K-Ras<sup>G12D</sup> Has a Potential Allosteric Small Molecule Binding Site
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Supporting Information

ABSTRACT: KRAS is the most commonly mutated oncogene in human cancer, with particularly high mutation frequencies in pancreatic cancers, colorectal cancers, and lung cancers [Ostrem, J. M., and Shokat, K. M. (2016) Nat. Rev. Drug Discovery 15, 771–785]. The high prevalence of KRAS mutations and its essential role in many cancers make it a potentially attractive drug target; however, it has been difficult to create small molecule inhibitors of mutant K-Ras proteins. Here, we identified a putative small molecule binding site on K-Ras<sup>G12D</sup> using computational analyses of the protein structure and then used a combination of computational and biochemical approaches to discover small molecules that may bind to this pocket, which we have termed the P110 site, due to its adjacency to proline 110. We confirmed that one compound, named K-Ras allosteric ligand KAL-21404358, bound to K-Ras<sup>G12D</sup>, as measured by microscale thermophoresis, a thermal shift assay, and nuclear magnetic resonance spectroscopy. KAL-21404358 did not bind to four mutants in the P110 site, supporting our hypothesis that KAL-21404358 binds to the P110 site of K-Ras<sup>G12D</sup>. This compound impaired the interaction of K-Ras<sup>G12D</sup> with B-Raf and disrupted the RAF-MEK-ERK and PI3K-AKT signaling pathways. We synthesized additional compounds, based on the KAL-21404358 scaffold with more potent binding and greater aqueous solubility. In summary, these findings suggest that the P110 site is a potential site for binding of small molecule allosteric inhibitors of K-Ras<sup>G12D</sup>.

As proteins belong to the small GTPase family and are involved in transmitting growth, survival, and proliferation signals within cells. As a GTPase, Ras cycles between a GTP-bound state and a GDP-bound inactive state, the transition of which is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Two regions of Ras proteins, switch I (residues 30–40) and switch II (residues 60–76), undergo substantial conformational changes and form effector–protein interaction surfaces upon GTP binding. In the GTP-bound active state, Ras interacts with effector proteins and activates downstream cellular signal transduction pathways, including the RAF-MEK-ERK, PI3K-AKT-mTOR, and Rap1GDS pathways. Oncogenic mutants of Ras are locked in an active signaling state: the constitutive activation of Ras downstream signaling results in sustained proliferation, metabolic reprogramming, inhibition of apoptosis, and other hallmarks of cancer.

There are three common RAS genes in humans: KRAS, HRAS, and NRAS. The frequencies and distribution of RAS gene mutations are not uniform among these three family members. KRAS is the most frequently mutated gene and is altered in 86% of RAS-altered cancers. G12, G13, and Q61 are three hot spot point mutations found around the RAS GTP binding site. Among these mutations, G12D mutations are predominant in pancreatic ductal adenocarcinoma and colon and rectal carcinomas. The high prevalence of KRAS mutations in cancers suggests it may be a potentially valuable drug target. However, there are still no effective inhibitors directly targeting K-Ras mutant proteins that are suitable for clinical use.

K-Ras is considered a challenging drug target for two main reasons. First, there does not seem to be a deep, hydrophobic pocket on the surface of K-Ras suitable for potent and selective small molecule binding; the only notable binding pocket on K-Ras is the nucleotide binding pocket, which binds GTP/GDP with picomolar affinity, making it an impractical target site for small molecule drugs. Second, K-Ras, like roughly 85% of other human proteins, exerts its biological effects via protein–protein interactions, which are often difficult to disrupt with small molecules, due to their large surface areas and the diffuse nature of the interactions between them. Despite these difficulties, direct K-Ras inhibitors have been explored using several strategies: (1) targeting G12C-specific K-Ras mutants with covalent, cysteine-reactive electrophilic inhibitors, (2) blocking K-Ras–effector interactions by developing small molecule and peptide inhibitors, (3) interrupting...
nucleotide exchange, including the K-Ras–GEF interaction and modification of the GTP binding site, and targeting potential allosteric regulatory sites. Here, we describe a strategy for targeting oncogenic K-Ras by combining computational methods and biochemical assays. We discovered a potential allosteric binding site, the P110 site, near the C-terminus of K-Ras<sup>G12D</sup>. The P110 site involves residues Arg97, Asp105, Ser106, Glu107, Asp108, Val109, and His166. By combining computational methods and biochemical assays, we identified an allosteric regulator of K-Ras<sup>G12D</sup>.

