RESEARCH ARTICLE

Selective Inhibition of Oncogenic KRAS Output with Small Molecules Targeting the Inactive State

ABSTRACT

KRAS gain-of-function mutations occur in approximately 30% of all human cancers. Despite more than 30 years of KRAS-focused research and development efforts, no targeted therapy has been discovered for cancers with KRAS mutations. Here, we describe ARS-853, a selective, covalent inhibitor of KRASG12C that inhibits mutant KRAS-driven signaling by binding to the GDP-bound oncoprotein and preventing activation. Based on the rates of engagement and inhibition observed for ARS-853, along with a mutant-specific mass spectrometry-based assay for assessing KRAS activation status, we show that the nucleotide state of KRASG12C is in a state of dynamic flux that can be modulated by upstream signaling factors. These studies provide convincing evidence that the KRASG12C mutation generates a “hyperexcitable” rather than a “statically active” state and that targeting the inactive, GDP-bound form is a promising approach for generating novel anti-RAS therapeutics.

SIGNIFICANCE: A cell-active, mutant-specific, covalent inhibitor of KRASG12C is described that targets the GDP-bound, inactive state and prevents subsequent activation. Using this novel compound, we demonstrate that KRASG12C oncoprotein rapidly cycles bound nucleotide and responds to upstream signaling inputs to maintain a highly active state. Cancer Discov; 6(3): 1–14. ©2016 AACR.

INTRODUCTION

Cancer genome sequencing efforts over the past 10 to 15 years have led to the identification of numerous oncogenes responsible for the development and maintenance of human cancers. These discoveries stimulated widespread oncogene-targeted drug-development efforts leading to the approval of a number of novel and efficacious targeted therapies, particularly against targets in the protein kinase family. Notably absent from the growing list of oncogenes with corresponding targeted therapeutics is the first to be discovered, and the most prevalent oncogene in human cancers, RAS. Cancers harboring RAS mutations remain essentially untreatable more than 30 years after the initial discovery of the oncogene.

RAS was for many years considered to be undruggable, but several recent reports have generated renewed interest in the development of direct RAS inhibitors (1). Molecules binding directly to RAS and inhibiting interaction with its activator SOS (2, 3) or effector RAF (4) have been reported. A third published approach specifically and covalently targeted the G12C mutation of KRAS with either electrophilic GDP mimetics (5) or electrophilic compouds targeting a novel allosteric pocket under the Switch II loop region (6). Compounds described in the latter work were found to inhibit RAS activity by blocking SOS-mediated nucleotide exchange and/or altering the relative affinity of KRAS for GDP versus GTP nucleotide. These innovative approaches demonstrated the presence of previously unknown binding pockets on the surface of RAS and provide a framework for continued efforts to develop novel RAS-targeted therapies.

Importantly, none of the recently presented KRAS-targeting approaches resulted in a compound with clearly demonstrated activity against mutant KRAS in cells. Here, we have focused on the novel Switch II pocket described by Ostrem and colleagues (6) and discovered a compound, ARS-853, with robust cellular activity against KRASG12C in the low micromolar range. A detailed biochemical and cellular characterization of the mechanism of action of ARS-853 revealed an unexpected plasticity in the nucleotide/activity state of KRASG12C. As opposed to the classic view of mutant KRAS as a constitutively active enzyme, we have discovered that the KRASG12C mutant rapidly cycles nucleotide and is thus responsive to upstream signaling inputs. These findings validate an unexpected strategy of targeting inactive mutant KRAS, and further provide a framework for exploring synergistic drug combinations and mechanisms of resistance to mutant KRAS inhibition.

RESULTS

Identification of Inhibitors That Selectively Engage KRASG12C Oncoprotein in Cells

In order to further characterize the cellular activity of the previously reported inhibitors and to enable the development of improved KRASG12C inhibitors with potent cellular activity, we developed an LC/MS-MS–based assay to directly and quantitatively determine engagement of KRASG12C in a cellular setting (Fig. 1A). Briefly, the decrease of the cysteine 12 (C12)–containing peptide from tryptic digests of KRASG12C-mutant cells following compound treatment is quantified relative to isotypic standard peptides. Using this assay we found that Compound 12 (6) did not show substantial KRASG12C covalent engagement in NCI-H358 (H358) cells, which harbor...
**Figure 1.** Identification of a covalent KRAS<sup>G12C</sup> inhibitor active in cells. **A,** schematic of the method for detecting small molecule engagement of KRAS<sup>G12C</sup> in cells by LC/MS-MS. Engagement is observed as a loss of the C12 containing tryptic peptide (KRAS<sup>G12C</sup> residues 6–16) relative to a control peptide (KRAS/NRAS residues 136–147). Each peptide is quantified relative to an internal standard (green and red, respectively) spiked in following the tryptic digest.

**B,** KRAS<sup>G12C</sup> signal intensity and LC/MS-MS extracted ion chromatograms of representative runs (upper right) from LC/MS-MS analyses of tryptic digests following 6-hour treatment of H358 cells (*, \(P < 0.05\); **, \(P < 0.01\)).

**C,** LC/MS-MS–extracted ion chromatogram showing adduct formation between the KRAS C12 tryptic peptide and ARS-107 in samples from **B**.

**D,** chemistry design scheme from Compound 12 to ARS-853.

**E,** KRAS<sup>G12C</sup> signal intensity and LC/MS-MS analyses of tryptic digests following treatment of H358 cells as indicated for 6 hours (#, \(P < 0.001\)).

