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Allele-specific inhibitors inactivate mutant KRAS G12C by a trapping mechanism

Piro Lito^{1,*}, Martha Solomon², Lian-Sheng Li³, Rasmus Hansen³, and Neal Rosen^{1,2,*}

¹Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, NY, USA

²Molecular Pharmacology Program, Memorial Sloan Kettering Cancer Center, New York, NY, USA

³Wellspring Biosciences, La Jolla, CA, USA

Abstract

It is thought that KRAS oncoproteins are constitutively active because their guanosine triphosphatase (GTPase) activity is disabled. Consequently, drugs targeting the inactive or guanosine 5'-diphosphate-bound conformation are not expected to be effective. We describe a mechanism that enables such drugs to inhibit KRAS^{G12C} signaling and cancer cell growth. Inhibition requires intact GTPase activity and occurs because drug-bound KRAS^{G12C} is insusceptible to nucleotide exchange factors and thus trapped in its inactive state. Indeed, mutants completely lacking GTPase activity and those promoting exchange reduced the potency of the drug. Suppressing nucleotide exchange activity downstream of various tyrosine kinases enhanced KRAS^{G12C} inhibition, whereas its potentiation had the opposite effect. These findings reveal that KRAS^{G12C} undergoes nucleotide cycling in cancer cells and provide a basis for developing effective therapies to treat KRAS^{G12C}-driven cancers.

Wild-type RAS guanosine triphosphatases (GTPases) cycle between an active, guanosine 5'-triphosphate (GTP)-bound, and an inactive, guanosine 5'-diphosphate (GDP)-bound, state (1, 2). This is mediated by nucleotide exchange factors, which catalyze the exchange of GDP for GTP, and GTPase-activating proteins, which potentiate a weak intrinsic GTPase activity (3). Cancer-causing mutations impair the GTPase activity of RAS, causing it to accumulate in the activated state (4–6). Despite the prevalence of these mutations, no therapies that directly target this oncoprotein are currently available in the clinic (7–9). A recently identified binding pocket in KRAS^{G12C} (10) now enables the discovery of compounds that potently inhibit KRAS-GTP or effector signaling by this mutant.

Here we characterize a novel compound, ARS853, designed to bind KRAS^{G12C} with high affinity (11). The structures of ARS853 and previously reported (10) compounds (cmpds) 6 and 12 are shown in fig. S1A. Treatment of KRAS^{G12C}-mutant lung cancer cells with

*Corresponding author. rosen@mskcc.org (N.R.); lito@mskcc.org (P.L.).

SUPPLEMENTARY MATERIALS

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Materials and Methods

Figs. S1 to S7

References (34–36)

ARS853 reduced the level of GTP-bound KRAS by more than 95% (Fig. 1A, 10 μ M). This caused decreased phosphorylation of CRAF, ERK (extracellular signal-regulated kinase), and AKT. In contrast, even at the highest concentration tested, compd 6 or 12 had only a minimal effect on pCRAF and pERK, without affecting KRAS-GTP levels (Fig. 1A and fig. S1B). ARS853 inhibited proliferation with an inhibitory concentration 50% (IC_{50}) of 2.5 μ M, which was similar to its IC_{50} for target inhibition (Fig. 1, A and B). ARS853 (10 μ M) inhibited effector signaling (Fig. 1C and fig. S1C) and cell proliferation (Fig. 1D and fig. S2) to varying degrees in six KRAS^{G12C} mutant lung cancer cell lines, but not in non-KRAS^{G12C} models (Fig. 1E and fig. S1, C and D). Similarly, it completely suppressed the effects of exogenous KRAS^{G12C} expression on KRAS-GTP levels, KRAS-BRAF interaction, and ERK signaling (fig. S1E). Inhibitor treatment also induced apoptosis in four KRAS^{G12C} mutant cell lines (Fig. 1, F to H). Thus, ARS853 selectively reduces KRAS-GTP levels and RAS-effector signaling in KRAS^{G12C}-mutant cells, while inhibiting their proliferation and inducing cell death.

