

Elevated CRAF as a Potential Mechanism of Acquired Resistance to BRAF Inhibition in Melanoma

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Abstract

Activating BRAF kinase mutations arise in ~7% of all human tumors, and preclinical studies have validated the RAF–mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase–ERK signaling cascade as a potentially important therapeutic target in this setting. Selective RAF kinase inhibitors are currently undergoing clinical development, and based on the experience with other kinase-targeted therapeutics, it is expected that clinical responses to these agents, if observed, will lead to the eventual emergence of drug resistance in most cases. Thus, it is important to establish molecular mechanisms underlying such resistance to develop effective therapeutic strategies to overcome or prevent drug resistance. To anticipate potential mechanisms of acquired resistance to RAF inhibitors during the course of treatment, we established drug-resistant clones from a human melanoma-derived cell line harboring the recurrent V600E activating BRAF mutation, which exhibits exquisite sensitivity to AZ628, a selective RAF kinase inhibitor. We determined that elevated CRAF protein levels account for the acquisition of resistance to AZ628 in these cells, associated with a switch from BRAF to CRAF dependency in tumor cells. We also found that elevated CRAF protein levels may similarly contribute to primary insensitivity to RAF inhibition in a subset of BRAF mutant tumor cells. Interestingly, AZ628-resistant cells demonstrating either primary drug insensitivity or acquired drug resistance exhibit exquisite sensitivity to the HSP90 inhibitor geldanamycin. Geldanamycin effectively promotes the degradation of CRAF, thereby revealing a potential therapeutic strategy to overcome resistance to RAF inhibition in a subset of BRAF mutant tumors. [Cancer Res 2008;68(12):4853–61]

Introduction

Genetic alterations that contribute to tumorigenesis can give rise to proteins that are essential for maintaining the enhanced growth and survival properties of tumor cells. Such “addiction” to individual oncogenic proteins seems to explain the exquisite clinical sensitivity of some tumors to various molecularly targeted kinase inhibitors (1). Thus, imatinib is highly effective in chronic

myelogenous leukemia (CML) cells that harbor the BCR-ABL translocation and gastrointestinal stromal tumors (GIST) with activating c-KIT or platelet-derived growth factor receptor mutations (2). Similarly, most non-small cell lung cancers (NSCLC) harboring an activating epidermal growth factor receptor (EGFR) kinase domain mutation are sensitive to the selective EGFR tyrosine kinase inhibitors (TKI) gefitinib and erlotinib (3–5).

As a result of cancer genome resequencing efforts, activating somatic mutations in BRAF have been identified in 60% of melanomas, 40% of thyroid cancers, and 20% of colon cancers (6). The most common *BRAF* mutation leads to a substitution of glutamic acid for valine at position 600 (V600E) within the activation segment of the BRAF kinase domain, which results in elevated kinase activity and stimulation of downstream mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK)–ERK signaling, consequently promoting tumor cell survival and proliferation (6–8). Therefore, inhibition of the BRAF pathway is considered to be a promising strategy for treating melanoma and other BRAF mutant cancers, and several selective kinase inhibitors that target the BRAF-MEK-ERK pathway are currently being developed (9, 10). In preclinical studies, inhibition of the MEK kinase effectively and specifically inhibits the growth of human tumor cell lines harboring activating BRAF mutations (9). Similarly, in a high-throughput tumor cell line profiling study, we have recently reported that AZ628, a selective and potent investigational small molecule RAF kinase inhibitor, is remarkably effective at inhibiting the growth of a specific subset of human cancer cell lines derived from melanomas, thyroid cancers, and colorectal cancers that harbor the BRAF V600E mutation (11).

Whereas various targeted kinase inhibitors have shown both preclinical and clinical activity, the application of these agents to large patient populations has clearly shown that, whereas initial clinical responses can be dramatic, rapid acquisition of drug resistance is a major limitation to the overall therapeutic efficacy of these drugs. Therefore, one of the major challenges associated with the broader use of these inhibitors is the elucidation of drug resistance mechanisms and the development of strategies to overcome or prevent resistance.

In CML, GIST, and NSCLC, acquired resistance to kinase inhibitors is frequently associated with either secondary kinase domain mutations, amplification of the gene encoding the target kinase, or mutational activation of genes encoding components of alternative survival pathways (12–18). Notably, each of these identified resistance mechanisms has been successfully modeled in cell culture using appropriate drug-treated cancer cell lines, indicating that such cell culture modeling can provide an effective system for identifying mechanisms of acquired drug resistance that are likely to arise clinically (16, 19, 20). This is important because the development of strategies to overcome drug resistance, which

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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will generally require considerable time, first requires the identification of relevant resistance mechanisms. Therefore, the ability to anticipate clinical mechanisms of acquired resistance to targeted kinase inhibitors is likely to greatly accelerate the development of strategies to overcome or prevent acquired drug resistance (21) and to reduce the current temporal gap between initial clinical successes and subsequent disease progression in the absence of available secondary treatment options.

Selective inhibitors of the RAF and MEK kinases are currently undergoing early-phase clinical testing (22–24). To anticipate potential mechanisms of acquired resistance to RAF inhibitors that could arise during the course of treatment, we established drug-resistant clones from a human melanoma-derived cell line that harbors the V600E activating BRAF mutation and exhibits exquisite sensitivity to AZ628, a selective RAF kinase inhibitor. In a subset of these clones, significantly increased expression of the BRAF-related CRAF protein seems to account for the acquisition of resistance to AZ628. Interestingly, the resistant clones, which have shifted their dependency from BRAF to CRAF, acquire substantial sensitivity to the HSP90 inhibitor geldanamycin. Geldanamycin effectively promotes the degradation of CRAF, thereby revealing a potential therapeutic strategy to overcome this resistance mechanism.

Materials and Methods

Cell culture and reagents. The human melanoma cell line M14 expressing the V600E BRAF mutation was kindly provided by Daphne Bell (NIH). M14 is a cell line from the NCI-60 cell line panel that has been extensively characterized (25). Cells were maintained at 37°C in a humidified atmosphere at 5% CO₂ grown in RPMI 1640 (Cellgro; Mediatech, Inc.) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2 nmol/L glutamine. AZ628 resistant M14 clones were maintained in the above-mentioned medium and 2 µmol/L of AZ628, except where otherwise indicated. The BRAF inhibitor AZ628 was synthesized by Astra Zeneca. It shows IC₅₀ values of ~30 nmol/L for BRAF V600E and wild-type CRAF and 100 nmol/L for wild-type BRAF and strong selectivity for RAF kinases among a panel of 150 tested kinases.³ Additional details regarding the structure and properties of AZ628 will be reported separately. The MEK inhibitor U0126 was purchased from Promega. Geldanamycin was acquired from Biomol International. The additional inhibitors were either obtained from the MGH pharmacy or were synthesized at the Dana-Farber Cancer Institute based on published structures.

