

# Sotorasib Is a Pan-RAS<sup>G12C</sup> Inhibitor Capable of Driving Clinical Response in NRAS<sup>G12C</sup> Cancers



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## ABSTRACT

KRAS<sup>G12C</sup> inhibitors, like sotorasib and adagrasib, potently and selectively inhibit KRAS<sup>G12C</sup> through a covalent interaction with the mutant cysteine, driving clinical efficacy in KRAS<sup>G12C</sup> tumors. Because amino acid sequences of the three main RAS isoforms—KRAS, NRAS, and HRAS—are highly similar, we hypothesized that some KRAS<sup>G12C</sup> inhibitors might also target NRAS<sup>G12C</sup> and/or HRAS<sup>G12C</sup>, which are less common but critical oncogenic driver mutations in some tumors. Although some inhibitors, like adagrasib, were highly selective for KRAS<sup>G12C</sup>, others also potently inhibited NRAS<sup>G12C</sup> and/or HRAS<sup>G12C</sup>. Notably, sotorasib was five-fold more potent against NRAS<sup>G12C</sup> compared with KRAS<sup>G12C</sup> or HRAS<sup>G12C</sup>. Structural and reciprocal mutagenesis studies suggested that differences in isoform-specific binding are mediated by a single amino acid: Histidine-95 in KRAS (Leucine-95 in NRAS). A patient with NRAS<sup>G12C</sup> colorectal cancer treated with sotorasib and the anti-EGFR antibody panitumumab achieved a marked tumor response, demonstrating that sotorasib can be clinically effective in NRAS<sup>G12C</sup>-mutated tumors.

**SIGNIFICANCE:** These studies demonstrate that certain KRAS<sup>G12C</sup> inhibitors effectively target all RAS<sup>G12C</sup> mutations and that sotorasib specifically is a potent NRAS<sup>G12C</sup> inhibitor capable of driving clinical responses. These findings have important implications for the treatment of patients with NRAS<sup>G12C</sup> or HRAS<sup>G12C</sup> cancers and could guide design of NRAS or HRAS inhibitors.

See related commentary by Seale and Misale, p. 698.

## INTRODUCTION

The RAS family of protooncogenes, which includes KRAS, NRAS, and HRAS, represents the most commonly mutated family of oncogenes in human cancer (1). Although KRAS

mutations represent the majority of RAS mutations present in approximately 18% of all cancers, NRAS mutations and HRAS mutations are found in approximately 3% and approximately 1% of all cancers, respectively. Despite the

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importance of mutant RAS as a potential therapeutic target in oncology, efforts to drug RAS directly were unsuccessful for almost 30 years. An important breakthrough arose through the development of KRAS<sup>G12C</sup> inhibitors, which exploit a covalent interaction with the mutant cysteine in KRAS<sup>G12C</sup> that is not present in wild-type KRAS to drive both binding affinity and selectivity (2). Clinical development of KRAS<sup>G12C</sup> inhibitors in patients with KRAS<sup>G12C</sup> cancers has demonstrated efficacy across a broad range of tumor types, leading to the FDA approval of the KRAS<sup>G12C</sup> inhibitors sotorasib and adagrasib in KRAS<sup>G12C</sup> non-small cell lung cancer (NSCLC; refs. 3–5). Together, the initial clinical experience with KRAS<sup>G12C</sup> inhibitors has proven that direct targeting of mutant RAS can drive meaningful clinical antitumor responses.

On the basis of these initial observations, multiple classes of KRAS inhibitors are under development, including mutation-specific KRAS inhibitors selective for other mutations (e.g., G12D), pan-KRAS or pan-KRAS-selective inhibitors that target nearly all KRAS mutations but spare NRAS and HRAS, and pan-RAS inhibitors that target all RAS isoforms and mutations (6–9). However, there has so far been limited development and progress focused on agents that can directly target mutant NRAS or HRAS, which are present in a smaller but still meaningful population of patients with cancer. Farnesyl transferase inhibitors have demonstrated activity in HRAS-mutant tumors, due to a unique dependency of HRAS on this posttranslational modification, again demonstrating that targeting mutant RAS signaling can drive clinical efficacy (10). However, no inhibitors that bind directly and selectively to NRAS or HRAS have been reported or entered clinical trials. Given clinical proof of concept that targeting mutant RAS can drive clinical responses, direct NRAS or HRAS inhibitors could have substantial impact.

Here, based on the high amino acid sequence conservation between the RAS isoforms, we tested available KRAS<sup>G12C</sup> inhibitors and observed that some demonstrate the ability to target G12C mutations in NRAS or HRAS. Notably, we find that sotorasib is a highly potent NRAS<sup>G12C</sup> inhibitor and show proof of concept that sotorasib can drive clinical antitumor responses in a patient with NRAS<sup>G12C</sup> cancer.

## RESULTS

### Sotorasib Can Inhibit NRAS<sup>G12C</sup> and HRAS<sup>G12C</sup>

The three RAS family isoforms—KRAS, NRAS, and HRAS—have highly conserved amino acid sequences (Fig. 1A), with most differences occurring in the hypervariable domain represented by the last 25 amino acids of each isoform. Remarkably, there are no amino acid differences in the first 86 amino acids of each RAS isoform, and only four amino acid differences in the first 120 amino acids of each RAS isoform, which includes all residues contributing to the switch-II binding pocket to which most KRAS<sup>G12C</sup> inhibitors bind. Thus, we hypothesized that some KRAS<sup>G12C</sup> inhibitors might show similar potency against NRAS<sup>G12C</sup> and/or HRAS<sup>G12C</sup> mutations.

