

Resistance to Selective BRAF Inhibition Can Be Mediated by Modest Upstream Pathway Activation

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Abstract

A high percentage of patients with BRAF^{V600E} mutant melanomas respond to the selective RAF inhibitor vemurafenib (RG7204, PLX4032) but resistance eventually emerges. To better understand the mechanisms of resistance, we used chronic selection to establish BRAF^{V600E} melanoma clones with acquired resistance to vemurafenib. These clones retained the V600E mutation and no second-site mutations were identified in the BRAF coding sequence. Further characterization showed that vemurafenib was not able to inhibit extracellular signal-regulated kinase phosphorylation, suggesting pathway reactivation. Importantly, resistance also correlated with increased levels of RAS-GTP, and sequencing of RAS genes revealed a rare activating mutation in KRAS, resulting in a K117N change in the KRAS protein. Elevated levels of CRAF and phosphorylated AKT were also observed. In addition, combination treatment with vemurafenib and either a MAP/ERK kinase (MEK) inhibitor or an AKT inhibitor synergistically inhibited proliferation of resistant cells. These findings suggest that resistance to BRAF^{V600E} inhibition could occur through several mechanisms, including elevated RAS-GTP levels and increased levels of AKT phosphorylation. Together, our data implicate reactivation of the RAS/RAF pathway by upstream signaling activation as a key mechanism of acquired resistance to vemurafenib, in support of clinical studies in which combination therapy with other targeted agents are being strategized to combat resistance. *Cancer Res*; 72(4): 969–78. ©2011 AACR.

Introduction

The *BRAF* oncogene is mutated in approximately 8% of all human tumors; however, the prevalence is much higher in melanoma, where a mutation is documented in more than 50% of all melanoma (1). Other tumor types with a substantial incidence of mutated BRAF include papillary thyroid (30%–70%), ovarian (15%–30%), and colorectal cancers (5%–20%; ref. 1). In more than 90% of cases, a single substitution of glutamic acid for valine in the BRAF kinase domain (V600E) is present and leads to RAS-independent constitutive activation of BRAF and downstream signal transduction in the mitogen-activated protein (MAP) kinase pathway (1–3). In melanoma cells BRAF^{V600E} causes deregulated proliferation by overcom-

ing the G₁ restriction point and causing cyclin D1 production in mid-G₁ (4). Notably, acquisition of the BRAF^{V600E} mutation seems to be an early event in melanoma development with a high percentage of premalignant melanocytic nevi also found to harbor the mutation (5).

Vemurafenib (RG7204, PLX4032) is an orally available, small-molecule inhibitor designed to specifically inhibit signaling from the *BRAF* oncogene (6). In *in vivo* and *in vitro* melanoma models, vemurafenib inhibits phosphorylation of MAP/ERK kinase (MEK) and extracellular signal-regulated kinase (ERK), leading to G₁ phase cell-cycle arrest and apoptosis (7–12). Phase I clinical studies have shown that vemurafenib treatment caused significant tumor regressions in a majority of metastatic melanoma patients with mutated BRAF (13). Importantly, tumor regressions were highly dependent on pathway blockade, with a high threshold required (6). For example, 60% inhibition was insufficient for tumor regression, whereas 90% inhibition often correlated with robust regression. Therefore, near the threshold relatively modest differences in pathway blockade can have large consequences on tumor response. Accordingly, tumor regrowth was frequently observed following initial tumor regression, presumably due to acquired resistance to vemurafenib. In the present study, we elucidate potential mechanisms underlying acquired resistance. Melanoma cell lines with acquired resistance to vemurafenib were established to model disease relapse associated with clinical resistance to vemurafenib in patients with

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melanoma. This model system was used *in vitro* and *in vivo* to understand molecular mechanisms of disease progression after initial response to vemurafenib (acquired resistance mechanisms) and subsequently to help identify potential combination therapies to prevent or mitigate disease relapse.

Materials and Methods

Cell culture, reagents, and transfection

A375 parental cell line was purchased from American Type Culture Collection (ATCC) and authenticated by exome sequencing (August 2010). All cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% of heat-inactivated FBS (HI-FBS; GIBCO/BRL) and 2 mmol/L L-glutamine (GIBCO/BRL).

Melanoma cell lines with acquired resistance to vemurafenib were generated by propagating parental A375 cells in increasing concentrations of vemurafenib (Hoffmann-La Roche, Inc.) to achieve chronic selection. Six cell lines with increased IC₅₀ values measured by MTT assay were isolated for further characterization. These cells were further propagated in growth medium containing 2.5 μmol/L vemurafenib.

Vemurafenib and RO5068760 were synthesized in house. MK-2206 was purchased from Selleck Chemicals.

A375 cell transfections were carried out 24 hours after seeding cells on 100-mm plates (~30%–40% confluent). The CRAF expression plasmid (OriGene Technologies, Inc.; RC201983) and KRAS wild-type plasmid (Biomx Technology; p1025) were transfected with FuGENE 6 (Roche Diagnostics Corporation) according to the manufacturer's protocol. Scrambled siRNA (ctrl siRNA), CRAF siRNA, and KRAS siRNA (Dharmacon Inc.; ON-TARGET plus Nontargeting Pool D-001810-10-05, ON-TARGET plus SMART pool L-003601-00-0005, NM_002880, and ON-TARGET plus SMART pool L-005069-00-0005, NM_004985, respectively) were transfected with DharmaFECT 1 (Dharmacon) according to the manufacturer's protocol.

Cellular proliferation and *in vitro* combination assays

Cellular proliferation assays were conducted as described previously (11).

In vitro study of the combination of vemurafenib and the MEK inhibitor RO5068760 or the AKT inhibitor was conducted using the procedure outlined earlier, using drug concentrations based on the IC₅₀ value of each drug as a single agent to yield optimal growth inhibition ranging from approximately 10% to 90%. The combined drug treatment maintained constant ratios of the 2 agents which were added simultaneously. Synergism, additive activity, or antagonism was determined by median effect analysis using the combination index (CI) calculated by the CalcuSyn software (Biosoft).

For transfected cells, 500 transfected cells (100 μL volume) were seeded in 96-well black-bottom plates in DMEM supplemented with 10% FBS. Six or 16 hours after seeding of siRNA or expression plasmid transfection, respectively, cells were treated with vemurafenib for 4 or 3 days, respectively, and cellular viability was measured by the CellTiter-Glo Assay (Promega Corp.) according to the manufacturer's instructions.