**MATERIALS AND EXPERIMENTAL DETAILS**

**Software and Computational Methods.** Molecular dynamics (MD) simulations, mixed solvent molecular dynamics (MxMD) simulations, molecular docking, and modeling were performed using Maestro (Schrödinger Suite), Molecular Operating Environment (MOE), and PyMOL. Chemical structures were drawn using ChemDraw Suite, Molecular Operating Environment (MOE), and TimTec. A fragment subset of the KAL scaffold was selected and screened. SiteMap (Schrödinger Suite) was run using the default setting. MD simulations were performed with Desmond Molecular Dynamics (Schrödinger Suite). SiteMap (Schrödinger Suite) was run using the default setting. MD simulations were performed with Desmond Molecular Dynamics (Schrödinger Suite). The total simulation time was 200 ns. Twenty representative clusters were then generated using the default setting of RMSD Based Clustering Of Frames From Desmond Trajectory (Schrödinger Suite). MxMD simulations were run by scripts. Ten protein cosolvent boxes for each of six probes (acetonitrile, isopropanol, pyrimidine, acetone, imidazole, and N-methylacetamide) were first generated by a shell script (see the Supporting Information). The map was finally generated by a "run_cosolvent_simulations.sh" script (see the Supporting Information). The P110 site was selected using Receptor Grid Generation (Schrödinger Suite) by specifying residue Pro110 as the center of the enclosing box. No constraints were defined. Different poses of compounds were generated by defining ionization states from pH 4.0 to 7.0 and stereoisomers using LigPrep (Schrödinger Suite). Ligand Docking (Schrödinger Suite) was then used to calculate the Glide Docking Scores. A lower score meant a higher binding affinity. WaterMap (Schrödinger Suite) was run by defining KAL-21404358 ligand docking in the P110 site. The simulation time was 2.0 ns.

**Molecular Cloning.** The KRAS<sup>G12D</sup> plasmid was previously described.<sup>14</sup> Binding-deficient mutants of the KRAS<sup>G12D</sup> plasmid were generated using a QuikChange XL site-directed mutagenesis kit. Primers were designed using the Agilent QuikChange Primer Design application and purchased from Integrated DNA Technologies: KRAS<sup>G12D</sup> Burgundy primer, S′ GAA GAT ATT CAC CAT TAT GGA GAA CAA ATT AAA AGA GTT AGG G 3′; KRAS<sup>G12D</sup> Burgundy reverse primer, S′ CTG AAT TCT TTT AAT TTG TTC TCC ATA ATG GTG AAT ATC TTC 3′; KRAS<sup>G12D</sup> Burgundy forward primer, S′ GAG TTA AGG ACT CTG CAG ATG TAC CTA TGG TCC 3′; KRAS<sup>G12D</sup> Burgundy reverse primer, S′ GGA CCA TAG GTA CTT CAG AGT CCT TAA CTC 3′; KRAS<sup>G12D</sup> Burgundy forward primer, S′ TAA GGA GTC TGA AGCT GT ACC TAT GGT CC 3′; KRAS<sup>G12D</sup> Burgundy reverse primer, S′ ACC ATTA GGT AC AGC T TCA GAG TCG TTA ACT C 3′; KRAS<sup>G12D</sup> Burgundy forward primer, S′ AGA TGT AGA TAT GGG CCT AG 3′; KRAS<sup>G12D</sup> Burgundy reverse primer, S′ AGG ACC ATTA TCT ACA TCT TC 3′. DNA sequencing was performed to confirm the amino acid sequence of the construct (GenWiz).

**Protein Expression and Purification.** The KRAS<sup>G12D</sup> construct was expressed in *Escherichia coli* BL21-Gold (DE3) cells (Strategene). An isolated colony was transferred to 8 mL of LB medium with 100 μg/mL ampicillin, and the inoculated culture was incubated while being shaken (225 rpm) at 37 °C for 4.5 h. The starter culture was added to 1 L of fresh LB medium with 100 μg/mL ampicillin. The culture was incubated while being shaken at 37 °C and 225 rpm until the OD<sub>600</sub> reached 0.6. The temperature was then decreased to 15 °C. Cells were incubated with 500 μM isopropyl β-D-thiogalactopyranoside (IPTG) while being shaken at 15 °C and 225 rpm overnight. The next day, the bacteria were harvested by centrifugation at 4000g for 20 min at 4 °C and the pellet obtained was ready for purification or stored at −20 °C.

