Combined Pan-RAF and MEK Inhibition Overcomes Multiple Resistance Mechanisms to Selective RAF Inhibitors

Steven R. Whittaker, Glenn S. Cowley, Steve Wagner, Flora Luo, David E. Root, and Levi A. Garraway

Abstract

RAF and MEK inhibitors are effective in BRAF-mutant melanoma but not in BRAF-mutant colorectal cancer. To gain additional insights into this difference, we performed a genome-scale pooled shRNA enhancer screen in a BRAF-mutant, RAF inhibitor–resistant colorectal cancer cell line exposed to the selective RAF inhibitor PLX4720. We identified multiple genes along the receptor tyrosine kinase (RTK)/mitogen-activated protein kinase (MAPK) signaling axis that, when suppressed, either genetically or pharmacologically, sensitized cells to the selective RAF inhibitor through sustained inhibition of MAPK signaling. Strikingly, CRAF was a key mediator of resistance that could be overcome by the use of pan-RAF inhibitors in combination with a MEK inhibitor. Furthermore, the combination of pan-RAF and MEK inhibitors displayed strong synergy in melanoma and colorectal cancer cell lines with RAS-activating events such as RTK activation, KRAS mutation, or NFI loss-of-function mutations. Combinations of selective RAF inhibitors, such as PLX4720 or dabrafenib, with MEK inhibitors did not incur such profound synergy, suggesting that inhibition of CRAF by pan-RAF inhibitors plays a key role in determining cellular response. Importantly, in contrast to the modest activity seen with single-agent treatment, dual pan-RAF and MEK inhibition results in the induction of apoptosis, greatly enhancing efficacy. Notably, combined pan-RAF and MEK inhibition can overcome intrinsic and acquired resistance to single-agent RAF/MEK inhibition, supporting dual pan-RAF and MEK inhibition as a novel therapeutic strategy for BRAF- and KRAS-mutant cancers. Mol Cancer Ther; 14(12): 2700–11. ©2015 AACR.

Introduction

Despite the impressive early successes with BRAF-targeted therapies in BRAF-mutant melanoma, intrinsic and acquired resistance presents an enormous challenge and ultimately leads to progressive disease (1). Furthermore, widespread, intrinsic resistance of BRAF-mutant colorectal cancers to selective RAF inhibitors has severely blunted the use of these agents in this context (2). This observation could be taken to suggest that colorectal cancers are indifferent to RAF inhibitors and that a BRAF mutation alone is not sufficient to inform drug selection in the clinic. However, BRAF has been shown to be a high-ranking dependency that discriminates between BRAF-mutant and BRAF wild-type cell lines, solidifying its status as a key oncogenic driver (3). These data are therefore consistent with a continued dependency on BRAF signaling for cell survival and validates it as a drug target in colorectal cancer. Furthermore, initial studies suggested that some cancers are able to evade the effects of BRAF inhibition and reactivate the MAPK pathway. Sustained MAPK pathway activity can be brought about by amplification of BRAF, overexpression of CRAF, mutation of NRAS, mutation of NFI, PDGFRB signaling, IGF1R/PI3K activity, MEK1 mutation, expression of MAP3K8/COT, or an altered transcriptional state (4–12). Surprisingly, second-site mutations of the BRAF gene have not yet been implicated in resistance to BRAF inhibition, despite demonstrating the potential to do so in preclinical models (13).

Therefore, to enable an unbiased and global assessment of potential RAF inhibitor combination therapies for BRAF-mutant cancers, we performed a pooled shRNA enhancer screen configured to identify genes, which when suppressed, sensitized cells to the effects of the selective RAF inhibitor PLX4720 (14). Candidate hits from the screen would then enable us to identify specific vulnerabilities in BRAF-mutant cancers that might indicate novel opportunities for combination therapies to maximize patient benefit.

