Cancer Therapy: Preclinical

Resistance to BRAF Inhibition in BRAF-Mutant Colon Cancer Can Be Overcome with PI3K Inhibition or Demethylating Agents


Abstract

Purpose: Vemurafenib, a selective inhibitor of BRAFV600, has shown significant activity in BRAFV600 melanoma but not in less than 10% of metastatic BRAFV600 colorectal cancers (CRC), suggesting that studies of the unique hypermethylated phenotype and concurrent oncogenic activation of BRAFmut CRC may provide combinatorial strategies.

Experimental Design: We conducted comparative proteomic analysis of BRAFV600E melanoma and CRC cell lines, followed by correlation of phosphoinositide 3-kinase (PI3K) pathway activation and sensitivity to the vemurafenib analogue PLX4720. Pharmacologic inhibitors and siRNA were used in combination with PLX4720 to inhibit PI3K and methyltransferase in cell lines and murine models.

Results: Compared with melanoma, CRC lines show higher levels of PI3K/AKT pathway activation. CRC cell lines with mutations in PTEN or PIK3CA were less sensitive to growth inhibition by PLX4720 (P = 0.03), and knockdown of PTEN expression in sensitive CRC cells reduced growth inhibition by the drug. Combined treatment of PLX4720 with PI3K inhibitors caused synergistic growth inhibition in BRAF-mutant CRC cells with both primary and secondary resistance. In addition, methyltransferase inhibition was synergistic with PLX4720 and decreased AKT activation. In vivo, PLX4720 combined with either inhibitors of AKT or methyltransferase showed greater tumor growth inhibition than PLX4720 alone. Clones with acquired resistance to PLX4720 in vitro showed PI3K/AKT activation with EGF receptor (EGFR) or KRAS amplification.

Conclusions: We show that activation of the PI3K/AKT pathway is a mechanism of both innate and acquired resistance to BRAF inhibitors in BRAFV600E CRC and suggest combinatorial approaches to improve outcomes in this poor prognosis subset of patients. Clin Cancer Res; 19(3); 657–67. ©2012 AACR.

Introduction

Outcomes in patients with colorectal cancer (CRC) have improved over the last decade, but this benefit has not extended to all subtypes of this disease (1). Less than 10% of patients with metastatic CRCs have tumors with a point mutation in BRAF, a component of the RAF/MEK/ERK signaling pathway (2, 3). Similar to other cancers, more than 95% of the BRAF mutations in CRC affect the V600 position of the protein, resulting in constitutive RAF/MEK/ERK pathway activation (4, 5).

BRAF-mutant CRC is associated with hypermethylation of CpG islands and minimal chromosomal instability, which is molecularly distinct from the traditional model of adenoma–carcinoma progression associated with loss of APC, KRAS, p53, and chromosomal instability (6–8). Patients with metastatic CRC with a BRAF mutation have a very poor prognosis, with median survival of only 10 months, as compared with 35 months for those with a wild-type BRAF (3, 9). Thus, in these patients, there is a critical need for more effective therapies.

Vemurafenib (PLX4032, Plexxikon/Roche) is a potent and selective inhibitor of the V600 mutant form of the BRAF protein. Vemurafenib, and its structural analogue PLX4720, has an IC50 of 31 nmol/L for the kinase activity of the BRAF

www.aacrjournals.org

Published OnlineFirst December 18, 2012; DOI: 10.1158/1078-0432.CCR-11-1446
Translational Relevance
Activating mutations in BRAF are present in several tumor types, including melanoma and colorectal cancer (CRC). Vemurafenib (PLX4032), a selective inhibitor of BRAF, has shown notable clinical benefit in patients with BRAFmut melanoma but had substantially less benefit in BRAFmut CRC. In this study, we show that the phosphoinositide 3-kinase (PI3K)/AKT pathway is activated to a greater extent in BRAFmut CRC tumors than in melanoma. We show that PI3K/AKT pathway activation results in de novo and acquired resistance to BRAF inhibition. This activation occurs through PIK3CA mutation or PTEN loss and is associated with the inherent CpG island phenotype associated with BRAFmut CRC through epigenetic silencing. We show that PI3K/AKT inhibitors or epigenetic therapy are synergistic with a BRAF inhibitor. These results provide rational therapeutic strategies for clinical studies in this poor prognosis subtype of CRC.