To test this hypothesis, we evaluated a range of commercially available KRAS<sup>G12C</sup> inhibitors in Ba/F3 cells engineered to express either KRAS<sup>G12C</sup>, NRAS<sup>G12C</sup>, HRAS<sup>G12C</sup>, or KRAS<sup>G12D</sup> as a negative control (Fig. 1B; Supplementary Fig. S1). Ba/F3

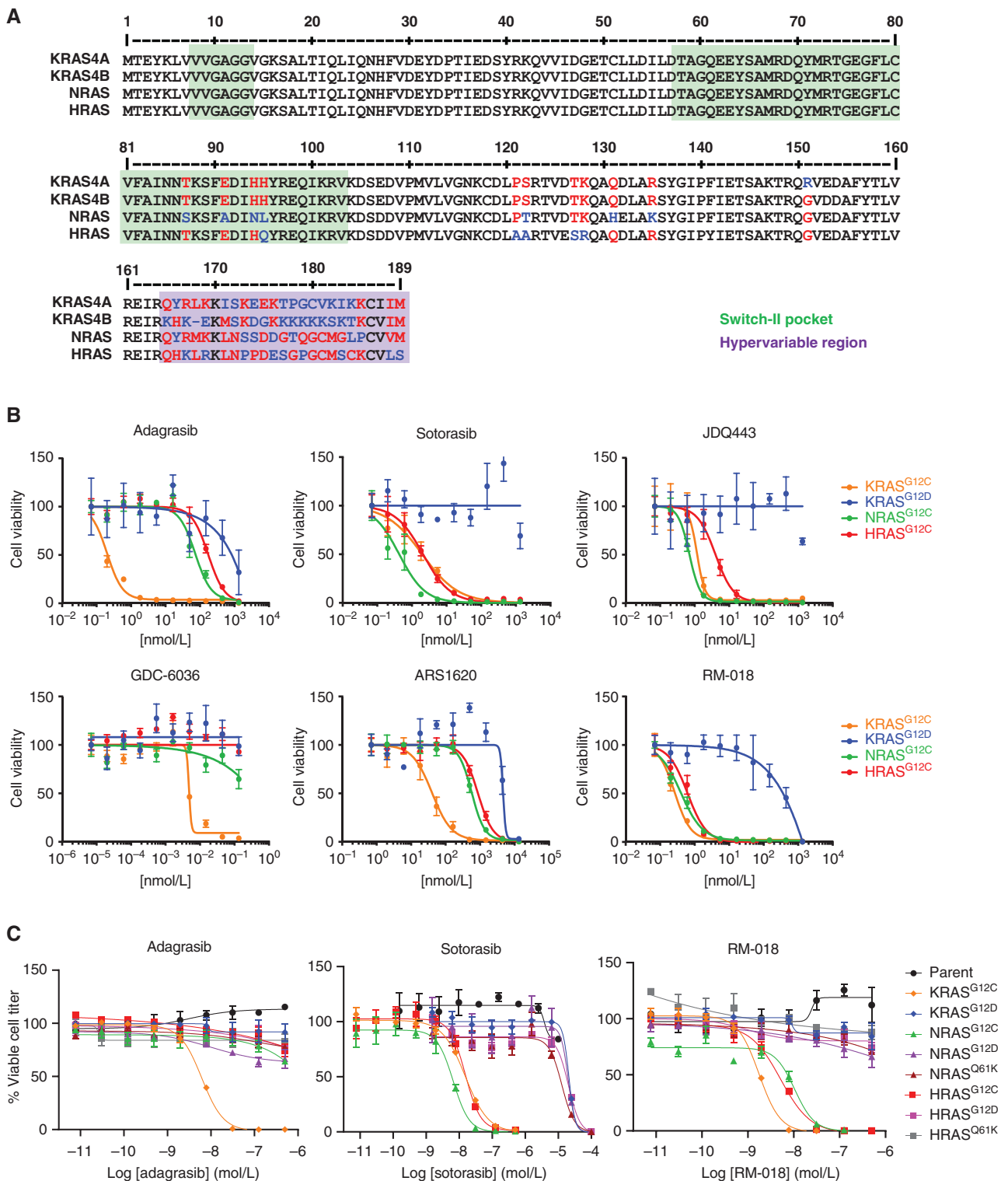
cells depend on exogenous IL3 for survival unless engineered to express oncogenic RAS, thus providing a robust isogenic system to interrogate RAS inhibitor sensitivity. All compounds potently inhibited KRAS<sup>G12C</sup>, as expected, and did not appreciably inhibit KRAS<sup>G12D</sup>, a negative control that lacks the mutant cysteine required for covalent binding. However, striking molecule-specific differences in the ability to target NRAS<sup>G12C</sup> and HRAS<sup>G12C</sup> were noted. Adagrasib demonstrated little activity against NRAS<sup>G12C</sup> and HRAS<sup>G12C</sup>, with an IC<sub>50</sub> similar to that observed with KRAS<sup>G12D</sup>. Similarly, GDC-6036 and ARS-1620 showed markedly reduced IC<sub>50</sub>s for NRAS<sup>G12C</sup> and HRAS<sup>G12C</sup> inhibition relative to KRAS<sup>G12C</sup>. Remarkably though, JDQ443 and sotorasib both demonstrated potent inhibition of NRAS<sup>G12C</sup> and HRAS<sup>G12C</sup>, comparable with that observed with KRAS<sup>G12C</sup>. The IC<sub>50</sub>s of JDQ443 for NRAS<sup>G12C</sup> and KRAS<sup>G12C</sup> were equivalent, with only a slight decrease in potency noted for HRAS<sup>G12C</sup>. Surprisingly, however, the IC<sub>50</sub> of sotorasib was five-fold more potent against NRAS<sup>G12C</sup> than for KRAS<sup>G12C</sup> and HRAS<sup>G12C</sup>, even though sotorasib was originally developed as a KRAS<sup>G12C</sup> inhibitor. Finally, the tri-complex RAS<sup>G12C</sup> inhibitor RM-018 (11, 12), which binds outside the switch-II pocket of RAS in a region where few amino acid differences exist between RAS isoforms, effectively inhibited NRAS<sup>G12C</sup>, HRAS<sup>G12C</sup>, and KRAS<sup>G12C</sup> with equal potency, as expected.

To evaluate further the differences in isoform selectivity between KRAS<sup>G12C</sup> inhibitors, we performed a detailed evaluation of adagrasib, sotorasib, and RM-018 against the G12C-, G12D-, and/or Q61K-mutated forms of KRAS, NRAS, and HRAS (Fig. 1C). As expected, none of these covalent inhibitors demonstrated inhibition of any RAS isoforms lacking the G12C mutation, confirming that their binding was still G12C-specific. Again, we observed that adagrasib only inhibited KRAS<sup>G12C</sup> and did not show any appreciable inhibition of NRAS<sup>G12C</sup> or HRAS<sup>G12C</sup>. RM-018 again inhibited all G12C-altered RAS isoforms with similar potency. However, sotorasib inhibited KRAS<sup>G12C</sup> and HRAS<sup>G12C</sup> with similar IC<sub>50</sub>s, but again showed greater potency with NRAS<sup>G12C</sup>.