Cellular proliferation assay. Approximately 50,000 or 25,000 cells were seeded in 12-well or 24-well plates, respectively, in medium supplemented with 5% FBS. After overnight incubation, the cells were treated with various concentrations of each drug. Fresh medium and drug were replaced every 2 d until the untreated control wells reached confluence. At this time point, the media were removed and the cells were fixed in 4% formaldehyde in PBS (Boston Bioproducts) for 20 min at room temperature. Cells were then washed twice with PBS and stained with a 1:5,000 solution of the fluorescent nucleic acid stain Syto60 (Molecular Probes). Quantitation of fluorescent signal intensity was carried out at 700 nm using an Odyssey IR Imager (Li-Cor Biosciences). Each experiment was performed in quadruplicate, and the results shown represent the average of the four values compared with untreated wells. Error bars represent SD of the four values from the mean. High-throughput cell growth/viability assays were performed as previously reported (11).

Protein detection. To collect protein lysates, cells were washed with PBS, scraped in lysis buffer (150 mmol/L NaCl, 1% NP40, 50 mmol/L Tris, 2 mmol/L EDTA, 10% glycerol, 5 µg/mL each of aprotinin, leupeptin, and

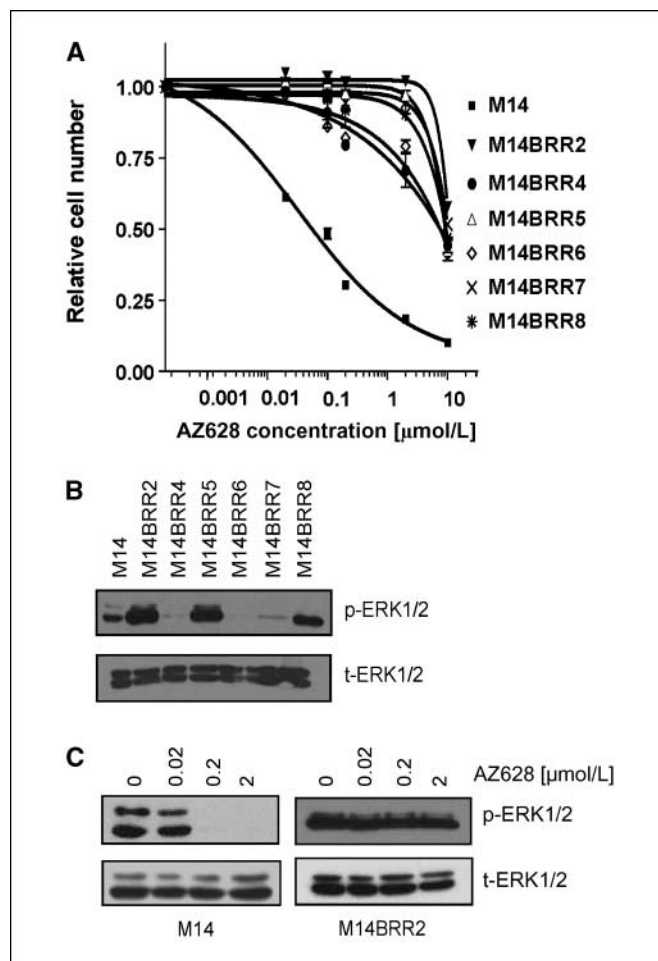


Figure 1. M14-derived AZ628-resistant (M14BRR) clones display elevated p-ERK1/2 levels and uncoupling of ERK signaling from BRAF. **A**, dose-response curves of M14 and six M14BRR clones treated with the indicated concentrations of AZ628. The fraction of viable cells is expressed relative to untreated controls. Error bars, SD from the mean. **B**, immunoblots with the indicated antibodies demonstrating that M14BRR clones exhibit elevated basal activation of ERK1/2 compared with parental M14 cells. Cell lysates from M14 and six independently generated M14BRR clones maintained in the presence of 2 µmol/L AZ628 were collected. **C**, immunoblots with the indicated antibodies demonstrating that M14BRR cells maintain ERK phosphorylation in the presence of AZ628. Cell lysates from M14 and AZ628-resistant clone (M14BRR2) were collected after 2-h treatment with the indicated concentrations of AZ628.

pepstatin, and 1 mmol/L each of NaF, Na₃VO₄, and phenylmethylsulfonyl fluoride) and incubated on ice for 40 min. The lysates were centrifuged at 14,000 rpm for 20 min, and the supernatant was collected. Protein concentration was measured with the bicinchoninic acid protein assay (Pierce), and proteins were resolved by SDS-PAGE. The gels were electroblotted onto polyvinylidene difluoride membranes (Hybond-P, Amersham). Antibody detection was performed with a chemiluminescence kit (Supersignal, Pierce). The ERK1/2, phosphorylated ERK1/2 (p-ERK1/2; T²⁰²/Y²⁰⁴), MEK, and ARAF antibodies were from Cell Signaling Technology. The CRAF and BRAF antibodies were from Santa Cruz Biotechnology. All antibodies were used at a 1:1,000 dilution.

Generation of AZ628-resistant M14 clones. Approximately, 10⁵ M14 cells were plated in each of three 10-cm dishes. After overnight incubation, the medium was removed and fresh medium was added, together with AZ628 at 2 µmol/L. Fresh medium containing drug was added to the cells every 3 d. Five weeks after drug selection, ~10 clones per dish appeared. Two to three clones per dish were isolated using cloning cylinders and propagated progressively in 3-cm, 6-cm, and 10-cm dishes maintained in

³ L. Drew, unpublished observation.

2 $\mu\text{mol/L}$ AZ628. All of the clones were confirmed to be resistant to AZ628 in a follow-up cell proliferation assay with Syto60 staining and quantitation.

DNA sequencing. Genomic DNA was isolated from the M14 parental cell line and AZ628-resistant clones using the Genra purification system according to the manufacturer's protocol. BRAF was amplified from genomic DNA by PCR. PCR products were purified using exonuclease I and shrimp alkaline phosphatase (U.S. Biochemical) followed by bidirectional sequencing using BigDye v1.1 (Applied Biosystems) in combination with an ABI3100 sequencer (Applied Biosystems). Primers used for sequencing of BRAF are listed in Supplementary Table S1. Electropherograms were analyzed using Sequence Navigator software (Applied Biosystems). All mutations were confirmed by at least two independent PCR amplifications.

Fluorescence *in situ* hybridization. Two-color fluorescence *in situ* hybridization (FISH) was performed on 3:1 methanol-acetic acid fixed cell lines using the following probes, according to the manufacturer's protocols: SpectrumOrange-labeled CRAF (BAC clones RP11-148M13 and CTD-2163C15, 3p25.1) and BRAF (BAC clones RP11-1065D4 and CTD-2516J12, 7q34), SpectrumGreen-labeled control probe for CRAF (BAC RP11-3616, 3q13.2), and SpectrumAqua-labeled centromeric probe for chromosome 7 (Vysis) that serves as a control probe for BRAF. Images were captured using an Olympus BX61 fluorescent microscope equipped with a CCD camera, and analysis was performed with Cytovision software (Applied Imaging).