protein with the V600E mutation, which is more than 10-fold lower than the IC_{50} for the wild-type BRAF protein (10). Vemurafenib achieved a response rate of 48% to 67% in the BRAF^{V600E} melanoma (11, 12). However, vemurafenib achieved a clinical response in only 1 of 21 patients with BRAF^{V600E} metastatic CRCs, suggesting important differences in the biology of BRAFmut CRCs in different cancer types (13).

To improve outcomes in patients with CRCs with a BRAF mutation, there is a critical need to better understand the mechanisms of resistance to BRAF inhibitors. Several studies have investigated mechanisms of resistance to selective BRAF inhibitors in melanoma (14–16). BRAF inhibition resistance has been shown to be mediated, in part, by EGF receptor (EGFR) in 2 recent publications, showing the importance of studies in CRC models (17, 18). We use comparative proteomic analysis of human melanoma and CRC cell lines and functional testing of BRAF-mutant CRC lines with primary (innate) and secondary (acquired) resistance to BRAF inhibition in vitro and in vivo to extend this line of research in colon cancer models. We hypothesized that the inherent CpG island hypermethylator phenotype and concurrent oncogenic activation of alternate pathways may impact sensitivity to BRAF inhibition. Our results reveal a role for the phosphoinositide 3-kinase (PI3K)/AKT pathway in resistance to BRAF inhibition in CRCs. In addition, we have identified novel combinatorial approaches to overcome resistance to BRAF inhibitors that are candidates for further testing.

Materials and Methods
Cell lines and inhibitors
A panel of BRAFmut CRCs and melanoma cell lines was used for comparative studies (Supplementary Table S1 for lines and conditions). The PLX4720-resistant Colo205 clone was a generous gift from Plexxikon and was grown in 5 μmol/L PLX4720. To establish the PLX4720-resistant HT29 clone, HT29 cells were continuously exposed to stepwise increasing concentrations of PLX4720: 0.1, 0.5, 1, 2, and 4 μmol/L until the surviving cells reached 90% confluency. One resistant clone was isolated from viable cells growing in medium with ≥4 μmol/L PLX4720 after 2-month selection and characterized by comparing the PLX4720 IC_{50} values to that of parental cells. PLX4720 (Plexxikon), LY294002 (EMD Chemicals), 5-azacytidine (Sigma-Aldrich), and GDC0941 (Selleck Chemicals) were dissolved in dimethyl sulfoxide as 10, 40,10, and 10 mmol/L stocks, respectively.

Proliferation and colony formation assays
Cells were seeded in a 96-well plate at a density of 2,000, allowed to attach for 24 hours at 37°C, and then treated with inhibitors for 72 hours, with quantification using MTS solution (Promega). For colony formation assay, cells were seeded in a 24-well plate at a density of 300, allowed to attach for 24 hours at 37°C, and then treated with PLX4720. The cells were maintained at 37°C for 2 weeks. Colonies of cells were then fixed with cold methanol for 25 minutes and stained with 1% crystal violet with quantification by ImageJ (NIH). Cell-cycle analysis was conducted within 1 hour after labeling with propidium iodide (EMD Chemical) on the FACS Canto machine (BD Biosciences) with BD FACS-Diva software.

Protein analysis
For reverse-phase protein array analysis (RPPA), proteins were isolated as described previously (19–21). Samples were analyzed for the expression of protein markers using RPPA-validated antibodies after correction of phospho-specific antibodies for expression levels of total protein and correlation for global protein content (21). Phospho-AKT (S473), AKT, phospho-p42/44 ERK (T202/Y204), p42/44 ERK1/2, phospho-GSK3β (T38, S42), phospho-p70S6K (T389), EGFR, PARP, and PTEN antibodies were purchased for immuno-blotting from Cell Signaling Technology. Anti-IGFBP-3 was from Santa Cruz Biotechnology. Anti-vinculin was obtained from Sigma-Aldrich.

