Abstract

The efficacy of selective BRAF inhibitors has now been established in the 50% of patients with metastatic melanoma whose tumors harbor activating mutations. However, for the vast majority of patients, responses persist for less than a year. In extensive preclinical investigations, researchers have focused on potential resistance mechanisms with the hope of identifying treatment strategies that can overcome resistance. Preliminary results suggest that reactivation of the mitogen-activated protein kinase (MAPK) pathway by several BRAF-independent mechanisms is the predominant pattern. However, MAPK pathway-independent mechanisms also seem to play a potential role. More definitive cataloging of resistance mechanisms in patients’ tumor samples is needed as combination regimens are being readied for clinical evaluation.

Clinical Trials

Vemurafenib

The current treatments for metastatic melanoma (i.e., interleukin-2 and dacarbazine) are largely ineffective because they provide no improvement in overall survival (11). Dacarbazine in particular has a response rate of only 15% to 20%. The discovery that >50% of melanomas harbor a mutation in the BRAF gene heralded the development of targeted therapies against the corresponding mutant, activated protein (6).

The small-molecule BRAF inhibitor vemurafenib showed promising results in a phase I trial (7). This multicenter phase I trial consisted of 2 phases: a dose-escalation phase and an extension phase. In the dose-escalation phase, investigators determined the recommended phase II dose to be 960 mg twice daily. Higher doses than this resulted in intolerable fatigue, rash, and arthralgias.

The small-molecule BRAF inhibitor vemurafenib also resulted in tumor shrinkage in most patients with mutated BRAF in a phase I trial (8). Despite the promising results of these trials and the demonstrated clinical benefit, responses are short-lived in many patients as a result of mechanisms that are not fully understood. Some studies have shed light on the molecular correlations of drug resistance (Fig. 2). Melanoma cell lines treated with BRAF inhibitors show rebound phosphorylated ERK (pERK) activation and escape from BRAF inhibition (9). Second-site mutations that confer resistance have not been observed in BRAF to date (10). It is crucial to gain a thorough understanding of the underlying mechanisms so that we can develop novel strategies to circumvent resistance and achieve more-prolonged responses.

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low invasive potential and no metastatic potential, and they can be easily excised. The purpose of the extension phase was to determine the response rate according to Response Evaluation Criteria in Solid Tumors (RECIST) in patients with metastatic melanoma harboring the \( \text{BRAF} \text{V600E} \) mutation. Patients with brain metastases were excluded from the study. Of the 32 patients in the extension cohort, 26 (81%) responded to treatment, with 68% of all patients having responses that were sufficiently durable to be confirmed on subsequent scans. Two patients showed a complete response, and 24 showed partial responses. Although some responses are transient, with a median progression-free survival (PFS) time of \( \sim 7 \) months (including both responders and non-responders), some patients show continued response for \( > 2 \) years (12).

The single-arm, multicenter, open-label phase II trial known as BRIM2 had a primary endpoint of overall response rate, with a target of 20%, and secondary endpoints of duration of response, PFS, and overall survival (13). A total of 132 previously treated patients with \( \text{BRAF}^{\text{V600E}} \)-positive metastatic melanoma were enrolled in the study. A companion diagnostic assay codeveloped with vemurafenib, known as the cobas 4800 \( \text{BRAF} \text{V600E} \) Mutation Test (Roche Molecular Diagnostics), was used to determine the \( \text{BRAF} \) mutational status of patients enrolled in the study. All patients were treated with 960 mg of vemurafenib twice daily until disease progression occurred. At the time of analysis, the median follow-up was 7 months. With a response rate of 52% (62/132), consisting of 59 partial responses and 3 complete responses, the trial met its primary endpoint. The median duration of response was 6.8 months, and the median PFS was 6.2 months. Consistent with the phase I trial, the most common side effects (\( > 25\% \)) were rash, fatigue, and arthralgias. The most common grade 3 adverse event was cutaneous squamous cell carcinoma (24.2%), which was excised without interfering with the trial.