Although NRAS<sup>G12C</sup> and HRAS<sup>G12C</sup> cancer cell models are limited, to further corroborate these findings, we evaluated the ability of sotorasib and adagrasib to inhibit NRAS<sup>G12C</sup> signaling in two additional models: (i) an isogenic cell line system in which KRAS<sup>G12C</sup> or NRAS<sup>G12C</sup> were exogenously expressed in 293T cells and (ii) the MOLT-4 leukemia cell line, which harbors an endogenous NRAS<sup>G12C</sup> mutation. In each model system, sotorasib, but not adagrasib, was able to inhibit NRAS<sup>G12C</sup> signaling (Supplementary Figs. S2A, S2B, and S3). Furthermore, sotorasib, but not adagrasib, exhibited covalent modification of NRAS<sup>G12C</sup> as evidenced by an upward shift in molecular weight upon Western blotting with an NRAS-specific antibody (Supplementary Fig. S2A and S2B).

### Isoform Selectivity of KRAS<sup>G12C</sup> Inhibitors Is Modulated by the Amino Acid at Position 95

We assessed the structural basis for these differences in isoform-specific RAS<sup>G12C</sup> inhibitory activity through comparative analyses of publicly available crystal structures (see Methods). As discussed above, the primary sequences of K-, N-, and HRAS are highly conserved; only four residues within 15Å of the switch-II binding pocket vary among the four family



**Figure 1.** Distinct RAS isoform selectivity profiles of KRAS<sup>G12C</sup> inhibitors. **A**, Amino acid sequence alignments of the four RAS family isoforms, including KRAS4A and KRAS4B, NRAS, and HRAS. Amino acid differences are shown in red and blue. Regions involved in the switch-II binding pocket are shaded in green, and the hypervariable region is shaded in purple. **B**, Ba/F3 cells engineered to express either KRAS<sup>G12C</sup>, NRAS<sup>G12C</sup>, HRAS<sup>G12C</sup>, or KRAS<sup>G12D</sup> were treated for 72 hours in the absence of IL3 with various concentrations of the indicated inhibitors. Viable cell titer was measured using CellTiter Glo. **C**, Ba/F3 cells engineered to express the various mutant forms of KRAS, NRAS, and HRAS were treated for 72 hours with a range of concentrations of the indicated inhibitors, and viable cell titer was assessed as in **B**.



members (Fig. 1A; Supplementary Fig. S4). Three of these differences are only present in NRAS: position 87 (Ser in NRAS, Thr in KRAS/HRAS), 91 (Ala in NRAS, Glu in KRAS/HRAS), and 94 (Asn in NRAS, His in KRAS/HRAS), whereas position 95 is different in all three RAS family isoforms (Leu in NRAS, His in KRAS, and Gln in HRAS).

Of these four isoform-variable positions of interest, only residue 95 is observed to make direct contact with switch-II pocket ligands). Notably, adagrasib (KRAS<sup>G12C</sup>-specific) and sotorasib (pan-RAS<sup>G12C</sup> inhibitor) show markedly different interactions with His95 in KRAS<sup>G12C</sup>. In binding KRAS<sup>G12C</sup>, adagrasib forms a direct hydrogen bond with His95 that is predicted to be relatively strong given the favorable geometry of the interaction. Moreover, the strength of this hydrogen bond is likely further enhanced by the presence of Glu62 and Tyr64, which are situated such that they shield this key interaction from solvent (Fig. 2A; Supplementary Fig. S5A). In contrast, sotorasib binds to KRAS<sup>G12C</sup> within the same cryptic groove but is not observed to interact with His95 and would therefore be predicted to be relatively insensitive to amino acid changes at that position. The importance of His95 in modulating isoform selectivity of KRAS<sup>G12C</sup> inhibitors is further supported by the fact that JDQ443, which like sotorasib is a pan-RAS<sup>G12C</sup> inhibitor, also does not form a direct hydrogen bond with His95 (Supplementary Fig. S5B). Conversely, ARS-1620 does form a direct hydrogen bond with His95 and exhibits a marked loss of potency for NRAS<sup>G12C</sup> (Supplementary Fig. S5C). Notably, the three switch-II-pocket-binding molecules in this study showing the greatest selectivity for KRAS<sup>G12C</sup>—adagrasib, GDC-6036, and ARS-1620—all share an unsubstituted core nitrogen that is well-positioned to form a hydrogen bond with His95. Furthermore, both adagrasib and GDC-6036 contain N-methylpyrrolidine moieties, which are likely key for orienting Glu62 and Tyr64 (Supplementary Fig. S6).

To more thoroughly explore the importance of the His95 interaction as well as other select amino acid differences in mediating distinctions in isoform selectivity, we performed reciprocal mutagenesis studies at the four amino acid positions within the switch-II binding pocket that differ between the RAS isoforms. When mutations corresponding to the NRAS amino acid sequence were introduced into KRAS<sup>G12C</sup>, mutations at positions 87, 91, and 94 had minimal effects on the activities of adagrasib and sotorasib (Fig. 2B). However, the H95 L mutation in KRAS<sup>G12C</sup> completely abrogated the activity of adagrasib. Conversely, the H95 L mutation actually increased the potency of sotorasib approximately five-fold, comparable with the greater potency observed for sotorasib in NRAS<sup>G12C</sup>. These data suggest that the presence of a leucine at position 95 may lead to increased potency of sotorasib, potentially through an enhanced hydrophobic interaction between the isopropyl pyridine substituent of sotorasib and Leu95 (Supplementary Fig. S7). Reciprocal mutations in NRAS<sup>G12C</sup> that introduced amino acids corresponding to the KRAS sequence further reinforced these observations, with minimal effects on potency observed with mutations at positions 87, 91, and 94 (Fig. 2C). However, mutations at position 95 again had striking effects, with a single L95H mutation in NRAS<sup>G12C</sup> capable of conferring activity of adagrasib at similar potency to that observed in KRAS<sup>G12C</sup>, despite

complete lack of activity in parental NRAS<sup>G12C</sup>. Similarly, in the reverse direction, the L95H mutation in NRAS<sup>G12C</sup> led to an approximately five-fold loss of potency for sotorasib. Similar patterns for inhibition of RAS–MAPK signaling were observed in an isogenic system, in which various NRAS<sup>G12C</sup> or KRAS<sup>G12C</sup> constructs with reciprocal mutations at amino acid positions 94 and 95 were expressed in 293T cells (Supplementary Fig. S3). Taken together, these reciprocal mutagenesis data suggest that a single amino acid difference at position 95 is the primary driver of isoform-specific differences in activity of KRAS<sup>G12C</sup> inhibitors.

