Supporting Information

Crystal Structure of a Human K-Ras G12D Mutant in Complex with GDP and the Cyclic Inhibitory Peptide KRpep-2d

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MATERIALS AND METHODS

Peptide synthesis. Peptide synthesis was conducted using Fmoc-chemistry on a Symphony X peptide synthesizer (Protein Technologies, Inc., Tucson, AZ, USA). The resin for peptide synthesis and N^{α}-Fmoc (and side chain) protected amino acids were purchased from Novabiochem-Merck Millipore (Darmstadt, Germany) and Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). The following side-chain protections were used: Arg(Pbf), Asp(OtBu), Cys(Trt), Ser(tBu) and Tyr(tBu). Solvents and other reagents were reagent-grade and were used further purification unless otherwise noted. N,N'-Diisopropylcarbodiimide (DIPCDI), OxymaPure, and trifluoroacetic acid (TFA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Piperidine was purchased from Watanabe Chemical Industries. Crude peptides were purified to homogeneity by reverse phase (RP)-high-performance liquid chromatography (HPLC) with the following conditions: YMC-Actus Triart Prep C8-S S-10 μm 20 nm column (30 × 250 mm); solvent gradient A, 0.1% TFA in water; B, 0.1% TFA in acetonitrile with gradient indicated below; flow rate, 15 mL/min; and UV detector, 220 nm. The purity of the products was characterized by analytical HPLC. Reverse phase analyzes were performed using a Shimazu gradient system with a YMC Triart C8 column (4.6 × 100 mm). The peptide molecular weights were confirmed by matrix-assisted laser desorption ionization−time of flight (MALDI−TOF) mass spectrometry (MS) on a Bruker Autoflex Speed system (Bruker Daltonics, Kanagawa, Japan).

Sieber amide resin (0.71 mmol/g, 140.8 mg, 0.1 mmol) was swelled with N-methyl-2-pyrrolidone (NMP). Sequential peptide chain elongation via Fmoc/OxymaPure®/DIPCDI (6 eq. of reagents) chemistry was performed in NMP, followed by N-terminal acetylation and vacuum drying on a Symphony X peptide synthesizer to yield the desired peptide resin: Ac-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-Cys(Trt)-Pro-Leu-Tyr(tBu)-Ile-Ser(tBu)-Tyr(tBu)-Asp(OtBu)-Pro-Val-Cys(Trt)-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-NH-Sieber amide resin (762 mg). The dried resin was suspended in 7 mL of TFA/m-

cresol/thioanisole/H_2O/triisopropylsilane/ethandithiol (80:5:5:5:2.5:2.5) and stirred for 180 min at room temperature. Diethyl ether was added to the reaction solution, after which the precipitate was centrifuged and the supernatant was removed. After repeating this washing procedure, the residue was extracted with an aqueous acetic acid solution and filtered to remove the resin. The filtrate was applied to preparative HPLC with a linear density gradient elution (60 min) was performed using eluent A/B ratios of 77/23−67/33 (eluent A: 0.1% TFA in water and eluent B: 0.1% TFA-containing acetonitrile) on a YMC-Actus Triart Prep C8-S S-10 μm 20 nm column (30 × 250 mm) with a flow rate of 15 mL/min. The product-containing fractions were collected and lyophilized to yield 100.4 mg of the desired peptide, Ac-Arg-Arg-Arg-Arg-Cys-Pro-Leu-Tyr-Ile-Ser-Tyr-Asp-Pro-Val-Cys-Arg-Arg-Arg-Arg-NH_2, as a white powder with a mass spectrum of (M + H) + 2562.41 (calc. 2562.40). The RP-HPLC elution time was 6.89 min, and the elution conditions were as follows: YMC Triart C8 column (4.6 × 100 mm), linear density gradient elution with eluent A/B ratios of 80/20−30/70 (25 min; eluent A: 0.1% TFA in water and eluent B: 0.1% TFA-containing acetonitrile), and a flow rate of 1 mL/min.