Materials and Methods

Cell lines and reagents

Cell lines were obtained from the ATCC or the National Cancer Institute (NCI) and cultured in DMEM (CellGro), 10% FBS (Gemini Bio-Products). Cells were passaged for less than 6 months after receipt and authenticated by short tandem repeat profiling: PLX4720, AZD6244, AZ628, RAF265, GSK1120212,
and GSK2118436 were purchased from Selleck Chemicals. MLN2480 was purchased from Chemiscene. Expression constructs for LacZ, KRASG12V, and MEKQ56P have been described previously (5, 11).

Cell proliferation assays

Cells were seeded into 96-well plates at a density permitting logarithmic growth for the duration of the experiment. The following day, compounds were added to the cells dissolved in DMSO/medium and incubated for 96 hours. CellTiter-Glo reagent (Promega) was then added to the wells, the plates were shaken for 15 minutes, and then luminescence was read using an Envision plate reader (Perkin-Elmer). GI50 values or % inhibition were calculated using GraphPad Prism. Where cell proliferation was determined relative to the starting cell titer, an untreated control plate was frozen at the time of treatment and inhibitor-treated plates were frozen following the 96-hour incubation. Plates were then thawed and CellTiter-Glo reagent was used as described to determine cell proliferation. For colony formation assays, cells were seeded at low density into 12-well plates. The following day, cells were treated with compounds and the medium/compound was replaced every 5 days. Following 7 to 10 days of treatment, cells were washed with PBS, fixed in 4% formaldehyde for 30 minutes, and then stained with 0.5% crystal violet solution. Unbound dye was removed by washing with excess water. Wells were photographed and the dye was resuspended in 10% cell lysis and Western blotting

Cells were lysed in SDS lysis buffer (1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris, pH 8.0). Protein concentration was determined using the BCA assay (Sigma). Proteins were resolved by SDS-PAGE and transferred to PVDF-F membranes (Millipore) using iBlot (Life Technologies). Blots were blocked with LI-COR blocking buffer and probed with the indicated antibodies overnight at 4°C. Detection of proteins was via IR dye-conjugated secondary antibodies and IR-fluorescence detection via the Odyssey system (LI-COR).

Pooled lentiviral shRNA screen

The conditions and procedure for pooled lentiviral shRNA screening have been described previously (11). Briefly, RKO cells were seeded into 12-well plates at a density of 3 × 104 cells per well, a total of 7.2 × 107 cells per replicate were infected with a 90,000 shRNA, pooled library at an MOI of 0.3 to 0.5. Cells were centrifuged at 2,000 rpm for 2 hours and medium changed immediately after. The next day, cells were pooled and selected in the presence of 1 µg/mL puromycin for 3 days. Cells were then passaged in the presence of either DMSO or 3 µmol/L PLX4720 for 16 population doublings. Cells were harvested by trypsinization and stored in PBS at −80°C. Extraction of gDNA, amplification of shRNA sequences, and screen deconvolution by massively parallel sequencing (Illumina) were performed as previously described (11). The depletion of shRNAs in the PLX4720-treated arm relative to the DMSO-treated arm was determined using the RIGER algorithm and this produced a statistically significant ranked list of genes according to the "second best shRNA" analysis in Gene-E (http://www.broadinstitute.org/cancer/software/GENE-E/).

shRNA/ORF lentiviral infection

Cells were seeded into 6- or 96-well plates as required. The following day, the medium was supplemented with 4 µg/mL polybrene (Millipore), and lentiviral particles were added to the cells to give an MOI of around 1. Plates were centrifuged at 2,000 rpm for 30 minutes and medium was changed immediately after. Seventy-two hours later, cells were treated with inhibitors as required. shRNA constructs used are as follows: shLuc TRC-N0000072243; shMET TRCN00000000393, TRCN0000121233; shPITPN1 TRCN0000005002, TRCN0000005003; shRAF1 TRCN0000001066, TRCN0000197115; shSHOC2 TRCN0000151603, TRCN0000154502; shNF1 TRCN0000039717. Further details are available via The RNAi Consortium portal http://www.broadinstitute.org/maiz/public/.