Tumor xenografts and treatment

For the A375 xenografts (parental and vemurafenib-resistant cell lines), 10 × 10⁶ cells were implanted subcutaneously on the right lateral flank of female SCID-beige mice and treatment was initiated after approximately 7 days. The vemurafenib microprecipitated bulk powder formulation used in clinical trials was described previously (11). Vehicle, vemurafenib, and RO5068760 were dosed orally once daily for 2 weeks.

Efficacy and safety endpoints

As described previously in Yang and colleagues (11).

Western blot analysis and RAS activity assay

The following antibodies were purchased from Cell Signaling Technology: anti-phospho-ERK1/2 (Thr202/Tyr204; #9101), anti-phospho-MEK1/2 (Ser217/221; #9121), anti-MEK1/2 (#9122), anti-cyclin D (#2926), anti-p-AKT (Ser473; #9171), and anticleaved PARP (#9541). Anti-ERK1/2 antibody (06-182) was purchased from Millipore. Anti-β-actin antibody (A5316) and anti-KRAS were purchased from Sigma. Anti-CRAF (BD610152) was purchased from BD Biosciences. Western blot analysis was conducted as described previously (11).

RAS-GTP pull down was carried out according to the manufacturer's protocol (Cytoskeleton Inc.).

Whole exome sequencing

Sequence capture was conducted with Nimblegen SeqCap EZ Human Exome Library SR (V1.2) at Roche Nimblegen according to the manufacturer's protocol. This assay enriches for approximately 35 Mb of coding sequence as annotated in the CCDS and MiRBase databases. SeqCap DNA from each of the 6 resistant and the one parental line was sequenced using the Illumina GAIIX. Each sample was sequenced with 2 lanes of single-end (SE) 75 bp and one lane of paired-end (PE) 2 × 75 bp sequence, by sequencing kits V.4 and generating approximately 10 Gbp of sequence per sample (range: 8.7–10.8 Gbp). Two lanes of 75 bp SE sequence were also generated in parallel for HapMap sample NA12752 to estimate accuracy of genotype calls. Sequence analysis was conducted with Illumina software (RTA base calling, Eland alignment, and CASAVA variant calling), using default parameters; with the exception that for CASAVA the SNP Max Ratio was set to 10 to allow for substantial aneuploidy in the cell lines. To generate a priority list of variants, we required each single-nucleotide polymorphism (SNP) to be present in both SE and PE sequences, to be absent in the parental line but present in 2 or more resistant lines, and to be predicted to be damaging using SIFT annotation or nonsense mutations (for detailed method, please see Supplementary Material).

Results

A375 melanoma cell lines with acquired resistance to vemurafenib show activation of ERK and AKT and increased expression of CRAF

The A375 melanoma cell line is driven by the *BRAF*^{V600E} oncogene and is exquisitely sensitive to proliferation inhibition

by the selective RAF inhibitor vemurafenib (Fig. 1A). To select for cells with acquired resistance, A375 cells were grown in the presence of serially increasing concentrations of vemurafenib.

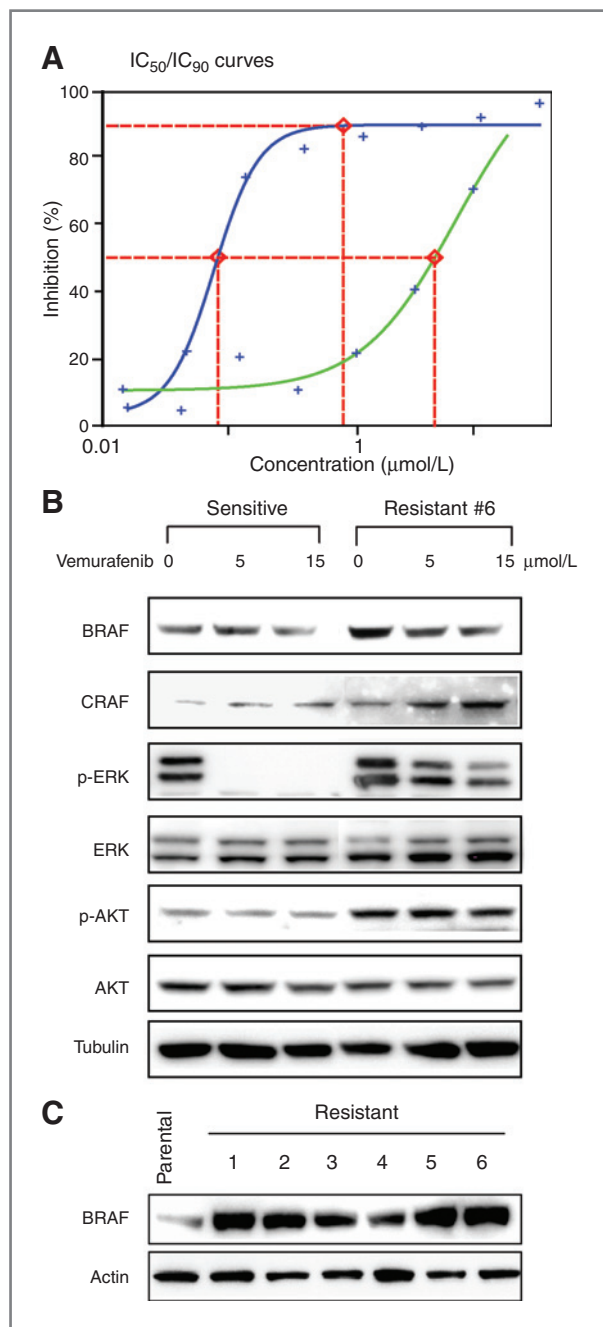


Figure 1. Comparison of the vemurafenib-resistant A375 melanoma cell lines to parental A375 cells. **A**, representative proliferation inhibition curve shows a 90-fold increase in IC_{50} value in vemurafenib-resistant cells (A375R1, green line) compared with parental A375 cells (blue line). **B**, Western blot analysis of the levels of BRAF, CRAF, p-ERK, ERK, p-AKT, and AKT in the vemurafenib-resistant A375 R6 cells and vemurafenib-sensitive parental A375 cells following 2-hour incubation with indicated concentrations of vemurafenib. **C**, Western blot analysis of basal level expression of BRAF protein in A375 parental and resistant cell lines.