Ac-Arg-Arg-Arg-Arg-Cys-Pro-Leu-Tyr-Ile-Ser-Tyr-Asp-Pro-Val-Cys-Arg-Arg-Arg-Arg-NH_2 (5 mg) was dissolved in 1.0 mol/L Tris-HCl buffer (pH 8.5, 4 mL) and acetonitrile (2 mL). DMSO (2 mL) was added to this solution, which was stirred for 36 h at room temperature. The solution was then filtered and applied to the preparative HPLC column, and a linear density gradient elution (60 min) was performed with eluent A/B ratios of 75/25−65/35 (eluent A: 0.1% TFA in water and eluent B: 0.1% TFA-containing acetonitrile) on a YMC-Actus Triart Prep C8-S S-10 μm 20 nm column (30 × 250 mm) at a flow rate of 15 mL/min. The product-containing fractions were collected and lyophilized to yield 3.1 mg of the desired KRpep-2d peptide, Ac-Arg-Arg-Arg-Arg-Cys(&)-Pro-Leu-Tyr-Ile-Ser-Tyr-Asp-Pro-Val-Cys(&)-Arg-Arg-Arg-Arg-NH_2 (disulfide), as a white powder with a mass spectrum of (M + H) + 2560.66 (calc. 2560.38). The elution time on RP-HPLC was 8.00 min, and the elution conditions were as follows: YMC Triart C8 column (4.6 × 100 mm), linear density gradient elution with eluent A/B ratios of 80/20−30/70 (25 min; eluent A: 0.1% TFA in water and eluent B: 0.1% TFA-containing acetonitrile), and a flow rate of 1 mL/min.

Recombinant protein production. Human K-Ras (Met1−Lys169, Gly12Asp) was prepared as described previously.[1] The corresponding DNA was amplified by PCR from a human cDNA library, and ligated into a pET21a vector (Merck Millipore, Darmstadt, Germany) with an N-terminus His−Avi tag. The protein was expressed in *Escherichia coli* BL21 (DE3) (NIPPON GENE, Toyama, Japan) via IPTG induction and purified on Ni-NTA (QIAGEN, Hilden, Germany) and Superdex200 columns (GE Healthcare, Milwaukee, WI, USA). For crystallization, TEV protease (Invitrogen, Carlsbad, CA, USA) was used to digest the His−Avi tag, and the sample was subsequently applied to a Ni-NTA column to remove the TEV protease and uncleaved pro-

tein. Final purification was achieved using a Mono Q column (GE Healthcare), and the purified protein was concentrated to 25 mg/mL by ultrafiltration and stored at −80°C.

Surface Plasmon Resonance (SPR). SPR biosensing experiments were performed on a Biacore S200 equipped with a Sensorchip SA at 25°C (GE Healthcare, Little Chalfont, UK) as described previously.[1] 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. The GDP- and GTP-bound forms of K-Ras were prepared by pretreatment with 1 mM GDP and GTP, respectively. For immobilization, each biotinylated K-Ras form was injected over the Sensorchip surface, yielding typical levels of approximately 5000 RUs. For the interaction study, HBS-P+ supplemented with 1% DMSO and 10 μM GDP or GTP was used as the running buffer. Serially diluted peptides were injected at a flow rate of 50 μL/min for 90 s, and subsequent dissociation was followed for up to 420 s. Data processing and analysis were performed using Biacore S200 evaluation software (GE Healthcare). Sensorgrams were double-referenced prior before global fitting of the concentration series to 1:1 binding with the mass transport model. The dissociation constant K_D was calculated as follows: $K_D = k_{off}/k_{on}$.

X-ray crystallography. The complex of K-Ras(G12D) with GDP and KRpep-2d was prepared by incubating 3-fold molar excesses of ligands on ice for 2–3 h prior to the crystallization experiments. The complex was crystallized from a reservoir solution containing 0.1 M Hepes (pH 7.5) and 50% (v/v) PEG 200 at 20°C via the sitting-drop vapor diffusion method. Prior to data collection, crystals were immersed in reservoir solution containing 30% ethylene glycol as a cryoprotectant and flash-frozen in liquid nitrogen. Diffraction data were collected from a single crystal using the DECTRIS Pilatus3-S6M PAD detector (Baden-Daettwil, Switzerland) with a BL-17A beamline (Photon Factory, Tsukuba, Japan) under a 100-K nitrogen cryostream. The diffraction data were reduced and scaled using HKL2000.[2] The structure was solved according to the molecular replacement method using Phaser[3] from the CCP4 software suite[4] and the K-Ras structure (PDB code 4QL3) as a search model. Refinement was performed using REFMAC5[5] and individual isotropic restrained B factors. Progress was monitored using R_{free}, and 5% of the data were set aside for cross-validation before refinement. For TLS refinement, the protein and ligands were set as a single rigid body.[6] Interactive model building was performed using COOT,[7] and the final models were validated using Molprobity.[8] All graphical figures were generated using PyMOL (Schrödinger LLC, Cambridge, MA, USA). A schematic diagram of protein–ligand interactions was generated using LIGPLOT.[9]