Short hairpin RNA constructs and lentiviral infection. Two short hairpin RNA (shRNA) species targeting sequences for BRAF and two for CRAF were expressed from the pLKO.1 lentiviral vector (BRAF target sequences, 5'GCAGATGAAGATCATCGAAAT3' and 5'CAGCAGTTACAAG-CCTTCAA33'; CRAF target sequences, 5'CATGAGTATTTAGAGGAAGTA3' and 5'GCTTCCCTATTCTCACATCAA3'). Cells were inoculated in 96-well plates and 6-cm dishes and incubated for 16 h until cells reach ~80% confluency. Cells were then infected with shRNA lentiviruses or control vector (pLKO.1) in the presence of polybrene 8 $\mu\text{g/mL}$ (hexadimethrine bromide, Sigma-Aldrich) under 1,200 $\times g$ gravity at 32°C for 60 min. Under

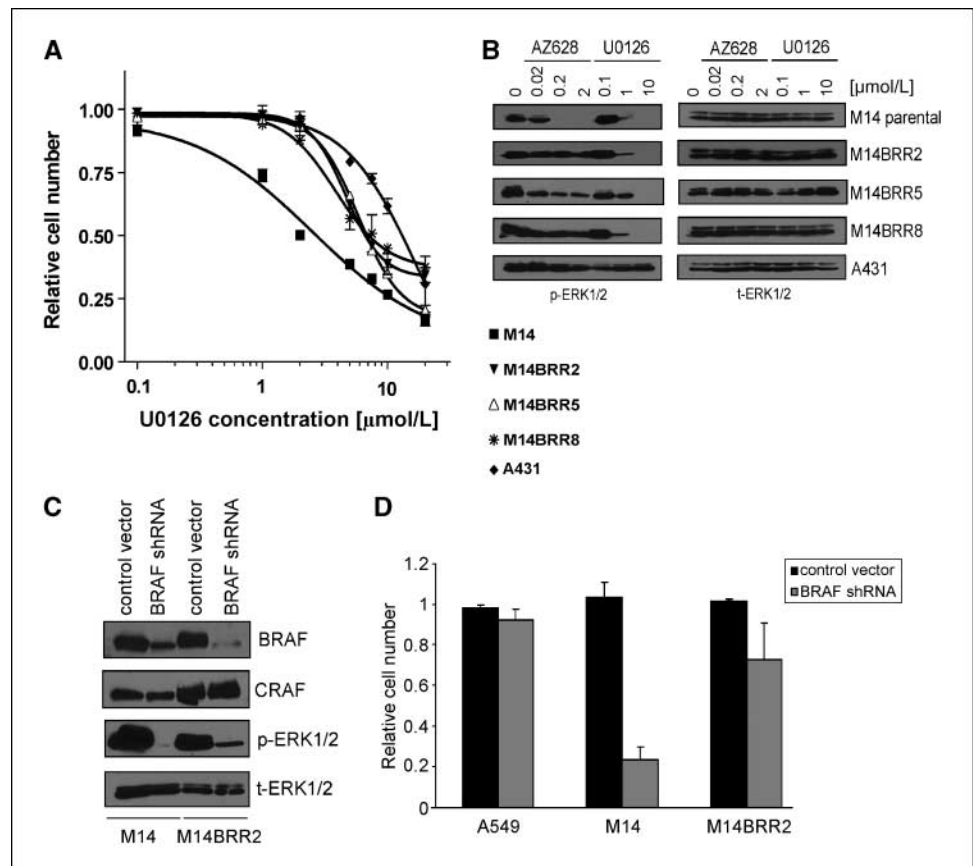
this condition, ~5 multiplicity of infection efficiency was achieved. After infection, cells were maintained in the presence of 2 $\mu\text{g/mL}$ of puromycin (Invitrogen) for an additional 4 to 6 d. A cell line resistant to pharmacologic RAF inhibition (A549) was used to show infection efficiency and specificity.

Quantitative PCR. Total RNA was isolated from M14 and M14BRR2 cells using RNeasy kit (QIAGEN). One-step reverse transcription and real-time PCR was performed using FastLane Cell kit (QIAGEN). The amount of CRAF amplicons was determined using the AB7500 quantitative PCR (qPCR) system (Applied Biosystems) with SYBR Green I as the fluorescence reporter dye and ROX as the passive reference dye. The amount of glyceraldehyde-3-phosphate dehydrogenase amplicons was determined similarly using QuantiTect primers (QIAGEN). The qPCR thermal profile was 50°C for 30 min for cDNA synthesis, 95°C for 15 min for hot-start activation of the antibody-neutralized DNA polymerase, followed by 45 cycles of denaturation (94°C, 15 s), annealing (60°C, 30 s), and elongation/data acquisition (72°C, 35 s). After completion of the thermal cycling, PCR product melting curves were obtained using the standard protocol of the AB7500 system. The C_t values were determined using manual settings recommended by the manufacturer of the FastLane reagent.

All specimens were analyzed in triplicate. CRAF primers used are as follows: forward 1 5'GCACGGAGATGTTGCAGTAA3', reverse 1 5'GCTACCA-GCCTTTCATTCG; forward 2 5'CTGTTCCAGGATGCCTGTT, reverse 2 5'GCTACTGGACAGGGCTGAAG.

CRAF expression construct. The CRAF 22W cDNA coding sequence within a pBABE retrovirus plasmid was a kind gift from Channing Der (University of North Carolina; ref. 26). Transfection of Phoenix Morpho cells with the retroviral vector was performed using the Fugene Transfection Reagent (Invitrogen). The collected retrovirus was used to infect M14 cells in the presence of polybrene (8 $\mu\text{g/mL}$) under 1,200 $\times g$ gravity at 32°C for 60 min. Cells were selected in puromycin (2 $\mu\text{g/mL}$), and resistant clones were used for analysis. M14 cells infected with an empty pBABE puro plasmid were used as control.

Figure 2. Proliferation of M14 AZ628-resistant (M14BRR) clones is dependent on MEK but not BRAF. **A**, dose-response curves of M14 and three M14BRR clones treated with the indicated concentrations of the MEK inhibitor U0126. The percentage of viable cells is expressed relative to untreated controls. Error bars, SD from the mean. The A431 cell line survival curve is shown as a negative control. **B**, AZ628-resistant cells retain sensitivity to U0126. Cell lysates from M14, AZ628-resistant clones, and A431 (negative control) were collected after treatment with the indicated concentrations of AZ628 or U0126 for 2 h. Immunoblotting analysis was performed using antibodies directed against the indicated proteins. **C**, effective depletion of BRAF protein by shRNA. M14 and M14BRR2 cells were infected with lentivirus containing control (pLKO.1 empty vector) or BRAF-specific shRNA. Cells were puromycin-selected and protein lysates were collected 4 d after the infection. Immunoblotting analysis was performed using antibodies directed against the indicated proteins. **D**, reduced dependency on BRAF in AZ628-resistant cells. Proliferation assay corresponding to cells in **C**. Control or BRAF-specific BRAF shRNAs were introduced in A549, M14, and M14BRR2 cells by lentiviral infection and a cell proliferation assay with Syto60 was performed 7 d later. The fraction of viable cells is expressed relative to untreated control. Error bars, SD from the mean.