Gene silencing by siRNA
Cells were seeded in 6-well plates at a density of 10^5 cells/well. The following day, cells were transfected with 50 nmol/L PTEN-targeting siRNA (L-003023-00-0005, Thermo Fisher Scientific) or nontargeting siRNA using DharmaFECT 1 transfection reagent, according to the manufacturer’s instructions.

Copy number and gene expression analysis
Parental and resistant cell lines were analyzed for copy number by single-nucleotide polymorphism (SNP) tiling (Affymetrix OncoScan) after DNA extraction as previously described (22–24). Total RNA was extracted with the Qiagen RNeasy Kit. Biotin-labeled cRNA samples for hybridization were prepared using Affymetrix followed by amplification, hybridization to Affymetrix HGU133plus2.
and imaging on GeneChip Scanner 3000 (Affymetrix), with data normalized by Robust Multi-Array Average (RMA) before analysis. Data are deposited in NCBI’s Gene Expression Omnibus with accession number GSE34299. Confirmatory FISH was conducted for EGFR as previously described (25, 26).

Animal studies

The HT29 or LS411N cells were injected subcutaneously (1 million per injection) in nude mice. In a variation of previous methyltransferase inhibitor murine studies, cells were pretreated in vitro for 4 weeks with 1 μmol/L 5-azacytidine (preprimed) or PBS before injection to accommodate the delayed epigenetics effects of methyltransferase inhibitors (27). When the tumor became visible, the mice were randomly grouped for treatment. MK2206 was dosed at 120 mg/kg per os 3 times per week. The irradiated PLX4720 diet was purchased from Scientific Diets at a concentration of 417 mg/kg. 5-Azacytidine was dosed at 0.8 mg/kg intraperitoneally 3 times per week.

Statistical analyses

Densitometry and colony counting were conducted using ImageJ v1.45s (NIH). Comparison of the relative sensitivity of the cell lines to PLX4720 on the basis of genotype was conducted using the Wilcoxon signed-rank test. Unpaired t tests were used for comparisons of cytotoxicity between conditions or cell lines. IC50 values, combination indices (CI; using the synergy methodology of Chou and Talalay), and IC90 isobolograms were calculated using CalcuSyn v2.0 (BioSoft; ref. 28).

Results

Comparison of levels of signaling proteins in CRC or melanoma cell lines

We first sought to determine whether the clinical efficacy of vemurafenib in patients with BRAF-mutant melanoma and CRCs is due to baseline differences in signaling pathways. To test this hypothesis, we compared the expression of a set of proteins and phospho-proteins that are components of various cell signaling pathways in a panel of human melanoma and CRC cell lines (Supplementary Table S1) by RPPA analysis. This analysis showed that BRAFmut CRC cell lines have higher activation of several proteins in the PI3K/AKT pathway, including p-AKT, p-P70S6K, and increased expression of eIF4E (Fig. 1A and B; Supplementary Table S3). This result is consistent with increased activation of the PI3K/AKT pathway in CRC cell lines as compared with melanoma cell lines. Conversely, CRC cell lines displayed lower levels of activation of the MEK pathway than BRAFmut melanoma lines, as indicated by lower levels of phosphorylated MEK, ERK, and RSK and lower levels of cyclin-D1 and Myc. Notably, HER2 and EGFR were expressed at higher levels in CRC than in melanoma.