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A **B**

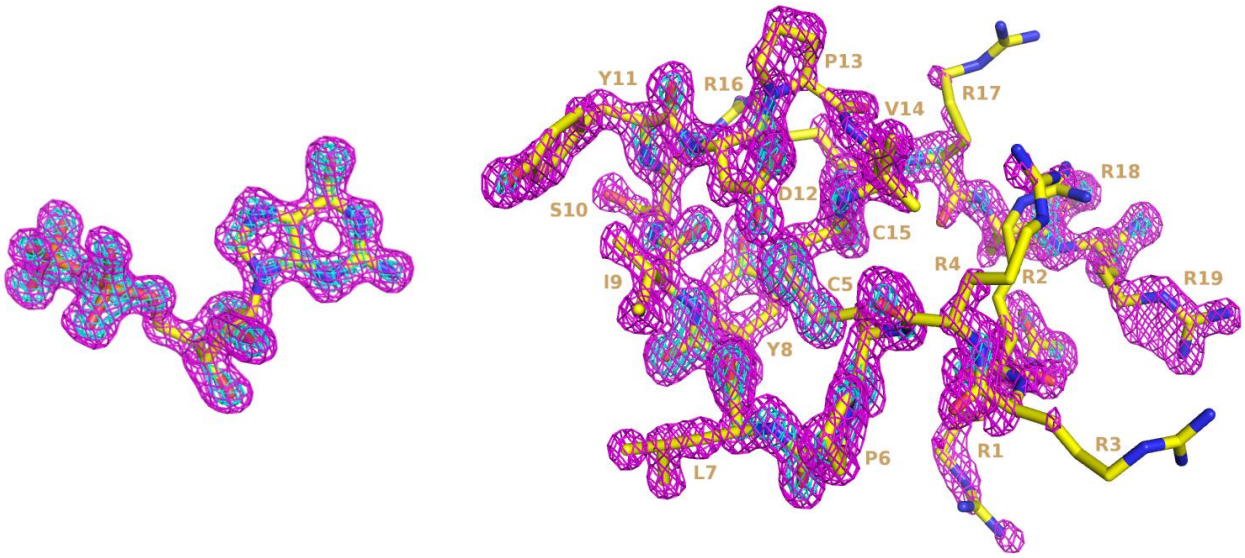


Figure S1. The F_{obs}–F_{calc} electron density omit map contoured at 3σ within the vicinity of (A) GDP and (B) KRpep-2d.