The pellet was resuspended in 25 mL of chilled lysis buffer [10 mM Tris (pH 7.5), 500 mM NaCl, 5 mM MgCl<sub>2</sub>, 5 mM imidazole, 2 mM TCEP, and Roche protease inhibitor cocktail]. The bacteria were lysed by sonication on ice for 6 min, and the lysate was centrifuged at 15000 rpm for 45 min at 4 °C to remove cell debris. The clarified lysate was incubated with Ni Sepharose 6 Fast Flow beads (GE Life Sciences) on a rotator at 4 °C for at least 1 h. The beads were washed with wash buffer [10 mM Tris (pH 7.5), 500 mM NaCl, 20 mM imidazole, 5 mM MgCl<sub>2</sub>, and 2 mM TCEP] to remove nonspecific binding. The protein was eluted with 10 mM Tris (pH 7.5), 500 mM NaCl, 250 mM imidazole, 5 mM MgCl<sub>2</sub>, and 2 mM TCEP. The protein was further purified using a gel filtration Superdex 200 column in FPLC buffer containing 25 mM Tris (pH 7.5), 100 mM NaCl, 5 mM MgCl<sub>2</sub>, and 2 mM TCEP. The fractions containing K-Ras<sup>G12D</sup> were pooled together and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The protein concentration was determined using absorbance at 280 nm with an extinction coefficient of 11920 M<sup>−1</sup> cm<sup>−1</sup>.

**Nucleotide Exchange.** Nucleotides in endogenous recombinant K-Ras<sup>G12D</sup> were exchanged with GDP or GTP using the following conditions: 50 mM Tris (pH 7.5), 100 mM NaCl, 5 mM MgCl<sub>2</sub>, and 2 mM TCEP. The nucleotide exchange was performed at 37 °C for 2 h, and the reaction was quenched with 10 μM GDP. The nucleotide exchange was monitored by analytical Superose 6 FPLC column in PBS buffer containing 25 mM Tris (pH 7.5), 100 mM NaCl, 5 mM MgCl<sub>2</sub>, and 2 mM TCEP. The fractions containing K-Ras<sup>G12D</sup> were pooled together and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The protein concentration was determined using absorbance at 280 nm with an extinction coefficient of 11920 M<sup>−1</sup> cm<sup>−1</sup>.
GppNHp using an EDTA loading procedure. K-RasG12D protein (final concentration of 70 μM) was incubated with a 70-fold molar excess of EDTA (final concentration of 5 mM) and a 70-fold molar excess of new nucleotide (final concentration of 5 mM) for 2 h at 30 °C. After incubation, the sample was placed on ice for 2 min and then MgCl₂ was added (final concentration of 65 mM) to stop the reaction. To remove excess unbound nucleotide, the sample was added to a NAP-5 column (GE Life Sciences) equilibrated with FPLC buffer and eluted with FPLC buffer at 100 μL per fraction. Eluted fractions were evaluated using the NanoDrop method to determine protein concentration.

Microscale Thermophoresis (MST). One hundred microliters of 200 nM K-RasG12D was combined with 100 μL of 100 nM RED-tris-NTA dye in PBS buffer with 3 mM DTT and 0.05% Tween 20 (PBSTBD buffer). The protein/dye mixture was incubated at room temperature for 30 min, followed by centrifugation for 4 min at 4 °C and 15000g. The compounds were arrayed across a 16-point dilution series in PBSTBD buffer and mixed in a 1:1 ratio with a labeled protein solution in a 20 μL volume. The reaction mixture were loaded into standard treated capillaries and analyzed with Monolith NT.115 (Nanotemper Technologies) at 60% LED power and 40% MST power with a laser-on time of 5 s. The Kₚ was calculated by taking the average of triplicate Fₚ₀ measurements at each concentration and fitting the data to a sigmoidal four-parameter fitting function in Prism (GraphPad Software). R-Ras, R-Ras₂, and Rap1A were purchased from ProSpecBio. K-Ras WT was purchased from Cell Biolabs. H-Ras WT was acquired from Enzo Life Sciences.