CRC cell lines with concurrent PIK3CA mutation or PTEN loss are more resistant to BRAF inhibition

To evaluate the role of PI3K/AKT pathway activation on sensitivity to BRAF inhibition, an extended panel of BRAFmut CRC cell lines characterized for PIK3CA and PTEN mutation status were tested for PLX4720 sensitivity (Fig. 2A). All of the BRAFmut CRC cell lines underwent

![Figure 1](image-url)
varying degrees of growth inhibition in response to PLX4720 treatment compared with untreated controls, whereas only minimal effects were observed in BRAFwt CRC cell lines. The extent of growth inhibition varied considerably across the cell line panel. Colo201, Colo205, and VACO-5 displayed a reduction in the number of viable cells after treatment. CRC cell lines with PI3KCA and PTEN activating mutations in PIK3CA and/or PTEN null/PIK3CA were more resistant to growth inhibition by PLX4720 than those cell lines without these alterations (P = 0.03 by Mann-Whitney signed-rank test). CRC cell lines without BRAF mutation are insensitive to this PLX4720, as shown in light gray. Melanoma cell lines are shown for reference. B, HT29, Colo205, and Vac05 cells were treated with transfection reagent alone (control) or together with PTEN siRNA (50 nmol/L) or nontarget siRNA for 48 hours and then lysed for immunoblotting. C, cells were treated with PTEN siRNA for 48 hours and then treated with 0.25 μmol/L PLX4720 for 2 weeks. HT29 contains an atypical and poorly characterized mutation (P449T) outside of the hotspots in the kinase and helical domains.

PI3K/AKT pathway modulates sensitivity of CRC cells to BRAF inhibitors.

Dual inhibition of mutant BRAF and PI3K synergistically inhibits growth of colon cancer cells

To further examine the impact of PI3K/AKT signaling on sensitivity to BRAF inhibitors, growth and proliferation of CRC cells were evaluated after treatment with PLX4720 and the PI3K inhibitors LY294002 or GDC0941. As shown in Fig. 3A, the combinations significantly reduced cell viability compared with single-agent treatment. In formal synergy studies, the combination of PLX4720 and LY294002 showed synergistic effects in inhibiting cell growth (CI = 0.34 and 0.62 at the IC50 for the combination in HT29 and RKO cells). A similar finding was observed with the combination of PLX4720 and GDC0941, which recapitulated the synergistic effects (CI = 0.42 in HT29). An isobologram for these combinations is shown in Fig. 3B. When used as single agents, PLX4720 and LY294002 caused accumulation of cells in the G1 phase of cell cycle. Cotreatment resulted in more pronounced G1 arrest than either
agent alone, indicating an additive effect of the combination on growth inhibition (\(P<0.05\) for each cell line, Fig. 3C). Combination treatment with PLX4720 and LY294002 also induced apoptosis in Colo205 and RKO cells as denoted by an increase in the sub-G1 population (Fig. 3C, Supplementary Fig. S3). Consistent with the flow cytometric data, cleaved PARP was detected in Colo205 and RKO cells but not in HT29 cells (Data not shown). To investigate whether the combination of PLX4720 and Akt inhibitor MK2206 has synergistic effects in vivo, we used the LS411N cell line, which is resistant to PLX4720 alone. Greater growth inhibition was seen with dual administration of PLX4720 and the Akt inhibitor, MK2206, than with the single administration of PLX4720 (\(P<0.01\)) or MK2206 (\(P<0.05\); Fig. 3D).

**Effect of PLX4720 and LY294002 on MAPK and AKT signaling**

We next investigated the effect of the combination therapy on signaling via the RAF/MEK and PI3K/AKT pathways in CRC cells. As expected, PLX4720 fully blocked ERK phosphorylation in HT29 and Colo205 cells, whereas LY294002 had minimal inhibitory effect (Fig. 4). After PLX4720 treatment, pAKT was increased in all 3 cell lines (range of 25% to 90% increase by densitometry) but decreased from control after the addition of the PI3K inhibitor. Intriguingly, RKO had only partial inhibition of pERK at 1 \(\mu\)mol/L (60% by densitometry); however, dual PI3K and BRAF inhibition reduced pERK further (90% inhibition). Similarly, more robust inhibition of pGSK3\(\beta\) was observed with dual inhibition than with single-agent LY294002, further indication of the interaction of the 2 pathways.