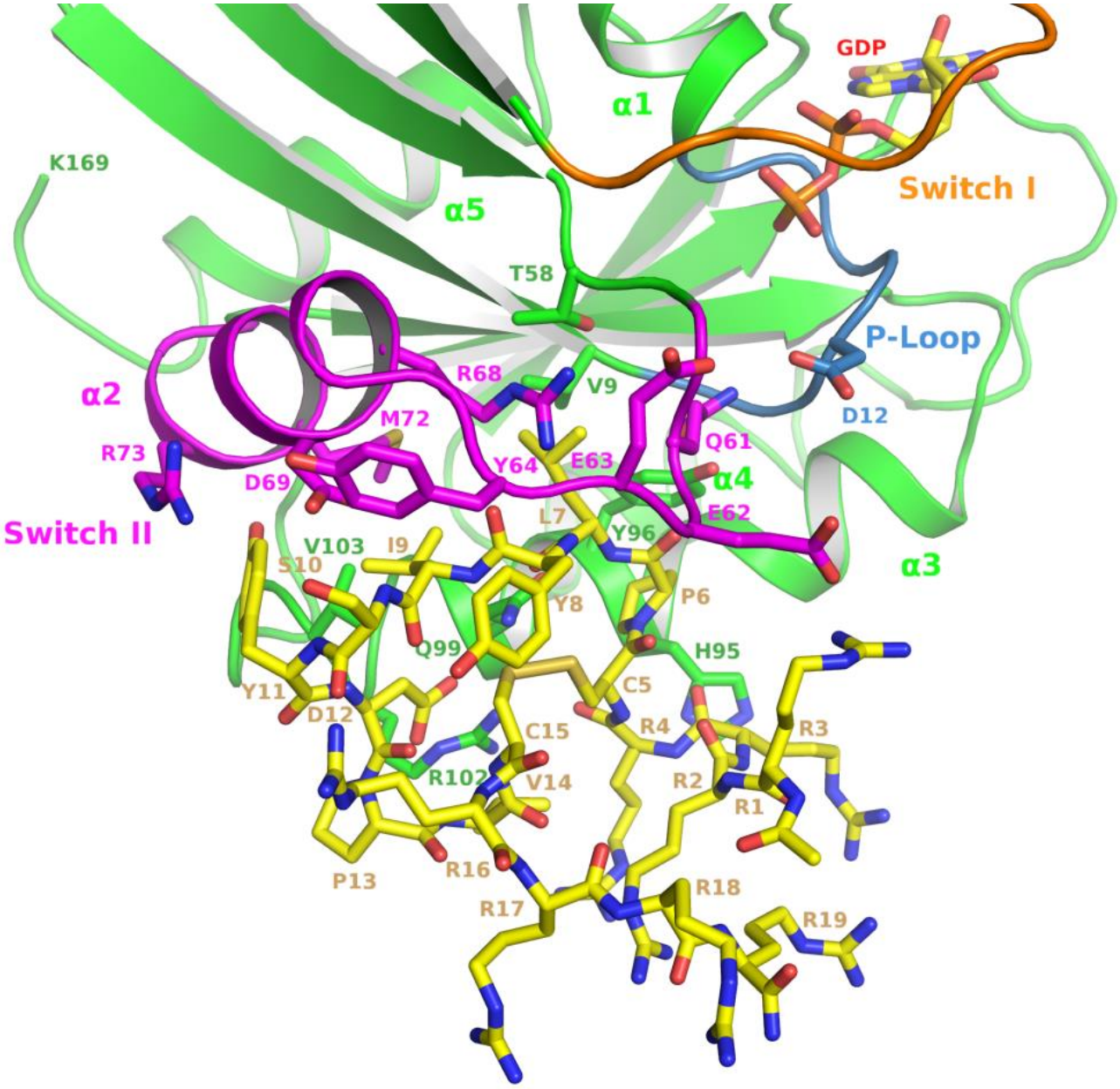


Figure S2. Close view of the binding interface within the K-Ras(G12D)−GDP−KRpep-2d complex. The peptides are depicted as yellow stick models, with Switch I, Switch II, and the P-loop colored orange, magenta, and blue, respectively.