**B–E,** error bars, mean ± SD (biologic replicates, \(n = 3\)).

**F,** crystal structure of ARS-853 bound to KRAS<sup>G12C</sup> highlighting key hydrogen bonds and hydrophobic interactions in the Switch II pocket. **G,** deconvoluted electrospray mass spectra of 2 \(\mu\)mol/L KRAS<sup>G12C</sup> (average MW 21,485) treated with ARS-853 (MW 432) for indicated times. ARS-853 covalently engages GDP-loaded KRAS<sup>G12C</sup> in a time-dependent manner, but not GMPPNP-loaded active KRAS<sup>G12C</sup>. **H,** EDTA-mediated nucleotide release assay with GDP-bound KRAS<sup>G12C</sup> protein prelabeled with the indicated inhibitor, and mant-GTP as excess incoming nucleotide. **I,** SOS-mediated nucleotide exchange assay with GDP-bound KRAS<sup>G12C</sup> protein prelabeled with the indicated inhibitor, and mant-GTP as incoming nucleotide.
the KRAS<sup>G12C</sup> mutation, even after a 6-hour treatment with 100 μmol/L compound (Fig. 1B).

To address the possible reasons for the lack of cellular efficacy observed for Compound 12, more potent Switch II pocket inhibitors were needed. With this aim, we performed iterative structure-based design of covalent KRAS<sup>G12C</sup>-targeted agents and tested candidates for their activity against purified, recombinant KRAS<sup>G12C</sup>, as well as their ability to engage KRAS<sup>G12C</sup> in cells. An early strong biochemical hit, ARS-107, exhibited promising KRAS<sup>G12C</sup> engagement in cells (Fig. 1B). Consistent with the loss of the C12 tryptic peptide of KRAS<sup>G12C</sup>, we detected a dose-dependent increase in a peptide species matching the adduct of ARS-107 with the C12 tryptic peptide (Fig. 1C). Structural and iterative structure activity relationship (SAR) evaluation of ARS-107 and related compounds suggested that the 5-chloro position on the phenyl ring was critical for engagement nucleotide exchange properties of KRAS<sup>G12C</sup> bound to ARS-853 further confirmed the structural predictions. Following reaction with ARS-853, GDP-bound KRAS<sup>G12C</sup> showed a dramatic decrease in EDTA catalyzed nucleotide release in the presence of either GTP or GDP as excess incoming nucleotides (Fig. 1H; Supplementary Fig. S2A–D). The extent of this bound Mg<sup>2+</sup>-GDP stabilization effect correlated with compound engagement potency across a set of ARS-853-related compounds (Fig. 1H; Supplementary Fig. S2A–D), suggesting cooperative binding between the Mg<sup>2+</sup>-GDP and the covalent inhibitor. This effect may be correlated structurally with the indirect Mg<sup>2+</sup> coordination observed in the crystal structure. ARS-853 and related analogues completely inhibited SOS catalyzed nucleotide exchange with either GDP or GTP as incoming nucleotide (Fig. 1I; Supplementary Fig. S2E–H), consistent with both Mg<sup>2+</sup> stabilization and general interference with Switch II function. Together, these data suggest an inhibitory mechanism of action whereby ARS-853 reacts specifically with GDP-bound KRAS<sup>G12C</sup>, and once bound prevents formation of the GTP-bound state through either intrinsic or catalyzed exchange mechanisms.

Before evaluating the effects of ARS-853 on cell functional readouts, we directly assessed its covalent selectivity across free cysteines in the proteome using an assay similar to that described by Wang and colleagues (ref. 7; Fig. 2A–B; Supplementary Fig. S3; Supplementary Table S4). Across the 2,740 surface-exposed cysteine residues profiled from 1,584 proteins, KRAS<sup>G12C</sup> was the most potently engaged target observed. Only two other targets, FAM213A and Reticulon-4 (RTN4), displayed significant engagement at doses lower than 30 μmol/L. Importantly, these two off-targets were also observed in A549 cells, which harbor the G12S oncogene and were chosen as a control line for subsequent studies (Fig. 2C).

**ARS-853 Inhibits KRAS<sup>G12C</sup> Oncoprotein Function in Cells**

We investigated the cellular effects of ARS-853 by monitoring the impact of treatment on active KRAS levels and RAS-mediated signaling in cells. ARS-853 treatment of KRAS<sup>G12C</sup> cells led to a dose-dependent and nearly complete inhibition of RAF-RBD pulldown and KRAS downstream signaling was determined by a proximity ligation assay (Fig. 3B). This method allows for the visualization of cellular KRAS–CRAF interactions through the proximity-dependent annealing and amplification of homologous DNA sequences present on antibodies to the two respective targets. Treatment of H358 cells by ARS-853 resulted in a significant loss of KRAS–CRAF interactions. Consistent with an inactive state of KRAS<sup>G12C</sup> once bound to ARS-853, downstream signaling through both MAPK (including pMEK, pERK, and pRSK) and PI3K signaling (pAKT) pathways was inhibited by ARS-853 in H358 and other KRAS<sup>G12C</sup> cell lines (Fig. 3A; Supplementary Fig. S4A–C). The inhibition of RAF-RBD pulldown and KRAS downstream signaling was sustained over a period of 72 hours (Fig. 3C), accompanied by G<sub>1</sub> cell-cycle arrest (Supplementary Fig. S4B), loss of Cyclin D1 and Rb expression, and an increase in the cell-cycle inhibitor p27 KIP1 (Fig. 3C; Supplementary Fig. S4A). In addition,
hallmarks of apoptosis, including cleaved PARP (Fig. 3A) and increases in sub-diploid DNA (Supplementary Fig. S4B), were observed in H358 cells following treatment with ARS-853. We observed no effects on RAF-RBD binding or downstream signaling in A549 cells (KRASG12S, Fig. 3A, right), and the inhibitory effects of ARS-853 in H358 cells could be rescued by ectopic expression of KRASG12V (Fig. 3D), highlighting the selectivity of ARS-853 for the KRASG12C oncoprotein.