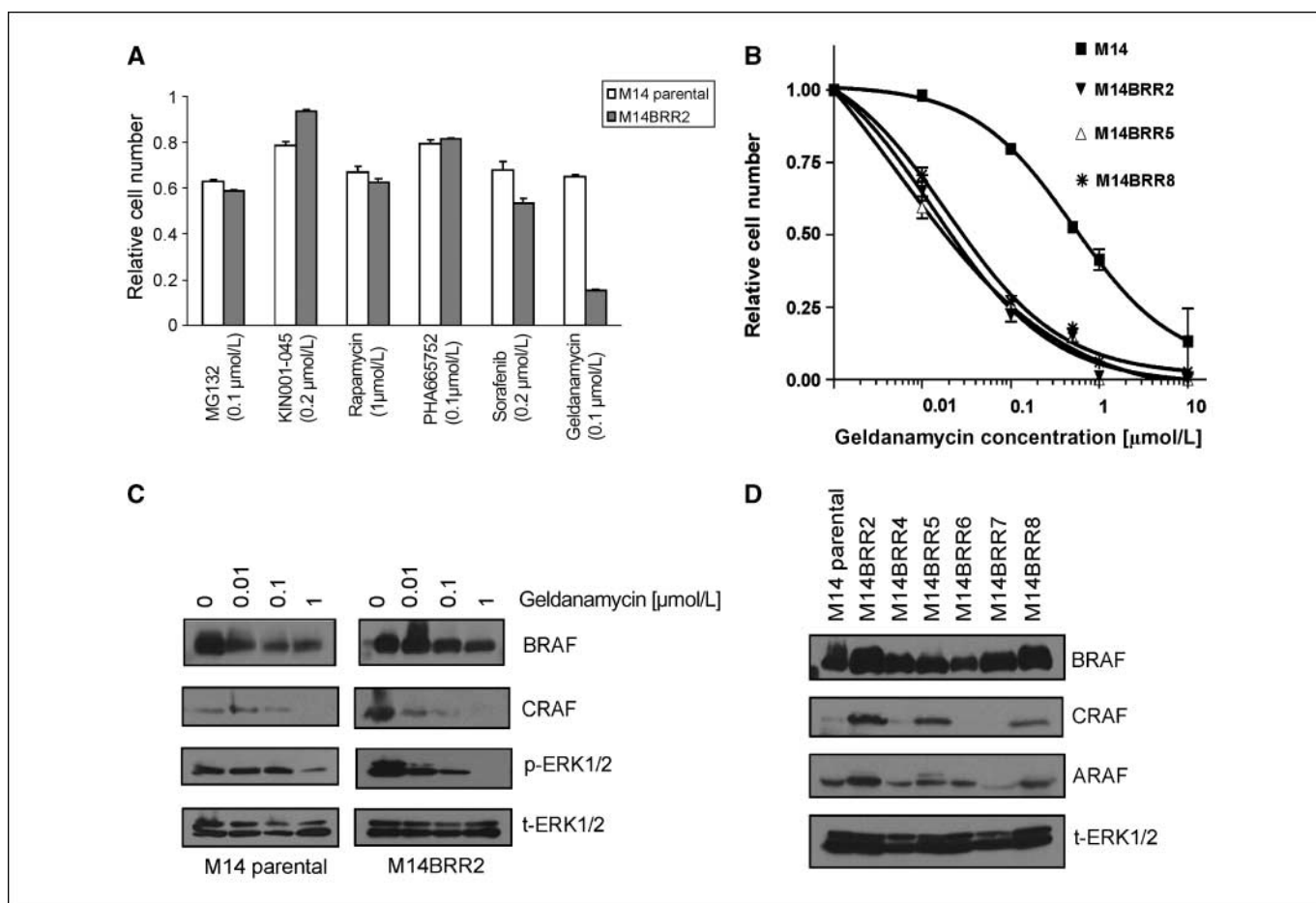


Figure 3. M14 AZ628-resistant clones express elevated CRAF and exhibit geldanamycin sensitivity. **A**, increased sensitivity of AZ628-resistant cells to geldanamycin. Survival curves of M14 and AZ628-resistant (M14BRR) clones treated with the indicated concentrations of the indicated drugs. MG132, proteasome inhibitor, nuclear factor- κ B inhibitor; KIN001-045, src inhibitor; rapamycin, mTOR inhibitor; PHA-665752, MET kinase inhibitor; sorafenib, multikinase inhibitor; geldanamycin, HSP90 inhibitor. **B**, AZ628-resistant clones exhibit increased geldanamycin sensitivity. Dose-response of M14 and three M14BRR clones treated with the indicated concentrations of geldanamycin. The fraction of viable cells is expressed relative to untreated controls. *Error bars*, SD from the mean. **C**, geldanamycin causes BRAF reduction and CRAF depletion in M14 and AZ628-resistant clones. Cell lysates from M14 and M14BRR2 cells were collected after treatment with the indicated concentrations of geldanamycin for 24 h. Immunoblotting analysis was performed using antibodies directed against the indicated proteins. **D**, elevated CRAF expression in a subset of AZ628-resistant M14-derived clones. Cell lysates from M14 and six M14BRR clones maintained in the presence of 2 μmol/L of AZ628 were collected. Immunoblotting analysis was performed using antibodies directed against the indicated proteins.

Results

Generation of melanoma cell line clones with acquired resistance to the RAF kinase inhibitor AZ628. To identify potential mechanisms of acquired resistance to a selective RAF kinase inhibitor, we used the M14 human melanoma-derived cell line. These cells harbor the V600E BRAF mutation and are exquisitely sensitive to the potent and selective RAF inhibitor AZ628 (11). Five weeks after continuously exposing M14 cells to 2 μmol/L AZ628, a concentration that rapidly promotes growth inhibition and cell death in the vast majority of treated cells, single cell-derived drug-resistant clones emerged at a frequency of ~1 in 10⁴ cells. Six of these clones were isolated and expanded for further characterization.

Morphologically, the M14-derived AZ628 resistant (M14BRR) clones are flat and epithelial-like when compared with the parental M14 cell line (Supplementary Fig. S1). Their growth properties are otherwise indistinguishable from the parental cells. A drug titration assay of cell viability showed that AZ628-resistant clones are ~100-fold more resistant to AZ628 than the parental cell line, exhibiting an IC₅₀ of ~10 μmol/L compared with 0.1 μmol/L for

the parental cell line (Fig. 1A). Similar results were observed with an alternative RAF-selective inhibitor that is currently undergoing clinical development (data not shown). DNA sequence analysis of the AZ628-resistant clones excluded the presence of any secondary mutations in BRAF, a potential mechanism reported to contribute to acquired resistance to other kinase inhibitors in other tumor types (12–14). Furthermore, the presence of the V600E activating BRAF mutation in the resistant clones (not shown) confirmed that they had not arisen from a contaminating subpopulation of cells.

Resistance to AZ628 is associated with elevated levels of the RAF downstream effector p-ERK1/2. To further investigate the mechanism underlying acquired AZ628 resistance in these cells, we performed immunoblotting studies of established RAF downstream effectors in the parental cell line and the resistant clones (Fig. 1B). In three of the resistant clones (M14BRR2, M14BRR5, and M14BRR8), basal activation of the downstream effector ERK1/2 was significantly increased relative to levels seen in the parental cell line. Therefore, we hypothesized that ERK1/2 might play a pivotal role in the mechanism of acquired resistance to AZ628, in at

least a subset of cases, and these three resistant clones were further characterized.