At the end of 3 months selection, 6 individual cell lines were isolated from the pool of resistant cells and characterized. As expected, each of these cell lines was highly resistant to the growth inhibitory effects of vemurafenib with IC_{50} values increased by 90- to 120-fold compared with the IC_{50} values observed in the sensitive parental cells (Fig. 1A and Supplementary Table S1). The vemurafenib-resistant cell lines are relatively cross-resistant to the 2 MEK inhibitors tested but not to other targeted agents tested including an AKT inhibitor, a cyclin-dependent kinase (CDK) inhibitor, and a dual PI3K/mTOR inhibitor (Supplementary Table S1). This acquired resistance model system was then used to investigate molecular mechanisms of disease progression after initial response to vemurafenib.

To further characterize the 6 resistant clones, signaling through the RAS/RAF and RAS/PI3K pathways was evaluated. In sharp contrast to the potent inhibition noted in the parental A375 cells at low concentrations of vemurafenib, phospho-ERK (p-ERK) levels in resistant cells were only modestly affected by high concentrations of vemurafenib (Fig. 1B and Supplementary Fig. S1). This observation recapitulates clinical findings, namely that p-ERK was reduced in tumor samples biopsied within 2 weeks of vemurafenib treatment; however, p-ERK was commonly reactivated at disease relapse (6). Therefore, it appears that the derived acquired resistant cell lines represent a relevant model system for exploring mechanisms of acquired resistance in patients.

CRAF protein levels and phospho-AKT (p-AKT) levels were increased in the vemurafenib-resistant cells compared with vemurafenib-sensitive cells (Fig. 1B). Thus reduced sensitivity to inhibition of the RAF/MEK pathway may be, in part, mediated by increased levels of CRAF protein, consistent with a previous study using a different RAF inhibitor (14). This acute increase in CRAF protein levels observed in the presence of vemurafenib could result from allosteric stabilization of the CRAF dimer (15, 16, 17). In addition, increased p-AKT levels suggest that alternative pathways may be simultaneously activated in the resistant setting. Importantly, V600E mutation was preserved in all resistant cell lines, and sequencing of the entire BRAF coding sequence revealed no additional mutations. Therefore, gatekeeper mutations do not account for ERK reactivation in this model system. In addition, Western blot analysis and quantitative PCR revealed an upregulation of BRAF mRNA and protein levels in the acquired resistant cells (Fig. 1C and Supplementary Figs. S2 and S3). Whether the increased mutated BRAF protein plays a role in conferring acquired resistance is being investigated. The absence of an increase in the levels of P-glycoprotein multidrug resistance (MDR) transporter (Supplementary Fig. S4) also indicates that upregulation of MDR activity does not contribute to resistance.

CRAF helps mediate acquired vemurafenib resistance in A375 melanoma cells

To further explore the role of elevated CRAF expression levels in resistance to vemurafenib, CRAF was depleted in the sensitive parental cells and in the 2 resistant cell lines using a CRAF-directed siRNA construct. As shown in Fig. 2A, expression of the siRNA construct downregulated CRAF protein

levels in both sensitive and resistant cells. Ablation of CRAF protein did not change the antiproliferative effect of vemurafenib on parental A375 cells but did increase the sensitivity of resistant cells to vemurafenib, by 7-fold (IC_{50} value shifted from 5.1 to 0.76 $\mu\text{mol/L}$) and 5-fold (IC_{50} value shifted from 8.7 to 1.3 $\mu\text{mol/L}$) for clones R1 and R6, respectively, as assessed by reductions in the IC_{50} values (Fig. 2B). Conversely, overexpression of CRAF protein with a transfected plasmid in the sensitive parental A375 cells (Fig. 2C) resulted in a more than 18-fold (IC_{50} value shifted from 0.03 to 0.55 $\mu\text{mol/L}$) increase in resistance to vemurafenib (Fig. 2D). This suggests that the upregulation of CRAF found in the resistant cell lines participates in the acquisition of resistance.

RAS-GTP levels are elevated and an activating KRAS mutation is acquired in vemurafenib-resistant cell lines

To further understand the role of increased RAS/RAF/MEK/ERK pathway activity in resistance, we also interrogated the pathway upstream of CRAF, directly measuring activated RAS using an assay that exploits the known specificity of the interaction between RAS-GTP and the RAS-binding domain of RAF (18). Because RAS binds to RAF in a GTP-dependent manner, determining the amount of RAS bound to RAF is a direct measure of RAS-GTP levels. As shown in Fig. 3A, intrinsic RAS-GTP levels in the resistant cell lines were substantially elevated compared with levels in the sensitive parental A375 cells.