**The DNA methyltransferase inhibitor 5-azacytidine sensitizes colon cancer cells to BRAF inhibition**

BRAF\(^{mut}\) colon cancer is strongly associated with the CpG island methylator phenotype (CIMP), which results in epigenetic silencing of multiple genes. To assess whether epigenetic effects were associated with innate resistance to
PLX4720, RKO, Colo205, LS411N, and HT29 were treated with the DNA methyltransferase inhibitor 5-azacytidine in combination with PLX4720. As shown in Fig. 5A, 5-azacytidine had minimal effect on colon cancer cell growth in vitro at the doses used. However, combination therapy resulted in greater inhibition than PLX4720 alone in all evaluated cell lines. Formal synergy analysis confirmed that the combination had synergistic effects (CI, 0.3–0.8 across the cell lines; Fig. 5B). In an HT29 xenograft, enhanced tumor growth inhibition was seen with 5-azacytidine (pre-primed in vitro tumor growth inhibition was seen with 5-azacytidine (pre-primed in vitro combined with PLX4720 administration in vivo) compared with either agent alone (P < 0.05 for both comparisons).

Given the relationship between AKT activation and BRAF inhibitor sensitivity, we evaluated the impact of 5-azacytidine therapy on pAKT in HT29 and LS411N cell lines. In both the cell lines, treatment with 1 or 2 µmol/L of 5-azacytidine for 72 hours resulted in decreased pAKT expression (Fig. 5C). As both PTEN and IGFBP3 have been reported to be under epigenetic regulation and are known modifiers of AKT signaling, we evaluated their expression in these 2 cell lines (29, 30). In HT29, PTEN was reexpressed in a dose-dependent manner (Fig. 5C). LS411N has a known truncating mutation in PTEN; no PTEN was detected in these cells. Conversely, IGFBP3 was increased in LS411N with 5-azacytidine treatment but was not detected in HT29. To assess whether the increase in PTEN expression may be an important mechanism of PLX4720 and 5-azacytidine synergy in HT29 cells, the combination therapy was repeated in HT29 cells first treated with siRNA to PTEN as previously described. Silencing PTEN was not sufficient to fully abolish the synergy seen with the combination, suggesting that additional epigenetically regulated proteins may also account for the observed synergy (Supplementary Fig. S4).

Acquired resistance to PLX4720 is associated with increased expression of activated AKT in human CRC cell lines

In the clinical trial of vemurafenib in patients with CRCs, minor responses and mixed responses were seen in almost half the patients, suggesting that resistance may not be solely due to inherent factors, but may also be due to rapidly acquired alterations. Therefore, analyses of the potential mechanisms of acquired resistance were undertaken by developing resistant clones of Colo205 and HT29 with IC_{50} values 5- to 10-fold above those of the parental lines (Fig. 6A). Unlike the complete inhibition of pERK with PLX4720 in the parental lines, retreatment of the resistant clones resulted in partial inhibition of pERK (Fig. 6B), consistent with previous findings with BRAF inhibitors in melanoma and MEK inhibitors in colon cancer (31). There were no acquired mutations in PIK3CA, NRAS, BRAF, or MEK1 or change in IGF1R, PDGFR, MET, or PTEN gene expression. Copy number analyses showed a gain in copy number in Chr7 (cytoband p11.2) in the HT29 cells and gain of a smaller region in Chr12 in Colo205 (p11.1-p12.1). Genes of potential relevance in these amplified regions include EGFR and PHKG1, a serine/threonine kinase, in 7p11.2, and in p11.1-p12.1, KRAS and RASSF8, a member of the Ras association domain family of tumor suppressors (Fig. 6C; Supplementary Fig. S5). EGFR amplification in HT29RC was confirmed by FISH (Supplementary Fig. S6). The increased copy number of EGFR and KRAS translated into increased gene expression in the respective cell lines (Fig. 6C) but not for RASSF8 and PHKG1 or other genes previously shown to affect BRAF inhibitor resistance, including the genes encoding for MEK1/2 (Supplementary Fig. S7). Colo205 also showed increased EGFR gene expression and protein levels independent from changes at the DNA level (Fig. 6D). Elevations in both phosphorylated