In contrast to the rapid inhibition of signaling by kinase inhibitors, inhibition of KRAS^{G12C} by ARS853 occurred slowly (Fig. 2A and fig. S3). In some cell lines, maximal inhibition of KRAS-GTP occurred in 6 hours; in others, in 48 to 72 hours. To understand this phenomenon, we examined the mechanism of KRAS^{G12C} inhibition in more detail. To determine whether ARS853 binds to the active or the inactive conformation of KRAS^{G12C}, we used differential scanning fluorimetry, which assays ligand-induced changes in protein thermal stability (12). Recombinant KRAS^{G12C} was loaded with either GTP γ S or GDP (fig. S4A) and then incubated with ARS853. Samples were incubated at increasing temperatures in the presence of a fluorescent dye that binds to hydrophobic surfaces exposed during thermal denaturation. ARS853 increased the amplitude of the thermal denaturation curve of KRAS^{G12C} loaded with GDP but not with GTP γ S (Fig. 2B and fig. S4B). ARS853 did not alter the denaturation curve of GDP-loaded KRAS^{WT} (fig. S4C). These data suggest that ARS853 preferentially interacts with inactive, or GDP-bound, KRAS^{G12C}.

KRAS mutants are thought to exist in a “constitutively” active (GTP-bound) state in cancer cells (13). Thus, inhibition of KRAS-GTP levels by a drug that preferentially interacts with GDP-bound mutant KRAS is puzzling. Codon 12 mutations disable the activation of RAS GTPase by GTPase-activating proteins (14–16). It is possible, however, that the basal GTPase activity of KRAS^{G12C} is sufficient to enable nucleotide cycling in cancer cells. Consequently, we hypothesized that binding of the inhibitor to KRAS^{G12C} traps it in an inactive (GDP-bound) conformation by reducing its susceptibility to exchange, which then results in the observed time-dependent reduction in cellular KRAS-GTP levels. For this to be the case, (i) inhibition by the drug should require KRAS^{G12C} GTPase activity. (ii) If KRAS^{G12C} GTPase activity is constant, the rate of RAS inhibition by the drug should depend on exchange factor activity. (iii) Regulating exchange factor activity should affect drug potency and the kinetics of inhibition.

We tested this hypothesis by using mutations that occur in cancer patients and affect the guanine nucleotide cycle of RAS (Fig. 3A and fig. S5A). A59G is a “transition state” mutant that abrogates GTPase activity (17, 18). When assayed for their GTPase activity (Fig. 3B), KRAS^{WT} and KRAS^{G12C} generated approximately three times as much hydrolysis product

as the control reactions, whereas KRAS^{A59G} or KRAS^{G12C/A59G} had little activity. Thus, KRAS^{G12C} has basal GTPase activity that is suppressed by the A59G mutation (see also Fig. 3C, lane 5 versus lane 1). ARS853 reduced KRAS-GTP levels and ERK phosphorylation in human embryonic kidney 293 (HEK293) or H358 cells engineered to express KRAS^{G12C} but not in those expressing KRAS^{G12C/A59G} (Fig. 3C and fig. S5B). The crystal structure of HRAS^{A59G} shows that Q61, a critical residue for GTP hydrolysis, is displaced in this mutant (fig. S5C). Q61L impairs *in vitro* GTP hydrolysis by HRAS (19), and, when expressed in HEK293 cells, KRAS^{G12C/Q61L} was less sensitive to ARS853 than KRAS^{G12C} (fig. S5D). Y64A both reduces RAS GTPase activity and impairs the interaction of HRAS with the active site of the guanine nucleotide exchange factor (GEF) SOS (18, 20, 21) (fig. S6, A to C). As expected, ARS853 caused only minimal inhibition of GTP-bound KRAS^{G12C/Y64A} or signaling driven by this mutant (fig. S6D). Thus, KRAS^{G12C} GTPase activity is required for its inhibition by ARS853.