Results

We profiled a panel of 20 BRAF-mutant melanoma and colorectal cancer cell lines for sensitivity to PLX4720 (Fig. 1A). The majority of colorectal cancer lines showed reduced sensitivity to the inhibitor compared with the melanoma lines. To determine whether this was related to inhibition of the MAPK pathway, we measured ERK1/2 phosphorylation following a 24-hour incubation with PLX4720 by in-cell Western (Fig. 1B). Compared with the melanoma lines, only one colorectal cancer line achieved near-complete suppression of phospho-ERK (COLO205) and was highly sensitive to PLX4720. The other lines tested all failed to reach >75% inhibition of phospho-ERK and, as demonstrated in clinical trials of vemurafenib, complete (>90%) suppression of the pathway was required for efficacy (15). Interestingly, when RKO cells were treated with 3 µmol/L PLX4720, ERK phosphorylation was inhibited by >90% at 1 to 2 hours, but this recovered during 4 to 24 hours to approximately 50% of controls (Supplementary Fig. S1A). Furthermore, the cells continued to proliferate in the presence of the drug (Supplementary Fig. S1B).

To understand what factors might permit reactivation of MAPK signaling in the presence of PLX4720, we conducted a pooled shRNA screen targeting >16,500 genes to identify which genes, when silenced, sensitized cells to the inhibitor (Fig. 1C). The RKO colorectal cancer cell line for this study, as it was readily

www.aacjrournals.org Mol Cancer Ther; 14(12) December 2015 2701

Published OnlineFirst September 8, 2015; DOI: 10.1158/1535-7163.MCT-15-0136-T

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Synergy between Pan-RAF and MEK Inhibition

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To understand what factors might permit reactivation of MAPK signaling in the presence of PLX4720, we conducted a pooled shRNA screen targeting >16,500 genes to identify which genes, when silenced, sensitized cells to the inhibitor (Fig. 1C). The RKO colorectal cancer cell line for this study, as it was readily
transduced with the lentiviral shRNA library, was highly resistant to PLX4720 (GI\textsubscript{50}, 17 μmol/L) and had a short doubling time (<24 hours). Moreover, these cells have previously been shown to possess a dependency on BRAF for proliferation [3]. The cells were infected with a pooled library of about 90,000 shRNAs, then cultured in the presence of either DMSO vehicle or 3 μmol/L PLX4720 (Fig. 1D). shRNAs depleted only in the presence of PLX4720 were identified by Illumina sequencing (11). The Pearson correlation between replicate samples for each experimental condition confirmed reproducibility of the screen result (Fig. 1E).

RNAi Gene Enrichment (RIGER; ref. 16) was used to rank genes according to the second best scoring shRNA per gene (Fig. 1F; Supplementary Table S1). The top-ranking gene was MET, which encodes the receptor tyrosine kinase (RTK) MET/HGFR, followed by MLL/KMT2A (encoding a lysine methyl-transferase) and PTPN11, which encodes the protein tyrosine phosphatase SHP2. We used the protein interaction tool DAPPLE (17) to identify possible connections between the top 300 genes in the screen (Supplementary Fig. S2). Notably, a node encompassing genes for several members of the MAPK pathway, the protein tyrosine phosphatases PTPN6 and PTPN11, and adapter proteins CRKL and GAB1, the protein tyrosine phosphatases PTPN6 and PTPN11, and several members of the MAPK pathway SHOC2, RAF1, MAP2K2 stood out due to the known functional relationships among the proteins encoded by these genes.