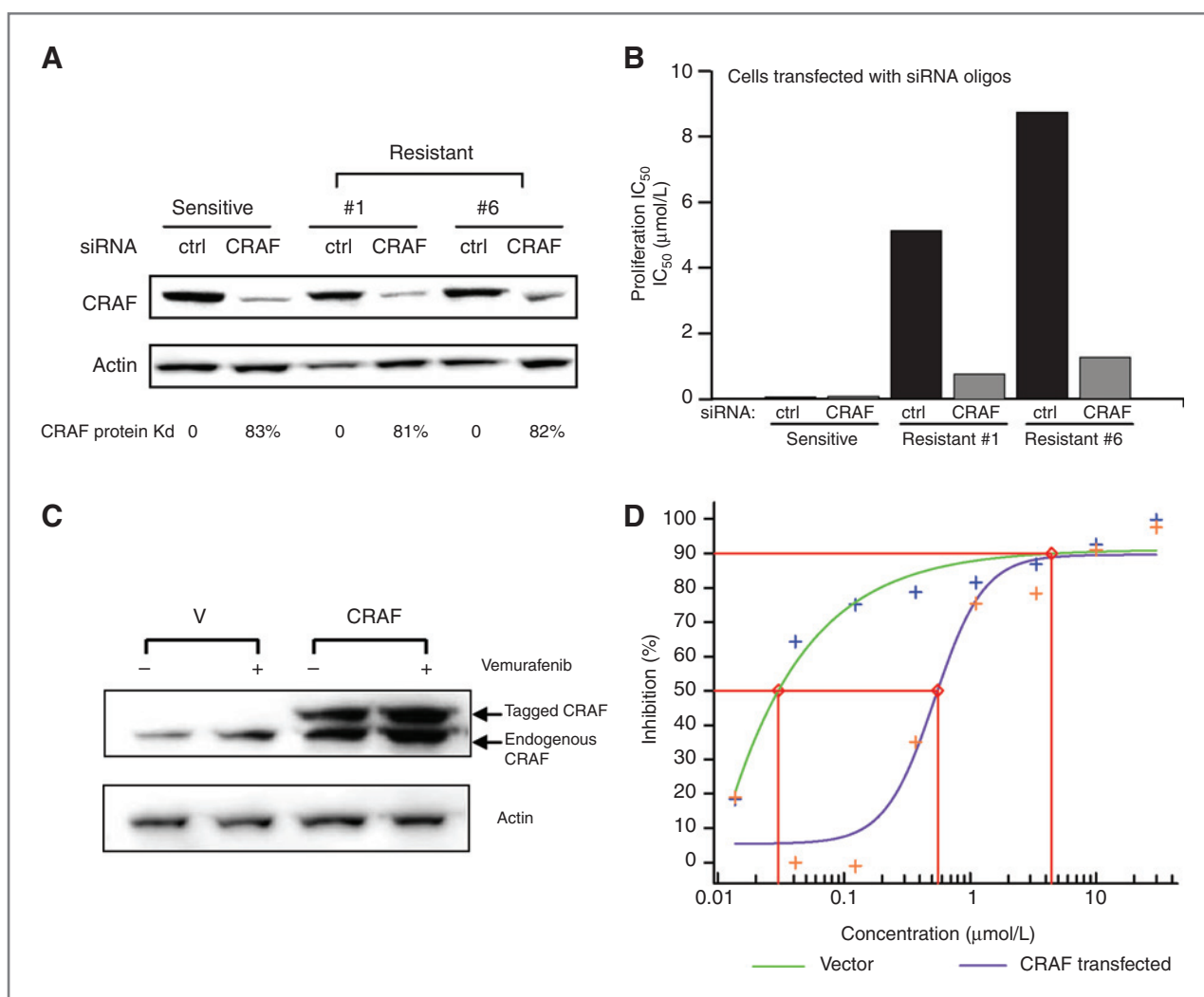
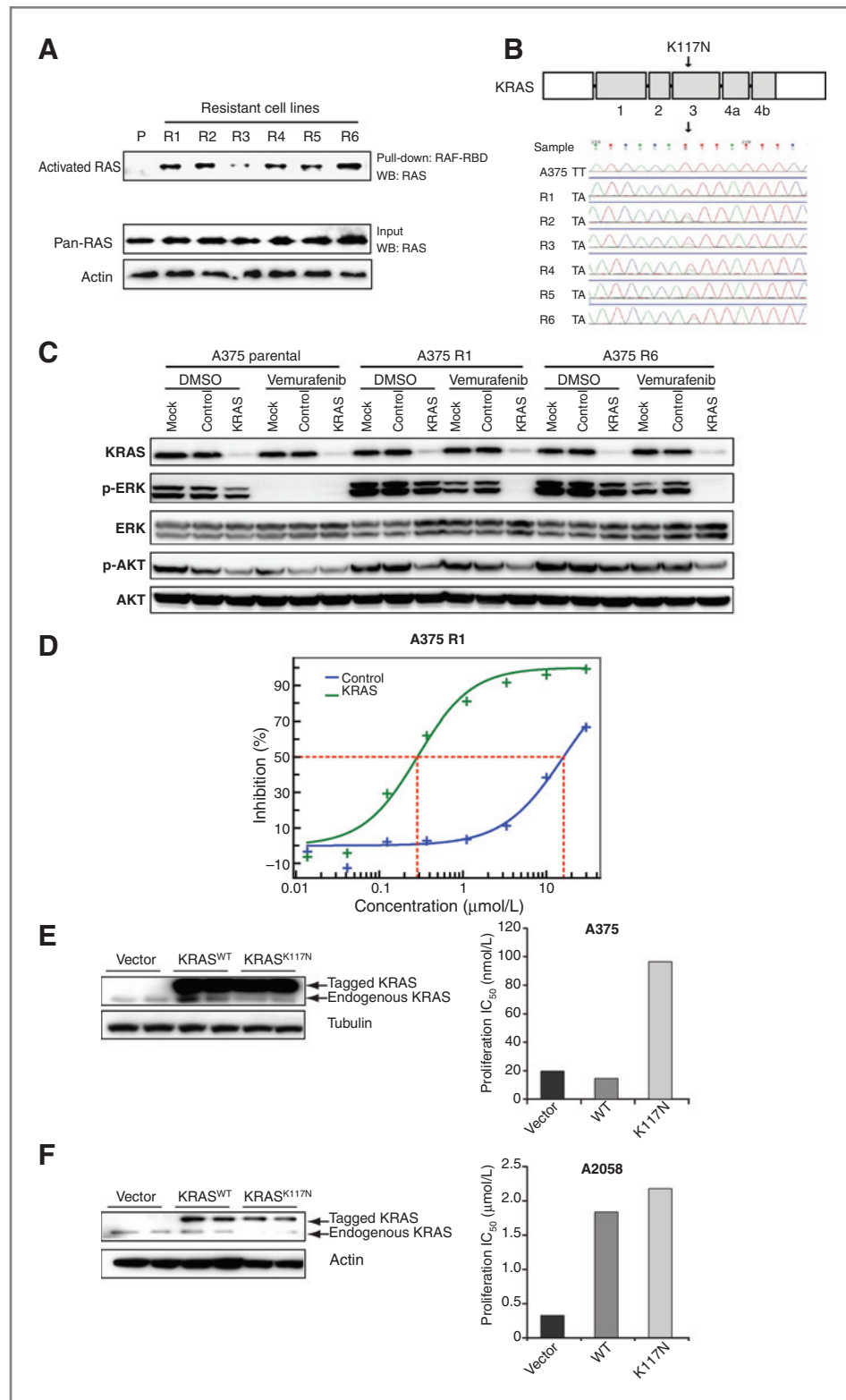


Figure 2. CRAF knockdown restores vemurafenib sensitivity in resistant clones, and CRAF overexpression confers resistance to vemurafenib in the parental sensitive A375 cells. **A**, Western blot analysis conducted with cell lysates collected 72 hours posttransfection show that CRAF protein expression is downregulated following transfection of a CRAF-directed siRNA construct in sensitive and resistant cell lines. **B**, effect of CRAF protein knockdown on cellular proliferation is shown in a bar graph of IC_{50} . Sensitive or resistant cells transfected with scrambled (ctrl) or CRAF siRNA were treated with various concentrations of vemurafenib for 4 days, and cell viability was measured to plot growth curves. **C**, Western blot analysis confirms overexpression of tagged CRAF protein in the sensitive parental A375 cells transfected with a CRAF expression plasmid. **D**, the sensitive A375 cells were transfected with vector plasmid or plasmid carrying *CRAF*. Sixteen hours after transfection, cells were treated with various concentrations of vemurafenib for 3 days, and cell viability was measured to plot growth curves.