The percentage of KRAS^{G12C} bound to ARS853 in cells was evaluated by mass spectrometry (Fig. 3D, dotted line). GTPase-impairing mutations A59G or Y64A diminished the cellular interaction of ARS853 with KRAS^{G12C} by ~75 and ~50%, respectively. This effect was inversely proportional to their effect on KRAS^{G12C}-GTP inhibition (Fig. 3D, solid line), supporting the conclusions that ARS853 interacts preferentially with GDP-bound KRAS^{G12C} in cells and that GTPase activity is required for the drug to bind and inhibit KRAS^{G12C}.

ARS853 reduced the interaction of KRAS^{G12C} with the catalytic subunit of SOS (Fig. 3E) and attenuated SOS-mediated nucleotide exchange by KRAS^{G12C} (fig. S6E). These findings suggest that ARS853 traps KRAS^{G12C} in a GDP-bound conformation by lowering its affinity for nucleotide exchange factors. It is therefore possible that nucleotide exchange activity modulates the effect of ARS853. We tested whether RAS mutations that affect nucleotide exchange alter the sensitivity of KRAS^{G12C} to ARS853 (Fig. 3F). Y40A, N116H, and A146V increase, whereas Y32S decreases, nucleotide exchange (22–24). Mutations that increase exchange attenuated the effect of ARS853 on KRAS^{G12C}-GTP, whereas Y32S caused a small augmentation of its effect (Fig. 3, G and H, and fig. S6, F and G). These data imply that potentiation of nucleotide exchange reduces sensitivity to ARS853, by lowering the levels of GDP-bound KRAS^{G12C} available for drug binding.

If the inhibitor works by the trapping mechanism suggested by these data (Fig. 4A), then cellular parameters regulating nucleotide exchange ought to modulate the inhibition of KRAS^{G12C}. Exposure to epidermal growth factor (EGF), an activator of SOS (25), delayed the inhibition of KRAS^{G12C} by ARS853 by several hours compared to inhibition in serum-free medium (Fig. 4B and fig. S7A). Treatment with an EGF receptor (EGFR) inhibitor (fig. S7, B and C) or SOS-specific small interfering RNAs (fig. S7D) had the opposite effect. We determined if EGFR signaling affects the inhibition of the mutant KRAS^{G12C} allele, by expressing hemagglutinin (HA)-tagged KRAS^{G12C} in EGFR-mutant PC9 cells, in which nucleotide exchange depends on EGFR (26, 27). The specific effect on the mutant allele was determined by pulling down active KRAS and then immunoblotting for HA-tagged KRAS^{G12C}. ARS853 inhibited GTP-bound KRAS^{G12C} more potently in the presence of gefitinib, as compared to its absence (fig. S7E). Furthermore, EGFR inhibition enhanced the

suppression of KRAS-GTP levels by ARS853 in both KRAS^{G12C} homozygous and heterozygous cells (fig. S7F). Receptor tyrosine kinase (RTK) signaling and nucleotide exchange are also subject to feedback inhibition by ERK or AKT (28). Mitogen-activated protein kinase kinase (MEK) or AKT inhibitors relieve this feedback and activated RTK signaling (29, 30). Inhibiting these pathways also attenuated the inhibitory effect of ARS853 (fig. S7, G and H). Taken together, these findings show that RTK-mediated nucleotide exchange regulates the kinetics and potency of KRAS^{G12C} inhibition in cancer cells.

The above results also suggest that pharmacologic inhibition of the exchange reaction, as achieved, for example, with inhibitors of RTK signaling, ought to potentiate the antiproliferative effect of KRAS^{G12C} inhibitors. To test this hypothesis, we evaluated the effects of 11 inhibitors targeting RTKs, or their downstream signaling pathways, in KRAS^{G12C}-mutant cell lines. In agreement with our model, inhibiting the tyrosine kinase activities of MET, SRC, or FGFR (fibroblast growth factor receptor) enhanced growth inhibition by ARS853 in H1972 and H2030 cells, whereas MEK, ERK, phosphatidylinositol 3-kinase (PI3K), or AKT inhibitor treatment did not have a significant effect (Fig. 4, C and D). In H2122 and Calu-1 cells, the effect of ARS853 was potentiated by concurrent inhibition of EGFR (Fig. 4D). Inhibition of EGFR also enhanced the induction of caspase activity by ARS853 (fig. S7D). These findings support another report showing that EGFR is required for KRAS-driven cancer formation (31) and suggest that the RTKs responsible for modulating the timing of KRAS^{G12C} inhibition in lung cancer vary from tumor to tumor. They also provide a rationale for selecting optimal combination treatments to achieve maximal KRAS^{G12C} inhibition in patients.