An additional study was conducted to investigate whether vemurafenib is associated with improved survival compared with dacarbazine, the standard of care. This study, a global, randomized, open-label, controlled, multicenter phase III trial known as BRIM3, accrued 675 untreated patients with \( \text{BRAF}^{\text{V600E}} \)-positive metastatic melanoma (14). The study met the primary endpoints of showing improvement in overall survival and PFS at the time of the interim analysis, which occurred 1 month after the last patient was accrued. With a median follow-up of just over 3 months, the interim analysis revealed that the vemurafenib group achieved a 6-month overall survival of 84%, compared with 64% in the dacarbazine group. On the basis of these extraordinary results, patients in the dacarbazine group were allowed to switch immediately to the vemurafenib group. The median PFS was 5.3 months for vemurafenib compared with 1.6 months for dacarbazine. Consistent with previous trials, the response rate of vemurafenib was 48% compared with
5% for dacarbazine, and the grade 3 or worse adverse effects consisted primarily of arthralgias (3%), rash (8%), fatigue (2%), and cutaneous squamous cell carcinomas or keratoacanthoma (20%).

Vemurafenib is the first personalized treatment for metastatic melanoma to show an improvement in overall survival, and it was approved by the U.S. Food and Drug Administration in 2011.

**Dabrafenib**

Dabrafenib (formerly known as GSK2118436) is a selective BRAF inhibitor that was designed for the treatment of BRAF-mutated cancers. Unlike the trial for vemurafenib, the phase I trial for dabrafenib included patients with other V600-position BRAF mutations, such as V600K and V600D, and patients with previously untreated brain metastases (8, 15). The dose-escalation phase of the trial determined the maximum tolerated dose to be 150 mg twice daily. At this level, the drug inhibited intratumoral phospho-ERK by >90%. Twenty patients, 18 of whom had metastatic melanoma, enrolled in the escalation phase of the trial and were treated with 150 mg twice daily. The response rate was 77% in patients with BRAF<sup>V600E</sup> mutations and 44% in patients harboring the BRAF<sup>V600K</sup> mutation. The preliminary PFS estimate was 8.3 months. The drug was well tolerated, with the only grade 3 events being fatigue (2%), fever (2%), headaches (2%), and squamous cell carcinomas. The inhibitor also showed evidence of activity in a cohort of 10 patients with brain metastases (15). Gadolinium-enhanced brain MRI for evaluation of intracranial lesions confirmed some degree of response in intracranial lesions in 9 patients. Shrinkage of brain metastases correlated with extracranial response. The success of this trial led to the development and execution of phase II and phase III trials.

The first attempt to develop a combination therapy involving BRAF inhibitors included other agents that target the MAPK pathway. Emerging evidence that some element of BRAF-inhibitor resistance is mediated by restored MAPK pathway activity prompted a phase I trial of dabrafenib in combination with the MEK inhibitor GSK1120212 (GSK212; ref. 16). Preclinical studies showed that the BRAF/MEK inhibitor combination was more active against BRAF-mutant cancer cells than either drug alone. Furthermore, it delayed emergence of resistance against dabrafenib and decreased the likelihood of squamous cell carcinomas. In a trial of 45 patients with BRAF<sup>V600E</sup>-positive solid tumors, the combination of GSK212 at 2 mg daily and dabrafenib at 150 mg twice a day, representing the maximum tolerated doses of each agent given individually, showed a favorable safety profile. Confirming preclinical studies, no squamous cell carcinomas developed at any dose level. Finally, in a group of 16 evaluable patients in the dose-escalation portion of the trial, 13 patients had partial responses and 3 had stable disease, for an overall response rate of 81%. On the basis of these promising results, the efficacy of the drug combination is being evaluated in a randomized phase II trial of patients with untreated metastatic melanoma.
Lessons learned from BRAF-inhibitor clinical development and strategies for optimal patient selection

The efficacy observed with vemurafenib and dabrafenib seems to be clearly related to the near-complete inhibition of the MAPK pathway achieved at tolerable doses. By comparison, sorafenib and RAF-265, 2 broad-spectrum kinase inhibitors (with BRAF being among the kinases inhibited), produced minimal evidence of tumor regression and achieved only modest MAPK pathway inhibition at their maximum tolerated doses (17, 18). [For a discussion about the pitfalls encountered in the development of targeted therapies, see the article by Bates and colleagues (19) in this CCR Focus section.] The selectivity of vemurafenib and dabrafenib likely accounts for the disparate results, because BRAF is inhibited to a far greater degree than other kinases.

