ABSTRACT

Purpose: Targeting RAF for antitumor therapy in RAS-mutant tumors holds promise. Herein, we describe in detail novel properties of the type II RAF inhibitor, LXH254.

Experimental Design: LXH254 was profiled in biochemical, in vitro, and in vivo assays, including examining the activities of the drug in a large panel of cancer-derived cell lines and a comprehensive set of in vivo models. In addition, activity of LXH254 was assessed in cells where different sets of RAF paralogs were ablated, or that expressed kinase-impaired and dimer-deficient variants of ARAF.

Results: We describe an unexpected paralog selectivity of LXH254, which is able to potently inhibit BRAF and CRAF, but has less activity against ARAF. LXH254 was active in models harboring BRAF alterations, including atypical BRAF alterations coexpressed with mutant K/NRAS, and NRAS mutants, but had only modest activity in KRAS mutants. In RAS-mutant lines, loss of ARAF, but not BRAF or CRAF, sensitized cells to LXH254. ARAF-mediated resistance to LXH254 required both kinase function and dimerization. Higher concentrations of LXH254 were required to inhibit signaling in RAS-mutant cells expressing only ARAF relative to BRAF or CRAF. Moreover, specifically in cells expressing only ARAF, LXH254 caused paradoxical activation of MAPK signaling in a manner similar to dabrafenib. Finally, in vivo, LXH254 drove complete regressions of isogenic variants of RAS-mutant cells lacking ARAF expression, while parental lines were only modestly sensitive.

Conclusions: LXH254 is a novel RAF inhibitor, which is able to inhibit dimerized BRAF and CRAF, as well as monomeric BRAF, while largely sparing ARAF.

Introduction

Activation of the RAS/RAF pathway due to oncogenic lesions occurs in more than one third of human cancers, most commonly via mutations in KRAS, NRAS, and BRAF (1, 2). Not surprisingly, significant effort has gone into developing inhibitors targeting different nodes in this pathway, most notably the RAF, MEK, and ERK kinases and more recently the KRAS G12C variant (3–7).

The requirement for RAS/RAF signaling in normal tissues limits the potential therapeutic index (TI) of RAF/MEK/ERK inhibitors. However, studies in mice and humans indicate that selective inhibition of RAF paralogs and/or oncogenic variants may enable the development of inhibitors with favorable TIs. First, in adult mice, combined deletion of BRAF and CRAF is tolerated, whereas combined removal of either MEK1/MEK2 or ERK1/ERK2 is lethal (8). Second, RAF inhibitors selective for V600 genetic variants have been developed, and tellingly these inhibitors have proven far more effective clinically than have MEK inhibitors, which equally inhibit both MEK1 and MEK2 (9–19). The simplest interpretation of differing clinical results for these two inhibitor classes is that mutant selectivity of the RAF inhibitors confers a favorable TI enabling relatively greater systemic drug exposure and correspondingly improved pathway suppression in tumors. Thus, agents that selectively target specific RAF isoforms or genetic variants should have improved TIs relative to agents that inhibit equally all family members of any given node in the pathway.

The mutant selectivity of BRAFV600E/D/K inhibitors precludes their use in KRAS-, NRAS-, and atypical BRAF–mutant tumors. Unfortunately, MEK inhibitors, which effectively inhibit signaling by mutant RAF in preclinical models, have proven largely ineffective in RAS-mutant tumors clinically (20). This failure likely results from two nonmutually exclusive sources. First, RAF activates multiple downstream pathways. Thus, although RAF/MEK/ERK is a major RAS effector pathway, in many tumors, additional pathways may be the major oncogenic driver, or provide redundancy, should RAF signaling be impaired (21). Second, the intricate feedback mechanisms that serve to control RAF pathway output in normal tissues, while disabled in BRAFV600E/K-mutant disease (22), are largely intact in RAS-mutant tumors. Thus, RAS-mutant tumors contain evolutionarily honed mechanisms that can buffer tumor cells against the effects of RAF pathway inhibitors (21, 23). This feedback effect, coupled with the relatively low systemic exposures...
Translational Relevance

The RAS/RAF/MEK/ERK pathway is activated in greater than 30% of human cancers, primarily via mutations in KRAS, NRAS, and BRAF. The development of highly selective and genetic context-specific RAF inhibitors has led to spectacular improvements in patients with BRAFV600E-mutant disease. Unfortunately, the BRAF V600E selectivity of these inhibitors prevents their utility in RAS-mutant tumors. Here, we describe an unexpected paralog selectivity for the type II RAF inhibitor, LXH254, that is currently undergoing clinical trials in RAS-driven tumors in combination with MEK and ERK inhibitors. LXH254 is a potent inhibitor of both monomeric and dimeric BRAF and CRAF, but a comparably poor inhibitor of ARAF. This paralog selectivity permits robust inhibition of dimeric BRAF and CRAF simultaneously with potentially improved therapeutic index. This tolerability profile has enabled vertical combinations with MEK and ERK inhibitors for the treatment of RAS-driven tumors in clinic.

Materials and Methods

Cell culture

HCT 116 RAF single- and double-knockout lines were generated using parental and BRAFV600E–/– cells from Horizon Discovery. All other cell lines were obtained from the BROAD/Novartis Cell Line Encyclopedia collection (24) and maintained in DMEM (MiaPaCa-2, 293T, and HEK-293), McCoy’s 5A (HCT 116), Eagle Minimal Essential Medium (Calu-6, HeyA8, and SK-MEL-2), or RPMI (all other lines) supplemented with 10% FBS (VWR Life Sciences).

Genetic context–specific RAF inhibitors has led to spectacular improvements in patients with BRAFV600E-mutant disease. Unfortunately, the BRAF V600E selectivity of these inhibitors prevents their utility in RAS-mutant tumors. Here, we describe an unexpected paralog selectivity for the type II RAF inhibitor, LXH254, that is currently undergoing clinical trials in RAS-driven tumors in combination with MEK and ERK inhibitors. LXH254 is a potent inhibitor of both monomeric and dimeric BRAF and CRAF, but a comparably poor inhibitor of ARAF. This paralog selectivity permits robust inhibition of dimeric BRAF and CRAF simultaneously with potentially improved therapeutic index. This tolerability profile has enabled vertical combinations with MEK and ERK inhibitors for the treatment of RAS-driven tumors in clinic.