We thus report an inhibitor that suppresses KRAS^{G12C} signaling and tumor cell growth by binding to the GDP-bound form of the protein. This reduces the levels of KRAS^{G12C}-GTP, because the protein retains GTPase activity and undergoes nucleotide cycling in cells. Some RAS mutants have low levels of basal GTPase activity *in vitro*; yet, perhaps owing to an inability to directly test such activity *in vivo*, these oncoproteins are assumed to be constitutively GTP-bound in cancer cells. The data presented here, however, indicate that inhibition by ARS853 requires GTPase activity and that nucleotide exchange activity is inversely related to the kinetics and/or magnitude of inhibition. The model suggests that the RAS inhibitor and exchange factors compete for binding to KRAS^{G12C}-GDP. Drug-bound KRAS^{G12C}-GDP is not susceptible to exchange and exits the cycle, thus lowering the levels of KRAS^{G12C}-GTP.

This model also predicts that genetic alterations that completely disable KRAS^{G12C} GTPase activity, or those that activate nucleotide exchange function, will confer resistance to this class of drugs. Careful selection of combination therapies may in turn prevent or delay the emergence of resistance by enabling a more potent inhibition of KRAS^{G12C}. The mechanism of action elucidated here establishes a basis for such combination therapies. Inhibitors of receptors dominantly responsible for activation of RAS nucleotide exchange are predicted to enhance activity, whereas inhibitors of downstream pathways that reactivate these receptors are likely to blunt inhibition of mutant KRAS.