We therefore decided to validate key genes in this node as mediators of resistance to BRAF inhibition. Compared with a control shRNA targeting luciferase, knockdown of MET/HGFR, PTPN11/SHP2, SHOC2, and RAF1/CRAF all sensitized RKO cells...
to PLX4720 (Fig. 2A) and permitted greater sustained suppression of ERK1/2 phosphorylation at 24 hours following treatment with 3 µmol/L PLX4720 (Fig. 2B). Suppression of most of these genes sensitized cells to BRAF inhibition in other colorectal cancer cell lines SW1417, LS411N, and WIDR (Supplementary Fig. S3); the exception was MET, whose sensitization effects were restricted to the RKO cell line. The sensitization of the RKO line by MET suppression is likely due to high expression of HGF by these cells, which activates MET signaling, thus creating a dependency on MET in the absence of signaling by oncogenic BRAF (18). We confirmed that inhibition of MET using crizotinib, SGX523, or foretinib in combination with PLX4720 resulted in near-complete inhibition of ERK1/2 phosphorylation and synergistic antiproliferative activity as determined using the Bliss independence model (Supplementary Fig. S4; ref. 19). Interestingly, inhibition of SHP2 using the tool compounds NSC87877 (20) or PHPS1 (21) in combination with PLX4720 also yielded greater antiproliferative activity than single-agent treatment and greater suppression of ERK1/2 phosphorylation, resulting in modest synergy

Figure 2.
Validation of candidate synthetic lethal genes. A, RKO cells were infected with individual lentiviral shRNA expression vectors targeting high-ranking genes from the primary screen or a control shRNA targeting luciferase. Cells were treated with increasing concentrations of PLX4720 for 4 days. Cell proliferation was determined using the CellTiter-Glo assay. GI50 values were determined using GraphPad Prism. B, RKO cells were infected as in A, then 72 hours after infection were treated with 3 µmol/L PLX4720 for 18 hours, and protein lysates were analyzed by Western blotting for the indicated proteins.
Thus, it seems likely that other RTKs, such as EGFR that has been demonstrated to confer resistance to BRAF inhibition in colorectal cancer cell lines (4, 22), also depend upon SHP2 to signal to the MAPK pathway and drive resistance. We confirmed that combined inhibition of BRAF and EGFR was synergistic in WiDr and SW1417 cells (data not shown).

Near-complete inhibition of ERK1/2 phosphorylation seemed essential to elicit an antiproliferative response in the PLX4720-resistant cell lines. Hence, we sought to examine drugs that were more likely to extinguish RAF/MEK/ERK signaling to this degree. We selected AZ628 for these studies, which is an inhibitor of BRAFV600E, BRAF, and CRAF (ref. 23; a so-called "pan-RAF" inhibitor). Notably, while PLX4720 is...
Synergy between Pan-RAF and MEK Inhibition

Consistent with this, both melanoma and colorectal cancer cell lines were generally more sensitive to AZ628 or the MEK inhibitor AZD6244 than PLX4720, although some lines still exhibited resistance (Fig. 3A). Therefore, we explored combination strategies using AZ628 and AZD6244 in PLX4720-resistant lines. The RKO cell line has a GI50 of 0.5 ± 0.04 μmol/L for AZ628 and 4.7 ± 0.9 μmol/L for the MEK inhibitor AZD6244 (Fig. 3B). However, when 10 nmol/L AZ628 was added to a titration of AZD6244, the GI50 value for AZD6244 dropped considerably to 240 ± 131 nmol/L and if the concentration of AZ628 was increased to 100 nmol/L, the GI50 value further decreased to 25 ± 9 nmol/L. Similarly, LOXIMVI cells, which have a GI50 value of 8.3 ± 3.6 μmol/L for AZ628, were sensitized to AZD6244 when treated with 10 and 100 nmol/L AZ628, resulting in the GI50 decreasing from 8.2 ± 1.1 μmol/L to 384 ± 129 nmol/L and 128 ± 39 nmol/L, respectively. Furthermore, in longer term colony formation assays, treatment of RKO and LOXIMVI cell lines with 300 nmol/L AZD6244 and 30 nmol/L AZ628 potently inhibited cell proliferation only when used in combination (Fig. 3C).