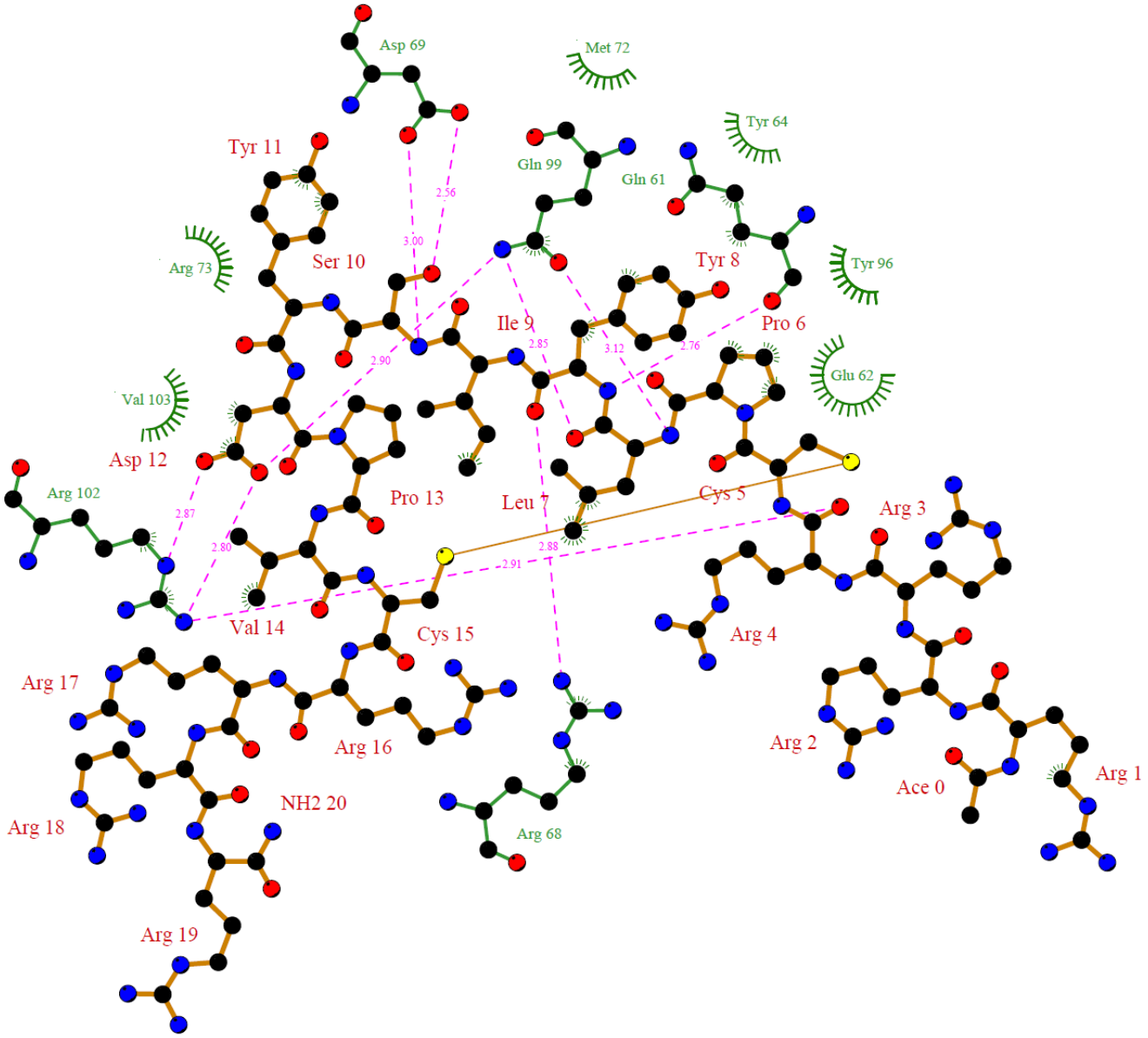


Figure S3. Schematic diagram of the K-Ras(G12D)−KRpep-2d interactions. Hydrogen bonds are depicted as dot lines.

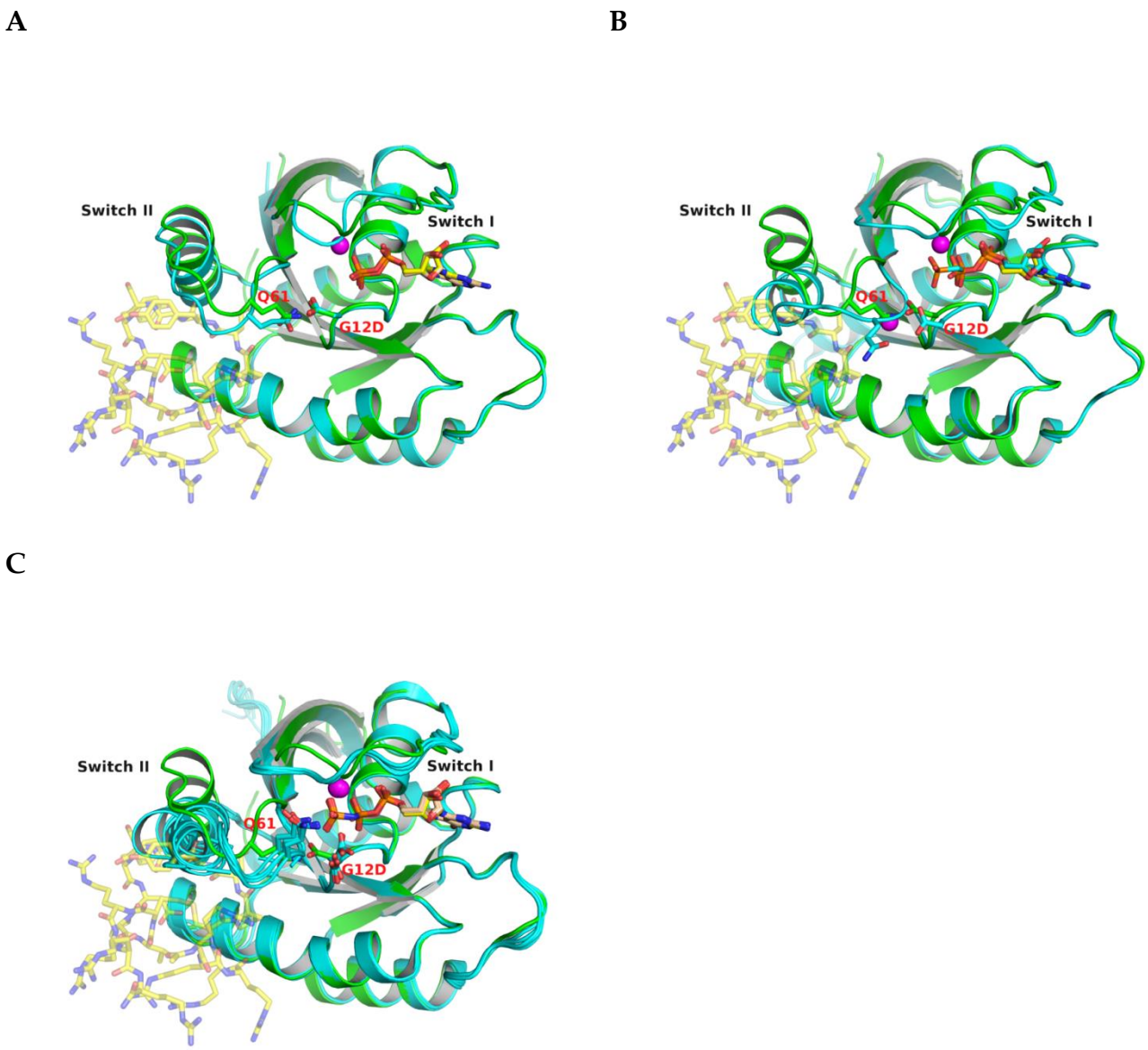


Figure S4. Structural comparison of the K-Ras(G12D)−GDP−KRpep-2d (green) complex with other K-Ras complexes (cyan). (A) The K-Ras(G12D) −GDP complex (PDB code 4EPR). (B) The K-Ras(G12D)−GMPPCP complex (PDB code 4DSN). (C) The K-Ras(G12D)−GMPPNP complex (PDB code 5USJ). All six monomers in the crystallographic asymmetric unit are superimposed. KRpep-2d is shown transparently for clarity. The magnesium ions are represented as magenta spheres.