KRAS^{G12C} mutations occur in 16% of lung adenocarcinomas (32), making them one of the most frequent activating genetic alterations in lung cancer, a disease responsible for approximately 1.6 million annual deaths worldwide. No effective inhibitor of mutant KRAS has yet been developed for use in the clinic. Our results suggest that KRAS^{G12C} allele-specific inhibitors, such as the one described here, merit investigation as potential therapeutics for KRAS^{G12C}-lung cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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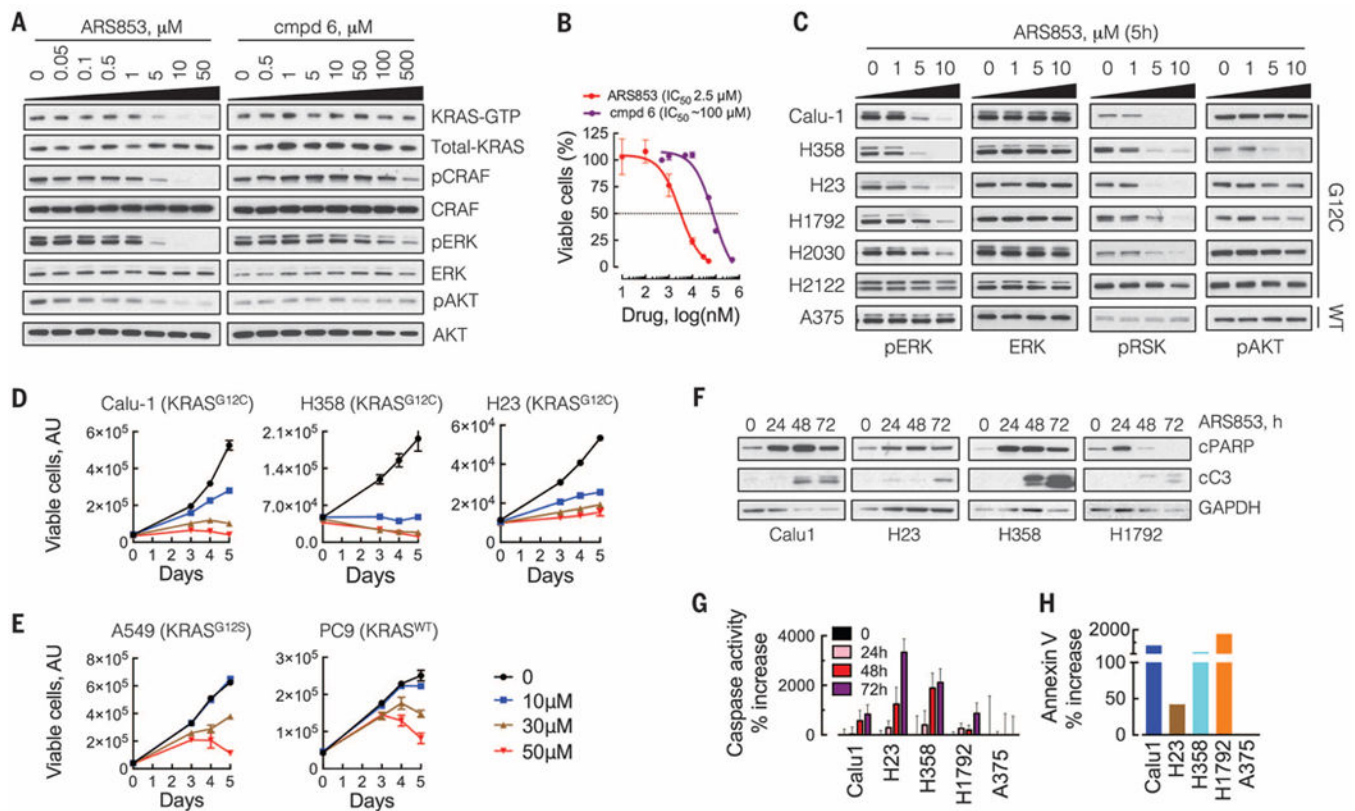


Fig. 1. Selective inhibition of KRAS^{G12C} signaling and cancer cell growth by ARS853
(A) KRAS^{G12C} mutant cells (H358) were treated with a novel (ARS853) or a previously described (compd 6) compound for 5 hours. The effect on the level of active, or GTP-bound, KRAS was determined by a RAS-binding domain pull-down (RBD:PD) assay and immunoblotting with a KRAS-specific antibody. The effect on ERK and AKT signaling was determined by immunoblotting with the indicated antibodies. A representative of at least two independent experiments for each compound is shown. **(B)** H358 cells were treated for 72 hours with increasing concentrations of the indicated inhibitors, followed by determination of viable cells by the ATP glow assay ($n = 3$ replicates). **(C)** The effect of ARS853 treatment on the inhibition of signaling intermediates in a panel of KRAS^{G12C}-mutant lung cancer cell lines. KRAS^{WT}A375 cells were used as a control. A representative of at least two independent experiments for each cell line is shown. **(D and E)** The cell lines were treated over time to determine the effect of ARS853 on the proliferation of G12C (D) or non-G12C (E) KRAS models ($n = 3$ replicates). **(F)** The effect of ARS853 treatment on the cleavage of apoptotic intermediates was determined by immunoblotting with the indicated antibodies. A representative of two independent experiments is shown. **(G)** Cell extracts from the indicated cell lines treated with ARS853 were subjected to a caspase activation assay with the Z-DEVD-AMC reporter substrate. The increase in fluorescence relative to untreated (0) is shown ($n = 3$ replicates). **(H)** Following treatment with ARS853 for 24 hours, the cells were stained with annexin V and evaluated by flow cytometry to determine the increase in annexin V-positive cells relative to untreated cells. Data in (B), (D), (E), and (G) are mean and SEM.