Thermal Shift Assay (TSA). A fluorescent thermal shift assay was used to validate the binding and confirm the success of the nucleotide exchange procedure of all GTPases used in the study. The assay was carried out in triplicate in Fast 96-well optical plates containing 5 μM protein and 5X SYPRO Orange dye (Invitrogen) in a total volume of 20 μL/well. Samples were heated at a rate of 3 °C/min from 25 to 95 °C, and protein unfolding was observed by monitoring the fluorescence of SYPRO Orange dye at 470 nm excitation and 623 nm emission using a ViaA7 real-time polymerase chain reaction machine (Applied Biosystems). K-RasG12D with dimethyl sulfoxide (DMSO) was used on the same plate as a reference for the shift in melting temperature of all GTPases used in the study. The assay was carried out in triplicate in Fast 96-well of the nucleotide exchange procedure of all GTPases used in the study. 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from ThermoFisher, catalog no. 78426). Unlysed cells and debris were pelleted for 15 min at 16000g and 4 °C. Samples were separated using SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The transfer was performed using the iBlot2 system (Invitrogen). Membranes were treated with Li-COR Odyssey blocking buffer for at least 1 h at room temperature and then incubated with a primary antibody (1:1000) in a 1:1 solution of PBS-T (PBS with 0.1% Tween 20) and Li-COR odyssey blocking buffer overnight at 4 °C. Following three 5 min washes in PBS-T, the membrane was incubated with secondary antibodies (1:3000) in a 1:1 solution of PBS-T and Li-COR Odyssey blocking buffer for 1 h at room temperature. Following three 5 min washes in PBS-T, the membrane was scanned using the Li-COR Odyssey Imaging System. Antibodies for pErk1/2, Erk1/2, pAkt Ser473, Akt, pan-Ras (Cell Signaling), and Raf-1 (Santa

Figure 1. P110 site and KAL-21404358 binding pose. (A) Model of K-Ras<sup>G12D</sup> (PDB entry 4DSN) with the P110 site colored red. The P110 site residues are Arg97, Asp105, Ser106, Glu107, Asp108, Val109, Pro110, Met111, Tyr137, Gly138, Ile139, Glu162, Lys165, and His166. (B) KAL-21404358 docking pose in the P110 site and its structure, docking score, chemical formula, mass, and molecular weight. (C) Detailed view of KAL-21404358 binding in the P110 site. Four potential hydrogen bonds are labeled with green lines. (D) KAL-21404358 superimposed with four high-energy water molecules colored red with ΔG > 2 and purple with ΔG > 1.
Cruz) were detected using a goat anti-rabbit or goat anti-mouse IgG antibody conjugated to an IRdye at 800CW and 680CW, respectively (Li-COR Biosciences).

## RESULTS

**Discovery of the P110 Site and KAL-21404358.** Given the challenges of directly targeting the nucleotide binding site and effector interaction surface, we hypothesized there might be allosteric pockets regulating the on and off cycle of K-Ras. To identify such sites, we first performed computational analysis of the K-RasG12D crystal structure (PDB entry 4DSN) using the SiteMap (Schrodinger Suites) prediction tool.21,22 The P110 site had the highest score for a potential small molecule binding site (r_sitemap_SiteScore = 0.92) and identified a binding site including residues Arg97, Asp105, Ser106, Glu107, Asp108, Val109, Pro110, Met111, Tyr137, Gly138, Ile139, Glu162, Lys165, and His166 (Figure 1A, colored red).

To explore the potential robustness of the P110 site across different protein conformations, we conducted similar analyses of other crystal structures of K-Ras (PDB entries 4EPR and 4OBE) and other Ras isoforms H-Ras (PDB entry 4L9W) and N-Ras (PDB entry 3CON). We found that the P110 site still appeared consistently in these simulations, of which one cluster (#6) showed the best SiteMap score of 1.06 (Figure 1D).