Consistent with its specific and complete ability to inhibit KRASG12C signaling, ARS-853 selectively inhibited the growth of H358 cells in culture (Fig. 4A–D). Growth inhibition in H358 cells could be rescued by ectopic expression of KRASG12V (Fig. 4A), and was observed for KRASG12C, but not for KRASG12V oncogenic transformation of NIH-3T3 fibroblasts (Supplementary Fig. S5) supporting the KRASG12C oncoprotein–specific activity of ARS-853. In further support of an on-target mechanism of action, a small series of active and inactive structural analogues of ARS-853 inhibited the two-dimensional/adherent (2-D) proliferation of KRASG12C-bearing H358 cells with a potency trend strongly correlating with biochemical and cellular target engagement (Supplementary Fig. S6; Supplementary Table S1).

Across a panel of cell lines, ARS-853 inhibited proliferation in 2-D growth assays in only a subset of the KRASG12C lines tested (Fig. 4B, left). Despite the lack of growth inhibition in some lines, we found that ARS-853 effectively inhibited RAF-RBD
Targeting Inactive KRASG12C Suppresses Oncogenic Signaling

pulldown of KRAS in all lines tested (Supplementary Fig. S4A). KRAS knockdown by shRNA in a subset of these cells showed a similar trend of KRAS independence in an adherent growth format (Fig. 4E; Supplementary Fig. S7A–C), supporting that KRASG12C lines not affected by ARS-853 in 2-D formats are generally not dependent on mutant KRAS for adherent growth. These findings are consistent with published studies showing that only a fraction of KRAS-mutated cell lines are dependent on the KRAS oncogene to support growth and survival in adherent/2-D assay formats (8–10).

KRAS dependence is well established to be more pronounced in 3-D and/or anchorage-independent settings than in adherent growth assays (10–12). In line with gene-targeted approaches (10–12), all tested KRASG12C cell lines were robustly inhibited by ARS-853 in soft-agar colony formation assays with an average ~2 μmol/L IC50 (Fig. 4C and D). Using an alternative 3-D assay format consisting of identical assay conditions to the 2-D format, but using ultra-low adherent plates, allowed for a more direct comparison of adherent and nonadherent growth across a broad panel of cells. In this 3-D ultra-low adherent format, we observed robust inhibition of all 11 KRASG12C cell lines tested (Fig. 4B, right). No inhibition of 13 non-KRASG12C cell lines was observed in either adherent or anchorage-independent assay formats (Fig. 4B). Together, the molecular specificity of ARS-853, along with its selective inhibition of active KRASG12C, downstream signaling, and KRASG12C cell line growth, support that this compound is a selective and relatively potent KRASG12C inhibitor.

ARS-853 Cellular Engagement Requires Rapid Cycling of Nucleotide on the KRASG12C Oncoprotein

The structural and biochemical effects of ARS-853 described above support a mechanism of KRASG12C inhibition that is...
in H358 cells demonstrated that high doses of ARS-853 (10 μmol/L) achieved engagement levels >90% within 2 hours. Time and dose dependence of the inhibition of active KRAS, and downstream signaling markers were consistent with the KRASG12C engagement data (Fig. 5B).

The cellular engagement kinetics for ARS-853 did not fit a typical/expected pseudo–first order curve, indicating the possibility that factors such as resting GDP-KRASG12C levels, and nucleotide cycling rates may be affecting engagement rates. Importantly, engagement of the off-target RTN4 protein (monitored simultaneously in the assay) showed a normal dose and time response at the high concentrations where KRAS engagement rates were saturated (Supplementary Fig. S8). To assess the rates of the underlying nucleotide cycling of KRAS, we fit the engagement dose/time-response data using a kinetic model including variable parameters for nucleotide exchange, GTP hydrolysis, and protein (monitored simultaneously in the assay) showed a normal dose and time response at the high concentrations where KRAS engagement rates were saturated (Supplementary Fig. S8). To assess the rates of the underlying nucleotide cycling of KRAS, we fit the engagement dose/time-response data using a kinetic model including variable parameters for nucleotide exchange, GTP hydrolysis, and

Patricelli et al.

Figure 4. Cellular activity and selectivity of ARS-853. A, T-REx H358 cells stably expressing LacZ/TO or FLAG-KRAS G12V/TO were cultured with doxycycline (10 ng/mL) 24 hours prior to treatment with indicated amounts of ARS-853. Effects on cell growth were monitored 5 days later by CellTiter-Glo. B, the effects on adherent cell growth (left) or 3-D ultra-low adherent cell growth (right) following 5-day treatment with ARS-853. C, the effects on anchorage-independent growth in soft agar following 14-day treatment with ARS-853. Error bars, mean ± SD [across at least 2 independent experiments (B) or biologic duplicate wells (C), respectively]. D, representative images of crystal violet–stained colony formation in soft agar displayed from [B, D], Biologic duplicate wells (C), respectively]. E, the effects of KRAS knockdown or ARS-853 treatment on growth in 2-D adherent or 3-D ultra-low adherent formats. Cells (20,000) stably transduced with lentivirus expressing shLuc or shKRAS were plated 48 hours following transduction in media containing 1 μg/mL puromycin in either 2-D adherent or 3-D soft-agar formats. Twenty-four hours following plating, cells were treated with or without ARS-853 (10 μmol/L). Growth was monitored after 10 to 14 days, and representative images of crystal violet-stained colony formation are depicted (see also Supplementary Fig. S7).