![Figure 4. Effect of PLX4720 and LY294002 on expression of signal proteins. HT29, Colo205, and RKO cells were treated for 2 hours with PLX4720, LY294002, or their combination at indicated concentrations. Cell lysates were subjected to Western blot analysis with antibodies against phosphorylated or total forms of AKT or ERK1/2, and antibodies against p-F70S6K, pGSK3β, or vinculin.](image-url)
ERK and total ERK were seen compared with parental cells (1.5- to 2.5-fold increases), but the ratio of pERK to ERK by densitometry did not change. Notably, expression of activated AKT increased in these chemoresistant clones (2.1- and 2.6-fold for HT29 and Colo205, respectively). Knockdown of EGFR in the resistant clone resulted in sensitivity to PLX4720 similar to that seen in the parental cell line (Supplementary Fig. S8).

Discussion

The identification of BRAF mutations in melanoma and CRCs, among other cancers, has led to rapid clinical testing of BRAF inhibitors, including the mutant BRAF isoform inhibitor vemurafenib. Unfortunately, the high response rates seen in melanoma have not extended to BRAFmut CRCs. Here, we have studied mechanisms that might account for lack of response to BRAF inhibitors in CRCs, with the goal of identifying rational clinical combinations with BRAF inhibitors.

Despite the fact that BRAFmut colon cancers and melanomas share the similar V600 mutation patterns, our study showed important differences in pathway activation and protein expression, including higher EGFR expression and AKT signaling in colon cancer cell lines than in melanoma cell lines. While there was not a correlation between EGFR expression and BRAF inhibitor sensitivity in the limited cell line panel, this type of analysis may be limited by the small number of BRAF-mutant cell lines available. However, cell lines with PIK3CA mutations and PTEN loss were more resistant to the BRAF inhibitors. Colo205, as a cell line lacking PIK3CA mutation or PTEN loss, appears to be as sensitive to BRAF inhibition as melanoma cell lines and may be an appropriate model for studying the rare subset of vemurafenib-sensitive CRCs. This result is consistent with previous work with MEK inhibitors, in which a correlation between AKT phosphorylation status and MEK inhibitor sensitivity has been seen in melanoma, further supported by knockdown of PTEN, leading to Akt activation and conferring resistance to MEK inhibitors (32, 33).

While the frequency of PTEN loss and/or PIK3CA mutations was high in the BRAFmut colon cancer cell lines, this phenotype is not seen as frequently in patient samples (34, 35). This likely represents a bias for tumors able to be established as cell lines, as seen in the overrepresentation of BRAFmut colon cancers and melanomas.
PIK3CA and KRAS dual mutations in colon cancer cell lines in contrast to primary tumors (36). A review of the COSMIC database for BRAF mut colon adenocarcinoma samples annotated for PIK3CA and PTEN shows co-mutation rates of 13% (25 of 194) and 22% (8 of 37), respectively, consistent with other published series (35, 37). However, PIK3CA and PTEN mutations represent only a subset of mechanisms whereby AKT pathway can be activated. Epigenetic events, including silencing of PTEN by promoter hypermethylation and overexpression and/or high basal activity of receptor tyrosine kinases such as EGFR, also increase activation of the PI3K pathway (36).