With both selective BRAF inhibitors, there is a wide range in the magnitude of tumor regression observed early in the course of therapy and the subsequent duration of response (12). It is becoming increasingly clear that larger or symptomatic disease burden at baseline predicts for a lower likelihood of significant tumor regression and a shorter duration of response (13). A minority of patients, and largely those with lesser disease burden at baseline, have maintained response for ≥2 years. However, the molecular underpinnings of this heterogeneity in outcome have not yet been determined. In preclinical models, it is clear that activity within the PI3K pathway or CDK4 pathways can limit the degree to which BRAF mutant melanoma cells are sensitive to selective BRAF inhibitors (20, 21). Robust biomarkers of activity in these pathways have not yet been developed for interrogation of tumor samples from patients treated with vemurafenib or dabrafenib. However, efforts are under way to develop phospho-specific antibodies and genetic analyses for this purpose.

Mechanisms of Resistance

COT drives resistance to RAF inhibition through MAPK pathway reactivation

Johannessen and colleagues (22) identified the kinase COT as a MAPK pathway agonist that drives resistance to PLX4720, a selective RAF inhibitor that is closely related to vemurafenib. The investigators expressed ~600 kinase open reading frame (ORF) clones individually in A375, a human BRAF(V600E) mutant melanoma cell line that is sensitive to PLX4720. The cell lines were screened for viability in the presence of 1 μM of PLX4720. Two candidate ORFs, COT and C-RAF (both of which are kinases that activate the MAPK pathway), conferred resistance to the inhibitor. This result was confirmed in multiple B-RAF(V600E) cell lines. Furthermore, overexpression of MAP3K8 (COT) and RAF1 (C-RAF) caused MAPK pathway activation, as evidenced by MEK and ERK phosphorylation. The level of ERK phosphorylation was comparable to the level produced by constitutively active MEK, the positive control. This observation suggests that COT activates the MAPK pathway through an MEK-dependent mechanism.

Johannessen and colleagues further characterized the role of COT in melanoma. They found through in vitro studies that the levels of COT and B-RAF(V600E) were inversely related, suggesting that oncogenic B-RAF antagonizes COT expression. Of interest, short hairpin RNA (shRNA)-mediated depletion of endogenous B-RAF, C-RAF, or both, even in the presence of PLX4720, had no effect on COT-mediated activation of the MAPK pathway. Together, these findings point to a model in which treatment with BRAF inhibitors selects for cells that express COT, a kinase that induces MAPK pathway activation in a RAF-independent manner. The identification of MAPK pathway-inhibitor–naive melanoma cell lines with robust COT expression (OUMS-23 and RPMI-7951) and sustained ERK phosphorylation in the presence of PLX4720 supports this model of de novo resistance. It is important to understand the phenomenon of insensitivity to initial RAF inhibition, or de novo resistance, because it is observed in ~10% of BRAF(V600E)-mutant metastatic melanomas (7).

Johannessen and colleagues (22) also provided evidence that COT may be involved in acquired resistance to RAF inhibitors. Biopsy samples from patients with metastatic B-RAF(V600E) melanoma undergoing treatment with vemurafenib showed higher COT expression during treatment than before treatment, and yet higher levels were detected in a relapse specimen. No additional mutations were detected in BRAF, NRAS, or KRAS. This study indicates that COT may be responsible for at least some cases of acquired resistance to vemurafenib in metastatic melanoma.

The investigators shed light on the mechanism of COT-dependent MAPK pathway activation. They found that COT-expressing cancer cell lines are refractory not only to RAF inhibitors but also to MEK1/2 inhibitors. These lines showed sustained ERK phosphorylation in the context of MEK inhibition, raising the possibility that COT may activate ERK through an MEK-independent mechanism as well as an MEK-dependent mechanism. Thus, ERK inhibition or direct COT inhibition may be needed to intercept this potential bypass mechanism.

Genetic analysis of a single patient’s tumor sample at the time of disease progression on vemurafenib and a separate sample from a tumor biopsy before treatment revealed the presence of an activating MEK1 mutation (23). This mutation was subsequently characterized biochemically to activate ERK in a BRAF-independent fashion and, thus, could conceivably account for restoration of MAPK pathway signaling. An ERK inhibitor might be able to intercept this particular mechanism, but that possibility has not yet been tested directly. Because this mechanism has only been identified in a single patient’s tumor sample, it is unclear whether it represents a prevalent mechanism in the larger population of patients whose tumors progress on BRAF-inhibitor therapy.
Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation

Nazarian and colleagues (10) set out to study the mechanism of resistance in 3 cell lines with acquired resistance to vemurafenib in vitro. First, they established that Braf (V600E) does not acquire secondary mutations during the evolution of resistance to vemurafenib in melanoma cell lines and patient samples.