One possible mechanism of increased RAS activity is acquisition or selection of activating mutations in RAS. We, therefore, conducted whole exome sequencing on the parental and

resistant lines, with particular interest in the sequencing result of the *NRAS*, *HRAS*, and *KRAS*. We used NimbleGen sequence capture technology to enrich for 1,97,218 exonic (and miRNA)

Figure 3. Vemurafenib-resistant cell lines display elevated RAS-GTP levels and harbor an acquired activating *KRAS* mutation. **A**, whole cell lysates of the parental sensitive and the 6 vemurafenib-acquired resistant cell lines were subjected to RAS-GTP pull down. RAS-GTP complex was revealed by immunoblotting with RAS antibody, showing elevated active RAS in all resistant cell lines. **B**, whole exome sequencing identified a single-nucleotide mutation (T→A) resulting in K117N amino acid substitution within exon 3 of *KRAS* in all 6 resistant cell lines but not in the parental line, which was confirmed by Sanger sequencing. **C** and **D**, vemurafenib-sensitive (A375 parental) or resistant (A375 R1 and A375 R6) cells were transfected with scrambled (control) or *KRAS* siRNA or mock transfected. Ninety-six hours posttransfection, cells were assessed via Western blot (WB) analysis (**C**) and cell viability assay (**D**). For Western blot analysis, cells were treated with vehicle (DMSO) or vemurafenib (parental 1 $\mu\text{mol/L}$, resistant clones 10 $\mu\text{mol/L}$) for 1 hour before lysis. For cell viability assay, A375R1 was treated with varying concentrations of vemurafenib 24 hours posttransfection, and cell viability was assessed 72 hours posttreatment (96 hours posttransfection). Growth curves were plotted for control-transfected cells (blue line) and *KRAS* knockdown cells (green line). **E** and **F**, A375 and A2058 melanoma cells were transfected with vector plasmid or plasmid carrying wild-type (WT) or K117N-mutated *KRAS* genes. Sixteen hours after transfection, cells were treated with various concentrations of vemurafenib for 3 days, cell viability was measured and proliferation IC_{50} value was calculated.



genomic regions and sequenced these to greater than 130-fold of median coverage on the Illumina GAI sequencer (detailed exome sequencing methods and additional results are included in Supplementary Tables S2 and S3). We identified a mutation in the *KRAS* gene resulting in a K117N substitution in *KRAS* protein (Fig. 3B). This uncommon mutation has been known for quite sometime to cause modest *KRAS* activation in biologic studies (19–22). To further evaluate the role of *KRAS* in the resistance to vemurafenib, genetic ablation of *KRAS* was conducted. Downregulation of *KRAS* protein was achieved using a *KRAS*-directed siRNA construct (Fig. 3C). *KRAS* downregulation had no effect on the vemurafenib sensitivity of the parental A375 cells assessed by inhibition of p-ERK and cellular proliferation, but caused increased sensitivity of the resistant cells to vemurafenib-mediated p-ERK inhibition (Fig. 3C) and decreased IC_{50} value for cellular proliferation in resistant cells (from 16 $\mu\text{mol/L}$ down to 0.26 $\mu\text{mol/L}$; Fig. 3D). Conversely, overexpression of the *KRAS*^{K117N} protein with a transfected plasmid in the sensitive parental A375 cells resulted in a 5-fold increase in resistance to vemurafenib (IC_{50} value shifted from 0.019 to 0.1 $\mu\text{mol/L}$; Fig. 3E). When the *KRAS*^{K117N} protein was overexpressed in another melanoma cell line, A2058, proliferation IC_{50} value was shifted from 0.32 to 2.2 $\mu\text{mol/L}$, corresponding to an approximately 7-fold increased resistance to vemurafenib (Fig. 3F). The potential of *KRAS*^{K117N} to elevate RAS activity was also assessed by comparison to a hotspot mutant RAS, *KRAS*^{G12V} in the activated RAS pull down assay. Both K117N and G12V mutations result in high levels of RAS-GTP than wild-type and vector-transfected controls. The hotspot G12V mutant shows slightly higher levels of RAS-GTP than the K117N mutant (Supplementary Fig. S5). Taken together, these data show that the mutated *KRAS*^{K117N} found in the resistant cell lines does play a role in the acquisition of resistance.

Identification of the *KRAS*^{K117N} mutation in the resistant cell lines was surprising for 2 reasons: *KRAS* mutations are rarely found in melanomas and nucleotide-binding mutations are exceedingly rare in all cancers. A plausible explanation may derive from the recently discovered pharmacodynamic analysis in vemurafenib-treated patients: tumor responses are exceptionally sensitive to small changes in pathway inhibition (6). Therefore, the mutation reported here could have the property of elevating pathway signaling just enough to overcome compound inhibition, perhaps reflecting the dynamics observed in relapsing patients. We, therefore, reasoned that additional pathway interference could restore sensitivity to vemurafenib.