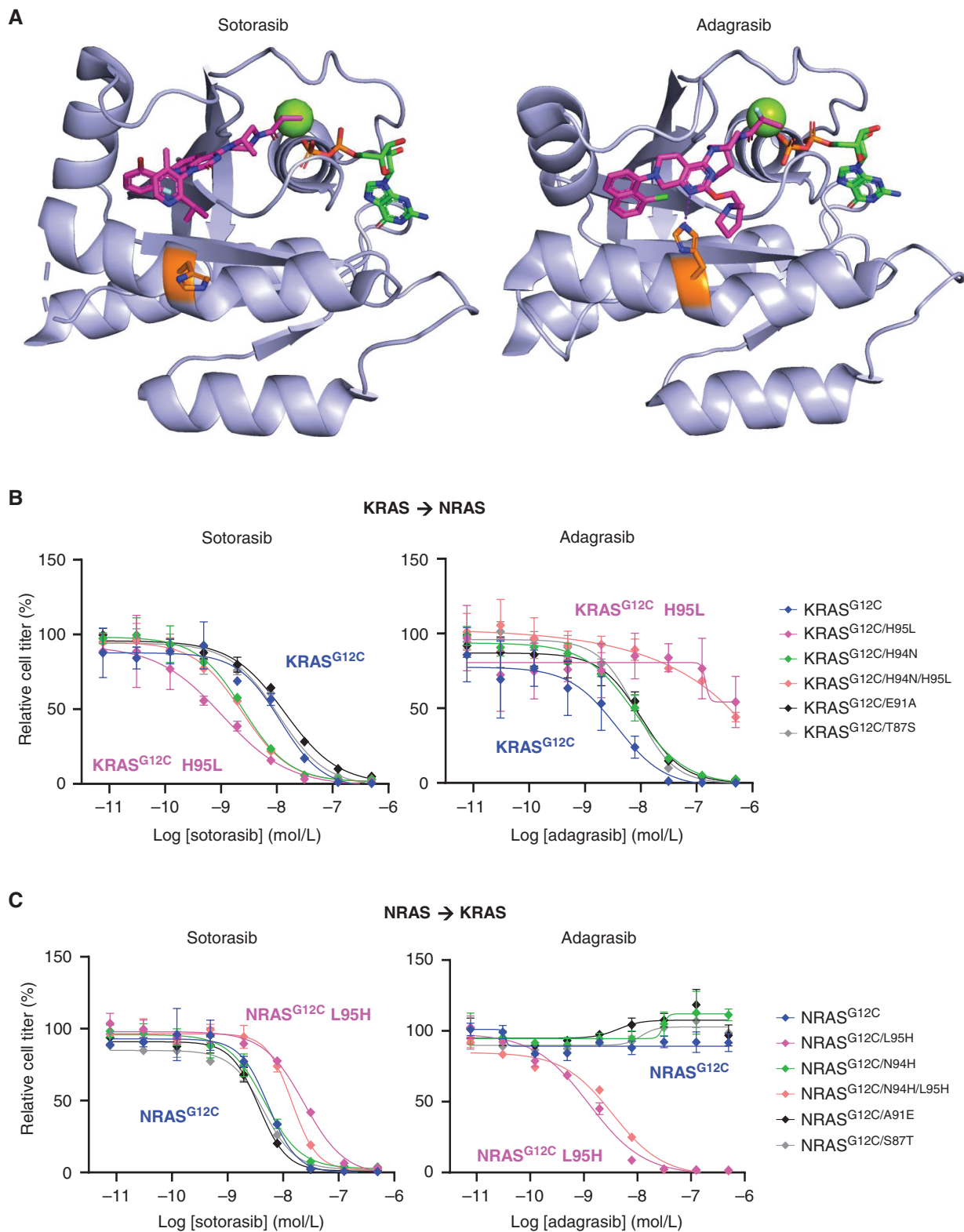
### Clinical Activity of Sotorasib in a Patient with NRAS<sup>G12C</sup> Colorectal Cancer

The observation that sotorasib can not only inhibit NRAS<sup>G12C</sup> but is actually a more potent inhibitor of NRAS<sup>G12C</sup> compared with KRAS<sup>G12C</sup> raises the possibility that sotorasib and other inhibitors that do not strongly rely on their interaction with His95 could be potential therapeutic agents for NRAS<sup>G12C</sup> cancers. Furthermore, the fact that sotorasib is an equipotent inhibitor of HRAS<sup>G12C</sup> and KRAS<sup>G12C</sup> further suggests that sotorasib could be used to target HRAS<sup>G12C</sup> cancers. To assess the frequency of NRAS<sup>G12C</sup> and HRAS<sup>G12C</sup> mutations in various tumor types, we evaluated 148,268 cancers in the AACR GENIE database (13). We observed that although NRAS<sup>G12C</sup> and HRAS<sup>G12C</sup> mutations are rarer than KRAS<sup>G12C</sup> mutations, these mutations are present in 0.08% and 0.01% of cancers, respectively, and are observed in specific cancer types, including melanoma, colorectal cancer, and leukemia (Fig. 3A and C).