surprising given the expectation that mutant KRAS would be locked in a fully active state. ARS-853 showed exquisite selectivity for the GDP-bound form of KRASG12C (Fig. 1G; Supplementary Table S3) which would likely exist at low levels in cells, yet the compound was clearly capable of achieving near-complete engagement and functional inhibition of cellular KRASG12C. To further investigate this surprising finding, we performed a series of studies to explore KRASG12C nucleotide cycling properties using ARS-853 as a “GDP-state titrant.” An acute time and dose-response analysis of cell engagement in H358 cells demonstrated that high doses of ARS-853 (>10 μmol/L) achieved engagement levels >90% within 2 hours (Fig. 5A). At these high doses, the engagement rate showed an initial burst (at 15 minutes) to an apparent rate-saturated level of ~45% engagement, followed by a slower rate of continued engagement that reached well over 90% by 2 hours. Time and
Targeting Inactive KRAS\textsuperscript{G12C} Suppresses Oncogenic Signaling

A good fit to the data, with half-lives for nucleotide release and hydrolysis of \(9.9\) and \(27\) minutes respectively and a rate constant for ARS-853 engagement with GDP-KRAS of \(140\) M\(^{-1}\)s\(^{-1}\). The value determined for KRAS G12C nucleotide exchange rate in cells is significantly faster than the intrinsic exchange rates determined by us (Supplementary Table S5) and others (13, 14). A recent comparison of KRAS position 12 mutant properties, however, found that KRAS\textsuperscript{G12C} hydrolyzed GTP with a half-life of \(23\) minutes (14), significantly faster than other mutants and consistent with our cellular characterization.

**KRAS\textsuperscript{G12C} GTP Levels Are Modulated by Upstream Signaling Factors**

The faster-than-expected nucleotide cycling of KRAS\textsuperscript{G12C} in cells prompted us to explore the intriguing possibility that mutant KRAS-GTP levels are regulated by the same signaling mechanisms that regulate wild-type (WT) RAS isoforms, such as growth factor receptor activation (Fig. 6A). Because H358 cells are heterozygous for \(\text{KRAS}^{\text{G12C}}\), we needed an assay capable of distinguishing the activation status of the \(\text{KRAS}^{\text{G12C}}\) oncoprotein from the WT-KRAS isoform. To achieve this goal, we developed a mass spectrometry–based approach using the RAF-RBD as in the traditional active RAS pulldown assay, but incorporating a GMPPNP-bound, stable isotope-modified KRAS\textsuperscript{G12C} (heavy KRAS G12C) protein as an internal reference. The heavy KRAS\textsuperscript{G12C} standard is added to lysates immediately prior to the RAF-RBD capture step, and a quantitative LC/MS-MS analysis focused on the residue 12 containing tryptic peptide (to differentiate WT and G12C isoforms) is used to determine the active fraction of endogenous (light) KRAS\textsuperscript{G12C} relative to the standard (RBD-MS assay; Supplementary Fig. S9A). By adding a GMPPNP-loaded heavy NRAS standard, we are also able to quantify the active fraction of the combined WT-RAS pool (Supplementary Fig. S9B). Following treatment with the EGFR inhibitor erlotinib, we observed a significant, time-dependent decrease in the KRAS\textsuperscript{G12C} GTP fraction (Fig. 6B and C; Supplementary Fig. S10). Using the general kinetic model for KRAS nucleotide cycling described above (Fig. 5A), the best fit of the erlotinib-induced loss of active KRAS suggested a GTP hydrolysis half-life of \(27\) minutes, consistent with the kinetic modeling of ARS-853 engagement. Following

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**Figure 5.** The kinetics of cellular G12C engagement and signaling inhibition with a GDP-state selective inhibitor demonstrates rapid nucleotide cycling of KRAS\textsuperscript{G12C} between GDP- and GDP-bound states. A, LC/MS-MS analysis of KRAS\textsuperscript{G12C} engagement following treatment of H358 cells (circles) overlaid with a fit (lines) based on nonlinear regression to a kinetic model depicted on the right. The kinetic model (right) incorporates parameters for KRAS\textsuperscript{G12C} nucleotide cycling with ARS-853 cellular engagement with best-fit values to the experimental data. B, Immunoblots showing kinetics and dose response of ARS-853 on KRAS-GTP by RBD pulldown (RBD-PD), and the effects on downstream RAS signaling activation markers for MAPK (pERK T202/Y204), and PI3K/mTOR (pAKT S473).
erlotinib treatment, we treated cells with ARS-853 and observed a significant increase in KRAS<sup>G12C</sup> engagement, consistent with an increase in the compound accessible GDP-bound KRAS<sup>G12C</sup> fraction (Fig. 6D). In contrast, when H358 cells were treated with EGF (50 ng/mL) to increase upstream signaling input to RAS, the active KRAS<sup>G12C</sup> fraction increased from 81% to 88%–91% (which would correspond to a ~2-fold decrease in the level of GDP-bound KRAS<sup>G12C</sup>), and ARS-853 engagement was significantly reduced (Fig. 6C and D). Furthermore, 24-hour pretreatment with trametinib, to induce
relief of negative feedback to RAS (15–17), increased HER3 activation (18, 19) and active KRASG12C levels while concomitantly reducing engagement (Fig. 6C and D). The effects of the inhibitor and growth factor–induced modulation of both KRAS-GTP levels and ARS-853 engagement were also observed in downstream signaling assays (Fig. 6E). Levels of active, GTP-bound WT-KRAS were increased, as expected by both EGF and trametinib treatments, but remained significantly lower than the mutant with all treatments (Fig. 6C). These results demonstrate that the activity of KRASG12C is not dictated only by intrinsic properties of the mutant protein, but rather can be modulated by signaling factors in a manner similar to WT-RAS isoforms, albeit with much higher active (GTP) fractions than WT-RAS isoforms.

