Co-targeting KRAS G12C and EGFR reduces both mutant and wild-type RAS-GTP

The combination of KRAS G12C inhibitors with EGFR inhibitors has reproducibly been shown to be beneficial. Here, we identify another benefit of this combination: it effectively inhibits both wild-type and mutant RAS. We believe that targeting both mutant and wild-type RAS helps explain why this combination of inhibitors is effective.

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The KRAS inhibitor, AMG-510 (brand name Lumakras, generic name sotorasib) recently obtained FDA approval for the treatment of non-small cell lung cancer patients who have the KRAS G12C mutation. Its utility on other forms of cancer with the G12C mutation is still being investigated. Colorectal cancer with the G12C mutation has appeared particularly intractable to G12C inhibition, motivating a desire to identify combination strategies and their mechanism of action.

Many different studies have identified the combination of EGFR inhibitors and KRAS G12C inhibitors as particularly effective in a variety of cancer types. That co-treatment with EGFR inhibitors and KRAS inhibitors would be effective was initially seen as counterintuitive. The first mechanism proposed to explain why the combination is effective was that blocking EGFR reduces the GTP-loading of KRAS G12C by RAS GEFs. As the G12C inhibitor in that study was also shown to only be capable of inhibiting the GDP bound form of RAS, increased levels of GDP bound KRAS G12C would in turn promote better inhibition of KRAS G12C.

Another reported mechanism involves counteracting the loss of negative feedback after KRAS G12C inhibition. Strong RAS pathway oncogenes drive strong signaling from ERK, which in turn results in the activation of negative feedback pathways. The treatment of the RAS pathway oncogene with a targeted therapy reduces the ERK signal and causes a loss of negative feedback, which may reactivate parts of the pathway. EGFR inhibitors have also been proposed to have a role in blocking the reactivation of EGFR after the loss of RAS pathway negative feedback. Whether reduced GTP-loading on KRAS G12C and reduced EGFR reactivation are the only mechanisms to explain the benefit of combining an EGFR inhibitor with KRAS G12C inhibitor is unclear.

We investigated the combination of KRAS G12C and EGFR inhibitors. We focus on the combination of AMG-510, the first KRAS G12C inhibitor to receive FDA approval, with cetuximab, the EGFR inhibitor that is regularly utilized for colorectal cancer patients. Our combination treatment drug dose response experiments detected low levels of proliferation for the combination of EGFR inhibitors with G12C inhibitors for all three of the KRAS G12C mutant cell lines (Fig. 1a). Using the excess over bliss score as a measure of synergy, we found that there was synergy for all three KRAS G12C cell lines, but not for KRAS G12V and KRAS WT controls (Fig. 1b). Overall, these experiments further support the idea that the combination of G12C and EGFR inhibitors may add value over G12C inhibitor alone.

In our recent work we demonstrated EGFR inhibitor sensitive KRAS mutants display reduced wild-type RAS-GTP upon treatment with EGFR inhibitors. To our knowledge, a role for WT RAS-GTP has not been evaluated for the combination of G12C and EGFR inhibitors. We therefore set out to experimentally evaluate how HRAS-, NRAS-, and KRAS-GTP levels change upon treatment with each inhibitor alone and in combination.

We observed profound suppression of KRAS-, NRAS-, and HRAS-GTP when the G12C mutant cells were treated with both cetuximab and AMG-510 (Fig. 1c, d). That KRAS-G12C-GTP is more effectively suppressed by the combination of a G12C inhibitor with an EGFR inhibitor has been observed previously in multiple studies. However, we also found this combination to suppress WT RAS-GTP (i.e. HRAS-GTP and NRAS-GTP). This suppression of WT RAS-GTP and KRAS-G12C-GTP was accompanied by an increased reduction in ERK phosphorylation (Fig. 1c).

We wanted to further investigate the phenomenon of co-targeting WT and mutant RAS-GTP. We have a unique tool for studying oncogenic RAS and its targeting: a mathematical model of RAS signal regulation that relates biochemical defects of RAS mutants to observable cellular levels of RAS-GTP. We have previously updated this model to study the early G12C inhibitors, and we desired to use the model to study the combination of EGFR and G12C inhibitors. To do this, we needed to updated our KRAS G12C and KRAS G12C inhibition model for AMG-510.