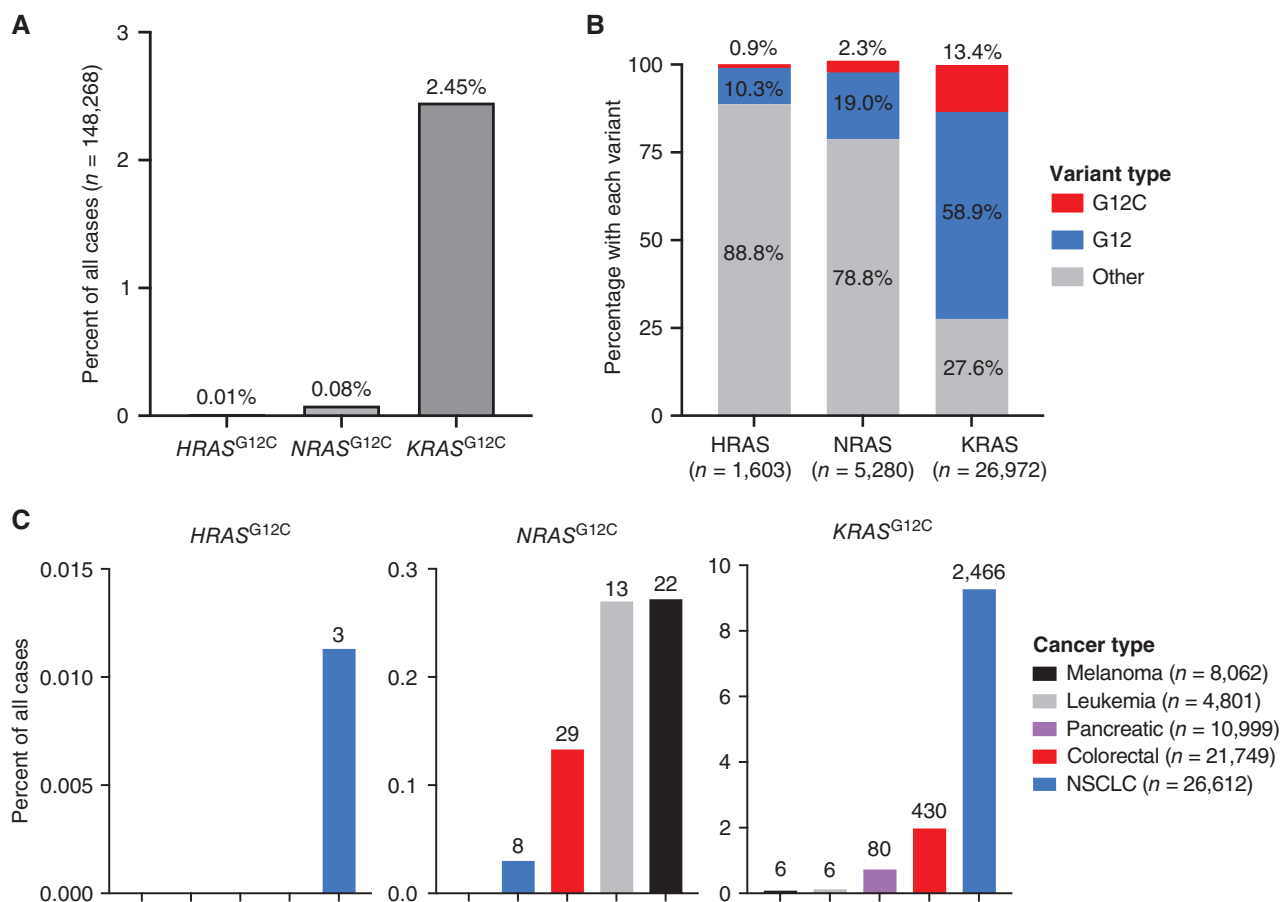
Given these findings, we predicted that sotorasib would have activity in NRAS<sup>G12C</sup> cancers. We identified a patient with colorectal cancer who harbored an NRAS<sup>G12C</sup> mutation and had exhausted all conventional therapy. Briefly, this 63-year-old man initially presented with vomiting, diarrhea, and marked anemia. A sigmoidoscopy demonstrated a mass with biopsy diagnostic of a microsatellite-stable, moderately differentiated adenocarcinoma. Staging evaluation with contrast-enhanced CT, MRI abdomen, and PET/CT showed for multiple hepatic metastases. A CEA was elevated at 84.7. Next-generation sequencing showed an NRAS<sup>G12C</sup> alteration. The patient received extensive palliative chemotherapy consisting of 5-fluorouracil (5-FU), leucovorin, and irinotecan (FOLFIRI) and eventually underwent sigmoid colectomy for locoregional progression (Fig. 4A). He was subsequently treated with 5-fluorouracil (5-FU), leucovorin, oxaliplatin (FOLFOX) with bevacizumab, capecitabine/bevacizumab, capecitabine, and FOLFIRI retreatment. He ultimately progressed after 23 cycles of FOLFIRI with imaging showing new peritoneal nodularity and mild increase in hepatic metastatic disease.

On the basis of the patient's NRAS<sup>G12C</sup> mutation, the lack of other standard treatment options, and our data above, we explored whether sotorasib therapy might be clinically beneficial in this setting. Colorectal cancer is known to have robust adaptive feedback signaling that leads to reactivation of the RAS–MAPK pathway after treatment with BRAF or KRAS<sup>G12C</sup> inhibitors (14–17). Preclinical and clinical studies have demonstrated that much of this feedback reactivation is mediated by EGFR. As a result, single-agent activity of





**Figure 2.** Structural basis of RAS<sup>G12C</sup> isoform selectivity. **A**, Crystal structures of sotorasib (6OIM) and adagrasib (6UTO) bound to KRAS<sup>G12C</sup> are shown making distinct interactions with His95 (orange). Each inhibitor is colored magenta, GDP and a conserved Mg ion are shown in green, and the key hydrogen bond is shown in purple. **B** and **C**, Reciprocal mutagenesis studies were performed to explore the effects of substituting the four amino acid differences within the switch-II binding pocket, with NRAS amino acids substituted into KRAS<sup>G12C</sup> (**B**) or KRAS amino acids substituted into NRAS<sup>G12C</sup>. Constructs were expressed in Ba/F3 cells and treated for 72 hours in the absence of IL3 with various concentrations of the indicated inhibitors. Viable cell titer was measured using CellTiter Glo.