One of the cell lines showed strong resistance to vemurafenib, as evidenced by elevated pMEK1/2 and pERK levels, compared with the other 2 lines. Gene expression profiles showed a distinct signature for the resistant line, which was consistent with persistent MEK-ERK activation. In contrast, the other 2 lines retained vemurafenib sensitivity, suggesting a common mechanism of resistance. Clustering of receptor tyrosine kinase gene expression profiles showed that the 2 vemurafenib-sensitive lines differed from the parental lines, based largely on the higher expression levels of a number of RTK genes, including PDGFRβ. Furthermore, PDGFRβ protein showed overexpression on Western analysis and activation-associated tyrosine phosphorylation in a phospho-RTK array.

Nazarian and colleagues (10) validated this in vitro study in vivo by using immunohistochemistry to analyze patient samples from clinical trials. In 4/11 resistant tumor samples, PDGFRβ was overexpressed compared with the pretreatment tumor in the same patients.

As for the resistant line, sequencing of NRAS showed an NRAS(Q61K)-activating mutation that was not present in the parental cell line. The investigators validated this finding in 2/16 acquired-resistance biopsy samples. Mutated NRAS is known to induce a switch from Braf to CRAF, explaining the persistent MAPK signaling observed in the resistant line in the setting of vemurafenib-inhibited Braf (24).

Further analyses using siRNA and gene overexpression confirmed these findings. As predicted, MEK-inhibitor sensitivity was low in PDGFRβ- and high in NRAS-mediated growth and survival pathways.

Acquired resistance to BRAF inhibitors mediated by a RAF kinase switch in melanoma can be overcome by cotargeting MEK and IGF-1R/Pi3K

RAF kinase switch. Villanueva and colleagues (25) explored the role of various RAF kinase isomers in the mechanism of acquired resistance to the BRAF inhibitor SB-590885. Braf knockdown with shRNA resulted in inhibition of ERK phosphorylation in 451Lu parental melanoma cells, whereas no effect was observed in resistant 451Lu cells, suggesting that ERK phosphorylation in these cells occurs via a BRAF-independent mechanism. In addition, BRAF knockdown using shRNA caused an increase in CRAF protein level in both parental and resistant cells. These findings suggest that CRAF is responsible for sustained ERK phosphorylation in resistant lines. However, knockdown of CRAF with shRNA in BRAF-inhibitor–resistant cells had no effect on ERK activity, indicating that 885-resistant cells can activate the MAPK pathway independently of BRAF and CRAF. Finally, simultaneous shRNA-mediated inhibition of CRAF and ARAF and inhibition of BRAF with 885 resulted in suppression of ERK phosphorylation, indicating that any RAF isoform can activate ERK and drive proliferation of melanoma cells resistant to BRAF inhibitors.

Following this conclusion, Villanueva and colleagues tested the effect of MEK inhibition on ERK activity. Surprisingly, they found that ERK in BRAF-inhibitor–resistant cells remained sensitive to MEK inhibition, but the effect on cells was primarily cytostatic (an increase in G0/G1 cell cycle arrest), and there was little effect on the level of apoptosis (a constant SubG1 fraction) as determined by flow cytometry. This finding suggests that additional prosurvival pathways play a role in acquired resistance to BRAF inhibitors.

IGF-1R. To study the role of prosurvival pathways, Villanueva and colleagues (25) analyzed RTK phosphorylation using antibody arrays. Their analysis suggested that some RTKs were differentially phosphorylated in resistant 451Lu melanoma cells compared with the parental lines. Only IGF-1R seemed to play a role in resistance, because inhibition of the receptor led to decreased viability of BRAF-resistant cells [see the review by Pollak (26) in this CCR Focus section for a discussion about IGF-1R–targeted therapies that may be relevant as part of a combination therapy for BRAF mutant tumors]. Flow cytometry revealed that the resistant melanoma cells showed increased surface expression of IGF-1R. No mutations in IGF-1R or changes in copy number were detected. Moreover, resistant cells showed sustained IGF-1R phosphorylation. IGF-BP-3 may be a factor involved in the regulation of the IGF-1R system. This protein sequesters the ligand IGF-1 and prevents its binding to the receptor, regulating IGF-1R activation. Analysis of IGF-BP-3 mRNA by quantitative reverse transcriptase PCR showed that levels were upregulated in parental cells but downregulated in resistant cells. Downregulation of IGF-BP-3 with a concomitant increase in IGF-1 levels may explain persistent IGF-1R activity in the resistant cells.