Coadministration of vemurafenib with a MEK inhibitor shows synergistic effects in the vemurafenib-resistant cells and xenograft models

The retention of the V600E mutation in resistant cell lines suggests that continued suppression by vemurafenib may be required to control cell proliferation; however, reactivation of the RAS/RAF signaling pathway may warrant combination with another agent that further inhibits ERK signaling to optimally resuppress the pathway and consequently overcome resistance. To test this hypothesis, we evaluated the effects of

combining vemurafenib and the MEK inhibitor, RO5068760 (23), in vemurafenib-resistant A375R6 cells. As shown in Fig. 4A, single-agent treatment with either vemurafenib or RO5068760 did not effectively inhibit ERK phosphorylation, as expected, as the resistant cells were also cross-resistant to MEK inhibitors (Supplementary Table S1). RO5068760 did cause partial inhibition of ERK phosphorylation, and the observation that this partial inhibition translated to minimal tumor growth delay supports the hypothesis that substantial pathway inhibition is required for efficacy. However, in combination, dual BRAF and MEK inhibition completely abrogated the constitutive upregulation of ERK phosphorylation, inhibited cell-cycle progression as assessed by cyclin D1 levels, and induced apoptosis evidenced by increased levels of BimEL and cleaved PARP in the resistant cells (Fig. 4A). Consistent with these findings, the combination of vemurafenib and RO5068760 resulted in more effective inhibition of cellular proliferation than either agent alone. The calculated CI values were less than 0.9 (range: 0.50–0.72) indicating synergy between the 2 drugs in blocking proliferation of the resistant cell lines R1 and R6. In addition, RAF/MEK inhibition shows greater synergy in the resistant cells than in the parental sensitive cells with CI values ranging from 0.79 to 0.96 (Fig. 4B). This synergism correlated to a synergistic effect in induction of apoptosis with combined RAF/MEK inhibition in resistant cells as compared with sensitive cells. Together, these data suggest that in the setting of vemurafenib resistance, addition of MEK inhibition to supplement ongoing inhibition of mutated BRAF is needed to resuppress ERK signaling sufficiently to inhibit tumor cell proliferation.

This *in vitro* synergy was confirmed *in vivo*, using xenograft studies. Of the various resistant clones, the A375R1 cell line showed growth kinetics that most closely matched the parental line and was selected for further testing. In the parental A375 tumor xenograft model, vemurafenib dosed at 12.5 mg/kg once daily produced 84% tumor growth inhibition (TGI) and at 25 mg/kg once daily achieved tumor regression (Supplementary Table S4). In contrast, in the vemurafenib-resistant A375R1 melanoma xenograft model, vemurafenib dosed at 50 mg/kg once daily achieved only minimal (39%) TGI (Fig. 5). Similarly, MEK inhibitor monotherapy produced minimal TGI ranging from 11% to 44% at doses up to 50 mg/kg daily (Fig. 5). This confirmed the cross-resistance between RAF and MEK inhibitors observed in the cellular proliferation study. However, using doses that on their own have minimal effects on tumor growth (50 mg/kg once daily), combination treatment with vemurafenib and the MEK inhibitor RO5068760 achieved substantially greater (>98%) antitumor activity than either agent alone (Fig. 5), suggesting that the MEK inhibitor restored sensitivity to vemurafenib in the vemurafenib-resistant melanoma xenograft model. Furthermore, these *in vivo* results support the importance of ongoing BRAF inhibition in combination with MEK inhibition to overcome resistance resulting from reactivated MAPK signaling. These results provide a rationale for combination clinical trials of vemurafenib with a MEK inhibitor to inhibit the development or restore the

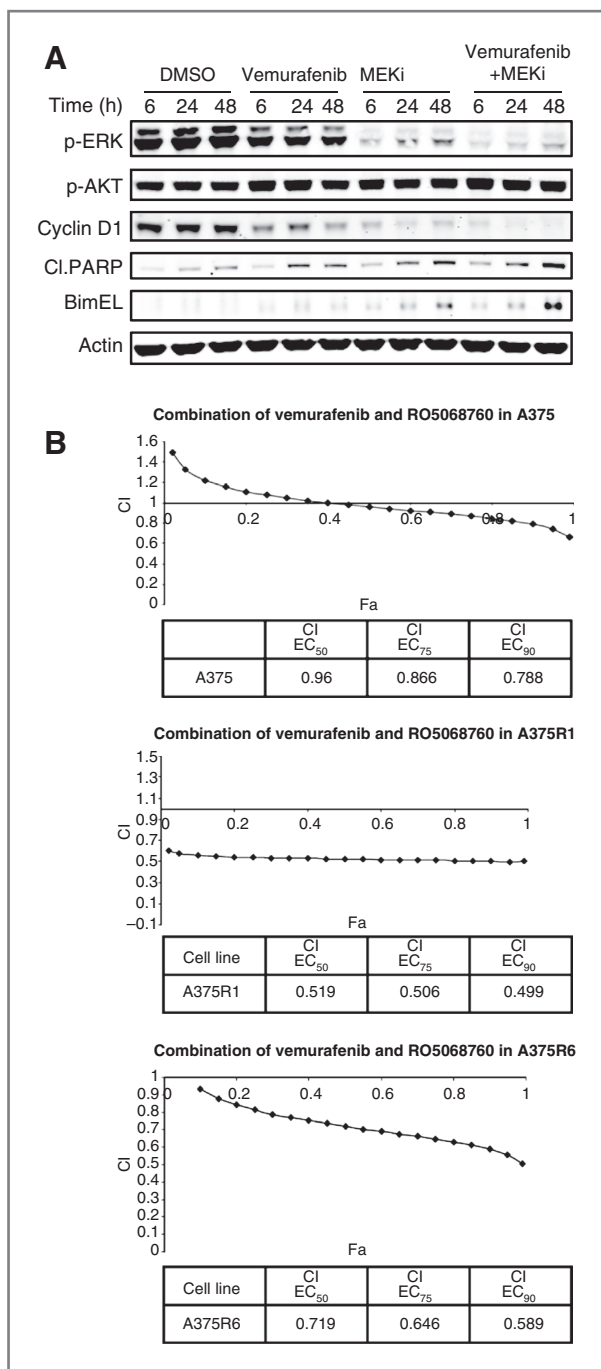


Figure 4. *In vitro* combination of vemurafenib with the MEK inhibitor (MEKi) RO5068760 shows synergism in vemurafenib-resistant cell lines. A, the A375R6 vemurafenib-resistant cells were treated with 7.5 μ mol/L vemurafenib and/or 1 μ mol/L RO5068760. Cells were harvested after 6, 24, and 48 hours of treatment and whole cell lysates were subjected to Western blot analysis for the levels of p-ERK, p-AKT, cyclin D1, BimEL, and cleaved PARP (Cl.PARP). B, CI-Fa graphs of combination studies with vemurafenib and RO5068760 in the parental sensitive A375 and vemurafenib-resistant cell lines A375R1 and R6. CI < 0.9 indicates synergy; CI = 0.9 to 1.1 indicates additivity; CI > 1.1 indicates antagonism between the 2 tested drugs; and Fa = fraction affected by the combination treatment.