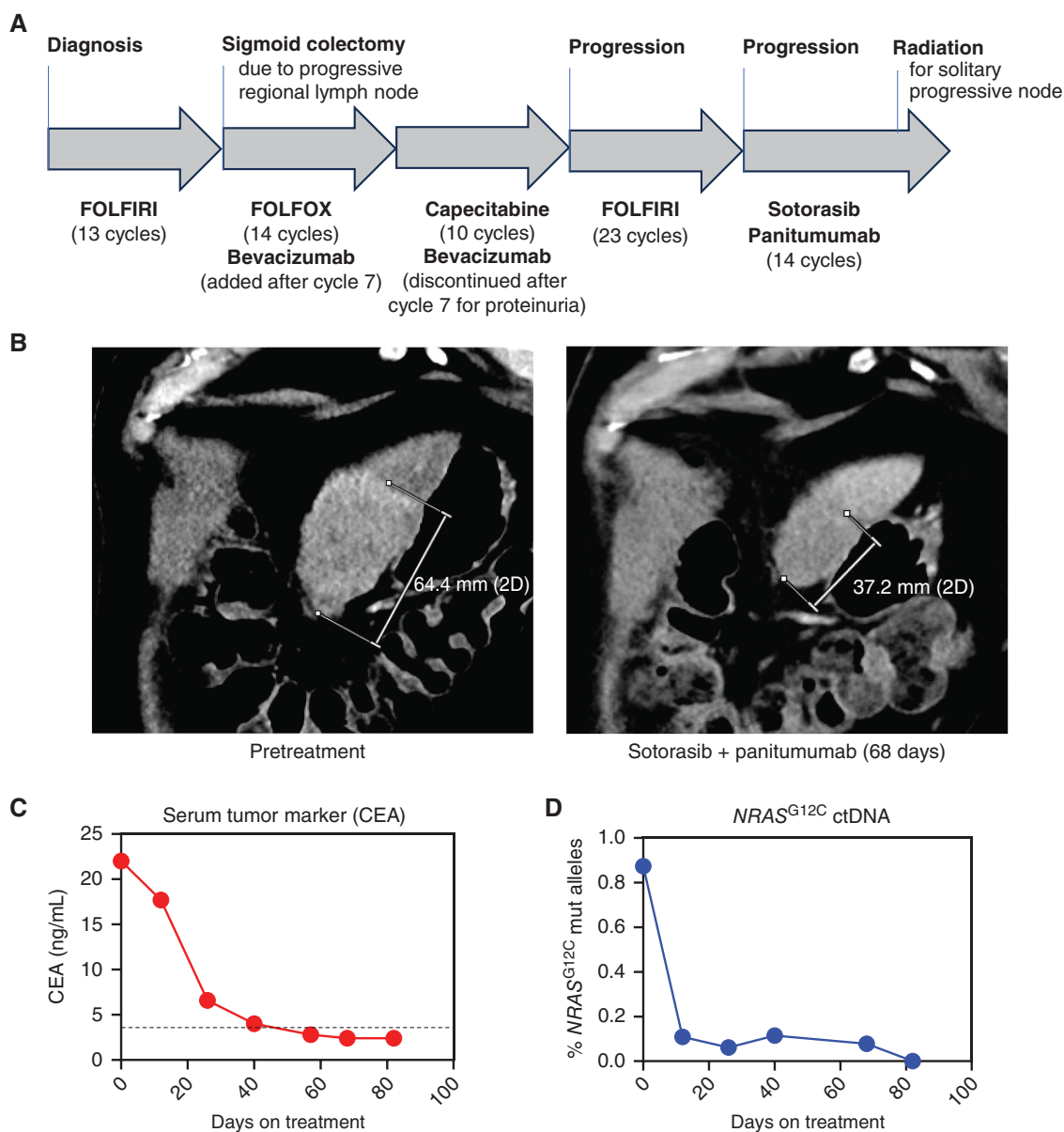
**Figure 3.** Frequencies of *NRAS*<sup>G12C</sup>, *HRAS*<sup>G12C</sup>, and *KRAS*<sup>G12C</sup> mutations in cancer. **A**, Percentage of *HRAS*<sup>G12C</sup>, *NRAS*<sup>G12C</sup>, and *KRAS*<sup>G12C</sup> mutations observed in 148,268 cancers from the AACR GENIE database. **B**, Of all cases with *HRAS*, *NRAS*, or *KRAS* mutations, the percentage with G12C mutations, other mutations of glycine 12 (G12), or other mutations not involving G12 are shown. **C**, Frequencies of *HRAS*<sup>G12C</sup>, *NRAS*<sup>G12C</sup>, and *KRAS*<sup>G12C</sup> mutations observed in specific cancer types. Actual numbers of cases with each mutation in specific diseases are indicated above each column.

BRAF and/or KRAS<sup>G12C</sup> inhibitors has been lower in colorectal cancer compared with other cancers, but combination therapy with an anti-EGFR antibody has led to improved response rates and clinical benefit (18–20). Thus, we elected to treat this patient with *NRAS*<sup>G12C</sup> colorectal cancer with the combination of sotorasib and the anti-EGFR antibody panitumumab based on established safety data from studies in patients with *KRAS*<sup>G12C</sup> colorectal cancer (21). After 2 months of treatment, repeat imaging showed a dramatic reduction in the size of the patient's liver metastases (Fig. 4B). In addition, the patient's serum carcinoembryonic antigen (CEA) tumor markers, which were elevated prior to treatment, normalized after the first month of therapy (Fig. 4C). We also evaluated levels of *NRAS*<sup>G12C</sup> in circulating tumor DNA (ctDNA) by droplet digital PCR (ddPCR), which similarly showed a dramatic reduction after initiation of treatment (Fig. 4D). Following cycle 12 of sotorasib/panitumumab, he received 40 Gy of radiation via MRI-guided stereotactic body radiotherapy to the solitary progressive mesenteric nodule. The patient remained on therapy with sotorasib and panitumumab and completed 14 treatment cycles. Therapy was well-tolerated with toxicities limited to grade 1 acneiform facial rash, grade 1 nausea, and grade 1 fatigue. Overall, these data provide

clinical proof of concept that sotorasib may be effective in patients with *NRAS*<sup>G12C</sup> cancers.