IGF-1R can promote melanoma development through both the MAPK and PI3 kinase pathways. Inhibition of IGF-1R in 885-resistant cells had no effect on ERK activation but suppressed phosphorylation of AKT, a PI3-kinase target. Consistent with these results, upregression of IGF-1 in Mel1617 parental cells led to increased phosphorylation of AKT, without a significant effect on ERK phosphorylation. These data suggest that persistent IGF-1R signaling stimulates PI3K/AKT activation in V600E mutant melanomas that are resistant to BRAF inhibitors. Resistance seems to be mediated by the antiapoptotic factor Mcl-1, which is expressed at high levels in resistant Mel1617 cells and is downregulated in the context of IGF-1R inhibition. An evaluation of other members of the Bcl2 family of apoptosis regulator proteins revealed that MEK activation induces BAD phosphorylation, a prosurvival event, and MEK inhibition stimulates BIM, a proapoptotic factor. Together, these data suggest that coinhibition of MEK and IGF-1R generates a protein profile that favors apoptosis in BRAF-inhibitor–resistant melanoma. In further support of this
hypothesis, 1 of 5 paired tissue sets from relapsed melanoma patients treated with the BRAF inhibitor vemurafenib showed increased IGF-1R expression and AKT phosphorylation by immunohistochemistry in the resistant sample relative to the pretreatment sample.

Conclusions

The selective BRAF inhibitors vemurafenib and dabrafenib have shown promising results in clinical trials, where they induced tumor shrinkage in most patients with BRAF-mutated metastatic melanoma. Although some patients respond to treatment for years, progression of disease in other patients makes the elucidation of resistance mechanisms an essential undertaking. Investigators have established several models of resistance to BRAF inhibitors, along with potential approaches to circumvent resistance. Emerging evidence suggests that mutations in the kinase domain of BRAF itself do not play a role in resistance. Instead, bypass mechanisms that restore MAPK signaling in a BRAF-independent manner or that activate alternative proliferation or survival pathways seem to be 2 common patterns (Fig. 3).

Most of the data supporting these mechanisms of resistance were derived from preclinical models, with relatively little corroboration in human tumor samples. For those mechanisms for which a somatic genetic alteration has not been identified, it remains to be fully established that the implicated signaling molecules are causally related and not an epiphenomenon associated with resistance. Such is the case for CRAF, COT, IGF, and PDGFR. It will be critical to characterize these and other potential mechanisms in tumor samples that have been procured at the time of disease progression and compared with a pretreatment sample. Although resistance mutations in BRAF have not yet been defined, a more thorough and unbiased genetic analysis is warranted. Because several mechanisms of escape have been described, it will be important to catalog which mechanism is present in an individual patient’s tumor following BRAF-inhibitor therapy and before entry into clinical trials designed to target resistance-conferring signaling molecules. Based on the available evidence, intervention with MEK, ERK, and (in the future) COT inhibitors is worthy of further evaluation in BRAF-inhibitor–refractory patients. However, for cases in which certain receptor tyrosine kinases or the PI3K pathway as a whole seem to be contributing to resistance, the possibility of switching to or adding selective inhibitors to target those entities should be explored. An equally valid approach would be the exploration of combination therapy regimens containing a BRAF inhibitor as well as an agent that would be anticipated to intercept one of the described resistance mechanisms. The problem with this approach currently is that there are no predictive molecular markers to indicate which mechanism of resistance would be likely to emerge in an individual patient.

A distinct strategy would be to explore combinations of BRAF-targeted therapy with systemic therapies targeting constituents of the tumor microenvironment. Combinations with antiangiogenics or immunotherapy seem to be justifiable on the basis of preclinical evidence suggesting that BRAF inhibitors affect melanoma cells in a way that might complement these approaches (27, 28).

The lofty goal of transforming melanoma from a disease with a poor prognosis into a disease that can be managed chronically now seems nearer, as the biology underlying sensitivity and resistance to BRAF inhibition becomes better understood.

Disclosure of Potential Conflicts of Interest

K.T. Flaherty is a consultant for Glaxo Smith Kline and Roche/Genentech. A. Alcalá disclosed no potential conflicts of interest.

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References