**Figure 2.** Validation of binding of KAL-21404358 to K-RasG12D. (A) MST assay of KAL-21404358 with GppNHp-bound K-RasG12D (KD = 88 ± 1 μM). Data are means ± the standard deviation (SD) of triplicate measurements and are representative of five independent experiments. (B) MST assay of KAL-21404358 with GDP-bound K-RasG12D (KD = 146 ± 2 μM) indicating selectivity toward GppNHp-bound vs GDP-bound K-RasG12D. Data are means ± SD of triplicate measurements and are representative of five independent experiments. (C) KAL-21404358 increases the melting temperature of K-RasG12D in a thermal shift assay. Data are means ± SD of triplicate measurements and are representative of two independent experiments. (D) KAL-21404358 lacks binding to P110 site mutants and has differential selectivity for K-RasG12D compared to other RAS family member proteins. Data are means ± SD of triplicate measurements and are representative of one independent experiment.
This suggests that this pocket can become even more accessible during the motion of the K-RasG12D protein.

MxMD simulations were then run with structure 4DSN using acetonitrile, isopropanol, pyrimidine, acetone, imidazole, and N-methylacetamide as organic probes. These organic probes can affect the conformation of K-RasG12D and reveal which sites can be accessible to small molecules of various chemotypes.23,24 The P110 site was found to contain all of these solvents as a hot spot, indicating its potential as a binding pocket for small organic molecules (Figure S1E).

We evaluated whether we could identify fragments or leadlike compounds predicted to bind with reasonable affinity to the P110 site. We tested 3.5 million compounds using the Glide docking algorithm (Schrödinger Suite), which generates a score in which the more negative the score, the higher the predicted affinity.25 Seventy-seven fragments with scores of less than −6.5 were obtained for further validation. Four rounds of biochemical screening using MST, a TSA, NMR line broadening, and HSQC NMR spectroscopy were used to select promising compounds from among these 77 candidates. KAL-21404358 was the most favorable compound, as it showed positive binding results in all four tests (Figures 1–3).

KAL-21404358 was predicted to interact well with the P110 site, with a Glide docking score of −7.37 (Figure 1B). A closer view of KAL-21404358 in the P110 site showed four potential hydrogen bonds (between the −NH in the hydroxyquinoline and the carboxyl group of Asp108, between the −OH in the hydroxyethyl group and the carboxyl group of Glu107, and two between the −OH in the hydroxyethyl group and the amine group of Arg97) as well as strong polar interactions (between the bridge of KAL-21404358 and the amide group of Glu107 and between the piperazinyl group and the amide group of Gly138) (Figure 1C).

WaterMap (Schrödinger Suite) was then used to estimate changes in the thermodynamic properties resulting from water molecule displacement by fragments binding in the P110 site.26,27 Four high-energy water molecules were predicted to be located in the P110 pocket superimposed upon KAL-21404358, which suggested that displacement of these water molecules may contribute to the binding free energy of KAL-21404358.

Figure 3. NMR validation of binding of KAL-21404358 to K-RasG12D. (A) NMR line broadening experiment of KAL-21404358 with an increased concentration of K-RasG12D (1:0, 1:0.3, 1:1, and 0:1 ratios). Peaks of hydrogens of quinolinol and the piperazinyl group (colored red) were broadened, indicating KAL-21404358’s binding to K-RasG12D. (B) HSQC NMR experiment showing conformational changes in switch I and switch II of K-RasG12D, which could be explained by P110 site’s allosteric effect. Blue represents GDP-bound K-RasG12D and red represents GDP-bound K-RasG12D with KAL-21404358 at a 1:7 concentration ratio. Residues changed upon binding with KAL-21404358 are labeled.
molecules would improve the binding affinity of KAL-21404358 even further (Figure 1D).

**Validation of Binding of KAL-21404358 to K-Ras<sup>G12D</sup>.**

MST and TSA were used as first-line screening methods. The \( K_D \) of KAL-21404358 for GppNHp-bound K-Ras<sup>G12D</sup> was 88 \( \mu \text{M} \), and the \( K_D \) with GDP-bound K-Ras<sup>G12D</sup> assessed by MST was 146 \( \mu \text{M} \) (Figure 2A,B). This suggests that KAL-21404358 has a slightly higher binding affinity for the GppNHp-bound form of K-Ras<sup>G12D</sup>. TSA experiments confirmed a 2.1 °C melting temperature shift, indicating binding of KAL-21404358 stabilizes GDP-bound K-Ras<sup>G12D</sup> to thermal denaturation, possibly further inhibiting activation of GDP-K-Ras<sup>G12D</sup> (Figure 2C).