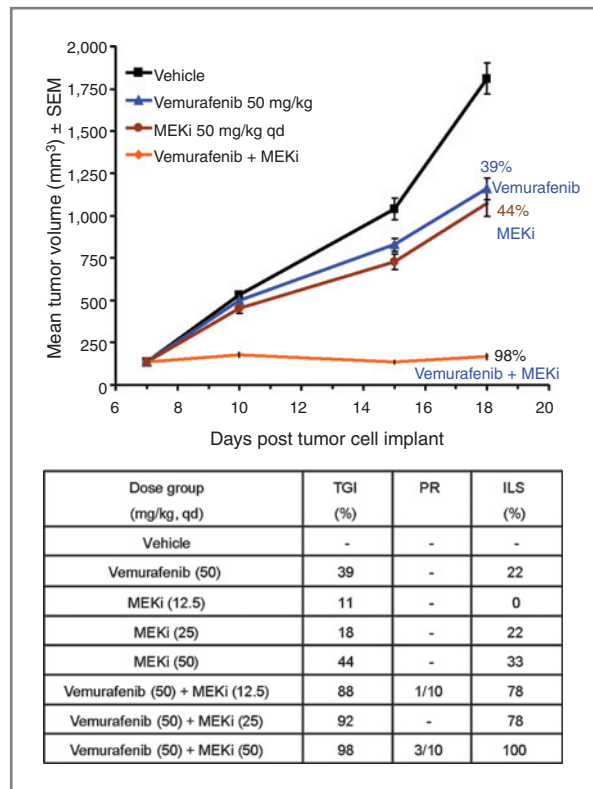


Figure 5. *In vivo* combination with vemurafenib and RO5068760 in the vemurafenib-resistant melanoma xenograft model. The combination of vemurafenib at 50 mg/kg once daily with increasing dosages of RO5068760 for 11 days achieved greater antitumor activity (TGI) and increased life span (ILS).

sensitivity of vemurafenib-resistant tumors to vemurafenib therapy by reestablishing blockade of the RAS/RAF/MEK/ERK pathway.

Combinations of vemurafenib with an AKT inhibitor show synergistic effects in vemurafenib-resistant cells

As previously mentioned, p-AKT levels were increased in the vemurafenib-resistant clones compared with vemurafenib-sensitive cells (Fig. 1B), suggesting that vemurafenib resistance may also be partly mediated by activating phosphoinositide 3-kinase (PI3K) signaling. Therefore, simultaneously targeting both BRAF and PI3K pathways may achieve greater proliferation control and overcome resistance. Indeed, *in vitro* combination with vemurafenib and an AKT inhibitor showed synergistic antiproliferative effects in the vemurafenib-resistant A375 R1 cells indicated by a CI value of 0.38 at ED₉₀ dose (Fig. 6A). We also monitored the pharmacodynamic effects of this combination. As shown in Fig. 6B, simultaneously targeting ERK and AKT signaling resulted in completely abrogation of the ERK and AKT phosphorylation, inhibition of cell-cycle progression indicated by decreased cyclin D1 and increased p27 levels and induction of apoptosis indicated by increased level of BimEL in the resistant cells (Fig. 6B). Additional studies are warranted to provide a rationale for clinical trials with vemurafenib and an AKT or a PI3K inhibitor to prevent the

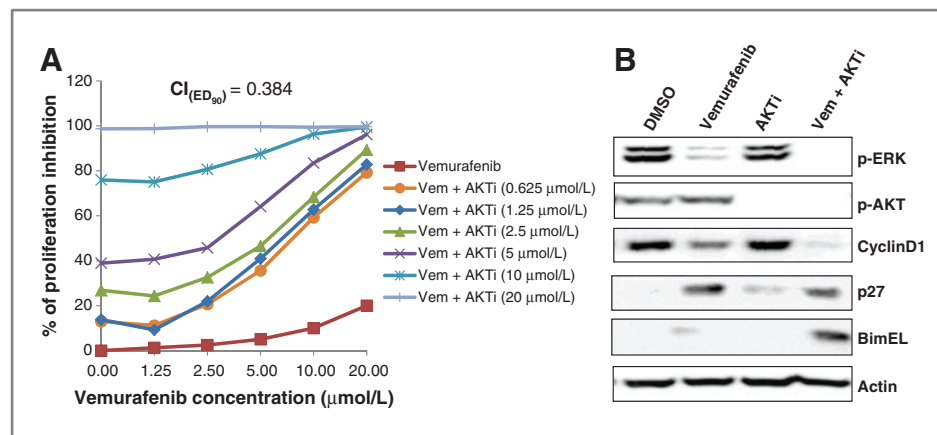


Figure 6. *In vitro* combination of vemurafenib (Vem) with the AKT inhibitor (AKTi) MK-2206 shows synergism in vemurafenib-resistant cell lines. **A**, *in vitro* proliferation assay of combination with vemurafenib and MK-2206 in the vemurafenib-resistant cell line A375R1. $CI < 0.9$ indicates synergy; $CI = 0.9$ to 1.1 indicates additivity; $CI > 1.1$ indicates antagonism between the 2 tested drugs. **B**, the A375R1 vemurafenib-resistant cells were treated with vemurafenib (10 $\mu\text{mol/L}$) and/or the AKT inhibitor (5 $\mu\text{mol/L}$) for 2 or 48 hours. Cells were harvested after the treatment and whole cell lysates were subjected by Western blot analysis for the levels of p-ERK, p-AKT (2 hours after vemurafenib treatment), cyclin D1, p27, and BimEL (48 hours after vemurafenib treatment).

development of resistance to or to restore the sensitivity to vemurafenib.

Discussion

Vemurafenib has shown extraordinary results in phase I clinical evaluation, with 81% of patients with BRAF mutant melanoma achieving a response (>30% tumor regression) to treatment (13). However, as noted with previous targeted anticancer therapies, despite remarkable initial response rates, sustained clinical utility is often compromised by emergence of acquired resistance. To develop effective therapeutic strategies to overcome or prevent such resistance, it is important to understand the underlying mechanisms of resistance.