RAF in vitro enzyme assays

Biochemical inhibition of ARAF, BRAF, and CRAF, shown in Fig. 1A, was performed as described for RAF09 in Shao and colleagues (25). In brief, the catalytically inactive MEK1C597R variant was used as a substrate for either full-length BRAF, full-length activated CRAF (Y340E/Y341E variant), or a full-length N-terminal GST–ARAF fusion. In all cases, LXH254 was preincubated with CRAF/BRAF/ARAF for 30 minutes prior to substrate addition/reaction initiation.

Biochemical activity of LXH254 in the extended panel of kinases, shown in Supplementary Table S1, was determined as described in Wylie and colleagues (26).

Generation of knockout cell lines

To generate RAF-knockout lines, clustered regularly interspersed short palindromic repeat (CRISPR) genome editing (25, 28) was used for all lines, except HCT 116. Guide sequences for ARAF (CCTGCCCAAGAAGCAACGCA), BRAF (ATATCAGTGTC-CAAACCAAT), and CRAF (TGGTGCACTAAAGATGTCCTA) were synthesized using standard chemistry and cloned into in-house generated lentiviral expression vectors. Lentiviral vectors were packaged in 293T cells with TransIT-T93 Transfection Reagent (Mirus, #MIR2700) using 5.4 μg of plasmid per 10-cm plate. Virus was harvested after 48 hours and passed through a 0.45-μm filter. Cell lines stably expressing Cas9 were incubated with lentivirus and 8 μg/mL Polybrene (Millipore, #TR-1003–G) for 24 hours, and subsequently cultured in media containing puromycin. Following selection in puromycin, reductions in target protein levels were assessed via immunoblotting and single clones with complete loss of target protein expression were isolated from pools via serial dilutions. HCT 116–knockout lines were generated in parental and BRAFV600E–/– lines from Horizon Discovery by transfection with zinc finger mRNAs against ARAF, BRAF, and CRAF purchased from Sigma.

Generation of cell lines expressing exogenous ARAF constructs

ARAFWT–FLAG, ARAFV600E–FLAG, ARAFK336M–FLAG, and ARAFV600E–FLAG cDNA constructs were synthesized and cloned into in-house generated lentiviral vectors and expressed from either a constitutive (pXP1704) or doxycycline-inducible (pXP1509A) EF1α promoter by GeneArt (Thermo Fisher Scientific). Lentivirus was packaged, harvested, and used to infect cells as described previously. Cells were cultured in media containing G418 MIA PaCa-2 until stable pools were generated. Protein expression was confirmed by immunoblotting and mutations by MassArray (Agena Biosciences).

Cell proliferation assays

Cells were seeded in 96-well plates (Corning, #3903), and 24 hours later, compound dilution series (1:3, starting at 10 μmol/L) was added to wells in duplicate or triplicate. All compounds were synthesized at Novartis Pharma AG. Plates were incubated for 72–120 hours, and then CellTiter-Glo Luminescent Cell Viability Assay (Promega, #G7575) was added to wells to assess ATP levels. All points were normalized to DMSO control samples when determining IC50 values and calculations were performed using GraphPad Prism 7.0.

High-throughput profiling of the cell line panel, described in Supplementary Fig. 1A, was performed as described in Barretina and colleagues (24).

pMEK1/2 and pERK1/2 Meso Scale Discovery assays

For Meso Scale Discovery (MSD) assays, cells were incubated with compound for 4 or 24 hours at 37°C and subsequently lysed for 30 minutes at 4°C. Assays were performed as described in the
manufacturer’s protocol for Phospho/Total ERK1/2 (MSD, #K15107D-1), phosphoprotein levels were normalized to DMSO-treated parental samples, and fold change was calculated based upon percentage phosphoprotein in each sample as described in the manufacturer’s protocol. Washout experiments, described in Fig. 1C, were performed as described in Shao and colleagues (25).

Protein immunoblots

Cells were plated in 6-well dishes (Corning, #3506) and treated with inhibitors for 4–72 hours. Cells were harvested in RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, #89900) containing 1 × Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific, #87885) and Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, #78420). Lysates were used for Western blot analysis with antibodies recognizing ARAF (Cell Signaling Technology, #4432), BRAF (Sigma-Aldrich, #HPA00132), CRAF (Cell Signaling Technology, #12552), CRAF (BD Biosciences, #610151), FLAG (Cell Signaling Technology, #8146), phospho-MEK1/2 (S217/S221) (Cell Signaling Technology, #9154), phospho-ERK1/2 (T202/Y204) (Cell Signaling Technology, #4370), and GAPDH (Millipore, #MAB374) diluted 1:1,000 or with β-actin antibody (Life Technologies, #AM4302) diluted 1:5,000.

Coimmunoprecipitations

For immunoprecipitations, shown in Fig. 1B, cells were seeded in 15-cm dishes and incubated with 1 μmol/L of the indicated compounds for 1 hour. Cell lysates were prepared in immunoprecipitation buffer [10 mmol/L Tris, pH 7.4, 50 mmol/L NaCl, 0.5% (volume/volume) NP-40, and 1 mmol/L EDTA] supplemented with 1 × protease and 1 × phosphatase inhibitor cocktails. Cleared lysates were normalized for protein concentration and incubated with BRAF (Sigma, #HPA001328), CRAF [Bethyl Laboratories, (A301-519A) (HCT 116) and Millipore, #04-739], or MEK (Millipore, #07-641) antibodies as indicated, overnight at 4°C. Protein A Ultra Link Resin (Thermo Fisher Scientific) was then added to each sample, incubated...
for 2 hours at 4°C, washed with immunoprecipitation lysis buffer, and eluted in SDS sample buffer. In all other immunoprecipitations, cells were treated +/− compound for 2–24 hours, cells were lysed in Pierce IP Lysis Buffer (Thermo Fisher Scientific, #87878) containing 1 × Protease (Thermo Fisher Scientific, #87785) and Phosphatase Inhibitor (Thermo Fisher Scientific, #78420) cocktails, and lysates were clarified by centrifugation. For each sample, 1–2 mg of lysate was incubated with antibodies to ARAF (Cell Signaling Technology, #4432), BRAF (Sigma-Aldrich, #HPA00132), or FLAG (Sigma-Aldrich, #F7425) and Protein G Sepharose Beads (GE Healthcare, #17-0618-01) for 3 hours at 4°C. Complexes were washed three times with IP lysis buffer and analyzed by immunoblotting.