We next tested the implications of the observed maintenance and regulation of KRASG12C nucleotide cycling on ARS-853 potency and efficacy in the setting of growth factor and drug combinations. When H358 cells were cultured in the presence of added EGF (50 ng/mL), the ARS-853 inhibition curve was right-shifted roughly 10-fold (Fig. 6F and G). Co-administration of erlotinib or afatinib with ARS-853 greatly potentiated growth arrest over single-agent ARS-853 treatment, even in the presence of added EGF (Fig. 6G; Supplementary Fig. S11A–C). In addition, combinations of ARS-853 with EGFR inhibitors more completely inhibited KRAS pulldown in the RBD assay, and downstream PI3K and MAPK signaling, accompanied by dramatic induction of apoptosis (Fig. 6H; Supplementary Fig. S11). The fact that complete inhibition of PI3K signaling can be achieved only by combined RTK and KRAS inhibition, leading to high levels of apoptosis, is consistent with published reports (20). Interestingly, we observed that the combination of trametinib with ARS-853 was less favorable based on its limited impact on growth arrest, RBD pulldown inhibition, PI3K pathway signaling, and apoptosis induction relative to ARS-853 alone. This may be due to counteracting effects of trametinib of reducing ARS-853 engagement and inhibiting MAPK signaling inhibition (Fig. 6G and H). These results highlight the implications of KRASG12C nucleotide cycling for inhibitors, like ARS-853, that target GDP-bound mutant KRAS. Although high levels of growth factor signaling may significantly reduce single-agent efficacy by such an approach, combinations with appropriate agents to mitigate upstream KRAS activation pressure can overcome this mechanism of resistance and provide significant benefit over a single-agent KRASG12C inhibitor (Fig. 7).

**DISCUSSION**

ARS-853 is the first direct KRAS inhibitor shown to selectively inhibit KRAS in cells with potency in the range of a drug candidate. Previously reported KRAS inhibitors have either shown insufficient potency for detailed cellular characterization (2, 3, 6), or exhibit dramatic deviations in potency across assays (4). The most potent covalent KRASG12C ligand reported previously (Compound 12) exhibited promising in vitro properties, but its cellular effects were less clear (6). Here, we demonstrate that Compound 12 is not capable of engaging KRASG12C in cells even at a relatively high dose and long incubation (100 μmol/L for 6 hours). However, by improving upon the groundbreaking approach of targeting the Switch II pocket of KRASG12C with covalent ligands, we have successfully identified a covalent inhibitor that demonstrates consistent low micromolar activity from biochemical and cellular engagement to KRASG12C activation, downstream signaling, and cell survival. Further, we have found that KRASG12C is the most potent covalent target of ARS-853 across more than 2,700 cellular proteins and consistently find that this compound exerts no effects on cellular signaling or growth in non-KRASG12C cells at concentrations up to 10-fold higher than its KRASG12C potency. These studies clearly establish that ARS-853 is a selective and highly efficacious KRASG12C inhibitor with low micromolar potency in cells.

Similar to the previously characterized Switch II pocket KRASG12C inhibitor, ARS-853 reacts only with the inactive (GDP-bound), but not the active (GTP-bound), state of KRAS. Mutant RAS proteins have been shown to exist predominantly in the GTP-bound state (21–24) and the overall “cycle time” (change of nucleotide due to either exchange or hydrolysis) of

**Figure 7.** Schematic models of the conventional (left) and proposed model (right) for the maintenance and modulation of active mutant KRAS levels. The proposed model presents mutant KRASG12C in a rapidly cycling state, whereby nucleotide exchange and hydrolysis occur and require signaling inputs (likely through exchange factors) to maintain high levels of the active, GTP-bound state. This model supports the following therapeutic opportunities as single agents or combinations: A, targeting the inactive-GDP-bound state; B, targeting the active-GTP-bound state (an area of assumed focus as sole strategy); and C, targeting the Ras Guanine nucleotide exchange factor (GEF), or (D) alternative upstream inputs that regulate RAS-GEFs.
ARS-853 binding to KRASG12C leads to a generally inactive signaling and proliferation studies clearly demonstrate that bound state once bound by ARS-853. Although this precise oncoprotein cycles its nucleotide state, allowing ARS-853 to achieve complete binding/inhibition over time as the oncoprotein cycles its bound nucleotide (Fig. 7, right).

Based on the structural and biochemical effects of ARS-853, particularly the dramatic stabilization of bound GDP preventing intrinsic and/or forced (EDTA) exchange and a complete block of enzymatic (SOS) exchange, we favor a mechanism of inhibition whereby KRASG12C is unable to achieve the GTP-bound state once bound by ARS-853. Although this precise mechanism is difficult to prove directly in cells, our cellular signaling and proliferation studies clearly demonstrate that ARS-853 binding to KRASG12C leads to a generally inactive state of the oncoprotein. ARS-853 is therefore a valuable pharmacologic tool to study KRAS function in cells that both complements and provides significant advantages over previously explored transcript and gene-targeted approaches (11, 12, 25). Perhaps most importantly, the rapid inhibition of mutant KRAS signaling by ARS-853 will enable the interrogation of the connectivity and responsiveness of mutant KRAS signals prior to the induction or relief of compensating feedback mechanisms (19, 25). This will be invaluable for understanding the mechanisms underlying the reported variability of KRAS dependence across cell lines observed by us and others (8–10). From our initial studies of KRASG12C-mediated signaling across cell lines (Supplementary Fig S4A) we have observed varying degrees of ARS-853 inhibition of downstream MAPK/PI3K signaling across the group of cell lines tested, suggesting that there are possible KRAS-independent mechanisms for maintenance of Ras signaling and that these inputs may contribute to differences in mutant KRAS dependence. Our studies with ARS-853 presented here have additionally confirmed that KRAS dependence is much more profound and universal in anchorage-independent settings. Future studies exploring the effects of KRASG12C inhibition on signaling in adherent and anchorage-independent settings may lead to a better mechanistic understanding of this feature of KRAS biology.