Next, we investigated the effects of combining AZD6244 and AZ628 on MAPK pathway activity. PLX4720-resistant RKO, LOXIMVI, and WIDR cells and PLX4720-sensitive A375 and COLO205 cells were treated with 1 μmol/L AZD6244 or 100 nmol/L AZ628 either alone or in combination for 16 hours (Fig. 4A). In the PLX4720-resistant lines, single-agent treatment failed to completely inhibit ERK1/2 phosphorylation and did not suppress the expression of cyclin D1, a MAPK-regulated protein. However, when the two compounds were combined, ERK1/2 phosphorylation was completely inhibited and cyclin D1 expression was markedly reduced. Strikingly, treatment with the relatively high concentration of 10 μmol/L PLX4720 did not inhibit either ERK1/2 phosphorylation or cyclin D1 expression and even in combination with AZD6244 did not appreciably inhibit MAPK pathway activity. PLX4720-sensitive cell lines showed good suppression of the MAPK pathway by single agents and the combination. Notably, the effects on MAPK signaling correlated well with the inhibition of cell proliferation (Fig. 4B) whereby in the PLX4720-resistant cell lines, only the combination of AZD6244 and AZ628 resulted in robust inhibition. Using the Bliss independence model, this combination proved to be highly synergistic across a broad range of concentrations and was greatest in lines resistant to single-agent treatment (Fig. 4C). For comparison, the combination of AZD6244 and PLX4720 did not potently inhibit cell proliferation, nor did it produce a strong synergistic effect in PLX4720-resistant lines (Supplementary Fig. S6).

Given that the combination of a pan-RAF inhibitor and a MEK inhibitor could drive a profound response, we sought to establish what factors were responsible for this. Accordingly, we screened a panel of melanoma and colorectal cancer cell lines and determined the sum excess over Bliss score across the range of concentrations tested. Strikingly, we observed that some of the cell lines that exhibited resistance to the single agents but were synergistically inhibited by the combination also had an activating mutation in either KRAS (SW480, SW837, DLD1, HCT116, HCT15) or loss-of-function mutations in NFI (LOXIMVI, HCT116, RKO, MeWo; Fig. 5A, ref. 24). All of these events could promote elevated RAS signaling, which may confer resistance to the aforementioned single agents. We did however observe KRAS- and NRAS-mutant cell lines (SW620 and SKMEL2, respectively) that did not display synergy, likely because they were more sensitive to single-agent treatment alone. RAS activity would be expected to promote CRAF activation (25) and potentially lead to sustained signaling in the presence of a selective RAF inhibitor such as PLX4720 (23, 26, 27). In this case, a greater dependency may exist on CRAF, which would be more efficiently targeted by AZ628 (7).

Therefore, to test the involvement of RAS in the response to pan-RAF/MEK inhibition, we introduced KRASG12V or shRNA targeting the RAS-GAP NF1 into the A375 melanoma cell line, which confer >200-fold or >50-fold resistance to PLX4720, respectively (11). The cells were then treated with AZD6244 and AZ628 alone or in combination and cell proliferation was assessed after 96 hours (Fig. 5B). In the control LaCz or shLuc cells, AZD6244 and AZ628 potently inhibited proliferation alone and in combination. In contrast, expression of KRASG12V or knockdown of NF1 conferred resistance to single-agent treatment but retained sensitivity to the combination of both drugs. The cells were also treated as above for 16 hours and MAPK pathway activity was assessed by Western blotting (Fig. 5C). In the control cells expressing LaCz or shLuc, both inhibitors reduced ERK1/2 phosphorylation and decreased the expression of cyclin D1. However, cells expressing KRASG12V or shRNA targeting NF1 maintained ERK1/2 phosphorylation and the expression of cyclin D1 when treated with a single agent but were acutely sensitive to the combination of both agents, resulting in loss of ERK1/2 phosphorylation and cyclin D1 expression.