A **B**

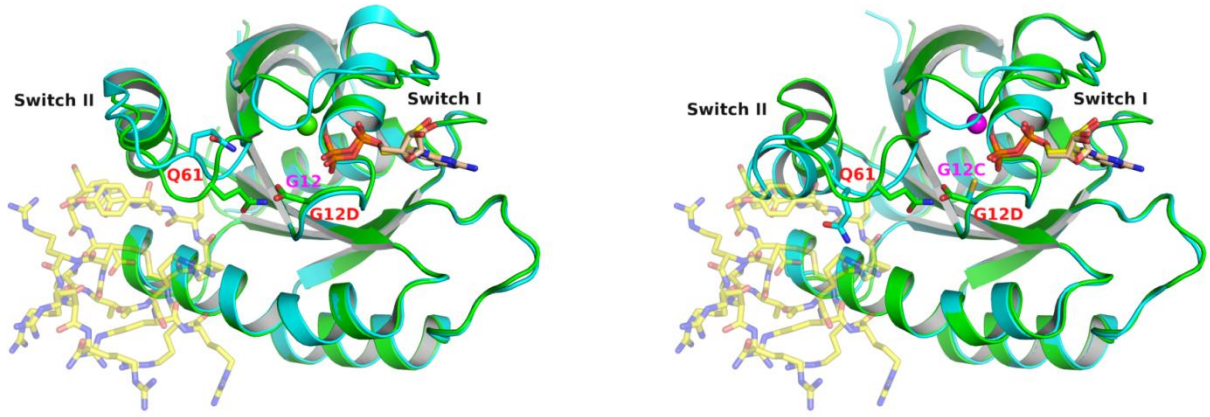
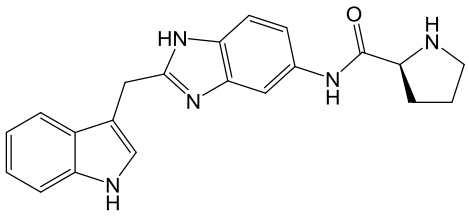
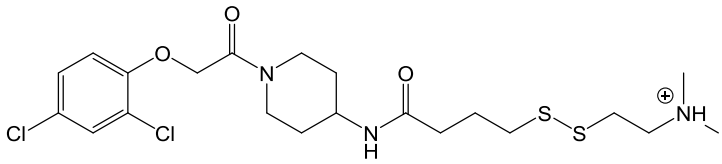


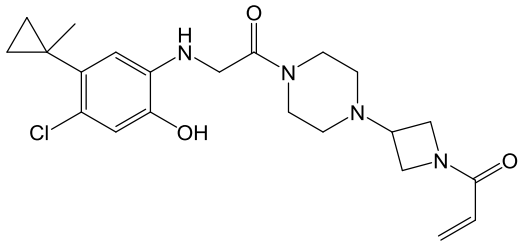
Figure S5. Structural comparison of the K-Ras(G12D)−GDP−KRpep-2d (green) complex with other K-Ras complexes (cyan). (A) The K-Ras(WT)−GDP complex (PDB code 4LPK). (B) The K-Ras(G12C)−GDP complex (PDB code 4LDJ). KRpep-2d is shown transparently for clarity. The magnesium and calcium ions are represented as magenta and green spheres, respectively.



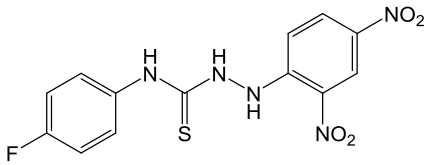
compound **13**



disulfide **6**



ARS-853



Kobe2601

Figure S6. Chemical structures of K-Ras inhibitors.