We next compared the effect of AZ628 treatment on RAF-dependent signaling on the M14 cells and drug-resistant derivatives. As we have previously reported, sensitivity to AZ628 is correlated with suppression of the downstream effector p-ERK1/2 after treatment of various melanoma-derived cell lines harboring BRAF activating mutations (11). Effective suppression of p-ERK1/2 levels was observed in the M14 parental cell line after treatment with increasing concentrations of AZ628. In contrast, p-ERK1/2 activity persisted at high levels in the resistant clones after AZ628 exposure, suggesting that sustained activation of ERK1/2 signaling may be critical to the maintenance of cell proliferation and survival in these cells and may play a role in conferring resistance to AZ628 (Fig. 1C and Fig. 2B).

ERK1/2 activation in AZ628-resistant clones is mediated by MEK. To determine whether activation of ERK1/2 in AZ628-resistant clones is mediated by the upstream ERK activator MEK, we assessed the sensitivity of the resistant clones to the selective MEK inhibitor U0126 (Fig. 2A). The IC_{50} of the resistant clones for U0126 ranged from 3 to 7 $\mu\text{mol/L}$ and was very similar to that of the M14 parental cell line (2 $\mu\text{mol/L}$). Biochemical analysis revealed that sensitivity to U0126 was consistent with the suppression of signaling to the downstream effector ERK1/2 in both the M14 parental cells and resistant clones (Fig. 2B). An unrelated human tumor cell line, A431 (negative control), was relatively insensitive to U0126 (IC_{50} of 10 $\mu\text{mol/L}$), and consistent with this, there was no detectable attenuation of p-ERK1/2 levels upon treatment with the inhibitor (Fig. 2B). Thus, persistent ERK1/2 activation in the AZ628-resistant clones seems to be mediated by MEK.

Sustained proliferation of AZ628-resistant clones is largely independent of BRAF kinase activity. We next explored the mechanism of persistent MEK-mediated ERK1/2 activation in AZ628-resistant clones in the presence of drug. Although we had excluded the role of a secondary BRAF mutation as a resistance

mechanism, it remained possible that reduced bioavailability of the inhibitor was involved in mediating resistance. A second possibility was that MEK-ERK activation was no longer driven by BRAF kinase activity, but instead involved another aberrantly activated intracellular pathway, as previously described in the setting of acquired resistance to other selective kinase inhibitors (16). To distinguish between these possibilities, we used lentivirus-mediated delivery of BRAF shRNAs to down-regulate the expression of BRAF in the resistant clones. Immunoblotting showed successful and specific depletion of BRAF protein using BRAF-directed shRNA in both the parental M14 cells and the M14BRR2 AZ628-resistant clone (Fig. 2C). As expected, knockdown of BRAF in the parental M14 cells resulted in a substantial decrease in cell viability, consistent with dependency on BRAF kinase activity in these cells (Fig. 2D). However, depleting BRAF protein in the M14BRR2 cell line or in the unrelated A549 tumor cell line (negative control) only had a very mild effect on cell viability, suggesting that these cells exhibited substantially reduced dependency on BRAF for their sustained survival (Fig. 2D). Moreover, whereas down-regulation of BRAF protein in the M14 parental cells resulted in complete abrogation of ERK1/2 activation, in the AZ628-resistant clones, p-ERK1/2 was only partially suppressed (Fig. 2C), suggesting an uncoupling of ERK1/2 from BRAF in the resistant clones. Taken together, these results strongly suggest that sustained survival of the AZ628-resistant clones is mediated through an alternative activated pathway that is largely independent of BRAF kinase activity.

AZ628-resistant clones express elevated CRAF. To identify the activated pathway that contributes to cell survival via sustained MEK-ERK signaling in the AZ628-resistant clones, we examined the sensitivity of these cells to a variety of small molecule inhibitors of cellular signaling pathways implicated in cancer (Fig. 3A). Among the many tested inhibitors, the resistant clones exhibited significantly increased sensitivity to the HSP90 inhibitor geldanamycin relative to the parental M14 cell line (Fig. 3A and B). HSP90

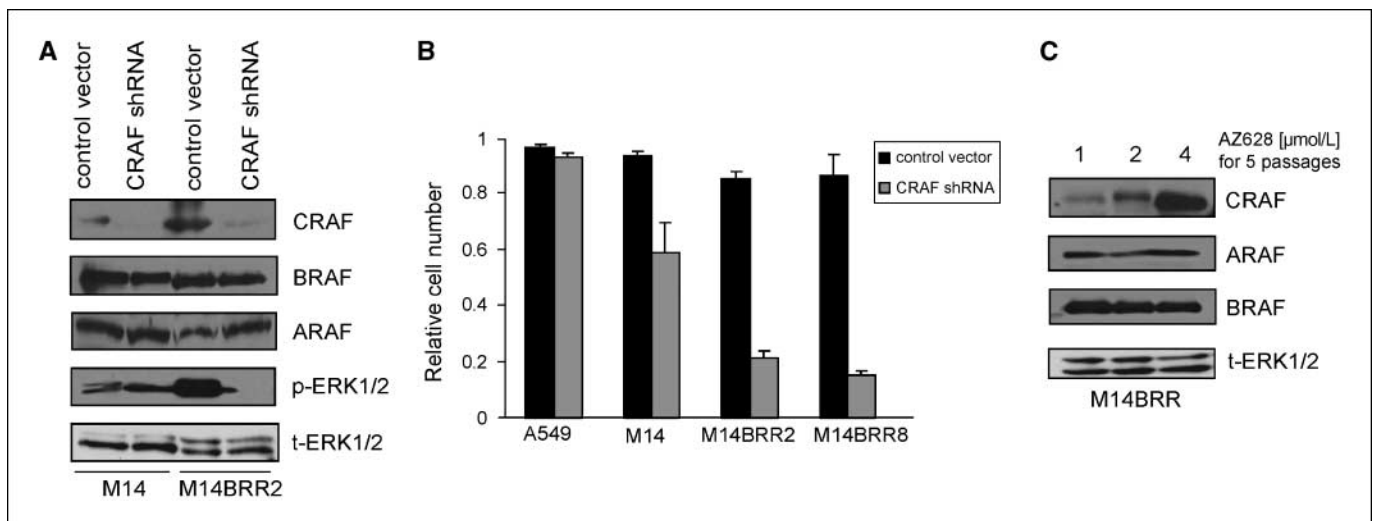


Figure 4. Proliferation of AZ628-resistant M14 clones is dependent on CRAF. **A**, down-regulation of CRAF in AZ628-resistant clones results in reduced p-ERK1/2. M14 and M14BRR2 cells were infected with a lentivirus control (PLKO.1 empty vector) or a virus expressing CRAF-specific shRNA. Cells were puromycin-selected and protein lysates were collected 4 d after the infection. Immunoblotting analysis was performed using antibodies directed against the indicated proteins. **B**, AZ628-resistant M14 cells are dependent on CRAF. Cell viability assay corresponding to **A**. Control or CRAF-specific shRNAs were introduced into A549, M14, M14BRR2, and M14BRR8 cells, and cell proliferation assays with Syto60 were performed 5 d later. The fraction of cells relative to untreated controls is expressed. Error bars, SD from the mean. **C**, CRAF levels in AZ628-resistant cells vary proportionately to the concentration of AZ628 in which cells are maintained. Cell lysates from M14BRR2 cells growing in the indicated concentrations of AZ628 for several passages were collected. Immunoblotting analysis was performed using antibodies directed against the indicated proteins.