**Figure 4.** KAL-21404358 inhibits the K-Ras<sup>G12D</sup>–B-Raf interaction and K-Ras<sup>G12D</sup>-dependent signaling. (A) A NanoBiT split luciferase assay showed that KAL-21404358 and its analogue KAL-YZ0965 disrupted the K-Ras<sup>G12D</sup>–B-Raf interaction. 3144 was used as a positive control. K-Ras<sup>G12D</sup> was fused to SmBiT; B-Raf was fused to LgBiT, and constructs were transfected in HEK293T cells. Luminescent signals were detected when K-Ras<sup>G12D</sup> bound to B-Raf. Ordinary one-way analyses of variance were conducted in Prism 7. Four asterisks indicate \( p < 0.0001 \). Three asterisks indicate \( p < 0.001 \). ns indicates \( p > 0.05 \). Data are means ± SD of quadruplicate measurements and are representative of three independent experiments. (B) KAL-21404358, KAL-YZ0965, and 3144 were screened against SmBiT-LgBiT to rule out nonspecific inhibition and toxicity. Data are means ± SD of triplicate measurements and are representative of three independent experiments. (C) L513 cells (with K-Ras<sup>G12D</sup>) were treated with KAL-21404358 as indicated, and the amounts of Raf-1–RBD-bound K-Ras<sup>G12D</sup> proteins were determined. Data are representative of two independent experiments. (D) Effects of KAL-21404358 on the abundance of phosphorylated Akt and total Akt (left) and phosphorylated Erk and total Erk (right) were determined at the indicated concentrations in L513 cells (with K-Ras<sup>G12D</sup> mutations). L513 cells from ATCC (catalog no. CRL-2134) were seeded 16 h prior to use in 10% FBS in RPMI-1640. The medium was then aspirated and replaced with serum-free medium containing KAL-21404358, and cells were incubated for 24 h. Relative intensities of phosphorylated forms vs total forms were quantified and are labeled. Data are representative of two independent experiments.
GppNHp-bound K-RasG12D did not cause a temperature shift (Figure S2A).

To test whether KAL-21404358 bound specifically to the P110 site, we constructed four mutants predicted to be deficient for binding to KAL-21404358: R97G, E107A, E107K, and E107N. The MST and HSQC NMR data showed that these mutants had no apparent chemical shifts, indicating specific binding to the P110 site.
To further elucidate how KAL-21404358 binds to K-RasG12D, we used NMR line broadening. The RasG12D, which is likely due to these being the interacting proteins, was tested for its ability to bind to KAL-21404358 using MST. Much weaker binding of KAL-21404358 was detected toward these other proteins, suggesting that KAL-21404358 has selectivity for K-RasG12D. We also examined the specificity of KAL-21404358 for K-RasG12D over K-RasWT, H-RasWT, Rap1a, R-Ras, and R-Ras2. Each protein was tested for its ability to bind to KAL-21404358 using MST. Much weaker binding of KAL-21404358 was detected toward these other proteins, suggesting that KAL-21404358 has selectivity for K-RasG12D.

Validation of the KAL-21404358 Binding Site and Binding Mode. To further elucidate how KAL-21404358 binds to K-RasG12D, we used NMR line broadening. The disappearance of hydrogens 1, 5, 6, 14, 15, 17, and 18 in the $^1$H NMR spectrum of the compound indicated binding to K-RasG12D, which is likely due to these being the interacting atoms on KAL-21404358. Analysis of the structure of KAL-21404358 suggested that it bound to K-RasG12D with the quinolinol and piperazinyl group, but not the neopentyl group, which was consistent with the computational prediction.

HSQC NMR was then used to identify the residues on K-RasG12D that change upon KAL-21404358 binding to test for possible allosteric effects. Conformational changes in the switch I and switch II regions (Asp33, Ser39, Leu56, Gly60, Met67, Thr74, and Gly75) were observed in GDP-bound K-RasG12D upon KAL-21404358 binding, suggesting an allosteric effect on the K-RasG12D conformation. However, KAL-21404358 interacted with only side chains of residues, as no backbone shifts near the P110 residues were observed in the HSQC NMR experiments.

Inhibition of the K-RasG12D–B-Raf Interaction. On the basis of KAL-21404358’s binding mode, we tested whether this compound could disrupt the interaction between K-RasG12D and B-Raf using a NanoBiT split luciferase assay. K-RasG12D was fused to SmBiT, and B-Raf was fused to LgBiT. The Raf–Raf interaction inhibitor 3144 was used as a positive control for validation of disruption of this interaction. Less K-RasG12D was detected after treatment of cells with KAL-21404358 (Figure 4C).