Given that the majority of resistance mechanisms to BRAF inhibitors involve either RAS activation by mutation, amplification, or upstream activation of RTKs, we posited that cells showing acquired resistance to PLX4720 would be sensitive to the combination of MEK and pan-RAF inhibition. We generated a PLX4720-resistant cell line by exposing A375 cells to 1 μmol/L PLX4720 for approximately 1 month and isolated resistant clones. This A375R10 clone was 23-fold less sensitive to PLX4720 than its parental pretreatment A375 cells (Supplementary Fig. S7A) and displayed a greater degree of CRAF S338 phosphorylation, a marker of CRAF activation promoted by RAS (28). Furthermore, the levels of phospho-MEK1/2, phospho-ERK1/2, and cyclin D1 were far less affected by PLX4720-treatment than compared with the sensitive, parental cells (Supplementary Fig. S7B). While resistant to treatment with single-agent AZD6244 or AZ628, the combination of these two compounds markedly inhibited proliferation of the cells (Supplementary Fig. S7C) and this correlated with greater suppression of MAPK pathway activity in the A375R10 cell line (Supplementary Fig. S7D).

To test the possibility that the antiproliferative effect of the drug combination was due to off-target effects of either inhibitor, we tested combinations of different pan-RAF and MEK inhibitors. The combination of RAF265 (29) and AZD6244, MLN2480 (30) and AZD6244, and of AZ628 and trametinib (GSK1120212; ref. 31) all resulted in highly synergistic activity in lines that were resistant to the single agents and this again correlated well with near-complete MAPK pathway inhibition (Supplementary Figs. S8–S10). We also tested the combination of AZD6244 and dabrafenib (GSK2118436; ref. 32) or trametinib and dabrafenib.
Figure 4.
Combinatorial inhibition of BRAF, CRAF, and MEK is synergistic in cell lines displaying intrinsic resistance to PLX4720. A, RKO, LOXIMVI, WiDr, A375, and COLO205 cancer cell lines were treated with 1 μmol/L AZD6244, 0.1 μmol/L AZ628, 10 μmol/L PLX4720, or a combination of AZD6244 with either AZ628 or PLX4720 for 18 hours. Cell lysates were analyzed by Western blotting for the indicated proteins. B, cell lines were treated as in A for 96 hours, and cell proliferation was assessed by CellTiter-Glo assay. C, cells were treated with a titration of AZD6244 versus a titration of either AZ628 or PLX4720 in combination for 96 hours. Cell proliferation was assessed using CellTiter-Glo and presented in the blue–red heatmaps. The green–red heat maps present the degree of synergistic interaction between the compounds, as determined using the “Bliss Independence model” where values greater than 0 indicate an effect greater than the combined fractional inhibition of the single agents, indicative of synergy.
Strikingly, while some modest synergy was observed between the MEK inhibitors and dabrafenib, the concentrations required to achieve a substantial reduction in proliferation were considerably higher than AZ628 and the degree of synergy was lower (Supplementary Fig. S11A). Interestingly, when we treated RKO cells with AZ628 and an inhibitor of ERK2, VTX-11e (33), little or no synergy was observed, despite potent inhibition of proliferation (Supplementary Fig. S11B). This is consistent with ERK2 activity being the critical integrator of RAS/RAF/MEK upstream signaling and that single-agent ERK2 inhibition may overcome the resistance exhibited to single-agent pan-RAF or MEK inhibition. This also underscores the specificity of the pan-RAF/MEK combination for its effect on the MAPK pathway. Importantly, we could rescue cells from the combination of AZD6244 and AZ628 by expression of the drug-resistant mutant MEK Q56P (5), which permitted sustained ERK1/2 phosphorylation and cyclin D1 expression in the presence of the inhibitors (Supplementary Fig. S12A) and restored cell proliferation (Supplementary Fig. S12B) consistent with MAPK pathway inhibition being the key effector of response to the combination.