Combination treatment with the BRAF inhibitor PLX4720 and PI3K/AKT inhibitors showed synergistic growth inhibition in the BRAF-mutant CRC cell lines, including an in vivo study with a cell line resistant to single-agent BRAF inhibition. Yang and colleagues presented a murine study of the combination of vemurafenib and the AKT inhibitor MK2206 in the RKO cell line (38). Similarly, Oikonomou and colleagues showed that pharmacologic suppression of the PI3K pathway further enhances the synergistic effect between TRAIL and PLX4720 in the same cell line (39). Given the selectivity of vemurafenib for the mutant form of BRAF, the combination of vemurafenib with a PI3K/AKT pathway inhibitor is anticipated to be tolerable in patients and is worthy of further development. While the choice of EGFR, AKT, pan-PI3K, or isoform-selective PI3K inhibitors to combine with BRAF inhibition would ideally be based, in part, on the individual mechanisms of PI3K pathway activation in BRAFmut CRCs.
(e.g., PI3K-β inhibitors in the setting of PTEN loss), this will need to be balanced by the practical limitations of study designs for this small population. In our studies, single-agent PLX4720 treatment failed to induce significant apoptosis in BRAF-mutant colorectal cell lines, in contrast to that seen in melanoma cell lines (10). One likely explanation for this disparity is the higher basal activation status of the prosurvival PI3K pathway in CRC cells. Consistent with this possibility, PTEN-null and AKT-activated melanoma cell lines undergo lower levels of apoptosis induction in response to PLX4720 treatment, supporting the concept that constitutive activation of the PI3K signaling pathways confers resistance mechanism to mutant BRAF inhibition (33, 40). While we showed that combination therapy with PI3K inhibitors was able to induce apoptosis in some cell lines, the level of apoptosis induction remains below that induced by single-agent BRAF inhibition in melanoma (10, 21). This result may be due to alternate survival signaling independent of MAPK and AKT pathways, for example, Src family signaling (41, 42). It is therefore possible that clinical trials of PI3K and BRAF inhibitors in colon cancer should be designed to detect prolonged stabilization of disease rather than objective responses.

CpG island promoter hypermethylation is present in nearly all BRAFmut CRCs, and the presence of BRAF mutation is used to confirm promoter hypermethylation of hMLH1 as a causative factor for nonfamilial microsatellite instability (43). The use of 5-azacytidine, a methyltransferase inhibitor currently under evaluation in CRCs, was shown to inhibit phosphorylation of AKT in a dose- and time-dependent manner and synergize with BRAF inhibition. Several genes in the PI3K/AKT pathway are commonly cited as being under epigenetic control, including IGFBP3, PIK3CG, and PTEN (29, 30, 44, 45). We show that potential mediators of the AKT inhibition include PTEN and IGFBP3, although additional effectors are likely given the pleiotrophic effects of methyltransferase inhibition. PTEN loss in melanoma is one of the potential cooperative events in BRAF mutant–associated transformation in melanoma, implying that a similar mechanism may be relevant in CRCs and driven by hypermethylation (46). These findings provide a possible colon cancer–specific approach for combination therapy. However, several months of demethylator therapy is usually required to fully inhibit aberrant methylation and such duration of therapy may be difficult to achieve in this rapidly progressive subset of metastatic CRCs. In our in vivo study, the pretreatment with 5-azacytidine in vitro significantly increased the efficacy with subsequent BRAF inhibition. Further efforts to optimize the epigenetic component of the therapy will be necessary before translation to the clinic (47).

In the clinical trial of vemurafenib in patients with CRCs, many patients appeared to derive transient and mixed benefit, manifesting as tumors with both regressing and progressing tumors. These findings suggest that resistance may not be solely due to inherent sensitivity but may also be due to rapidly acquired resistance factors. Hence, the acquired resistance cell lines may still be relevant to the tumor biology seen in patients with colon cancer. As described previously in melanoma, acquired resistance to BRAF inhibition is commonly associated with restored ERK activation through multiple mechanisms, including overexpression or use of alternate MAPK family members or RAF isoforms (14–16). Consistent with these previous findings, we observed partial restoration of pERK in PLX4720 resistant colon cancer cell lines, which has been attributed, in part, to overexpression of EGFR in recently publications (17, 18). Consistent with this, EGFR expression was increased in both resistant cell lines, through both copy number changes and transcriptional/translational mechanisms. Intriguingly, our model shows an apparent predilection to amplification events, instead of acquired mutations, suggesting that mechanisms of resistance may vary by tumor type, even in the setting of similar oncogenes and selective pressure. KRAS amplification has also been seen in cell lines after selective pressure from EGFR inhibition (48). A recent publication similarly showed the role of receptor tyrosine kinases in activating PI3K and inhibiting apoptosis despite MEK inhibition in KRASmut CRCs (49). Given these findings and the relevance of EGFR signaling in CRCs, dual EGFR and BRAF inhibition may represent an additional therapeutic possibility and an opportunity for further preclinical study, as suggested by others (17, 18).