Cell-based kinase assays
In-cell kinase selectivity profiling was performed by KiNativ. Briefly, HCT 116 cells were treated with LXH254 for 2 hours at 10 μmol/L, and then lysates were processed, probe labeled, and analyzed by LC/MS-MS, as described previously (29). For immunoprecipitate (IP)-kinase assays, cells were cultured in compound for 4–24 hours prior to protein immunoprecipitation, as described above. IPs were washed once with IP lysis buffer and twice with 1 × Kinase Buffer (Cell Signaling Technology, #9802), and protein-bound beads were incubated in 2 × kinase buffer with 250 μmol/L ATP (Cell Signaling Technology, #9804) and 0.5 μg MEK1 (Millipore, #14-420) or MEK1-K97M (Proqinase, #0785-0000-1) for 30 minutes at 30°C. Beads were then washed three times with IP lysis buffer and prepared for immunoblotting as described above.

In vivo studies
Mice and statement of welfare
Outbred athymic (nu/nu) female mice (“HSD: Athymic Nude-nu”; Charles River Laboratories) and SCID Beige mice (Charles River Laboratories) were allowed to acclimate in the Novartis NIBRI animal facility with access to food and water ad libitum for a minimum of 3 days prior to manipulation. Animals were handled in accordance with Novartis Animal Care and Use Committee regulations and guidelines.

Cell culture and in vivo efficacy
All cell lines were shown to be free of Mycoplasma species and murine viruses in the IMPACT VIII PCR Assay Panel (IDEXX RADIL, IDEXX Laboratories Inc). In all cases, cells were harvested at 80%–95% confluence with 0.25% trypsin-EDTA (Gibco, #25200-056), and then lysates were processed, probe labeled, and neutralized with growth medium. Following centrifugation for 5 minutes at 1,200 rpm, all cells, except HEY-A8 cells (PBS), were resuspended in either cold Hank’s Balanced Salt Solution (HBSS; Gibco, #14175-095, HCT 116 and A375) or cold HBSS and an equal volume of Matrigel Matrix (Corning, #354234, all other cells). Cells were then injected in 100–200 μL volumes into either the right or left flanks of female nude mice, with the exception of MEL-JUSO cells, which were injected into SCID beige mice. Total cell numbers injected per mouse were 2 × 10^6 (HCT 116 and HEY-A8), 5 × 10^6 (MIA PaCa-2, HPAF-II, A375, Hs944.T, and SK-MEL-30), and 1 × 10^7 (MEL, JUSO, Calu-6, and PC-3). Five to 7 mice were used for each group.

Xenograft qPCR
Tumors were homogenized and RNA was extracted using the Qiagen RNeasy Mini Qiacube Kit (Qiagen, catalog no., 74116) per the manufacturer’s instructions. For RT-qPCRs, 4 μL/well of a 10 μg/μL suspension of RNA was loaded into 384-well plates along with 16 μL of Master Mix (Qiagen, catalog no. 204645), containing primers for hDUSP6 (Life Technologies, catalog no., Hs00737962) and hRPLPO (Life Technologies, catalog no., 4326314E), and reverse transcriptase. Reactions were run for 20 minutes at 50°C, 15 minutes at 95°C, followed by 45 cycles of 45 seconds at 94°C and 45 seconds at 60°C.

Gene expression analysis
ARAF RNA sequencing (RNA-seq) expression, as well as BRAF, NRAS, KRAS, and HRAS mutation data from all The Cancer Genome Atlas (TCGA) Pan-Cancer Atlas studies were downloaded from the cbioPortal data viewer (https://www.cbioportal.org/). RNA-seq by expectation-maximization (RSEM) values were converted to transcripts per million (TPM) and log, transformed. Only samples with ARAF expression were considered. Differential expression normalization was performed using Limma/VOOM for differential expression in R (30).

Structural modeling
The in-house X-ray structure (PDB entry: 6N0P) of LXH254 bound to BRAF was used in conjunction with an X-ray structure of CRAF (PDB entry: 3OMV) to build a homology model of ARAF with the in-house X-ray structure (PDB entry: 6N0P) of LXH254 bound to BRAF. This model was extensively refined with explicated solvent molecular dynamics simulations at 300 K, 1 atm, AM1BCC/ELF charges, TIP3P water, and ff14SB (32) force field with the PARM@FROSST (33) extension within the AMBER (34) suite of simulation tools. Ten-nanosecond simulations for each system were run and time average structures were extracted.

Results
LXH254 inhibits both monomorphic and dimeric RAF and promotes RAF dimer formation
LXH254 is a potent inhibitor of the BRAF and CRAF kinases, inhibiting their catalytic activity at picomolar (pmol/L) concentrations in biochemical assays (35). Consistent with LXH254 being a type II inhibitor, LXH254-bound BRAF adopts an inactive DFG out, αC-helix conformation compared with the vehicle control group is reported, unless otherwise stated.

Drug formulation
LXH254 was dosed orally in MEPC4 vehicle [45% Cremophor RH40 + 27% PEG400 + 18% Corn Oil Glycerides (Mainise CC) + 10% ethanol] for all experiments, except PC3, where LXH254 was formulated in 90% PEG400 + 10% Tween80. MEPC4 stock was diluted 5 × (1:4 with de-ionized water) prior to dosing. Trametinib was dosed orally as a suspension in 0.5% HPMC and 0.2% Tween80 in distilled water at pH 8. Trametinib was stirred overnight and protected from light at room temperature prior to dosing.