Modification of the KAL-21404358 Scaffold. To optimize the properties of KAL-21404358, we designed and synthesized a series of close structural analogues. We divided KAL-21404358 into four functional groups: the hydroxyquinoline (blue), the bridge (green), the amine group (black), and the neopentyl group (red) (Table 1). First, we hypothesized that the neopentyl group might be replaced to improve properties of the compounds, because (1) it did not interact with the P110 pocket in the computational models and (2) this group is hydrophobic, decreasing the aqueous solubility of the compound. We thus identified and tested seven KAL-21404358 analogues (red background) that had replacements of the neopentyl group only. The binding affinities of these compounds, as assessed by MST, were not improved, consistent with a lack of interaction at this site. These replacements did not generate new interactions between compounds and the receptor, likely because this group is facing the solvent. We next focused our attention on the...
hydroxyquinoline moiety, which fits well into the P110 pocket. Our hypothesis was that adding functional groups or changing the hydroxyquinoline moiety might form new hydrogen bonds between KAL-21404358 analogues and K-RasG12D, thereby improving binding. Six KAL-21404358 analogues (blue background) were identified and tested, among which analogue KAL-11067146 (bold) showed more potent binding in the MST assay (Table 1). However, this analogue did not affect the K-Ras G12D conformation in an HSQC NMR experiment, suggesting a loss of allosteric inhibitory activity. Computational docking did not show the presence of a hydrogen bond formed by the addition of the fluorine (Figure 5A), consistent with the NMR result.

Next, we added a carbonyl group to the bridge region to increase the number of hydrogen bond interactions with K-RasG12D and also to increase the hydrophilicity of KAL-21404358. Considering the difficulty of synthesizing the hydroxyethyl piperazinyl moiety, we decided to replace it with other cyclic amines. Three analogues were synthesized, and one was commercially available, among which KAL-PHB6002, KAL-PHB6003, and KAL-2241124388 exhibited more potent binding in the MST assay. Computational docking showed that KAL-PHB6002 fit well into the P110 site, as an example of this series of analogues (Figure 5B). The chemical shifts of these compounds in HSQC NMR experiments were similar to those of KAL-21404358 but not as apparent (Figure S3A). No backbone chemical shifts of the P110 site were observed. Computational docking showed that KAL-PHB6002 fit well into the P110 site, as an example of this series of analogues. The addition of an amide group as the bridge could form two potential hydrogen bonds with K-RasG12D, stabilizing the binding of KAL-PHB6002 (Figure 5C). KAL-PHB6002, KAL-PHB6003, and KAL-2241124388 together with KAL-55883121 (similar and better binding affinity with KAL-21404358 in the MST assay) were thus selected for testing in the NanoBiT K-RasG12D-B-Raf interaction assay. We detected

<table>
<thead>
<tr>
<th>Functional sites</th>
<th>Residues</th>
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<tbody>
<tr>
<td>P110 site</td>
<td>Arg97, Asp105, Ser106, Glu107, Asp108, Val109, Pro110, Met111, Tyr137, Gly138, Ile139, Glu162, Lys165, and His166.</td>
</tr>
<tr>
<td>P-loop</td>
<td>Gly10, Ala11, Asp12, Gly13, Val14, Gly15, Lys16, Ser17</td>
</tr>
<tr>
<td>Switch I</td>
<td>Asp30, Glu31, Tyr32, Asp33, Pro34, Thr35, Ile36, Glu37, Asp38, Ser39, Tyr40</td>
</tr>
<tr>
<td>Switch II</td>
<td>Gly60, Glu61, Glu62, Glu63, Tyr64, Ser65, Ala66, Met67, Arg68, Asp69, Glu70, Thr71, Met72, Arg73, Thr74, Gly75, Gly76</td>
</tr>
<tr>
<td>AllostERICALLY affected with binding of KAL-21404358</td>
<td>Asp33, Ser39, Leu56, Gly60, Met67, Thr74 and Gly75</td>
</tr>
</tbody>
</table>