Previously described KRAS G12C inhibitors are believed to lock the KRAS G12C in the GDP-bound, inactive state and render it unable to interact with proteins that specifically bind to the GTP-bound conformation of RAS. We investigated whether AMG-510 also disrupts interactions between the KRAS-G12C protein with CRAF and NF1, which we assessed with a Bioluminescence Resonance Energy Transfer (BRET) assay. We observed KRAS-G12C treated with AMG-510 interacted much less with CRAF (Fig. S1A), and that KRAS-G12C treated with AMG-510 interacted much less with NF1 (Fig. S1B). In contrast, AMG-510 had no effect on the interactions between KRAS-G12V with CRAF and NF1. We further confirmed that AMG-510 disrupts these interactions in a G12C specific manner with a co-immunoprecipitation assay (Fig. S1C,D). Thus, we concluded that the structure and equations of our
The combination of AMG-510 and cetuximab results in suppression of wild-type RAS-GTP. a Cellular proliferation as measured by the MTT assay for SW48 G12C (KRAS\textsuperscript{G12C/WT}), SW48 KRAS G12V (KRAS\textsuperscript{G12V/WT}), SW48 (KRAS\textsuperscript{WT/WT}), SW1463 (KRAS\textsuperscript{G12C/G12C}), SW837 (KRAS\textsuperscript{G12C/WT}) Caco-2 (KRAS\textsuperscript{WT/WT}), and SW403 (KRAS\textsuperscript{G12V/WT}) treated with combinations of cetuximab and AMG-510 for 48 h. Heatmaps present average values from three separate experiments. b Calculated Excess over Bliss (EOB) synergy scores for the data in a. c Active RAS measurements by RBD-WB for SW387, SW1463, SW403, and Caco-2 cells treated either with vehicle, 1 \(\mu\)g/ml cetuximab, 250 nM AMG-510 or both 1 \(\mu\)g/ml cetuximab and 250 nM AMG-510 for 48 h. Results are representative of three separate experiments. d Mean RAS-GTP levels from three RBD-WB pull down assays with standard deviation. Statistical significance was determined by performing one-way ANOVA followed by post-hoc Tukey’s test for multiple comparisons. P-values are indicated within each panel comparing combination treatment to cetuximab and AMG-510 monotherapies.

Our previous work, we found that Neurofibromin (NF1) must be present and functional for a KRAS mutant cell to respond to cetuximab\textsuperscript{13–19}. We hypothesize at the low doses of AMG-510 and cetuximab, that both mutant KRAS-G12C-GTP and WT RAS-GTP are diminished together to reduce the MAPK signaling cascade to a level where cell proliferation is halted. AMG-510 covalently modifies KRAS G12C to be in a GDP-bound state, thus reducing the affinities for both RAF and NF1 (Fig. S1A–D). We propose KRAS G12C activity is reduced due to the direct targeting of AMG-510, whereas the WT RAS activity is depleted due to the reduced GEF activity from cetuximab, paired with the excess free NF1. We tested whether NF1 is necessary for this combination therapy approach by treating cells harboring KRAS G12C with a combination of AMG-510 and cetuximab in the presence or absence of NF1 knockdown. It was observed that WT RAS-GTP, ERK phosphorylation and cell proliferation were rescued in the combination treatment when NF1 was diminished (Fig. 3a, b). To ensure that regulation of WT RAS activity is
not due to off target effects of AMG-510, we recapitulated KRAS G12C inhibition by loss of function assays with KRAS siRNA in SW1463 cells (KRAS G12C/G12C). We observed that specifically knocking down KRAS (homozygous G12C cell line) with KRAS siRNA resulted in a partial loss of WT RAS-GTP, but was not sufficient to impede ERK phosphorylation, or cellular proliferation (Fig. 3c, d). In contrast, loss of KRAS G12C paired with cetuximab resulted in robust decreases in WT RAS activity, depleted phosphorylated ERK and inhibited cellular proliferation (Fig. 3c, d). Loss of KRAS G12C by siRNA phenocopies inhibition by AMG-510, indicating regulation of WT RAS is not the result of an off-target process (Fig. 3c, d). It was consistently observed that loss of KRAS G12C activity by AMG-510 or by siRNA knockdown resulted in partial reductions of WT RAS-GTP. We hypothesize that reductions of KRAS G12C-GTP results in increased free NF1 to exert negative regulation on the WT RAS population. To test this, SW1463 (KRAS G12C/G12C) were treated with either AMG-510 or KRAS siRNA in the presence or absence of NF1 siRNA. Partial loss of WT RAS-GTP was observed in both AMG-510 and KRAS knockdown but was rescued when NF1 was knocked down, indicating NF1 is the key mediator of the mutant-WT cross talk (Fig. 3e, f). Thus, this data suggests an additional mechanism of synergy for the combination of EGFR inhibitors and G12C inhibitors. Direct targeting of KRAS G12C with AMG-510 ablates mutant signaling and mutant sequestration of NF1. The free NF1 then partially reduces WT RAS-GTP and the remaining WT RAS-GTP can be ablated with upstream inhibition with EGFR inhibitors. The combined decrease in KRAS-G12C-GTP and WT RAS-GTP is then what ultimately leads to decreased cellular proliferation.