The observation that KRASG12C activity levels are responsive to growth factor stimulation and inhibition supports further exploration of approaches targeting potential upstream KRAS inputs. The interaction of KRAS with its activator SOS (2, 3, 26–28), for example, may be a valid approach to suppress KRAS oncogenic signaling, at least in the setting of KRASG12C-mutant tumors. Additionally, combination regimens of KRASG12C-targeted agents with inhibitors of targets upstream of KRAS may specifically enhance efficacy in KRASG12C-harboring tumors. Importantly, although we have shown here that EGFR signaling can support active KRASG12C levels, it is likely that the specific signaling inputs to KRAS will be cell type–specific. Thus, choosing the most effective combination agent may require an understanding of tumor-specific signaling vulnerabilities upstream of KRAS. Finally, the finding that KRASG12C behaves in a manner distinct from expectations based on historic studies of more commonly explored RAS mutants (Q61K, G12V, G12D) suggests that general assumptions of mutant RAS properties should be directly explored across the spectrum of oncogenic RAS mutations. KRASG12C may not be the only mutant RAS protein whose behavior deviates from the classic view of oncogenic RAS as a constitutively and fully active protein.

ARS-853 takes advantage of the unexpected nucleotide cycling features of KRASG12C detailed above and represents the first direct KRAS inhibitor to achieve cellular potency in the range of a drug candidate. Further optimization will be required to generate compounds suitable for in vivo studies, but this work presents a significant step toward a direct KRAS inhibitor for the treatment of patients with the KRASG12C mutation, which comprises 20% of lung cancers.

METHODS

Cell Lines and Reagents

Human cancer cell lines were purchased from the ATCC or the Korean Cell Line Bank (HCC-1171) and maintained at 37°C in a humidified atmosphere at 5% CO2 and grown in RPMI-1640 or DMEM growth media (Gibco) supplemented with 10% fetal bovine serum (Gibco), 50 units/mL penicillin, and 50 μg/mL streptomycin (Gibco). NCI-H358, NCI-H23, A549, and A375 cell line identities were confirmed using the CellChek 16 (IDEXX Bioresearch), an SNP-based profile authentication service, before studies were conducted. Cell lines utilized within the embodied work were purchased from ATCC (August 2014) and were carried for no longer than 12 cell passages. Erlotinib, afatinib, and trametinib were purchased from LC Laboratories. Recombinant human EGF was purchased from Life Technologies. All antibodies were purchased from Cell Signaling Technologies, except as follows: KRAS-specific antibody (clone C-19), NRAS-specific antibody (clone C-20), and HRAS-specific antibody (clone C-20) were from Santa Cruz Biotechnology; Ras-specific antibody (clone EPR3255) was from Abcam; and CRAF-specific antibody was from BD Biosciences.

Protein Expression and Purification

KRASG12C, WT, and G13C proteins were expressed in E. coli as truncated (1-169), hexahistidine-tagged forms as described by Ostrem and colleagues (6). For crystallography studies, the KRASG12C protein was expressed in E. coli and purified as described, including removal of the hexahistidine tag (6). Proteins used in biochemical studies were expressed and purified as described (6), except that the TEV cleavage (hexahistidine tag removal) and ion exchange chromatography steps were omitted. The resulting proteins were >95% pure as judged by SDS-PAGE. For SOS nucleotide exchange, the catalytic domain (594-170) and purified as described (6), except that the TEV cleavage (hexahistidine tag removal) and ion exchange chromatography steps were omitted. The resulting proteins were >95% pure as judged by SDS-PAGE. For SOS nucleotide exchange, the catalytic domain (594-170) and purified as described (6).
supplemented with 20% glycerol, flash-frozen, and stored in liquid nitrogen prior to obtaining diffraction data at beamline 5.0.1. (100 K nitrogen stream, wavelength = 0.9774 Å) at the Berkeley Lab Advanced Light Source. Data were initially processed with iMosfilm, solved by molecular replacement using Phaser and refined to the indicated statistics using Refmac (29). The refined model showed no Ramachandran outliers, and 98.5% of the residues were in the favored region (30).

**KRAS Biochemical Modification Assay**

GDP-loaded, hexahistidine-tagged, truncated (1-169) KRAS proteins (G12C, WT, G13D, as indicated) at 2 μM final concentration were incubated with the test compounds at the doses and time points indicated in a buffer containing 20 mM/L HEPES pH 7.5, 150 mM/L NaCl, 1 mM/L MgCl₂, and 1 mM/L DTT. Reactions were quenched by adding formic acid to 0.2%. The extent of covalent modification was determined by liquid chromatography, electrospray mass spectrometry analysis of the intact proteins on either a time of flight (TOF; Agilent 6530) or Q-Exactive (Thermo) mass spectrometer.