is a chaperone protein required for conformational stability of various proteins, including mutant V600E BRAF and the BRAF-related RAF family member CRAF (27–30). Therefore, we examined levels of the three RAF isoforms in these cells after geldanamycin treatment. As previously reported (28, 29), we observed that geldanamycin promotes a reduction in BRAF protein levels, although the decline in CRAF protein levels in these cells was notably more substantial, associated with a virtually complete elimination of CRAF protein in both geldanamycin-treated M14 parental cells and the resistant clones (Fig. 3C). A role for CRAF in the AZ628-resistant cells was further supported by the observation that some of the resistant clones expressed significantly elevated basal levels of CRAF relative to the M14 parental cells (Fig. 3D), whereas BRAF and ARAF protein expression was not detectably changed. As expected, the AZ628-resistant clones that expressed elevated CRAF protein levels (M14BRR2, M14BRR5, and M14BRR8) correspond to the clones demonstrating increased p-ERK1/2 levels (Fig. 1B).

FISH analysis of interphase chromosomal spreads revealed that the increased expression of CRAF in the AZ628-resistant cells was not associated with CRAF gene amplification (Supplementary Fig. S2). Similarly, RT-qPCR analysis showed indistinguishable CRAF mRNA levels in both the parental cell line and the resistant clones (Supplementary Fig. S3). Taken together, these data suggest that resistance to AZ628 was associated with increased CRAF protein levels that cannot be attributed to gene amplification or increased CRAF gene transcription. Thus, changes in CRAF protein levels in the resistant cell lines seem to reflect a posttranscriptional regulatory mechanism.

Survival of AZ628-resistant cells is dependent on CRAF. To confirm that proliferation and survival of the AZ628-resistant clones is truly mediated by CRAF, we used an RNA interference approach. M14 cells and the AZ628-resistant clones were infected with a lentiviral vector expressing shRNA designed to specifically target CRAF. Immunoblotting showed specific and effective down-regulation of CRAF after infection (Fig. 4A). We observed a

considerable reduction in cell proliferation after CRAF down-regulation in the AZ628-resistant clones (M14BRR2 and M14BRR8), whereas the effect on M14 cells was significantly less (Fig. 4B). Furthermore, ERK1/2 activation was completely suppressed by CRAF depletion in the resistant clones (M14BRR2), but not in the M14 parental cells (Fig. 4A), suggesting that ERK1/2 activation is tightly coupled to CRAF in the AZ628-resistant cells.

During the course of characterizing the AZ628-resistant clones, we observed that varying the concentration of AZ628 in which cells were propagated led to a corresponding change in their expression of CRAF protein. Thus, after five passages in 4 $\mu\text{mol/L}$ AZ628, a significant increase in CRAF protein expression was observed (relative to cells propagated in 2 $\mu\text{mol/L}$ AZ628), whereas there was a relative decrease in CRAF levels in cells growing in 1 $\mu\text{mol/L}$ AZ628 (Fig. 4C). Significantly, ARAF and BRAF protein levels were unchanged under these conditions. Such a correlation between AZ628 concentration and CRAF protein levels is consistent with a “compensatory” regulation of CRAF expression to permit cell growth and survival in the presence of AZ628.

To confirm a causal role for increased CRAF expression in the AZ628-resistant phenotype, we determined that stable overexpression of CRAF in the M14 parental cells confers significant resistance to AZ628 (IC_{50} , 2 $\mu\text{mol/L}$) when compared with M14 cells transfected with a vector control (IC_{50} , 100 nmol/L ; Fig. 5A–C). However, we note that CRAF-overexpressing M14 cells were not as resistant to AZ628 as the clones that were initially selected in 2 $\mu\text{mol/L}$ AZ628 (IC_{50} 10 $\mu\text{mol/L}$). Taken together, these results suggest that elevated CRAF expression is a potential mechanism of acquired resistance to continuous AZ628 exposure, leading to sustained activation of ERK1/2.

Elevated CRAF can confer primary insensitivity to a RAF inhibitor. In a high-throughput profiling study of AZ628 sensitivity across 500 human tumor-derived cell lines, we recently showed that, whereas sensitivity to AZ628 is highly correlated with the presence of the activating BRAF V600E mutation, a small subset of cell lines exhibit primary insensitivity to AZ628 despite harboring

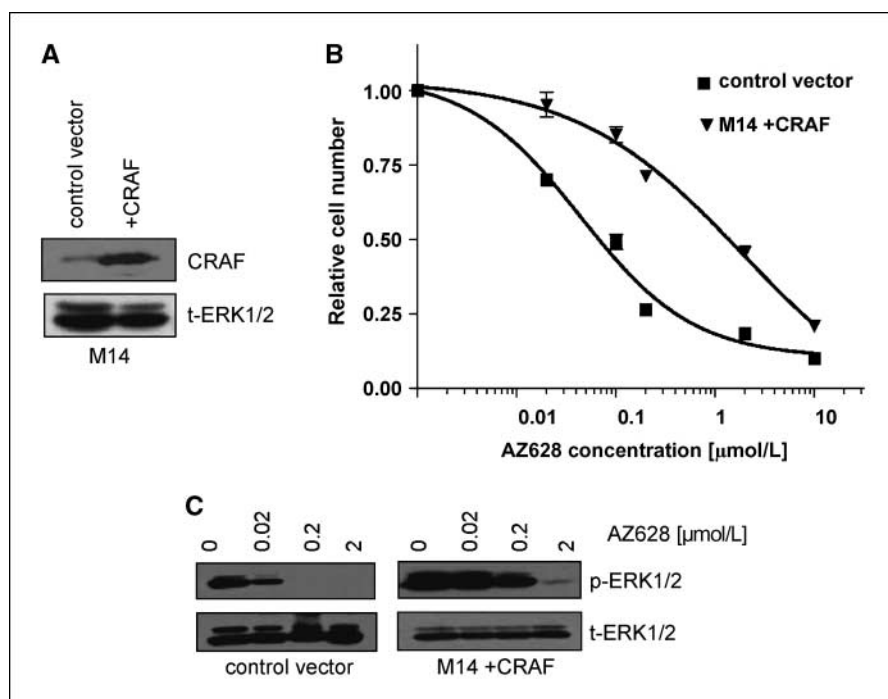


Figure 5. CRAF overexpression can confer resistance to RAF inhibition. **A**, CRAF cDNA or control (pBabe empty vector) were introduced into M14 parental cells. Cell lysates were collected and immunoblotting analysis was performed using antibodies directed against CRAF and t-ERK 1/2 (loading control). **B**, M14 cells expressing exogenous CRAF exhibit reduced sensitivity to AZ628. Dose-response curves corresponding to cells in **A**. M14 + CRAF and M14 + pBabe control vector cells were treated with the indicated concentrations of AZ628. The fraction of viable cells is expressed relative to untreated controls. *Error bars*, SD from the mean. **C**, M14 cells expressing exogenous CRAF exhibit reduced suppression of ERK1/2 activation after AZ628 treatment. Cell lysates from M14 + pBabe control vector and M14 + CRAF were collected after treatment with the indicated concentrations of AZ628 for 2 h. Immunoblotting analysis was performed using antibodies directed against the indicated proteins.