Fig. 2 Mathematical modeling of KRAS G12C inhibition by the KRAS G12C and EGFR inhibitors finds the biophysical properties of the RAS network proteins and the RAS and EGFR inhibitors are sufficient to explain the co-targeting of wild-type and mutant RAS.

a Schematic of model of RAS regulation and KRAS G12C inhibition. Arrow colors indicate the type of reactions: gray, biochemical reactions that regulate the RAS nucleotide binding state; green, RAS production; red, RAS degradation.

b Computational model-predicted levels of total RAS-GTP for the combination of KRAS G12C inhibition and EGFR inhibition.

c Computational model-predicted Excess over Bliss (EOB) synergy scores based on predicted total RAS-GTP for the combination of KRAS G12C inhibition and EGFR inhibition.

d Model-predicted levels of mutant RAS-GTP and WT RAS-GTP for the combination of KRAS G12C inhibition and EGFR inhibition for a modeled KRAS G12C heterozygous network.

e Model-predicted EOB synergy scores based on predicted mutant RAS-GTP levels and WT RAS-GTP levels.
Two processes have previously been demonstrated to contribute to the benefits of co-targeting KRAS G12C and EGFR: increased loading of the G12C inhibitor and targeting EGFR after negative feedback pathways are released. Our work presents a third benefit of this combination: combined targeting of WT and mutant RAS. That wild-type RAS signaling is frequently elevated in the presence of mutant RAS has extensive experimental evidence, yet it is an aspect of RAS signaling that is often overlooked when mutant RAS cancers are considered.
METHODS

Cell line models and culture
SW837, SW1463, SW403, Caco-2, SW48 cells and SW48 isogenic counterparts were cultured in RPMI 1640 medium supplemented with fetal bovine serum (FBS) (10%), penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM). All cells were grown in indicated medium and incubated at 37 °C in 5% CO2 unless indicated otherwise in experimental methods. SW48 cells were obtained from Horizon Discovery. SW837, SW1463, SW403, Caco-2 cells were obtained from the American Type Culture Collection.

siRNA knockdown
Cells were plated in adherent culture plates containing siRNA and Lipofectamine RNAi Max (13778150, Thermo Fisher) with a concentration of 10 pmol siRNA per 10,000 cells in optiMEM reduced medium (per manufacturers instruction). siRNAs used were KRAS siRNA (S7940, Thermo Fisher), Control siRNA (AM4611, Thermo Fisher) and NF1 siRNA (S77341, Thermo Fisher). Drug treatments were initiated after cells attached (24 h). 48 h after drug treatment cells were used for active RAS pull down (RBD assay), western blot analysis or MTT proliferation assay. siRNA’s are validated proprietary assays generated and sold by Thermo Fisher and all information on assays can be obtained by the assay ID.

Proliferation assay
Cells (5000 per well) were seeded in 96-well plates in complete media. Treatments were initiated after the cells were attached (24 h). At the appropriate time points, cell viability was determined by MTT assay; (5 mg/ml in phosphate-buffered saline) was added to each well followed by incubation at 37 °C for 2 h. The formazan crystal sediments were dissolved in 100 μl of dimethyl sulfoxide, and absorbance was measured at 590 nm using a Tecan Infinite 200 PRO plate reader. Each treatment was performed in eight replicate wells and repeated three different times.