**Nucleotide Exchange Assays**

KRASG12C protein (truncated, hexahistidine tagged) was loaded with the indicated nucleotide (GDP or mantGDP) as described (6). For the exchange assays with incoming mant-nucleotide, 12 μL of the prepared protein (1.25 μM) in reaction buffer containing 1 μM/L of the indicated incoming nucleotide (mantGDP and mantGTP) was added to wells of a low-volume black-bottom plate. For studies with mant-nucleotide-loaded protein, 12 μL of the prepared protein (1.25 μM) in reaction buffer containing 1 μM/L of the indicated incoming nucleotide (GDP or GTP) was added to the plate. Exchange reactions were initiated by the addition of 3 μL of SOS (10 μM/L), or 3 μL of EDTA (20 μM/L), or buffer control as indicated, and the fluorescence was monitored for 45 minutes at 60-second intervals as described (6).

**G12C Cell Engagement Assay**

Cells (35 × 10⁶) adhered overnight were treated with compound at the indicated concentration and incubation time. After treatment, cells were washed twice with PBS buffer, and proteins were extracted using a buffer containing 9 mol/L urea, 10 mmol/L DTT, and 50 mmol/L ammonium bicarbonate, pH 8. Following iodoacetamide alklylation and trypsin digestion, the samples were analyzed by targeted LC/MS-MS analysis on a Dionex RSLCnano LC (Thermo Scientific) coupled with a Q-Exactive (Thermo) mass spectrometer. A detailed description of the calculations used to determine the RAS-GTP fraction can be found in Supplementary Methods.

**RBD-MS Assay**

Samples were prepared as described for the RAS-GTP pulldown assay by Western blotting above with the following modifications. Immediately before cell lysis, 50 ng each of heavy isotopic labeled, GMPNP-loaded KRASG12C and WT-NRAS protein standards were added to the lysis buffer. After lysis, 10% of the cell lysate was removed for precapture MS analysis, and the remaining 90% was precipitated with glutathione beads. The captured material was eluted with MS elution buffer (0.1% SDS, 10 mM/L DTT). Both the RBD eluted samples and precapture lysate samples were acetone precipitated, resuspended in a buffer containing 9 mol/L urea, 10 mmol/L DTT, and 50 mmol/L ammonium bicarbonate, pH 8, and subjected to trypsin digest and MS analysis as described above for the G12C cell engagement assay. A detailed description of the calculations used to determine the RAS-GTP fraction can be found in Supplementary Methods.

**Lentivirus Generation and shRNA Constructs**

For mutant KRAS overexpression, codon-optimized cDNAs encoding N-terminal FLAG-tagged human KRAS (G12V and G12C) were cloned into pLenti6.2/V5-DEST gateway vector or the T-REX Gateway vector pLenti6.3/TO/VS-DEST (LifeTech). For inducible ectopic expression of KRAS or LacZ for rescue experiments, NCI-H358 cells were stably transduced with the tetracycline repressor−based backbone vector pLenti3.3/TR from the ViraPower HiPerform T-REX Gateway vector kit as recommended by the manufacturer’s instructions (referred to as T-REX NCI-H358 cells) and were utilized for both signaling and proliferation assay in Figs. 3D and 4A. MISSION shRNA TRC1.5 vector clones in the pLKO.1-puro backbone for KRAS and Luciferase were purchased from Sigma. The clone IDs for shRNAs are as follows: shKRAS #1 (TRCN00000033260), shKRAS #2 (TRCN00000033262), and shLuciferase (SHC007). Lentiviral particles were packaged in HEK293FT cells using the ViraPower Lentiviral expression system (LifeTech) and transduced at an multiplicity of infection of 5 using standard recommended procedures. Cells were spinfected in the presence of 5 to 8 μg/mL polybrene and centrifuged at 600 ×g for 30 minutes at 37°C. Following spinfection cultures were replenished with fresh media.

**3-D Agar-Based Colony Formation Assay**

Cells (10,000–20,000) were seeded in 0.35% soft agar (SeaPlaque GTG agarose; Lonza) cultures (1 mL of 0.8% base, 1 mL of 0.35% cell layer, 1 mL of liquid top layer in 6-well plates). Cells were allowed to rest overnight in 3-D before treatment with compound. For compound treatment, DMSO or ARS-853 was supplied into the top liquid media layer. For shRNA experiments conducted in Fig. 3F, cells were seeded 48 hours after...
transduction with indicated lentiviruses in media containing 1 μg/mL puromycin either in 2-D or 3-D agar–based formats. After 12 to 14 days, colonies were stained with 0.05% crystal violet, and foci formation was scored with colony counting software (NIH Image, particle analyzer).

Cell Proliferation Assays

For experiments conducted in 2-D adherent formats or 3-D ultra-low adherent formats, cells (800–1,200 per well) were seeded in standard tissue culture–treated 96-well plate format (Corning Costar #3903) or ultra-low attachment surface 96-well plate format (Corning Costar #3474). The day after plating, cells were treated with serial dilutions of indicated inhibitors. Five days later, cellular viability was assessed using CellTiter-glo (Promega) according to the manufacturer's instructions. IC50 calculations were performed in GraphPad Prism Version 6.0. For shRNA experiments conducted in Fig. 3F, cells (20,000) for the 2-D format were seeded into tissue culture–treated 6-well plates following lentiviral transduction as described above.