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in conformation, and treatment of cells with LXH254 promotes BRAF/CRAF heterodimer formation most likely due to antagonism of the autoinhibited state of the kinase, which enables RAS-mediated dimer formation (35, 36). We further extended characterization of RAF inhibition by LXH254 in several ways. First, we measured the biochemical inhibition of ARAF, BRAF, and CRAF by LXH254 (Fig. 1A). Consistent with values reported previously (35), LXH254 inhibited BRAF and CRAF with IC50 values of 0.2 and 0.07 mol/L, respectively. Inhibition of ARAF, while robust, occurred with a higher IC50 value of 6.4 nmol/L. Second, the ability of LXH254 to promote B/CRAF heterodimer formation of endogenous proteins was examined in three RAS-mutant cell lines via IP-Western blot analysis (Fig. 1B). In all instances, both LXH254 and the type II RAF inhibitor, LY3009120 (37) promoted heterodimer formation as judged by analysis of pull-downs using either BRAF- or CRAF-directed antibodies. MEK1 and MEK2 were not detected in either BRAF or CRAF IPs, however, both BRAF and CRAF were detected upon compound treatment following immunoprecipitation with MEK1/2-directed antibodies. Finally, we compared the relative ability of LXH254 to inhibit monomeric BRAFV600E with that of its potently active homologous BRAFV600E in A-375 cells compared with RAS-induced wild-type RAF dimers in HCT 116 KRASG12D cells. For this assessment we used an approach described by Yao and colleagues (22), in which cells were preincubated and subsequently washed following treatment with 1 μmol/L encorafenib such that inhibition likely reflects LXH254 binding to the second protem in RAF dimers unoccupied by encorafenib. In contrast to the approximately 600-fold greater inhibition of monomeric BRAFV600E versus wild-type dimeric RAF by dabrafenib, which we reported previously, LXH254 displayed similar inhibition of monomeric BRAFV600E and wild-type dimeric RAF (IC50 for p-ERK levels of 59 and 78 nmol/L in A-375 and HCT 116 cells, respectively, Fig. 1C).

An issue that has potentially limited the efficacy of type II RAF inhibitors in clinic is a lack of selectivity for RAF versus other kinases (37–39). LXH254 displayed a high degree of selectivity for BRAF and RAF-1 (CRAF) relative to other kinases, as judged by KINOMescan (35). We further interrogated the selectivity of LXH254 in two orthogonal screening formats. First, in biochemical assays using purified proteins, LXH254 had IC50 values >10 μmol/L against 60 of 62 non-RAF kinases, with only MAPK14 (p38α) and ABL1 having IC50 values below 10 μmol/L (2.1 and 4.1 μmol/L, respectively; Supplementary Table S1). Second, we profiled LXH254 using the KiNATiv platform, wherein kinase selectivity is determined in cells via competition with an ATP competitive covalent probe followed by mass spectrometry (29). In HCT 116 cells incubated for 2 hours with 10 μmol/L LXH254, the only kinases where probe binding was inhibited >80% were BRAF (90%) and CRAF (88%; Fig. 1D; Supplementary Table S2). Beyond BRAF and CRAF, only two, ARAF (58%) and p38α (66%), of the additional 184 kinases, detected in this cell line, demonstrated >50% inhibition of probe binding. High selectivity for BRAF and CRAF, and to a lesser extent for p38α, in HCT 116 cells was consistent with results from both the biochemical and KINOMEscan profiling (35). Moreover, weaker binding of LXH254 to p38α in both binding assay formats corresponds to relatively poor biochemical inhibition of this kinase (IC50 = 2.1 μmol/L), relative to BRAF (0.0002 μmol/L) and CRAF (0.0007 μmol/L). Collectively, these data indicate that within the kinase, LXH254 is highly selective for RAF kinases.

**In vitro and in vivo activity of LXH254**

We next profiled 291 cell lines for sensitivity to LXH254 in a high-throughput format (Supplementary Table S3). Using IC50 value cutoffs ranging from 1 to 2.5 μmol/L, concentrations that approximate Cavg concentrations in clinic (data not shown), we found that cell lines harboring BRAFV600E, as well as activating mutations in NRAS, were enriched in the subset of sensitive cells. KRAS mutants were only weakly enriched, with cells lacking RAS/RAF mutations being predominantly insensitive (22/146 with an IC50 > 2 μmol/L; Supplementary Fig. S1A; Supplementary Table S3). In the case of wild-type cell lines, in an insensitive model tested, LXH254 effectively suppressed RAF signaling, suggesting that some insensitivity may result from a lack of pathway dependence (Supplementary Fig. S3F). To identify potential marker of either resistance or sensitivity in MAPK-altered models, we performed differential gene expression analysis, in both the entire set of cell lines, as well as the KRAS-, NRAS-, and BRAF-mutant subsets. Unfortunately, this analysis did not reveal any predictors of sensitivity beyond melanoma lineage makers in the BRAF subset, which was likely a consequence of the high fraction of BRAFV600E models derived from this lineage (Supplementary Table S9). Cell line sensitivities to LXH254 were very similar to those previously obtained for the type II RAF inhibitor, RAF709 (ref. 25; Supplementary Fig. S1B).

We subsequently examined the antitumor effects of LXH254 in a set of BRAF-, NRAS-, and KRAS-mutant xenograft models, as well as a RAS/RAF wild-type model. In general, and concordant with cell line sensitivity in vitro, models harboring BRAF mutations either alone (e.g., BRAFV600E) or coincident with either activated NRAS (SK-MEL-30) or KRAS (HEYA8 and 1855HCOX), either repressed or demonstrated prolonged stasis following treatment with LXH254 (Fig. 2A–C; Supplementary Table S7). In contrast to RAF-mutant models, and similar to in vitro cell line data, the majority of KRAS-mutant models, as well as the RAS/RAF wild-type model, displayed best modest responses to LXH254, although there were notable outliers (e.g., Calu-6 cells, Fig. 2D; Supplementary Table S7). When examined in a subset of models, pathway inhibition was consistent with antitumor results (Fig. 2A–D).