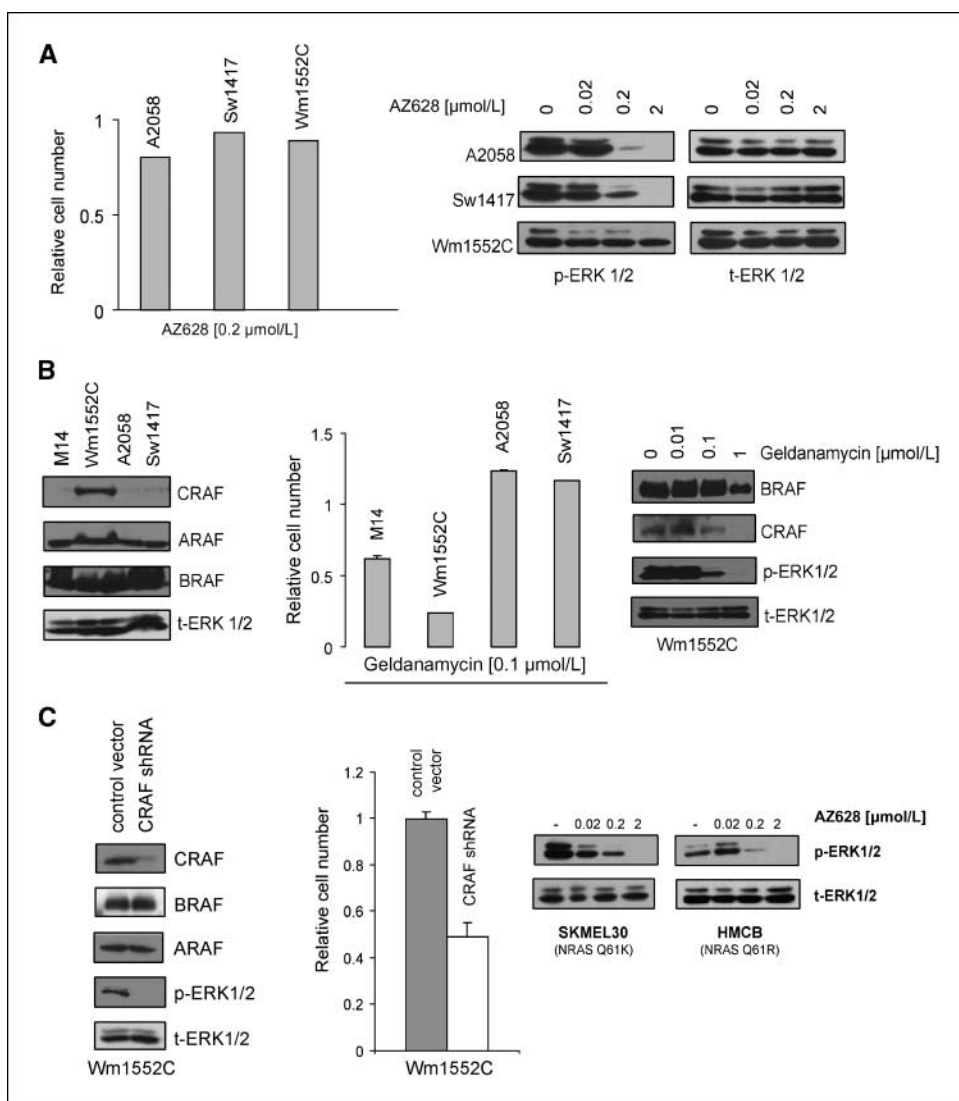


Figure 6. CRAF overexpression can confer insensitivity to RAF inhibition. *A, left*, AZ628 insensitivity among three BRAF mutant tumor cell lines. Three AZ628-insensitive BRAF mutant cell lines (A2058, Sw1417, and Wm1552C) were treated with 0.2 $\mu\text{mol/L}$ AZ628, and a proliferation assay with Syto60 was performed 72 h later. The fraction of viable cells is expressed relative to untreated controls. *Error bars*, SD from the mean. *Right*, AZ628 fails to suppress p-ERK1/2 in Wm1552C cells. Cell lysates from three AZ628-insensitive cell lines were collected after treatment with the indicated concentrations of AZ628 for 2 h. Immunoblotting analysis was performed using antibodies directed against p-ERK1/2 and t-ERK1/2 (loading control). *B, left*, CRAF levels are relatively high in Wm1552C cells. Cell lysates from M14 and three AZ628-insensitive cell lines were collected. Immunoblotting analysis was performed using antibodies directed against the indicated proteins. *Middle*, Wm1552C cells exhibit geldanamycin sensitivity. M14 and three AZ628-insensitive cell lines (Wm1552C, A2058, Sw1417) were treated with 0.1 $\mu\text{mol/L}$ geldanamycin, and a proliferation assay with Syto60 was performed 72 h later. The percentage of viable cells is expressed relative to untreated controls. *Error bars*, SD from the mean. *Right*, geldanamycin treatment causes CRAF depletion and suppresses ERK1/2 activation in Wm1552C cells. Cell lysates from Wm1552C were collected after treatment with the indicated concentrations of geldanamycin for 24 h. Immunoblotting analysis was performed using antibodies directed against the indicated proteins. *C, left*, CRAF depletion by shRNA suppresses ERK1/2 activation in Wm1552C cells. Wm1552C cells were infected with a lentivirus control (PLKO.1 empty vector) or a virus expressing CRAF-specific shRNA. Cells were puromycin-selected, and protein lysates were collected 4 d after the infection. Immunoblotting analysis was performed using antibodies directed against the indicated proteins. *Middle*, CRAF depletion by shRNA inhibits cell growth in Wm1552C cells. Cell viability corresponding to cells in left panel. Control or CRAF-specific shRNAs were introduced in Wm1552C cells and a cell proliferation assay with Syto60 was performed 5 d later. The fraction of viable cells is expressed relative to untreated controls. *Error bars*, SD. *Right*, immunoblots demonstrating that AZ628 effectively suppresses ERK phosphorylation in two different melanoma cell lines that harbor activated *NRAS* alleles.

the V600E BRAF mutation (11). To determine if elevated CRAF expression could potentially account for insensitivity to BRAF inhibitors in such lines, we further characterized the three most AZ628-insensitive cell lines harboring BRAF V600E mutations, A2058, Sw1417, and Wm1552C (Fig. 6A). p-ERK1/2 activity was not significantly suppressed by exposure to AZ628 in one of these three AZ628-insensitive cell lines (Wm1552C) suggesting that, as in the M14-derived AZ628-resistant clones, activated ERK1/2 was uncoupled from BRAF in this cell line (Fig. 6A).