Proximity Ligation Assay

Protocols and reagents for the proximity ligation assay were based on the DuoLink In Situ Staining Kit (Sigma, DUO92014). Cells were allowed to adhere overnight onto 18-well microslides (iBidi, #81826) in a humidity slide chamber. Following compound treatment, cells were washed with PBS and fixed with 3.7% paraformaldehyde for 15 minutes at 37°C in a humidity slide chamber. Cells were permeabilized (0.1% triton X-100) for 30 minutes at room temperature and stained with anti-KRAS antibody (clone C-19; Santa Cruz Biotechnology, 1:50) and anti-CRAF (BD Biosciences, 1:50) overnight at 4°C in a humidity slide chamber. Following a 3x wash, secondary DuoLink PLUS and MINUS PLA probes (Sigma, DUO92002 and DUO92004) were added for 2 hours at 37°C. Following three washes, a ligation and amplification reaction was performed following the manufacturer's instructions. In brief, the oligonucleotide-conjugated secondary antibodies were ligated together with a ligation reaction of 45 minutes at 37°C, and a rolling circle PCR amplification reaction for 100 minutes at 37°C (GFp version). Slides were subsequently air-dried for 1 hour at room temperature and mounted with ProLong Gold antifade (LifeTech) media with DAPI. The slides were imaged using a 40x objective on a Fluorod Xl microscope (LifeTech). KRAS–CRAF interactions were quantified using DuoLink In Situ Image Tool software (Sigma) from merged TIFF color images of both the PLA signal and nucleic channel (DAPI stain). A minimum of 200 cells were quantified from three independent replicate wells for each treatment condition.

Cysteine Selectivity Profiling

NCI-H358 cells (1 × 106) were seeded overnight in 6-well plates and treated with compound for 4 hours at the indicated concentration. Cells were trypsinized and washed with PBS buffer. Cells were resuspended in a buffer containing 25 mmol/L Tris–HCl, pH 7.5, 150 mmol/L NaCl, 1% NP-40, 5% glycerol and protease inhibitor. Following probe sonication, cell extracts were treated with 100 μmol/L iodoacetamide desthiobiotin for 1 hour at room temperature to label surface-exposed cysteines. After probe labeling, proteins were acetone precipitated and resuspended in a buffer containing 9 mol/L urea, 10 mmol/L DTT and 50 mmol/L ammonium bicarbonate, pH 8, processed, and digested with trypsin as described for the G12C cell engagement assay. The eluate from the Zeba desalting plates was diluted 1:1 with 100 μL of 25 mmol/L Tris–HCl pH 7.4, 150 mmol/L NaCl, and 0.1% NP-40, and the desthiobiotinylated peptides were enriched using high-capacity Streptavidin agrose beads (30 μL per sample, Thermo #20359). The beads were incubated with the sample for 1 hour and washed sequentially with 25 mmol/L Tris–HCl pH 7.4, 150 mmol/L NaCl, 0.1% NP-40, PBS, and water. Enriched peptides were eluted with 150 μL of 50% acetonitrile and 0.1% TFA, dried using a Savant SPD100 centrifugal evaporator (Thermo Scientific), and stored at −20°C until LC/MS-MS analysis. A detailed description of the LC/MS-MS analysis procedure can be found in Supplementary Methods.

NIH-3T3 KRAS Transformation

3T3 cells (2 × 106) transduced with indicated mutant KRAS lentiviruses were selected for 1 week with 1 μg/mL blasticidin and were subsequently plated in a top layer of 0.35% soft agar (SeaPlaque GTG agar; Lonza), cultured, and treated as described.

Cell-Cycle Analysis by Flow Cytometry

Cells plated in 6-well plates were treated for 72 hours as indicated. One hour prior to harvest, 2 μmol/L 5-ethyl-2′-deoxyuridine (EdU) was added to cultures to assess cycling cells. Cells were subsequently trypsinized, collected (including unattached cells), and washed with PBS. Cells were then fixed with 4% paraformaldehyde, permeabilized with saponin-based solution, and stained for EdU incorporation using the Click-iT EdU flow cytometry assay kit (LifeTech C10632) using the Alexa Fluor 488 azide per the provided instructions. DNA content was monitored by the addition of FarRed Fluor Violet stain (LifeTech) 20 minutes prior to acquisition. Cell-cycle analysis was performed with a Millenyi MACSQuant flow cytometer, and indicated populations were quantified using FlowJo software, V10.

Chemical Synthesis of Compounds

See supplementary information and Supplementary Fig. S12A–B.

Disclosure of Potential Conflicts of Interest

M.P. Patricelli reports receiving commercial research support from Janssen Biotech and has ownership interest (including patents) in Wellspring Biosciences LLC. M.R. Janes has ownership interest (including patents) in Wellspring Biosciences LLC. L.-S. Li reports receiving commercial research support from Janssen Biotech, Inc. and has ownership interest (including patents) in Wellspring Biosciences LLC. M.P. Patricelli, M.R. Janes, and R. Hansen has ownership interest (including patents) in Wellspring Biosciences LLC. T. Ely reports receiving commercial research support from Janssen Biotech Inc. and has ownership interest (including patents) in Wellspring Biosciences LLC. J.M. Kucharski reports receiving commercial research support from Janssen Biotech, Inc. and has ownership interest (including patents) in Wellspring Biosciences LLC. U. Peters reports receiving commercial research support from Janssen Biotech, Inc. and has ownership interest (including patents) in Wellspring Biosciences LLC. U. Peters reports receiving commercial research support from Janssen Biotech Inc. and has ownership interest (including patents) in Wellspring Biosciences LLC. A. Babbar reports receiving commercial research support from Janssen Biotech Inc. and has ownership interest (including patents) in Wellspring Biosciences LLC. P. Ren has ownership interest (including patents) in Wellspring Biosciences LLC and is a consultant/advisory board member for the same. Y. Liu has ownership interest (including patents) in Wellspring Biosciences LLC and is a consultant/advisory board member for the same. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Targeting Inactive KRASG12C Suppresses Oncogenic Signaling

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