**Loss of ARAF expression sensitizes RAS-mutant cells to LXH254**

On the basis of the dual observations that (i) LXH254 had reduced binding of ARAF relative to BRAF and CRAF in the KiNATiv assay and

### Table 1. p-ERK MSD results in cells lacking ARAF, BRAF or CRAF expression.

<table>
<thead>
<tr>
<th>Cell line variant</th>
<th>SK-MEL-30</th>
<th>MEL-JUSO</th>
<th>HCT 116</th>
<th>COR-L25</th>
<th>Mia PaCa-2</th>
<th>Mia PaCa-2 (24 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>90</td>
<td>77</td>
<td>12</td>
<td>889</td>
<td>687</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>ARAFΔ</td>
<td>60</td>
<td>10</td>
<td>5</td>
<td>43</td>
<td>85</td>
<td>170</td>
</tr>
<tr>
<td>BRAFΔ</td>
<td>650</td>
<td>94</td>
<td>18</td>
<td>1,072</td>
<td>262</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>CRAFΔ</td>
<td>300</td>
<td>531</td>
<td>357</td>
<td>1,358</td>
<td>3,627</td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>

Note: p-ERK IC50 results as measured by MSD.
(ii) RAS mutants, particularly KRAS, are largely insensitive to LXH254, we hypothesized that insensitivity might stem from poor ARAF inhibition by LXH254. To test this hypothesis, we used CRISPR-Cas9 or zinc finger nucleases to functionally delete ARAF, BRAF, or CRAF from a series of RAS-mutant cell lines, including two NRAS-mutant melanoma cell lines (MEL-JUSO and SK-MEL-30), and three KRAS-mutant cell lines (COR-L23, MIA PaCa-2, and HCT 116; Supplementary Fig. 2). Loss of ARAF expression resulted in 3- to 11-fold increases in sensitivity to LXH254 relative to parental cell lines (Fig. 3; Supplementary Table S3). Loss of BRAF did not alter sensitivity to LXH254 in the wild-type BRAF cell lines; however, loss of BRAF expression in NRAS-mutant SK-MEL-30 cells, which also harbor a kinase-impaired class III variant (BRAF^{D287H}; ref. 40), greatly reduced sensitivity to LXH254 (14-fold). Loss of CRAF either did not change (MIA PaCa-2 and COR-L23) or only slightly altered (HCT 116) sensitivity to LXH254 relative to parental cell lines in all models tested (Fig. 3; Supplementary Table S3). Loss of BRAF did not alter sensitivity to LXH254 in the wild-type BRAF cell lines; however, loss of BRAF expression in NRAS-mutant SK-MEL-30 cells, which also harbor a kinase-impaired class III variant (BRAF^{D287H}; ref. 40), greatly reduced sensitivity to LXH254 (14-fold). Loss of CRAF either did not change (MIA PaCa-2 and COR-L23) or only slightly altered (HCT 116) sensitivity to LXH254 to KRAS-mutant models; however, CRAF loss in both NRAS-mutant cell lines reduced sensitivity to LXH254 by 3- to 9-fold. In contrast, loss of any of the three RAF paralogs did not consistently alter sensitivity to either the MEK1/2 inhibitor, trametinib, or ERK1/2 inhibitor, ulixertinib (Supplementary Tables S4 and S8).

We then examined the effect of loss of the various RAF proteins on pathway suppression by LXH254. Cells were treated with increasing concentrations of LXH254 for 4 hours, and in some cases for 24, 48, and 72 hours, and p-ERK levels were determined using the MSD platform, and/or Western blot analysis using antibodies that detect phosphorylated ERK1/2. Loss of ARAF improved pathway suppression by LXH254 relative to parental cells and cells lacking either BRAF or CRAF (Table 1; Supplementary Fig. S3A). Similarly, reduced pathway inhibition occurred in cases where loss of BRAF (SK-MEL-30) or CRAF (SK-MEL-30 and MEL-JUSO) expression decreased sensitivity to LXH254 (Table 1). Moreover, in both HCT 116 cells and MIA PaCa-2 cells, where p-ERK levels were monitored beyond 4 hours, the difference in p-ERK reduction by LXH254 between cells lacking versus expressing ARAF increased further (Table 1; Supplementary Fig. S3A). Thus, increased sensitivity to LXH254 in cells lacking ARAF corresponds to improved pathway suppression. Reduced, albeit transient pathway inhibition by LXH254 in cells lacking CRAF suggests that in the absence of CRAF, ARAF might play a larger role in signaling. To test this, we immunoprecipitated ARAF and BRAF from parental and CRAF-deficient MIA PaCa-2 cells following 2 and 24 hours of incubation with 1 μmol/L LXH254. In parental cells, increasing amounts of BRAF and CRAF copurified with immunoprecipitated ARAF over time after LXH254 treatment (Supplementary Fig. S3B). Similarly, increasing amounts of both BRAF and, more prominently, CRAF copurified with BRAF in cells lacking CRAF expression. However, in the absence of CRAF, greater amounts of BRAF/ARAF heterodimers were present at all times despite similar efficiencies of immunoprecipitation. Moreover, increased levels of BRAF/ARAF heterodimers in CRAF-deficient cells following the 2-hour LXH254 treatment corresponded to failure of LXH254 to inhibit signaling. These data are consistent with a model where LXH254 has reduced ability to suppress MAPK signaling driven by ARAF and further that the contribution of ARAF to MAPK signaling increases in the absence of CRAF expression.