In summary, this work highlights important differences between CRC and melanoma and implicates the PI3K/AKT pathway in innate and acquired resistance to BRAF inhibition. Further, we showed that a demethylating agent inhibits the PI3K pathway and also synergizes with PLX4070 in BRAF-mutant CRC cell lines. Targeting EGFR and PI3K are viable combination strategies worthy of immediate clinical implementation in doublet or triplet regimens, whereas combinations with DNA methylation inhibition will require additional optimization of schedule to maximize likelihood of success in the clinic. In addition, the differential, clinical, and biologic response of BRAF-mutant CRCs and melanoma to BRAF inhibitors has provided an important warning about treatment selection done solely on the basis of gene mutations without regard to the broader molecular context of the tumor.

Disclosure of Potential Conflicts of Interest
J.M. Mariadason has commercial research support from Roche Pharmaceuticals. G.B. Mills has commercial research grants from AstraZeneca, Celgene, Cytogenics, Eiselis/Sanoﬁ, GSK, Roche, and Wyeth/Pfizer/Puma; has ownership interest (including patents) in Catena Pharm, PIV Ventures, and Spindle Top Ventures; and is a consultant/advisory board member of AstraZeneca, Catena Pharm, Tau Therapeutics, Critical Outcome Tech, Daichi Pharm, Targeted Molecular Diagnostics LLC, Foundation Medicine, Han AllBio Korea, Komen Foundation, Novartis, and Symphogen. J. Desai has a commercial research grant from Roche. M.A. Davies has commercial research grants from GlaxoSmithKline, Genentech, Merck, AstraZeneca, and Oncolyteigen and is a consultant/advisory board member of GlaxoSmithKline, Novartis, and Genentech. S. Kopetz has a commercial research grant from Roche and is a consultant/advisory board member of Roche and GSK. No potential conﬂicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: G. Powis, G. E. Gallick, S. Kopetz
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.M. Mariadason, C.C. Tsao, R. Lemos, Jr, F. Dayani, Z.-Q. Jiang, I.I. Wistuba, G. Powis, J. Desai, M.A. Davies, S. Kopetz

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Mao, F. Tian, J.M. Mariadason, C.C. Tsao, F. Dayani, G.B. Mills, G.E. Gallicchi, S. Kopetz

Writing, review, and/or revision of the manuscript: M. Mao, F. Tian, J.M. Mariadason, C.C. Tsao, F. Dayani, W.V. Borrman, G. Bollag, C.B. Mills, G. Powis, J. Desai, G.E. Gallicchi, M.A. Davies, S. Kopetz

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F. Tian, C.C. Tsao, Z.-Q. Jiang, X.M. Tang, S. Kopetz

Study supervision: S. Kopetz

References

33. Paraiso KH, Xiang Y, Rebecca VW, Abel EV, Chen A, Munko AC, et al. PTEN loss confers BRAF inhibitor resistance to melanoma cells

Grant Support

The study was supported by CA136980 (S. Kopetz), CA172670 (S. Kopetz and G. Powis), and the Cancer Center Support Grant CA16672, including the Translational Chemistry Core.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 10, 2011; revised November 13, 2012; accepted November 21, 2012; published OnlineFirst December 18, 2012.

Mao et al.
Resistance to BRAF Inhibition in BRAF-Mutant Colon Cancer Can Be Overcome with PI3K Inhibition or Demethylating Agents

Muling Mao, Feng Tian, John M. Mariadason, et al.


Updated version
Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-11-1446

Supplementary Material
Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2012/12/18/1078-0432.CCR-11-1446.DC1

Cited articles
This article cites 49 articles, 24 of which you can access for free at: http://clincancerres.aacrjournals.org/content/19/3/657.full.html#ref-list-1

Citing articles
This article has been cited by 20 HighWire-hosted articles. Access the articles at: /content/19/3/657.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.