Western blot analysis
Cell lysates were generated using lysis buffer (Thermo Fisher Scientific, 1862301) containing protease inhibitor cocktail (Cell Signaling Technology) and incubated on ice for 1 h, with brief vortexing every 5 min. The total protein concentration was determined by Pierce Protein assay (Thermo Fisher Scientific). Protein samples were resolved by electrophoresis on 12% polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation) for 20 min at 25 V with the Trans-Blot Turbo (Bio-Rad Laboratories). The blots were probed with the appropriate primary antibody and the appropriate fluorophore-conjugated secondary antibody. All antibodies were diluted to 1/1000 in Licor intercept antibody diluent (927–66003, Licor). The protein bands were visualized using the Licor Odyssey M imaging station (Licor Biosystems). Comparative changes were measured with Licor Image Studio software from three independent experiments. Comparisons were made by normalizing to endogenous loading control for internal reference, and to control treatment for external reference. All uncropped, unprocessed western blot scans with molecular weight markers are deposited in the Source Data file. All western blots and images of bands within a panel are from the same experiment. Protein bands within cropped images are from the same experiment, and all blots were probed, imaged, and processed in parallel. Antibody dilutions, reagents, and drug sources were sourced from the same stocks across experimental replicates. Supplementary Figs. 3–10 include the full, un-cropped images of all blots and includes full molecular weight markers.

AMG-510 NF1 coimmunoprecipitation
HEK293T cells were individually transfected or co-transfected with the expression plasmid for NF1-Flag, WT KRAS-GFP, or G12C KRAS-GFP. Cells were also simultaneously treated with either vehicle or 500 nM of AMG-510 for 24 h. Cells were harvested in IP Lysis/Wash Buffer (0.025 M tris-HCl, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, and 5% glycerol; pH 7.4 and 1× protease inhibitor) 24 h after transfection and treatment. Whole-cell lysates (500 μg) were precleared for 0.5 h using Control Agarose Resin slurry (Thermo Fisher Scientific). Immunoprecipitation was performed by first incubating 800 μl of HEK293T NF1-Flag precleared lysate with 200 μl of either WT KRAS-GFP, G12V KRAS-GFP or G12C KRAS-GFP precleared cell lysate. The final steps of the coimmunoprecipitation were performed using the Pierce Immunoprecipitation Kit (Thermo Fisher Scientific) with 10 μg of immobilized anti-NF1 antibody (Santa Cruz Biotechnology, CA). A total of 500 μg of the cell lysate was added and incubated at room temperature under rotary agitation for 45 min. At the end of the incubation, the complexes were washed five times with lysis buffer. The western blot was probed with mouse monoclonal NF1 antibody (Santa Cruz Biotechnology) and mouse monoclonal RAS antibody (Thermo Fisher Scientific). All antibiotics were diluted to 1/1000 in Licor intercept antibody diluent (927–66003, Licor).

Bioluminescence Resonance Energy Transfer (BRET) assay
Human embryonic kidney HEK293T cells were grown in DMEM/10% FBS without antibiotic treatment. Cells were seeded at 5 × 100 cells per well in a 96-well white opaque Perkin Elmer microplate. Twenty-four hours after seeding, cells were co-transfected with either a constant concentration of 0.1 μg of NF1-NanoLuc pcDNA expression plasmid or CRAF RBD-NanoLuc pcDNA expression plasmid with 0.2 μg of GFP-tagged KRAS mutant with 0.25 μl of Lipofectamine 3000 per well following the manufacturer’s protocol (Thermo Fisher Scientific). Twenty-four hours later, medium was aspirated from each well and 25 μl of Nano-Glo Live Cell Reagent was added to each well per the manufacturer’s protocol (Promega). Plates were placed on an orbital shaker for 1 min at 300 rpm. After incubation, the plate was read on the Tecan Infinite M200 PRO with LumioColor Dual Setting with an integration time of 1000 ms. BRET ratio was calculated from the dual emission readings. BRET ratio was plotted as a function of the RAS-GFP/NF1-NanoLuc plasmid ratio. BRET assays were repeated three times, each with eight biological replicates.
Excess over bliss score
Cell proliferation index was converted to fraction affected (fA)(1—
personable viable) = fA. The predicted value (C) was calculated for each
dose where A corresponds to fraction affected for cetuximab and B
corresponds to fraction affected for AMG-510: C = (A + B) – (A x B).
Excess over Bliss (EOB) was calculated as: EOB = (fA/A + B) – C × 100,
where fA/A+B corresponds the fraction affected of combination of
same dose of both Cetuximab and AMG-510.