Significantly, immunoblot analysis revealed relatively high levels of CRAF protein in the Wm1552C cell line (Fig. 6B). Wm1552C is a melanoma-derived cell line that harbors the BRAF V600E mutation and is highly refractory to AZ628 treatment (IC_{50} , 10 $\mu\text{mol/L}$), as well as to an alternative RAF inhibitor (data not shown). Interestingly, in a high-throughput screen of 500 tumor cell lines for geldanamycin sensitivity, Wm1552C scored as being exquisitely sensitive to geldanamycin when compared with the other AZ628-insensitive cell lines, consistent with a potential role for elevated

CRAF in maintaining cell proliferation in Wm1552C cells (Fig. 6B and data not shown). Consistent with this possibility, immunoblot analysis of geldanamycin-treated Wm1552C cells showed markedly reduced CRAF protein levels and suppression of ERK1/2 activation (Fig. 6B).

To confirm that Wm1552C cells are in fact dependent on CRAF, we used the lentiviral-mediated CRAF shRNA to reduce CRAF protein in these cells. After efficient depletion of CRAF in Wm1552C cells, a significant decrease in viability was observed (Fig. 6C). Taken altogether, our findings suggest that elevated CRAF levels may also represent a mechanism that confers primary insensitivity to RAF kinase inhibition in a subset of tumor cells harboring the BRAF V600E mutation. Because it has been reported that, in NRAS mutant melanomas, ERK activation is mediated largely through CRAF (8), we also examined the ability of AZ628 to suppress ERK phosphorylation in two different melanoma lines that harbor activated NRAS alleles. We found that, unlike in the AZ628-resistant M14 cells in which AZ628 fails to suppress ERK activation, AZ628 treatment efficiently attenuates ERK activation in the NRAS mutant melanoma cells, suggesting distinct roles for CRAF in the activation of ERK in these two settings (Fig. 6C).

Discussion

Kinase-targeted drugs have emerged as an important new class of cancer therapeutics, with demonstrated clinical efficacy in multiple tumor contexts. However, treatment with such agents is invariably associated with the eventual emergence of drug resistance, which remains the most significant limitation of such therapies. Therefore, it is important to identify mechanisms underlying acquired drug resistance, as well as to use preclinical models to reveal resistance mechanisms that are likely to be observed clinically to accelerate the development of strategies to overcome or prevent such resistance. Here, we have described a cell culture model of acquired resistance to RAF inhibition in a BRAF V600E mutated melanoma cell line with exquisite sensitivity to the selective RAF kinase inhibitor AZ628. Inhibitors of this class are currently undergoing early clinical testing, and consequently, it will be quite some time before mechanisms of acquired drug resistance can be established from clinical specimens, assuming that these agents produce clinical benefit. Our findings have revealed a potential mechanism underlying acquired resistance to a selective RAF kinase inhibitor and, furthermore, have shown that HSP90 inhibition may be an effective therapeutic strategy to overcome such resistance. Because HSP90 inhibitors are currently undergoing clinical evaluation as cancer therapeutics (30–32), our findings may prompt another potential application of such agents. Notably, a recent report showed that HSP90 inhibition effectively promotes tumor regression in a mouse transgenic model of EGFR, in which the T790M TKI-resistance mutation was expressed (33). Such findings, together with our findings, implicate HSP90 inhibitors as potentially important drugs in a variety of drug resistance settings.

Previous preclinical cell culture–based studies have shown that most BRAF mutant tumor cells are in fact “addicted” to BRAF and that signaling to the downstream effector ERK, via the MEK kinase, is a critical pathway by which BRAF drives cell proliferation and survival in such tumors (6, 7, 9, 34). Our finding that M14 melanoma cells are growth inhibited by both BRAF and MEK kinase inhibitors is consistent with those observations. Moreover, the sustained ERK activation seen in drug-treated M14-derived AZ628-resistant cells is also consistent with a critical role for this

pathway in these melanoma cells. Notably, not all of the individual clones exhibit the same signaling properties, suggesting that multiple distinct mechanisms of acquired resistance can be established through such modeling, and further study will certainly be required to reveal such alternative resistance mechanisms in those clones.

We focused on the subset of AZ628-resistant clones in which ERK signaling is sustained in the presence of the inhibitor. Interestingly, these cells exhibit significantly elevated expression of CRAF, which seems to involve a posttranscriptional mechanism. Although this finding suggests that these cells may have switched their dependency from BRAF to CRAF, it is noteworthy that AZ628 inhibits the BRAF and CRAF isoforms somewhat equally *in vitro*.³ Therefore, it is possible that increased CRAF protein levels decrease the bioavailability of drug within cells by virtue of increased intracellular concentration of drug-binding targets. Similarly, increased expression of the BCR-ABL kinase, frequently as a result of specific gene amplification, in imatinib-resistant cases of CML may contribute to resistance through a similar drug titration mechanism (13).

The increased expression of CRAF in the AZ628-resistant cells suggests that these cells may have shifted their dependency from BRAF to CRAF. Notably, this switch does not necessarily require CRAF kinase activity, as previous reports have shown kinase-independent functions for CRAF (35, 36). The fact that the AZ628-resistant cells can be growth inhibited by shRNA-mediated knockdown of CRAF, but much less so by knockdown of BRAF, is consistent with a shift in their dependency from BRAF to CRAF. Previous studies have shown a physical interaction between BRAF and CRAF, with BRAF promoting CRAF-mediated ERK activation through the formation of BRAF/CRAF heterodimers, thereby demonstrating an intimate relationship between these two proteins in regulating the ERK pathway (8, 37, 38). Moreover, recent studies have revealed a role for cyclic AMP (cAMP) signaling in a switch from BRAF to CRAF dependency for MEK-ERK signaling in BRAF mutant melanoma cells (39). Thus, it is possible that the acquisition of AZ628 resistance in M14 melanoma cells similarly involves altered cAMP signaling. It is worth noting that the parental M14 cells also display some CRAF dependency, as revealed by gene knockdown studies, and that the M14 cells transfected with CRAF are not as resistant to AZ628 as the selected clones. Thus, the collective data point to a critical balance between BRAF and CRAF in parental M14 cells that drives ERK signaling, which is substantially affected by the relative levels of these proteins. However, the precise mechanism by which CRAF levels are increased to disrupt this balance in the drug-resistant clones is currently unknown.

Molecular mechanisms of acquired resistance to kinase inhibitors can also contribute to primary insensitivity to such treatment in some cases. For example, the T790M EGFR mutation that seems to account for acquired resistance to EGFR TKIs in about half of TKI-responsive patients who subsequently relapse has also been detected in a small subset of untreated tumors (40–42). Similarly, amplification of the gene encoding the MET tyrosine kinase, which occurs in a small percentage of EGFR TKI-treated NSCLCs, has also been detected in NSCLCs demonstrating primary insensitivity to treatment with EGFR TKIs (11). Based on our preclinical findings, CRAF overexpression may similarly represent both a mechanism of acquired drug resistance and primary drug insensitivity in a subset of cases. The fact that tumor cell lines demonstrating elevated CRAF protein are highly geldanamycin-sensitive, irrespective of whether they were derived through acquired resistance or not, suggests that

elevated CRAF protein may constitute a tumor biomarker that predicts response to HSP90 inhibition, as well as a lack of response to BRAF kinase inhibitors, in both of these clinical settings.

Disclosure of Potential Conflicts of Interest

L. Drew: employee, AstraZeneca. The other authors disclosed no potential conflict of interest.

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Elevated CRAF as a Potential Mechanism of Acquired Resistance to BRAF Inhibition in Melanoma

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