Figure 6. Location of the P110 site in relation to effector domains. (A) The P110 site (orange) is in the allosteric lobe, opposite the functional P loop (blue), switch I (red), and switch II (green) domains that constitute the active site for GTP hydrolysis and effector protein binding. The left one shows the surface, whereas the right one shows the structure. (B) Residues in the P110 site, P loop, switch I, and switch II and those allosterically affected with binding of KAL-21404358 are listed and compared. Key residues that undergo conformational changes upon binding are shown in bold.
K-Ras\(^{G12D}\) has been considered a challenging target over the past 30 years. Here, we explore a strategy for discovering small molecule inhibitors that directly bind to this oncogenic K-Ras mutant. This strategy started with computational design, leading to discovery of a potential binding pocket. The P110 site is in the allostERIC lobe, which is opposite of the functional P loop (phosphate binding loop, residues 10−17), switch I (effector binding region, residues 30−40), and switch II (effector binding region and GEF and GAP binding region, residues 60−75) regions, which constitute the active site for GTP hydrolysis and interaction sites for effector proteins, including Raf, PI3K, RalGDS, and GAP (Figure 6A).\(^{29,30}\)

Despite the distance from the P110 site to effector domains, residues in switch I and switch II undergo conformational changes upon binding of KAL-21404358, causing disruption of K-Ras\(^{G12D}\) signaling activity (Figure 6B).

We used a tiered set of computational and biochemical assays to evaluate whether compounds can bind to this site and, if so, what effect they have on the K-Ras\(^{G12D}\) protein. The first-line screening consisted of computational ligand docking to select compounds predicted to bind in this site. The second screening filter involved the use of MST and TSA to validate binding to K-Ras\(^{G12D}\) experimentally. The third tier was NMR line broadening and a HSQC NMR binding assay to further discover the binding mode and location and to assess allosteric effects of binding. The fourth tier was a Ras functional assay involving direct interactions with Raf and two well-established cellular signaling pathways.

KAL-21404358 was found to be the best candidate from this set of assays. KAL-21404358 was further validated to specifically bind to K-Ras\(^{G12D}\) in the P110 site. Although the binding affinity was moderate, KAL-21404358 was able to disrupt the K-Ras−B-Raf interaction and Akt and Erk signaling pathways at high concentrations. GTP-bound K-Ras\(^{G12D}\) exists in two distinct conformations, state 1 and state 2, where state 1 has a lower binding affinity for effectors.\(^{31−33}\) On the basis of our experimental results, we hypothesize that KAL-21404358 binds to GTP-bound K-Ras\(^{G12D}\) state 1 and thus shifts the protein equilibrium from state 2 toward state 1 (Figure 7). KAL-21404358 can also bind to the GDP-bound (inactive) K-Ras\(^{G12D}\) in this inactive GDP-bound state by reducing its probability to nucleotide exchange, thus decreasing the amount of GTP-bound K-Ras\(^{G12D}\) (Figure 7). Thus, the P110 pocket, according to this model, is slightly more pronounced in state 1 of the GTP-bound protein but is also present in the GDP-bound protein.

To optimize this scaffold, a high-resolution structure is likely needed. We attempted to obtain such a crystal structure without success, likely due to the low binding affinity and low solubility of KAL-21404358. Nonetheless, we did synthesize a series of analogues to define the structure−activity relationship and found that addition of a carbonyl group to the bridge enhances the binding affinity because of the formation of new hydrogen bonds and increases the hydrophilicity of KAL-21404358. The binding affinities of those analogues were improved as shown by the MST assay, but their ability to disrupt K-Ras−B-Raf interaction was not enhanced. This remains to be studied further, especially by using structural biology approaches.

In summary, these findings suggest that the P110 site is a potential allosteric regulatory site for targeting oncogenic K-Ras proteins. KAL-21404358 is the first small molecule candidate for this site. This provides a new strategy for discovering small molecule allosteric inhibitors of KRAS\(^{G12D}\).
H.F., J.M.C., and B.R.S. designed, performed, and analyzed the results of computational analyses. H.F. and B.R.S. designed, performed, and analyzed the results of molecular cloning, protein expression, purification, MST, TSA, NMR, and cell culture studies. B.R.S., P.H.B., and Y.Z. designed, performed, and analyzed the results of synthetic chemistry. B.R.S. and M.M.D. designed, performed, and analyzed the results of the NanoBiT split luciferase assay. B.R.S. supervised the project. H.F. and B.R.S. wrote the manuscript, with input from other authors.

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Notes
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