]. The SAH-SOS1 peptide bound to both WT K-Ras and mutants with equal affinity, and the binding activity was not

Abbreviations: K-Ras, V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; SOS, son of sevenless; PPI, Protein-protein interaction.

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dependent on the presence of GDP or GTP. Instead of using natural protein sequences, Pei et al. identified artificial cyclic peptide inhibitors from a random peptide displayed beads library [9–11]. They prepared recombinant K-Ras(G12V) as a fusion protein with glutathione S-transferase (GST), and introduced a chemical label to GST *via* fluorescent dye Texas Red on a Lys. By using this fluorescent labeled K-Ras mutant, 6×10^6 various cyclic peptides were screened, and sequences binding K-Ras with submicromolar affinity were identified.

These approaches successfully generated K-Ras inhibitors. However, Cys-reactive small molecules present concerns regarding undesirable side effects due to their potential for promiscuous inhibition. Moreover, the aforementioned peptide inhibitors did not display sufficient inhibition activities and showed poor selectivity toward mutated K-Ras. In this study, we focused on K-Ras(G12D) as the target molecule, since G12D is the most common substitution in many K-Ras-driven cancers and the side-chain structure/size of Asp has greater potential for selectivity compared to other substitutions such as Cys (G12C) or Val (G12V). We screened random peptide libraries displayed on T7 phage against recombinant K-Ras(G12D) in GDP states. By using phage display, we can screen 10^{11} distinct clones, which is much greater than that included in Pei et al.'s aforementioned peptide beads library. Furthermore, we thoroughly subtracted phages bound to WT K-Ras in the phage panning process. As a result, we successfully discovered K-Ras(G12D)-selective inhibitory peptides. Here, we demonstrate the notable selectivity and inhibition activities of the peptides to K-Ras(G12D) through cell-free and cell-based assays.

2. Materials and methods

2.1. Preparation of recombinant K-Ras proteins

Human KRAS(Met1–Lys169) (NCBI Reference Sequence: NM_004985) DNA sequence was isolated from human cDNA clone (GeneCopoeia, Rockville, MD) and was ligated into a pET21a vector (Merck Millipore, Darmstadt, Germany) with a C-terminus His-Avitag. Expression plasmids were co-transfected with the BirA expression plasmid, which is constructed internally and encodes a biotin protein ligase, into *E. coli* BL21(DE3) (Nippon Gene, Toyama, Japan). Protein expression was induced with 0.1 mM IPTG, followed by addition of 50 μM D-biotin and culture for 16 h at 16 °C. Cells were harvested by centrifugation, suspended in lysis buffer (50 mM Tris (pH 8.0), 1 mM DTT, 150 mM NaCl, 5 U/mL Nuclease), and centrifuged at 15000 × *g* for 20 min. The proteins were purified by NiNTA superflow column (QIAGEN, Hilden, Germany) and HiLoad 26/60 Superdex 200 pg column (GE Healthcare, Piscataway, NJ).

2.2. Phage library construction and panning

T7 phage libraries displaying random peptides, which were generated by mixed-oligonucleotides as template DNA, were constructed by using T7Select 10-3 vector from Merck Millipore, according to methods described previously [12,13]. Biotinylated Avitagged K-Ras protein was preincubated with 1 mM GDP in reaction buffer (0.5% BSA, 10 mM $MgCl_2$ in PBS) at 4 °C overnight to prepare the GDP-form, and then immobilized onto Dynabeads M280 streptavidin (SA) (Invitrogen, Carlsbad, CA). After washing the beads by PBS containing 0.1% Tween20 (PBST), the beads were incubated with phage libraries for 1 h with 1 mM GDP and 50 μg/mL non-tagged WT K-Ras in reaction buffer, and subsequently washed with PBST. The bound phages were eluted with 1% SDS and transfected into *E. coli* BLT5615 cells (Merck Millipore) in log-phase growth for phage amplification. After bacteriolysis, phages were recovered from the culture supernatant by centrifugation and PEG-precipitation, dissolved in PBS, and used for the next round of panning.

2.3. Synthetic peptides

Linear peptides were prepared by standard Fmoc-based solid phase peptide synthesis followed by HPLC purification.

For disulfide formation, a linear peptide (6 mg) was dissolved in 1.0 mol/L Tris-HCl buffer (pH 8.5, 4.5 mL) and acetonitrile (3 mL). DMSO (3 mL) was added to the solution, and the mixture was stirred for 36 h at room temperature. The reaction mixture was diluted with H_2O and the reaction product was purified by preparative HPLC. Fractions containing the product were collected and lyophilized to obtain the desired cyclic peptide.

2.4. Peptide binding evaluation by SPR

SPR biosensing experiments were performed on Biacore3000 and BiacoreS200 equipped with Sensorchip SA at 25 °C (GE Healthcare).

For immobilization, HBS-P+ (10 mM Hepes, 150 mM NaCl, 0.05% surfactant P20, pH 7.4, GE Healthcare) was used as the running buffer. Apo-, GDP-, and GTP-form K-Ras were prepared by pretreatment with 5 mM EDTA, 1 mM GDP, or 1 mM GTP, respectively. For immobilization, each biotin-K-Ras was injected over the sensorchip surface. Typical immobilization levels were around 5000 RUs.

For the interaction study, HBS-P+ supplemented with 1% DMSO and with/without 10 μM GDP or GTP was used as a running buffer. Peptides diluted in series were injected at a flow rate of 50 μL/min for 120 s, and the dissociation was thereafter followed for up to 240 s. Data processing and analysis were performed by Bioevaluation software ver. 4.1.1 and BiacoreS200 evaluation software (GE Healthcare). Sensorgrams were double-referenced prior to global fitting the concentration series to 1:1 binding with the mass-transport model. Dissociation constant K_D was calculated from the following equation $K_D = k_{off}/k_{on}$.

Competition experiments were performed by sequential injection of peptide solutions either individually, or as mixtures of two peptides, each for 120 s at a flow rate of 50 μL/min. When peptides occupied different sites, the response observed for the mixture was the sum of the 2 individual responses observed for the peptides.

2.5. In vitro enzyme assay

BODIPY-FL-GDP, Terbium-labeled streptavidin (Tb-SA), and human SOS1 protein (Exchange Domain 564-1049) were purchased from Life Technologies (Carlsbad, CA), Cisbio (Codolet, France), and Cytoskeleton (Denver, CO), respectively.

TR-FRET assay was carried out using 384-well plates (784075, Greiner Bio-One, Frickenhausen, Germany) and the signal was measured using an EnVision plate reader (PerkinElmer, Waltham, MA). The solution in each well was excited with a laser (λ = 337 nm) reflected by a dichroic mirror (D400/D505), and fluorescence from Tb and BODIPY were detected through two emission filters (CFP 486 nm for Tb, Emission 515 nm for BODIPY). Biotin-K-Ras mutants (WT, G12C, and G12D) were diluted to 2 μM in EDTA buffer (20 mM HEPES, 50 mM NaCl, 10 mM EDTA, and 0.01% (w/v) Tween20) and preincubated for 30–60 min at room temperature. The EDTA pretreated K-Ras proteins were diluted to 3 nM in Ras assay buffer (20 mM HEPES, 50 mM NaCl, 10 mM $MgCl_2$, 0.01% (w/v) Tween20) containing 0.5 nM Tb-SA and 30 nM BODIPY-GDP and further incubated for over 6 h at room temperature. Various concentrations of 45 nL peptides (100-fold concentration) in DMSO were dispensed in each well of the assay plate using

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