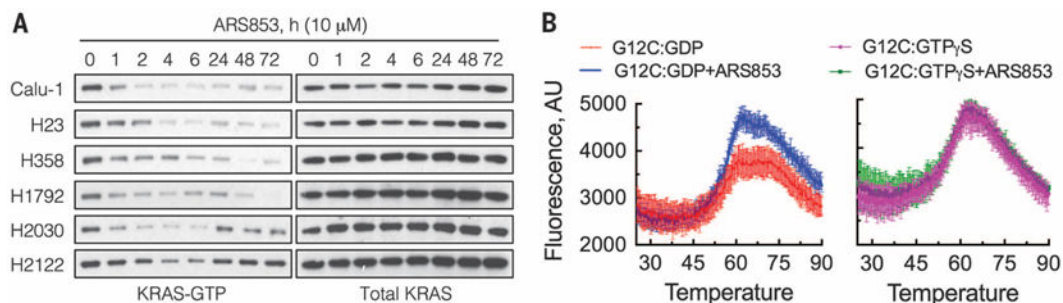


Fig. 2. Inhibition of active KRAS levels despite an interaction with GDP-bound KRAS^{G12C}
(A) KRAS^{G12C}-mutant lung cancer cell lines were treated with ARS853 (10 μM) over time, and cellular extracts were analyzed to determine the effect on KRAS-GTP as in Fig. 1A. **(B)** Recombinant KRAS^{G12C} was loaded with GDP or nonhydrolyzable GTP γ S and then reacted with ARS853 at a 1:1 molar ratio for 1 hour, at room temperature. The samples were analyzed by differential scanning fluorimetry to determine the shift in thermal denaturation curve induced by ARS853 binding ($n = 4$ replicates for GDP and $n = 3$ replicates for GTP γ S). Mean and SEM are shown.