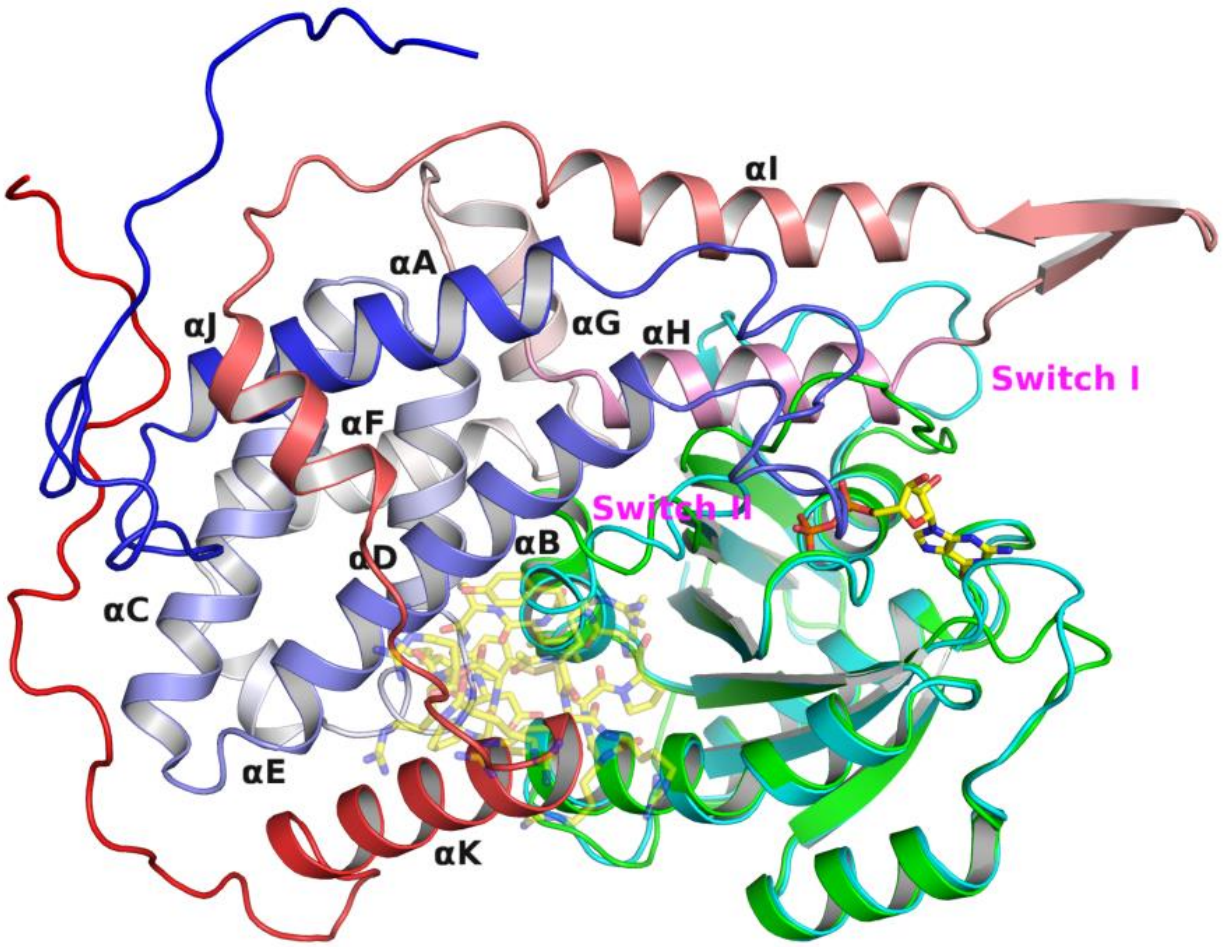


Figure S7. Structural comparison of the K-Ras(G12D)−GDP−KRpep-2d (green) complex with the H-Ras−SOS complex (cyan) (PDB code 1NVW). KRpep-2d is shown transparently for clarity. Only the cdc25 domain of SOS (residues 750−1046) is drawn for clarity. The carbon atoms of the cdc25 domain of SOS are drawn with a spectrum of colors ranging from blue to red.