**ARAF-mediated resistance to LXH254 is dependent on both ARAF kinase activity and ability to form dimers**

To determine whether ARAF-mediated LXH254 insensitivity depends on ARAF catalytic function or the ability to form RAF dimers,
we reintroduced either N-terminally FLAG-tagged wild-type ARAF, kinase dead ARAF (K336M or D447A), or dimer-deficient ARAF (R362H) variants into cells lacking endogenous ARAF. In all models examined, reintroduction of wild-type ARAF reverted sensitivity to LXH254 to levels seen in parental cells (Fig. 4A and B). In contrast, expression of either kinase dead variants, K336M (Fig. 4A and C) and D447A (Fig. 4B and C), or dimer-deficient variant, ARAF<sup>R362H</sup> (Fig. 4B), did not revert sensitivity to LXH254. Concordant with sensitivity to LXH254, overexpression of wild-type ARAF, but neither kinase dead nor dimer-deficient ARAF, reduced pathway suppression by LXH254 (Supplementary Table S5). Failure of kinase dead and dimer-deficient variants to complement loss of endogenous ARAF did not result from poor transgene expression, as protein levels of all introduced ARAF variants exceeded the expression of endogenous ARAF, although it should be noted that kinase dead variants were consistently expressed at lower levels than either wild-type or dimer-deficient versions of ARAF (Supplementary Fig. S2).

To ensure that kinase dead and dimer-deficient RAF variants lacked kinase activity and failed to form heterodimers, respectively, we performed IP-kinase assays using material isolated from RAS-mutant cell lines expressing the various FLAG-tagged ARAF variants. In these experiments, the MEL-JUSO, HCT 116, and MIA PaCa-2, but not COR-L23, cell lines lacked endogenous ARAF expression due to CRISPR/Cas9 editing. ARAF was immunoprecipitated using antibodies directed against the FLAG epitope from either untreated cells, or cells treated for 24 hours with LXH254, and RAF kinase activity was assessed using recombinant MEK1 as a substrate for the immunoprecipitated material and monitoring production of phosphorylated MEK1 (p-MEK1) using a phospho-MEK1 specific antibody. IP-kinase assays using precipitates from all ARAFwt-expressing cells produced p-MEK1 (Supplementary Fig. S3D). Cells expressing ARAFK336M and ARAF<sup>D447A</sup> either produced significantly less (COR-L23) or failed to generate detectable p-MEK1 despite similar efficiencies of ARAF immunoprecipitation. In all cell lines expressing exogenous wild-type and kinase dead ARAF variants, treatment with LXH254 promoted heterodimer formation between ARAF and both BRAF and CRAF, with the exception of MEL-JUSO cells, where only ARAF-CRAF heterodimers were detected. In contrast, IPs from HCT 116 and MEL-JUSO cells expressing ARAF<sup>R362H</sup> were largely devoid of both BRAF and CRAF and generated very little p-MEK1. These data indicate that kinase dead and dimer-impaired variants lack catalytic activity and heterodimer forming capability, respectively. Failure of

**Figure 3.**
Loss of ARAF expression sensitizes RAS-mutant cell lines to LXH254. Shown are proliferation curves for five cell lines and derived variants lacking expression of either ARAF, BRAF, or CRAF. Cell line identity and RAS (or RAF) mutation status are shown above each graph. Concentrations of LXH254 (nmol/L) and percentage of cells remaining relative to an untreated control, as judged by ATP levels, are given on the x-axis and y-axis, respectively. Effects of LXH254 on proliferation were determined after either 3 (MEL-JUSO, SK-MEL-30, HCT 116, and MIA PaCa-2) or 5 (COR-L23) days of incubation with LXH254.
these variants to restore LXH254 insensitivity indicates that both catalytic function and the ability to dimerize are required for ARAF-mediated resistance to LXH254.

To investigate further differential inhibition of RAF paralogs by LXH254, we generated isogenic cell lines using CRISPR/Cas-9 that expressed only one of the three RAF genes. Clonal isolates expressing only ARAF (BRAF<sup>D</sup>/CRAF<sup>D</sup>) or CRAF (ARAF<sup>D</sup>/BRAF<sup>D</sup>) were generated in HCT 116, MIA PaCa-2, and MEL-JUSO cells. BRAF-only cells were more difficult to generate, and while MIA PaCa-2 (ARAF<sup>D</sup>/CRAF<sup>D</sup>) cells were generated, BRAF-only HCT 116 cells maintained a low level of CRAF (ARAF<sup>D</sup>/CRAF-low) and we were unable to generate BRAF-only MEL-JUSO cells (Supplementary Fig. S2). Consistent with results in cells lacking ARAF (Fig. 4), BRAF-only and CRAF-only cells were 5- to 10-fold more sensitive to LXH254 in 3-day growth assays than parental cells (Fig. 6A). After 4 hours of treatment, LXH254 effectively inhibited signaling in BRAF- and CRAF-only cells, with IC<sub>50</sub> values below 100 nmol/L (Fig. 6B). In contrast, in ARAF-only cells, signal inhibition required higher drug concentrations with IC<sub>50</sub> values measured at >1,500 nmol/L in all cell lines (Fig. 6B; Supplementary Table S6). Moreover, treatment of all ARAF-only cell lines with LXH254 resulted in modestly increased p-ERK (25%–50%) at LXH254 concentrations ranging from 10 to 370 nmol/L in a manner reminiscent of paradoxical activation described for type 1/1.5 RAF inhibitors (41–43). As described previously for cells lacking ARAF (Fig. 3; Table 1), pathway reactivation over time after LXH254 treatment in BRAF- and CRAF-only cells was reduced relative to parental cells (Supplementary Fig. S3E).