Active RAS pull-down assay
Isolation of active RAS-GTP was performed using the Active Ras
Pull-Down and Detection Kit (Thermo Fisher Scientific) following
the manufacturer’s protocol. RAS abundance was measured by
Western blot. Western blot analysis of RBD pull-down lysates was
performed with mouse anti-KRAS antibody (WH0003845M1,
Sigma), rabbit anti-NRAS (ab167136, Abcam), rabbit anti-HRAS
(ab32417, Abcam), and mouse anti-GAPDH (sc-4772, Santa Cruz
Biotechnology). Input lysates were analyzed with mouse anti-pERK
(675502, Biolegend) and rat anti-ERK (686902, Biolegend). All
antibodies were diluted to 1/1000 in Licor intercept antibody
diluent (927–66003, Licor).

Mathematical model
The RAS model utilized here is an extension of our original RAS
model16 that was extended to include protein production and
protein degradation17. We developed this extended model to
study situations where protein turnover must also be con-
sidered, such as to study the effects of KRAS G12C inhibitors. The
modeled half-life for RAS proteins (24 h) is consistent with
recent measurements8. We use this version of the model to
study combined treatment with KRAS G12C inhibitors and EGFR
inhibitors. For both models, we model KRAS G12C to have
impaired intrinsic GTPase activity (kGTPase = 72% of the value of
kGTPase for WT RAS)15, GAP mediated activation (kGAP) equal
to zero, with the assumption that codon 12, 13, and 61 RAS
mutations are insensitive to GAPs, but with the GAP bound
KRAS G12C-GTP protein still capable of intrinsic GTPase activity,
and with a lower affinity for binding to RAS effectors
(modeled as slightly faster dissociation from effectors,
κd,effectort = 120% of the value of κd,effectort for WT RAS)23, with
all of these values based on previously published, experimental
studies as cited. Based upon our separate detection of impaired
binding to NF115, we also modeled NF1 binding with an
impaired binding to KRAS G12C (Km,GAP = 1000% of the value of
Km,GAP for WT RAS). EGFR inhibitor dose responses are simulated
with the assumption that EGFR mediated activation of RAS is
driven by the recruitment of GEFs like SOS1 and SOS2 to
actiavated EGFR, and that EGFR inhibition results in a reduction
of this GEF mediated activation of RAS-GTP, as we have done
previously13. KRAS G12C inhibition is modeled as done
previously17, but with new second order KRAS G12C inhibitor
covalent binding parameters specific for AMG-5101. Model
simulations are used to determine steady-state levels of total
RAS-GTP (KRAS-GTP + NRAS-GTP + HRAS-GTP) as a measure of
RAS pathway signal output. Our model is a set of Ordinary
Differential Equations (ODEs) that we solve numerically using
the Python package SciPy. Code can be found at https://
github.com/StitesLab/KRASG12C-WT.

Statistical analysis
All data are reported as mean values ± standard deviation and
were analyzed using Prism 8 software (GraphPad Software,
Incorporated, La Jolla, CA, USA). Statistical significance testing
between two conditions was done using unpaired two-tailed t-

test, assuming equal variances, and from at least three independent experiments. Statistical significance for three or
more conditions was calculated via one-way ANOVA followed by
post hoc Tukey’s test for multiple comparisons. Significances
were reported as *P < 0.05, **0.01, 0.001, 0.0001 and are reported
in each figure.

Ethics statement
No animals, no patients, and no patient information was
included as part of this study. Experimental work was done in
accordance with approval of the organizations Institutional
Biosafety Committee (IBC).

Reporting summary
Further information on research design is available in the Nature
Research Reporting Summary linked to this article.

DATA AVAILABILITY
Source data used are available from the corresponding author upon reasonable
request.

CODE AVAILABILITY
Our model is a set of Ordinary Differential Equations (ODEs) that we solve numerically using
the Python (v3.8.8) package SciPy (v1.6.2). All code utilized to numerically solve
the mathematical model and generate the figures in the manuscript can be
downloaded at: https://github.com/StitesLab/KRASG12C-WT. The Python code is
provided as a Jupyter notebook (v6.3.0). All needed parameter values are included
within the code and described in the methods section.

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**AUTHOR CONTRIBUTIONS**

T.M. and E.S. designed the experiments and study. T.M., A.G., and J.L. performed the experiments with assistance from L.S.H. T.M. performed the statistical analysis. M.T. and E.S. performed computational analyses. T.M., M.T. and E.S. wrote the manuscript with input from the other authors. The authors declare that they have no competing interests.

**COMPETING INTERESTS**

The authors declare no competing interests.

**ADDITIONAL INFORMATION**

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