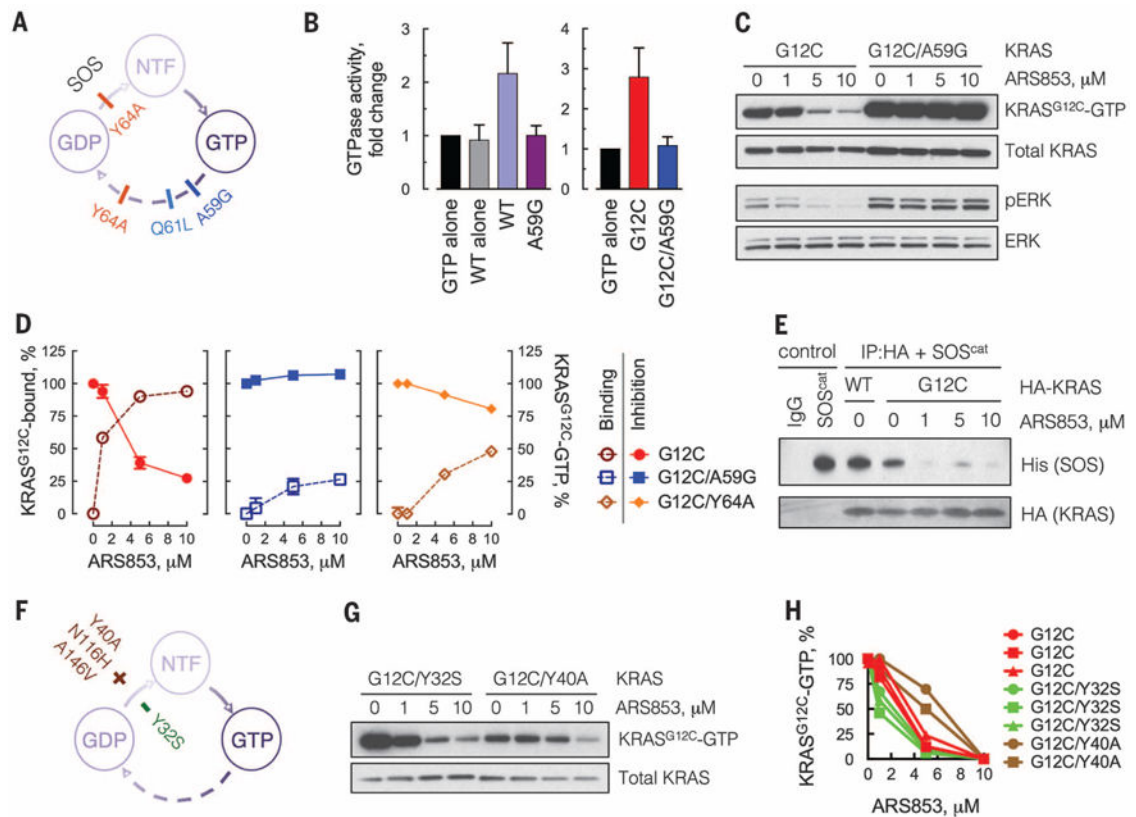


Fig. 3. Inhibition of KRAS^{G12C} requires GTPase activity and is attenuated by nucleotide exchange

(A) Schematic of the mutations used to block the GTPase activity of KRAS. NTF, nucleotide free. (B) KRAS mutants, expressed and affinity purified from HEK293 cells, were subjected to a GTPase reaction. The orthophosphate product was measured by the Cytophos reagent and expressed as fold change over GTP alone ($n = 3$, mean and SEM). (C) HEK293 cells expressing HA-tagged KRAS constructs were treated with ARS853 for 5 hours. Cell extracts were evaluated by RBD:PD and immunoblotting with an anti-HA antibody, to determine the effect on GTP-bound mutant KRAS^{G12C}. A representative of three independent experiments is shown. (D) Extracts from cells expressing the KRAS constructs treated as shown were evaluated by mass spectrometry to determine the percentage of KRAS^{G12C} bound to the drug. KRAS^{G12C}-GTP levels in the same extracts were determined as in (C) and quantified by densitometry ($n = 3$, mean and SEM). (E) HEK293 cells expressing the constructs shown were treated with ARS853 for 5 hours. HA-tagged KRAS was immunoprecipitated (IP) and subjected to a binding assay with the catalytic domain of SOS (SOS^{cat}). (F) Schematic of the mutations used to biochemically increase (+) or decrease (-) the nucleotide exchange function of KRAS^{G12C}. (G) HEK293 cells expressing the indicated constructs were analyzed as in (C). (H) The level of active KRAS-GTP was quantified by densitometry. Each replicate is shown. A similar result was obtained in H358 cells (fig. S6F). The baseline level of KRAS^{G12C/Y40A} detected by the RBD:PD is low because the Y40 mutation impairs the interaction of RAS with RAF (33). See also the effects of N116H and A146V mutations, which increase nucleotide exchange without affecting RAS-RAF interaction (fig. S6G).