In the current study, melanoma cell lines with acquired resistance to vemurafenib were established by culturing sensitive parental cells under continuous vemurafenib selection to model disease relapse associated with vemurafenib treatment in patients with melanoma. This method simulates the chronic selective pressure that occurs during drug treatment in the clinic and has successfully identified clinically relevant mechanisms of resistance to other agents (24, 25). In addition, compared with newer, alternative methodologies to identify targets responsible for drug resistance such as synthetic lethal screens using short hairpin RNA (shRNA) or siRNA libraries, this method is faster and more economical. This system was, therefore, used to understand molecular mechanisms of disease relapse after initial response to vemurafenib and subsequently to identify potential combination therapies to prevent or mitigate emergence of progression.

Consistent with the *ex vivo* findings in tumor samples from the phase I clinical trial of vemurafenib (6, 13), vemurafenib exposure led to reduced ERK phosphorylation in the sensitive parental A375 cell line. In contrast, p-ERK levels were elevated in resistant cell lines and insensitive to vemurafenib inhibition, consistent with early clinical observations of p-ERK reactivation at disease relapse. Therefore, continued dependence on

the RAS/RAF/MEK/ERK pathway may be an important contributor to tumor cell growth in resistant BRAF^{V600E}-positive melanoma. Notably, the V600E mutation was retained and no additional mutations were identified in the entire coding region of BRAF gene. However, it was found that BRAF protein levels were upregulated in the acquired resistant cells. Similar observations with BCR-ABL-driven tumors were noted in imatinib-resistant cell lines (26, 27) and relapsed patients (28). Whether the upregulation of mutant BRAF protein plays a role in conferring acquired resistance to clinical vemurafenib treatment is a subject of further investigation.

Investigation of the RAS/RAF/MEK/ERK pathway upstream of CRAF revealed that RAS-GTP levels were elevated in vemurafenib-acquired resistant cells. Whole exome sequencing revealed a K117N mutation in KRAS. While this mutation is uncommon in human tumors (19–22), it has been known for quite sometime to cause RAS activation in biologic studies (19). Discovery of this mutation provides a plausible mechanism for acquired resistance, as it has recently been shown that oncogenic RAS confers resistance to RAF inhibitors (15, 16, 17). Indeed, an oncogenic NRAS allele was identified in 2 of 16 (both came from the same patient) tumor biopsies taken at disease relapse from patients with melanoma who received vemurafenib therapy (29). Activation of RAS by mutations could, therefore, explain the elevated levels of RAS-GTP observed in our resistant cell lines, which subsequently could recruit CRAF (or BRAF) to the membrane and potentially stabilize and activate the RAF proteins. Sequencing the KRAS coding region of 15 relapsing tumor samples revealed no mutations in the KRAS gene, suggesting the KRAS mutation may happen much less frequently than NRAS mutations in melanoma. Sequencing additional relapsing tumors may help solidify the conclusion. Nonetheless, perhaps the key point of the current discovery is that modest upstream pathway activation is sufficient for vemurafenib resistance. This is consistent with the findings in patient tumors (6): increasing signaling by less than 50% could be sufficient to bypass the inhibitor. It will be particularly

interesting as more samples are analyzed to see whether perturbations in proteins involved in RAS activation will be found in relapsed tumors. This discovery provides optimism that relapsed tumors can be resensitized to vemurafenib by combining with a second inhibitor of the same pathway.

Accordingly, combining vemurafenib with the MEK inhibitor RO5068760 restored sensitivity to vemurafenib in the vemurafenib-resistant cell lines. Notably, as single agents, neither inhibitor effectively blocked ERK phosphorylation in resistant cell lines; but in combination, these agents completely abrogated the elevation of ERK phosphorylation, inhibited cell-cycle progression, and induced apoptosis. Indeed, the synergistic induction of apoptosis was greater with combined RAF/MEK inhibition in the resistant cells compared with the sensitive cells. The potential downstream signaling change that may result in this differential apoptosis induction is being investigated. Importantly, synergistic antitumor activity was also observed with this combination in the vemurafenib-resistant melanoma xenografts, whereas activity with either agent alone was minimal. Therefore, addition of MEK inhibition to tonic BRAF inhibition seems to be sufficient to resuppress ERK activity in the resistant setting. These findings, together with preclinical evidence that combined inhibition of BRAF and MEK abrogates the emergence of resistance (30), support the clinical evaluation of combination therapy strategies incorporating MEK inhibition with BRAF inhibitors to combat emerging resistance.

In the course of preparation of this report, 3 reports were published on vemurafenib resistance mechanisms, showing that resistance to vemurafenib can be mediated by COT (MAP3K8), an ERK upstream component, or receptor tyrosine kinase (RTK) such as platelet-derived growth factor receptor β (PDGFR β) and insulin-like growth factor-1 receptor (IGF1R; ref. 29, 31, 32). We, therefore, analyzed the expression levels of COT, PDGFR β , and IGF1R in our resistant cell lines by Western blotting and quantitative PCR. There were no changes in protein expression or mRNA levels of IGF1R in our resistant cell lines compared with the parental cells (Supplementary Figs. S2 and S3). There were modest changes in the mRNA levels of PDGFR β and COT; however, the changes did not show a consistent trend of increased expression, and the protein expression levels were too low for detection by Western

blotting in all cell lines. On the basis of these expression data, it appears unlikely that upregulation of COT, PDGFR β , or IGF1R are potential resistance mechanisms in our model system. The upregulation of p-AKT levels in our vemurafenib-resistant cells appeared to result from the acquisition of mutant KRAS^{K117N}. This was supported by data showing the reduction of p-AKT with KRAS knocked down using siRNA in the vemurafenib-resistant cells (Fig. 3C). *In vitro* combination studies with vemurafenib and an AKT inhibitor showed synergistic antiproliferative effects in the vemurafenib-resistant cell lines. Further investigation of combinations between vemurafenib and PI3K pathway inhibitors in xenograft models is warranted to provide a rationale for conducting combination clinical trials with such agents in patients with BRAF mutant tumors with deregulated PI3K signaling.

It is becoming apparent that the development achieving sustainable remissions with molecularly targeted anticancer therapies will require preemptive attention to potential escape pathways. The results of this study further support this concept and provide a rationale for clinical trials incorporating coadministration of vemurafenib with MEK or PI3K pathway inhibitors to prevent or delay the emergence of resistance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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