To further characterize the strong synergistic response produced by the combination of the pan-RAF and MEK inhibitors, we measured proliferation relative to the number of cells present at the commencement of treatment. When treated with single agents, cells were still able to proliferate, albeit at a reduced rate (Fig. 6A). Strikingly, when used in combination, we observed a net loss of cells relative to the starting number consistent with cell death. We therefore assessed cytotoxicity using the Cytotox-Glo assay, which measures protease activity associated with cell death. The combination of AZ628 and AZD6244 led to significantly increased cytotoxicity compared with the single agents (Fig. 6B; Supplementary Fig. S13). The induction of apoptosis by the drug combination was further confirmed by the detection of cleaved PARP following a 72-hour exposure to the inhibitors (Fig. 6C). Therefore, while exposure to single-agent pan-RAF/MEK inhibition was able to
reduce, but not stop, cell proliferation; the combination of both agents resulted in significant apoptosis.

**Discussion**

Although BRAF is a clear driver of melanoma and colorectal cancers, attempts at targeting this oncogene have delivered mixed responses due to intrinsic or acquired resistance mechanisms (34). Our shRNA screening approach identified multiple potential candidates for RAF inhibitor–based combinatorial therapies. Strikingly, our analysis of genes that promote resistance to PLX4720 indicated enrichment for genes within the RTK/MAPK pathway, including MET, ERBB3, NTRK2, GAB1, CRKL, SHP2, SHOC2, MAP2K2, and CRAF. These combinations have clear mechanistic rationales; for example, MET inhibition overcomes signaling induced by overexpression of the MET ligand HGF, as others and we have observed (18, 35). Similarly, inhibition of SHP2 is consistent with blocking RTK-mediated signaling that may compensate for inhibition of BRAF, through relief of negative feedback (36, 37). Furthermore, inhibition of SHP2 may have wider use than inhibition of specific RTks, as multiple RTks are known to depend upon SHP2 to mediate mitogenic signaling and have been implicated in resistance to BRAF inhibition (e.g., EGFR, WIDR, SW1417, and LOXIMVI cells; refs. 4, 22, 38) and MET (RKO cells; refs. 18, 35, 39). However, more potent and selective inhibitors of the SHP2 phosphatase are needed before this strategy can be tested clinically. We also identified SHOC2, a protein that mediates the activation of CRAF by RAS, as a candidate target to block RTK- or RAS-driven resistance to BRAF inhibition (40, 41). Targeting protein–protein interactions with small molecules is technically challenging but may be an alternative to targeting specific RAS mutants directly as recently described (42). Interestingly, our data also show that loss of CRAF sensitizes cells to inhibition of BRAF, consistent with CRAF being activated by selective RAF inhibitors either through relief of negative feedback mechanisms, so-called “paradoxical activation” or relief of auto-inhibition (26, 36, 43). Notably, RKO cells have multiple loss-of-function mutations in NF1 and have activated MET due to high expression of the MET ligand HGF, which could cooperate to increase RAS activation and promote activation of CRAF. Taken together, the data suggest that reactivation of the MAPK pathway in a CRAF-dependent manner plays a major role in the manifestation of resistance to BRAF inhibition. In light of these observations, we targeted the MAPK pathway with the combination of a pan-RAF inhibitor and a MEK inhibitor to fully suppress signaling. Combined treatment with AZD6244 and AZ628 resulted in a profound synergistic inhibition of cell proliferation. In comparison, the combination of AZD6244 and PLX4720 or trametinib and dabrafenib failed to show strong synergy. We hypothesized that the synergistic activity of combinations with AZ628 was due to inhibition of both BRAF and CRAF (7). Pan-RAF inhibitors such as AZ628 reportedly retain potency against CRAF in cells whereas inhibitors such as PLX4720 do not (23). This is because kinase assays conducted at low ATP concentrations in vitro suggest that PLX4720 is equipotent against BRAFV600E and CRAF. However, in the cellular environment with physiologically relevant ATP concentrations of 1 mmol/L, the potency of PLX4720 against CRAF is dramatically reduced because the K_m(app) for ATP is much higher for BRAFV600E than for wild-type CRAF (14, 23). Thus, in cells, the wild-type proteins are relatively resistant to PLX4720 when compared with the...
BRAF\textsuperscript{V600E} mutant. Furthermore, both PLX4720 and AZ628 can promote Raf dimerization, but only PLX4720 causes paradoxical MAPK pathway activation that is only partially reversed by MEK inhibition, consistent with our observation that PLX4720 in combination with AZD6244 displays no synergy and has a limited antiproliferative effect (23, 26). Other mechanisms have recently been described whereby MEK is less susceptible to MEK inhibitors when activated by CRAF than when it is activated by BRAF\textsuperscript{V600E} (44). This may also explain why despite being downstream of both BRAF and CRAF, the MEK inhibitor AZD6244 was only able to reduce ERK1/2 phosphorylation by about 50% when used as a single agent, perhaps also, in part, due to relief of negative feedback (36). Consistent with this, we have previously shown that moderate Ras activation by NF1 loss can confer resistance to AZD6244 in BRAF-mutant melanoma (11). Therefore, AZ628 inhibits BRAF and CRAF, prevents MEK activation, and maintains sensitivity to the MEK inhibitor, resulting in greater pathway inhibition and synergistic effects on proliferation. It should be noted that AZ628, RAF265 and MLN2480 are all type II inhibitors, whereas PLX4720, vemurafenib, and dabrafenib are type I inhibitors. It is conceivable that the inactive confirmation induced by type II inhibitor binding is less favorable for transactivation of Raf dimers, as correct positioning of the DFG motif is required to form the “hydrophobic hydrophobic spine” as found in the active conformation (45, 46). Thus, a reduced ability to transactivate Raf dimers translates into more complete pathway inhibition with type II inhibitors versus type I inhibitors. Overall, our complimentary pharmacologic and genetic approaches support the concept that more effective targeting of wild-type CRAF drives the synergy observed between pan-Raf and MEK inhibitors.