## DISCUSSION

This study provides initial proof of concept that sotorasib, although originally developed as a *KRAS*<sup>G12C</sup> inhibitor, is a potent inhibitor of *NRAS*<sup>G12C</sup> that can potentially be used to treat patients with *NRAS*<sup>G12C</sup> cancers. Furthermore, our preclinical data suggest that certain other *KRAS*<sup>G12C</sup> inhibitors that do not strongly rely on their interaction with His95 (i.e., JDQ443) may also potentially inhibit *NRAS*<sup>G12C</sup> and could therefore also be evaluated in this population. Although we treated and observed a clinical response in only a single patient, when taken together with a similar recently published case report of a patient with *NRAS*<sup>G12C</sup> colorectal cancer who also responded to treatment with sotorasib and panitumumab (22), these observations collectively provide support for the potential clinical efficacy of sotorasib in patients with *NRAS*<sup>G12C</sup> cancers. Although *NRAS*<sup>G12C</sup> mutations are rare (Fig. 3), these data suggest that sotorasib should be further evaluated as a treatment option for these patients when identified.



**Figure 4.** Clinical response in a patient with *NRAS*<sup>G12C</sup> colorectal cancer treated with sotorasib and panitumumab. **A**, Graphical representation of the patient's treatment history. **B**, CT scans taken pretreatment and after 68 days of combined therapy with sotorasib and panitumumab with measurements of the dominant metastatic liver lesion shown. **C**, Serial serum CEA tumor marker measurements were obtained before and throughout treatment. The dotted horizontal line represents the upper limit of normal (3.5 ng/mL). **D**, The mutant allele fraction of *NRAS*<sup>G12C</sup> was assessed in serial ctDNA specimens collected before and throughout therapy by ddPCR.

Our data also demonstrate that sotorasib can inhibit *HRAS*<sup>G12C</sup> with similar potency to *KRAS*<sup>G12C</sup>. Although *HRAS*<sup>G12C</sup> mutations are rare and no patients with *HRAS*<sup>G12C</sup> cancer have yet been treated with sotorasib, these preclinical data suggest that sotorasib and/or other *KRAS*<sup>G12C</sup> inhibitors that are not isoform-restricted might also be effective in these patients. These findings also have implications for other current or future mutation-selective *KRAS* inhibitors, such as *KRAS*<sup>G12D</sup> inhibitors (9), which are currently in clinical trials. Our data, as well as other recent reports (23, 24), suggest that it is possible that some mutation-selective inhibitors may retain the ability to inhibit a specific RAS mutation

across all RAS isoforms, thereby expanding the population of patients that may benefit. This could certainly have broader impact for mutations, such as G12D, that represent more common *NRAS* and *HRAS* mutations, and thus novel mutation-selective inhibitors should be evaluated for their ability to inhibit a specific mutation in the context of each RAS isoform going forward.

Our study has several limitations that affect the generalizability of the conclusions. First, it involves clinical evaluation of only a single patient. Although this patient did respond to therapy, supporting a potential role for sotorasib in this patient population, further study in a larger patient cohort



will be needed to assess its utility as a possible treatment strategy for *NRAS*<sup>G12C</sup> cancers. Second, our patient was treated with the combination of sotorasib and the anti-EGFR antibody panitumumab rather than single-agent sotorasib based on historical experience in colorectal cancer that suggests that coinhibition of EGFR can block adaptive feedback signaling and improve efficacy specifically in patients with colorectal cancer. Because there is extensive evidence that *NRAS*-mutant colorectal cancer does not respond to anti-EGFR antibodies alone, it is unlikely that the efficacy observed was due uniquely to panitumumab. Still, future evaluation of sotorasib monotherapy in *NRAS*<sup>G12C</sup> tumor types that are more likely to respond to single-agent inhibition (e.g., melanoma or NSCLC, based on experience with *KRAS*<sup>G12C</sup> inhibitors) would provide a more conclusive demonstration of clinical potential and would be warranted. Finally, as *NRAS*<sup>G12C</sup> and *HRAS*<sup>G12C</sup> models are limited, our analysis was restricted to a limited number of available or engineered models, and more extensive evaluation of patient-derived models and more detailed biochemical studies would be warranted.

Importantly, our study has potential implications for the design of inhibitors of *KRAS* and/or other RAS isoforms. Structural and reciprocal mutagenesis data suggest a mechanistic basis for RAS isoform selectivity of switch-II pocket inhibitors and the important role for interactions with the amino acid at position 95. Indeed, recent studies have demonstrated the potential for *KRAS* isoform-specific inhibitors (also called pan-*KRAS* or pan-*KRAS*-selective inhibitors), which have been shown to utilize a key interaction with His95 (8). The purpose of such inhibitors would be to effectively target the full spectrum of *KRAS* mutations while leaving *NRAS* and *HRAS* signaling in normal cells unaffected to provide for a tolerable therapeutic index. This is also supported by prior data that H95 mutations can drive acquired resistance to adagrasib (which our data confirm is highly selective for *KRAS*<sup>G12C</sup> vs. *NRAS*<sup>G12C</sup> or *HRAS*<sup>G12C</sup>), but not other less isoform-selective inhibitors like sotorasib (25). Understanding these key structure activity relationships can help drive the design of desired inhibitor profiles.

This study is also one of the first demonstrations that direct targeting of mutant *NRAS* can lead to clinical response. These data provide strong support for the development and clinical evaluation of direct *NRAS* inhibitors as a potential novel therapeutic class. The increased potency seen with sotorasib in the presence of Leu95, either native in *NRAS* or when substituted into *KRAS*, suggests a potential avenue for *NRAS* isoform selectivity that could be further exploited to generate *NRAS* isoform-selective inhibitors similar to the *KRAS* isoform-selective inhibitors discussed above and capable of inhibiting the full spectrum of more common *NRAS* mutations. Enhancing the potential hydrophobic interaction with Leu95 could be one avenue to achieve this desired selectivity profile to spare inhibition of signaling through all RAS isoforms in normal cells as a potential means of achieving favorable therapeutic index. Together, our data highlight the potential for clinical efficacy in patients with *NRAS*<sup>G12C</sup> cancers with currently available *KRAS*<sup>G12C</sup> inhibitors like sotorasib, and potentially also agents in clinical trials such as JDQ443 and RMC-6291 (the investigational *KRAS*<sup>G12C</sup>-selective tri-complex inhibitor, for which RM-018

is a representative preclinical tool; ref. 12), and point to a potential path for designing direct *NRAS* inhibitors capable of benefitting a broader patient population.