To compare the effects of LXH254 on signaling with that of a type 1.5 inhibitor, we treated parental and individual RAF paralog-expressing MIA PaCa-2 cells for 4 hours with the type 1.5 RAF inhibitor, dabrafenib, and measured the effects on p-ERK levels by MSD. Consistent with previous results reported in RAS-mutant models, treatment of MIA PaCa-2 cells with dabrafenib resulted in increased p-ERK levels to a peak approximately 4-fold greater than baseline at concentrations up to 3 µmol/L (Fig. 6C).
exceeding 3 μmol/L, p-ERK levels declined, returning to original baseline levels at dabrafenib concentrations of 10 μmol/L. Dabrafenib exerted distinctly different effects on p-ERK levels in cells expressing only one of each of the three RAF proteins. In CRAF-only cells, treatment with dabrafenib resulted in a similar pattern of pathway activation to parental MIA PaCa-2 cells, although activation was subtly greater at all concentrations up to approximately 600 nmol/L. In cells expressing only BRAF or ARAF, increases in p-ERK levels were also observed, however, increases were modest (~50%) relative to what occurred in parental and CRAF-only cells. These data are consistent with previous reports suggesting that CRAF activation is the major driver of paradoxical activation (17, 41, 44). Comparing the effects of signaling indicated that while both drugs similarly and modestly induced paradoxical activation in ARAF-only–expressing cells, LXH254, but not dabrafenib, is able to inhibit wild-type BRAF and CRAF in cells (Fig. 6B and C).

Figure 5.
Kinase-impaired and dimer-deficient ARAF variants lack in vitro kinase activity and have reduced interactions with BRAF and CRAF, respectively. Shown are Western blots using the indicated antibodies performed on either whole-cell lysates (WCL) or material immunoprecipitated using a FLAG-directed antibody (IP-FLAG) from COR-L23 (A), MIA PaCa-2 (B), MEL-JUSO (C), and HCT 116 (D) cell lines. In the cases of COR-L23 and MIA PaCa-2 cells, exogenously expressed ARAF variants were under the transcriptional control of the doxycycline (dox)–regulated TET-3G promoter. Concentrations of LXH254 used for cell line pretreatment are shown above each sample. For the IP-kinase assay, purified recombinant His6x-tagged MEK1 and ATP were added to immunoprecipitated material.
RAS mutants lacking ARAF are more sensitive to LXH254 in vivo

We next determined the effect of ARAF ablation on sensitivity to MAPK inhibitors in vivo. Tumors formed from parental and ARAF-deleted variants of the HCT 116, MIA PaCa-2, and MEL-JUSO models were treated with vehicle, 0.3 mg/kg every day trametinib, or 50 mg/kg twice a day LXH254. Doses of trametinib and LXH254 were selected that matched AUC values for the approved dose of trametinib (2 mg, twice a day) or the recommended phase II dose of LXH254 (400 mg, twice a day). ARAF-deleted xenografts either grew with similar kinetics (HCT 116 cells) or slightly slower (MIA PaCa-2 and MEL-JUSO) than their parental counterparts, indicating that ARAF ablation has at most modest effects on the fitness of these models. In all parental models, LXH254 and trametinib exerted similar effects on tumor growth, resulting in a slow growth phenotype, which was most pronounced in MEL-JUSO cells (Fig. 7A–C). Treatment with LXH254 in the ARAF knockouts led to complete regression of HCT 116 and MEL-JUSO and near-complete regression of MIA PaCa-2 xenografts. Several tumors from each KRAS-mutant model regrew after prolonged treatment, including three of six from the HCT 116 and five of six of the MIA PaCa-2 tumors (Supplementary Fig. S4A–S4C). Initial analysis of these variants by Western blot analysis suggested that at least two tumors (MIA PaCa-2, M2, and M5) became resistant due to RAF pathway reactivation, with reactivation in one case potentially attributable to restoration of ARAF expression (M5). Lack of clear pathway reactivation in the other six tumors suggested resistance occurred via a MAPK bypass mechanism (Supplementary Fig. S4D). Strikingly, two of three of the regressed HCT 116 tumors failed to regrow after cessation of drug treatment consistent with complete eradication of the tumor (Supplementary Fig. S4A). In contrast, variants lacking ARAF expression had similar sensitivities to trametinib as parental MIA PaCa-2 cells.

These data indicate that RAS-driven activation of BRAF and CRAF is inhibited sufficiently by LXH254 to eradicate tumors, and conversely that poor ARAF inhibition is a critical contributor to the relative insensitivity of RAS-mutant models to LXH254. Accordingly, we tested whether cotreatment of tumors with LXH254 and low doses of trametinib, aimed at inhibiting residual ARAF-driven signaling, improved antitumor effects in MIA PaC-2 cells. Combining LXH254 with either 50% (0.3 mg/kg, every 2 days) or 10% (0.03 mg/kg once a day) doses of trametinib improved efficacy relative to both LXH254 and a full dose of trametinib (0.3 mg/kg, twice a day), although efficacy was inferior to that seen for single-agent LXH254 in cells lacking ARAF.

Figure 6.

RAS-mutant cells expressing only ARAF are resistant to LXH254. Shown are proliferation (A) and p-ERK (B) inhibition curves for parental (closed blue circles) HCT 116, MIA PaCa-2, and MEL-JUSO cell lines, as well as variants lacking BRAF and CRAF expression (closed maroon triangles), ARAF and BRAF expression (closed light blue triangles), and ARAF and CRAF expression (closed green squares), following treatment with increasing concentrations of LXH254. In all cases, changes in p-ERK levels following 4 hours of treatment of MIA PaCa-2 cells with increasing concentrations of either LXH254 or dabrafenib are shown in C.
Combining LXH254 with even small amounts of an MEK inhibitor can result in significant antitumor effects.

Discussion

LHX254 is a type II RAF inhibitor with a high degree of selectivity for BRAF and CRAF, which predominantly binds the inactive, DFG-out kinase configuration, and promotes the formation of BRAF/CRAF heterodimers (ref. 35; Fig. 1; Supplementary Tables S1 and S2). LXH254 treatment also promoted MEK1/2–B/CRAF interactions in IPs using MEK-directed antibodies, however, this interaction was not reciprocated in IPs using RAF-directed antibodies. This discrepancy may be an artifact due to the choice of RAF antibodies used for the IPs. Alternatively, this may reflect the existence of a substantial pool of dimerized RAF that is not complexed with MEK1/2.

Here, we further describe an unexpected paralog selectivity of this molecule, wherein it is a relatively poor inhibitor of ARAF. Poor ARAF inhibitory activity in cells was somewhat unanticipated on the basis of biochemical analysis that indicated single-digit nmol/L inhibition of the ARAF kinase in cell-free assays. LXH254 did not translate to robust inhibition in the cellular setting. Ablation of ARAF expression in five of five RAS-mutant cell lines resulted in more potent antiproliferative effects and RAF/MEK/ERK signal suppression by LXH254 (Fig. 3; Table 1).