Our data show the strongest synergy in cell lines that were resistant to the single agents and also harbored Ras activating mutations such as KRAS (SW480, SW587, DLD1, HCT116, HCT15) or mutation/activation of upstream regulators of MAPK pathway activation that is only partially reversed by MEK inhibitors (49, 53, 54), it will be important to do so in combination with MEK inhibitors to see if such treatment is well tolerated and whether it yields the striking responses described herein.

**Disclosure of Potential Conflicts of Interest**

L.A. Garraway reports receiving a commercial research grant from Novartis, has ownership interest (including patents) in Foundation Medicine, and is a consultant/advisory Board member of Novartis, Foundation Medicine, and Boehringer Ingelheim. No potential conflicts of interest were disclosed by the other authors.

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**Grant Support**

This study was supported by grants to L.A. Garraway from the National Cancer Institute (5P50CA127003-05) STAR Cancer Consortium (14-A444), the NIH New Innovator Award (DP2OD002750), and the Melanoma Research Alliance.

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Received February 12, 2015; revised August 5, 2015; accepted August 30, 2015; published OnlineFirst September 8, 2015.

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Mol Cancer Ther; 14(12) December 2015

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2710 Mol Cancer Ther; 14(12) December 2015 Molecular Cancer Therapeutics


Synergy between Pan-RAF and MEK Inhibition


Molecular Cancer Therapeutics

Combined Pan-RAF and MEK Inhibition Overcomes Multiple Resistance Mechanisms to Selective RAF Inhibitors

Steven R. Whittaker, Glenn S. Cowley, Steve Wagner, et al.

Mol Cancer Ther 2015;14:2700-2711. Published OnlineFirst September 8, 2015.

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