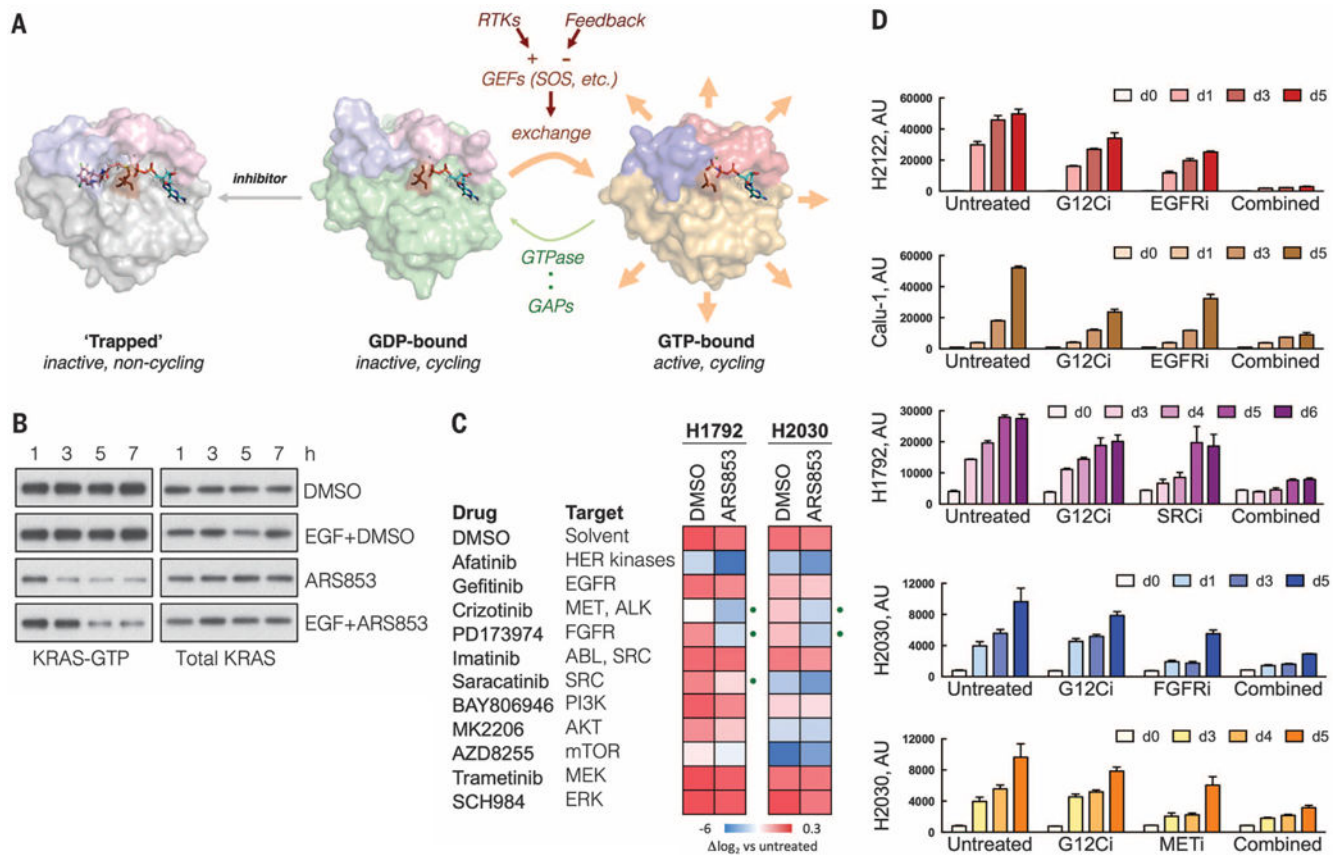


Fig. 4. Tyrosine kinase activation of nucleotide exchange modulates KRAS^{G12C} inhibition in cancer cells

(A) A schematic of the trapping mechanism by which ARS853 targets KRAS^{G12C}-dependent signaling and the role of RTK- or feedback-regulated nucleotide exchange in this process. Structures were adapted from Protein Data Bank accessions 4luc (inhibitor-bound KRAS^{G12C}-GDP), 4ldj (KRAS^{G12C}-GDP), and 4l9w (HRAS^{G12C}-GTP). (B) H358 cells were serum starved overnight followed by treatment with ARS853 (10 μ M), with or without EGF (100 ng/ml) for the indicated times, to determine the effect on KRAS-GTP. A representative of two independent experiments is shown. (C) The indicated cell lines, selected because of their relative insensitivity to KRAS^{G12C} inhibition, were treated with inhibitors of RTK signaling, alone or in combination with ARS853 for 5 days, in order to determine the most effective combinations. The effect of treatment is shown relative to the proliferation of untreated cells. The dots indicate combination treatments resulting in significantly more pronounced inhibition, compared to either drug alone ($n = 3$ replicates, mean). DMSO, dimethyl sulfoxide. (D) The effect of inhibitors targeting tyrosine kinases on the antiproliferative effect of KRAS^{G12C} inhibition in KRAS^{G12C} mutant cell lines ($n = 3$ replicates, mean and SEM). The concentrations of the inhibitors used in (C) and (D) are noted in Materials and Methods.