The mechanistic basis for the failure of LXH254 to inhibit ARAF is unknown and insights into a structural explanation are hampered by the lack of a crystal structure for ARAF. However, as suggested here (Supplementary Table S8) and elsewhere (37), failure to inhibit ARAF may not be a property of all type II RAF inhibitors. Simulations of ARAF structure using information available for BRAF and CRAF (see Materials and Methods) suggest a possible explanation for reduced activity of LXH254 against ARAF (Supplementary Fig. S5B). The terminal hydroxyl group of the alkane chain of LXH254 forms a hydrogen bond with the backbone carbonyl of F595 in BRAF (and CRAF) in the DFG-out configuration of the protein (35). Simulations of the ARAF structure place this carbonyl shifted to a position more proximal to the hydroxyl group on LXH254, which would likely eliminate this hydrogen bond, instead creating a steric clash. Interestingly, RAF709, a similarly poor inhibitor of ARAF (Supplementary Table S8) also forms a hydrogen bond with the same carbonyl on F595, albeit via a water intermediary, which is also predicted to be lost in ARAF. An understanding of the basis for the decreased inhibitory activity of LXH254 against ARAF requires further study.

Figure 7.

RAS-mutant cell lines lacking ARAF are sensitive to LXH254 in vivo. Shown are changes in tumor volume until the time of T/C determination results for HCT 116 (A), MIA PaCa-2 (B and D), and MEL-JUSO (C) models. Parental models are denoted with solid lines and variants lacking ARAF expression with dashed lines. Vehicle treated animals are shown in black (open squares), 0.3 mg/kg trametinib daily treated in blue (open triangles), and 50 mg/kg LXH254 twice a day in red (open circles). D, The MIA PaCa-2 model treated as in B, but with additional combinations of 50 mg/kg LXH254 twice a day with either 0.3 mg/kg once every 2 days (open purple hexagons) or 0.03 mg/kg daily (open green diamonds) trametinib. *, P = 0.0007; **, P = 0.0001, against vehicle by one-way ANOVA.
Roles for ARAF in promoting signal transduction in a scaffold rather than catalytic capacity have been described previously (45). However, we found that ARAF-mediated insensitivity to LXH254 was dependent on ARAF having both kinase activity and the ability to form dimers, strongly suggesting that failure of LXH254 to inhibit ARAF catalytic activity while ensconced as an active member of RAF signaling complexes underlies this phenotype. In RAS-mutant tumors engineered to express only ARAF, LXH254 paradoxically activated signaling to a similar degree, and with a similar bell-shaped dose–response curve to the type 1.5 inhibitor, dabrafenib (Fig. 6). In contrast, in RAS-mutant cells expressing only BRAF or CRAF, LXH254 potently inhibited signaling, whereas dabrafenib caused pathway activation, most notably in cells expressing CRAF. Collectively, these data suggest a model where LXH254 potently inhibits dimeric BRAF and CRAF, but paradoxically activates ARAF-containing dimers (Supplementary Fig. S3A).

In the phase 1 dose escalation trial of LXH254, very few partial responses to single-agent LXH254 were observed (46). This trial was enriched for KRAS-mutant tumors (~30%) and consistent with preclinical data presented herein; the majority of patients with KRAS-mutant disease progressed on treatment, although a minority of these patients displayed stabilization of disease. On the basis of the findings here, LXH254 might have single-agent activity only in RAS-mutant tumors with either low expression or utilization of ARAF. However, no correlation was found between ARAF mRNA expression and sensitivity to LXH254, either in the entire panel of cell lines profiled, or within specific RAS/RAF-mutated subgroups in vitro (Supplementary Fig. S1C). Moreover, analysis of RAS-mutant tumors in TCGA did not reveal a clearly identifiable subset of RAS-mutant tumors with intrinsically low ARAF expression (ref. 47; Supplementary Fig. S1D). Thus, the primary utility of LXH254 is likely to be in combination with additional agents targeting the MAPK pathway.

Consistent with this hypothesis, we found that adding even low doses of trametinib to a full dose of LXH254 improved antitumor activity relative to single-agent effects in the Mia PaCa-2 model (Fig. 7D). The observation that adult mice tolerate simultaneous loss of BRAF and CRAF (8) suggests that poor ARAF inhibition may improve LXH254 tolerability relative to, for example, MEK and ERK inhibitors, thereby facilitating the achievement of drug exposures wherein both BRAF and CRAF are strongly inhibited. Such improved tolerability might also facilitate combinations with additional MAPK inhibitors for the treatment of RAS-mutant tumors, resulting in improved pathway suppression and antitumor effects. In support of this idea, LXH254–anchored MAPK combinations, such as those described in Fig. 7, display a superior tolerability/efficacy relationship when compared with single-agent treatments in preclinical species (manuscript in preparation). Moreover, combinations between LXH254 and the MEK inhibitor, trametinib, and ERK inhibitor, LTT462, are currently in dose expansion in patients with a variety of RAS/RAF pathway alterations.

Authors’ Disclosures
M. Jaskelioff reports employment with Novartis Institutes for Biomedical Research during the conduct of this study. J.A. Engelman reports employment with Novartis and equity in Novartis. D.D. Stuart reports other from Novartis during the conduct of the study and outside the submitted work, as well as a patent for drug combinations pending to Novartis. V.G. Cooke reports a patent for WO2018/203219 pending, as well as employment with Novartis and ownership of Novartis stock. G. Caponigro reports other from Novartis Institutes for Biomedical Research outside the submitted work. No disclosures were reported by the other authors.

Authors’ Contributions

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References
21. Simanshu DK, Nissley DV, McCormick F. RAS proteins and their regulators in
23. Lake D, Correa SA, Muller J. Negative feedback regulation of the ERK1/2 MAPK

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