## METHODS

### Patient Care and Specimen Collection

All patient data and peripheral blood draws for plasma isolation were collected in accordance with Institutional Review Board-approved protocols, to which patients provided written informed consent, and all studies were conducted in accordance with the Declaration of Helsinki. The patient was treated with sotorasib and panitumumab, both approved by the FDA, off-label with informed consent, and the patient's insurance company covered the cost of this therapy. Sotorasib was administered as 960 mg once daily. Panitumumab was administered at a dose of 6 mg/kg intravenously once every 2 weeks. Imaging studies, including CT scans as well as serum CEA tumor marker measurements, were obtained as part of routine clinical care.

### Cell Lines and Reagents

Parental Ba/F3 cells were maintained in DMEM supplemented with 10% FBS and 10 ng/mL IL3. Oncogene-addicted Ba/F3 cells and HEK-293T cells were maintained in DMEM supplemented with 10% FBS. MOLT-4 cells were cultured in RPMI1640 medium (ATCC, #30-2001) with 10% FBS. All cell lines were grown in the presence of penicillin-streptomycin at 37°C and 5% CO<sub>2</sub>. All cell lines were obtained from ATCC or from the MGH Center for Molecular Therapeutics cell line collection, which performs routine short tandem repeat genotyping and *Mycoplasma* testing, and all cell lines were maintained in culture for less than 6 months from receipt. Sotorasib, JDQ443 (opnurasib), and GDC-6036 (divarasib) were purchased from MedChemExpress. Adagrasib and ARS-1620 were purchased from Selleck Chemicals. RM-018 was kindly provided by Revolution Medicines.

### Plasmid Construction and Establishment of Ba/F3 Line Series

Plasmids for *KRAS*<sup>G12C</sup>, *NRAS*<sup>G12C</sup>, and *HRAS*<sup>G12C</sup> were purchased from Addgene. pDONR223\_KRAS\_p.G12C and pDONR223\_NRAS\_p.G12C were a gift from Jesse Boehm, William Hahn, and David Root (pDONR223\_KRAS\_p.G12C: Addgene plasmid # 81667; <http://n2t.net/addgene:81667>; RRID:Addgene\_81667, pDONR223\_NRAS\_p.G12C: Addgene plasmid # 81660; <http://n2t.net/addgene:81660>; RRID:Addgene\_81660; ref. 26). Hs.HRAS G12C was a gift from Dominic Esposito (Addgene plasmid # 83182; <http://n2t.net/addgene:83182>; RRID:Addgene\_83182). The insert genes were subcloned into pMXs-Puro Retroviral Expression Vector, and the mutagenesis was performed for constructing the appropriate plasmids used in the study. Series of Ba/F3 lines were established as previously described (11).

### Cell Viability Assay

Cells were seeded in 96-well plates at 5–10 × 10<sup>3</sup> cells/well and incubated for 24 hours. Each cell was treated with a serial dilution of drugs for 72 hours, and then the cell viabilities were measured CellTiter-Glo (Promega) following manufacturer's instructions.

### Structural Modeling

Publicly available crystal structures showing the interaction between the drugs and protein were downloaded from the RCSB Protein Data Bank, and the computational structural modelings were performed using PyMol (The PyMOL Molecular Graphics System) and MOE (Chemical Computing Group). Models used in this study were: 6UT0 (adagrasib), 6OIM (sotorasib), 7R0M (JDQ443), and 5V9U (ARS-1620).

## Analysis of the Frequency of RAS<sup>G12C</sup> Variants

Mutational data were obtained from the AACR GENIE database.

## Cell-free DNA Isolation and Droplet Digital PCR

Cell-free DNA was isolated from plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen). For droplet digital PCR (ddPCR) analysis, DNA template (up to 10  $\mu$ L for a total of 20 ng) was added to 10  $\mu$ L of ddPCR Supermix for Probes (Bio-Rad) and 2  $\mu$ L of the custom primer/probe mixture. This reaction mix was added to a DG8 cartridge together with 60  $\mu$ L of Droplet Generation Oil for Probes (Bio-Rad) and used for droplet generation. Droplets were then transferred to a 96-well plate (Eppendorf) and then thermal cycled with the following conditions: 10 minutes at 95°C, 40 cycles of 94°C for 30 seconds, and 55°C (with a few grades of difference among assays) for 1 minute followed by 98°C for 10 minutes (ramp rate 2°C/second). Droplets were analyzed with the QX200 Droplet Reader (Bio-Rad) for fluorescent measurement of FAM and HEX probes. Gating was performed on the basis of positive and negative controls, and mutant populations were identified. The ddPCR data were analyzed with QuantaSoft analysis software (Bio-Rad) to obtain fractional abundance of the mutant DNA alleles in the WT/normal background. The quantification of the target molecule was presented as the number of total copies (mutant plus WT) per sample in each reaction. Fractional abundance is calculated as follows: F.A. % = [Nmut/(Nmut + Nwt)]  $\times$  100, in which Nmut is the number of mutant events and Nwt is the number of WT events per reaction. ddPCR analysis of normal control plasma DNA (from cell lines) and no-DNA template controls were always included. Probe and primer sequences are available upon request.

## Data Availability

Accession numbers for publicly available crystal structures used in this study are provided in the respective figure legends. Raw data are available upon reasonable request from the corresponding authors.

## Authors' Disclosures

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## Authors' Contributions

**D.A. Rubinson:** Conceptualization, formal analysis, validation, investigation, methodology, writing—original draft, writing—review and editing. **N. Tanaka:** Conceptualization, formal analysis, investigation,

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## Note

Supplementary data for this article are available at Cancer Discovery Online (<http://cancerdiscovery.aacrjournals.org/>).

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