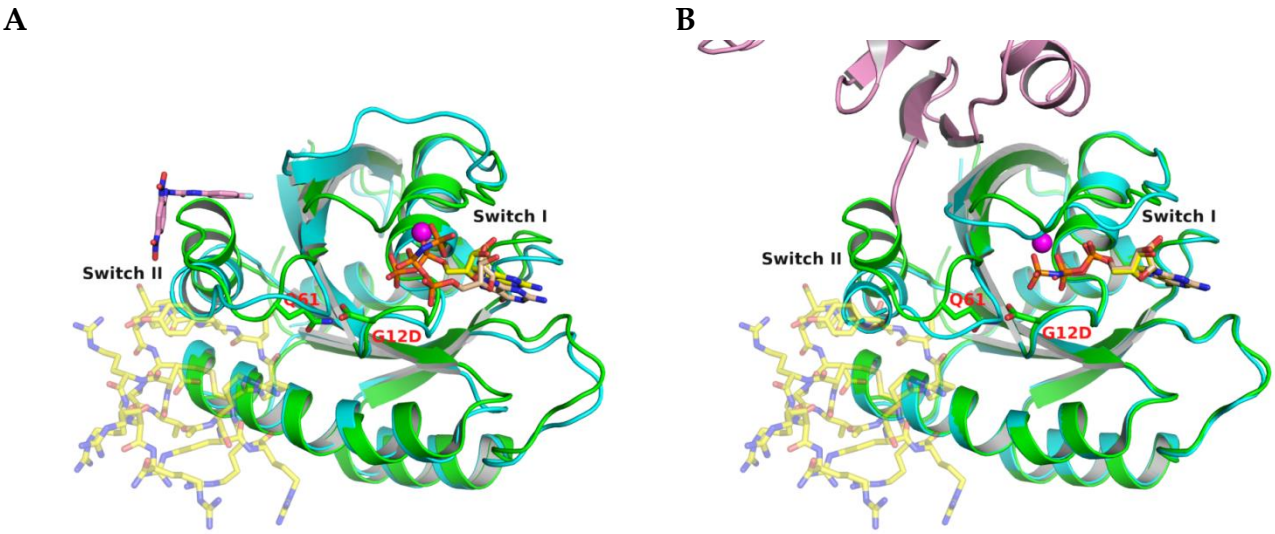


Figure S8. Structural comparison of the K-Ras(G12D)−GDP−KRpep-2d (green) complex with other K-Ras complexes (cyan). (A) The H-Ras(T35S)−GMPPNP−Kobe2601 complex (PDB code 2LWI). (B) The H-Ras−GMPPNP−Raf-RBD complex (PDB code 4G0N). The magnesium ions are represented by magenta spheres. KRpep-2d is shown transparently for clarity. The carbon atoms of Kobe2601 and Raf-RBD are colored pink.

Table S1. Data Collection and Refinement Statistics

Crystal	K-Ras(G12D)−GDP−KRpep-2d
Data collection	
Space group	$P3_121$
Unit cell dimensions	
a, b, c (Å)	51.5, 51.5, 129.6
α, β, γ (°)	90, 90, 120
Resolution (Å)	50−1.25 (1.27−1.25)
Unique reflections	56366 (2780)
Redundancy	9.3 (7.3)
Completeness (%)	100.0 (99.7)
I/σ	54.4 (1.9)
R_{sym} [a]	0.041 (0.964)
Molecules in ASU	1
Refinement	
Resolution (Å)	45−1.25 (1.28−1.25)
Reflections	53531 (3866)
R_{work} [b]	0.171 (0.273)
R_{free} [b]	0.194 (0.293)
Number of atoms	
Protein	1431
Ligand/Ion	211
Water	138
Average B factor (Å^2) [c]	19.1
Rms deviation from ideal geometry	
Bond lengths (Å)	0.011
Bond angles (°)	1.592
Ramachandran plot (%) [d]	
Preferred regions	97.7
Allowed regions	2.3
Outliers	0.0
PDB code	**5XCO**

[a] $R_{sym}=\Sigma_h\Sigma_i|I(h)_i-<I(h)>|/\Sigma_h\Sigma_i<I(h)>$, where <I(h)> is the mean intensity of symmetry-related reflections. [b] $R_{work}=\Sigma||Fobs|-|F_{calc}||/\Sigma|F_{obs}|$. R_{free} was calculated for randomly chosen 5% of reflections excluded from refinement. [c] B-factor includes contributions from TLS parameters. [d] Calculated with Coot. Values in parentheses are those for the highest resolution shell.

Table S2. Binding Affinities of KRpep-2d and Amino-acid Substituted Derivatives

Modification from KRpep-2d	K_D (nM)					
	K-Ras−GDP			K-Ras−GTP		
	G12D	G12C	WT	G12D	G12C	WT
(KRpep-2d)	8.9	35	58	11	250	200
$Arg^{1,2,18,19}$ → deletion (KRpep-2) [a]	51	480	700	100	920	1300
$Arg^{1,2,3,4,16,17,18,19}$ → deletion	770	680	> 1000	> 1000	> 1000	> 1000
$Arg^{1,19}$ → D-Arg	10	290	140	11	560	260
Pro^{6} → Ala	55	520	560	260	> 1000	> 1000
Leu^{7} → Ala	920	> 1000	> 1000	> 1000	> 1000	> 1000
Tyr^{8} → Ala	89	740	910	190	> 1000	> 1000
Ile^{9} → Ala	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
Ser^{10} → Ala	78	570	990	160	> 1000	> 1000
Tyr^{11} → Ala	220	> 1000	> 1000	470	> 1000	> 1000
Asp^{12} → Ala	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
Pro^{13} → Ala	26	170	320	33	750	670
Val^{14} → Ala	8.9	38	83	9.9	240	210

[a] Reference 1