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Resistance to BRAF Inhibitors: Unraveling Mechanisms and Future Treatment Options

Jessie Villanueva, Adina Vultur, and Meenhard Herlyn

Abstract
The mitogen-activated protein kinase (MAPK) pathway has emerged as a central target for melanoma therapy due to its persistent activation in the majority of tumors. Several BRAF inhibitors aimed at curbing MAPK pathway activity are currently in advanced stages of clinical investigation. However, their therapeutic success is limited by the emergence of drug resistance, as responses are transient and tumors eventually recur. To develop effective and long-lasting therapies for melanoma patients, it is essential to understand the mechanisms underlying resistance to BRAF inhibitors. Here, we briefly review recent preclinical studies that have provided insight into the molecular mechanisms of resistance to BRAF inhibitors and discuss potential strategies to treat drug-resistant melanomas. Cancer Res; 71(23); 7137–40. ©2011 AACR.

Introduction
Current therapies for the treatment of metastatic melanoma, the most lethal form of skin cancer, offer limited clinical benefit. Patients with advanced disease have a poor prognosis and a 5-year survival rate of less than 20% (1). In the past decade, however, the mitogen-activated protein kinase (MAPK) pathway began to take center stage in melanoma therapy because it is commonly activated in tumors through mutations in BRAF, NRAS, receptor tyrosine kinases (RTK), and G-coupled protein receptors, or by growth-factor-mediated stimulation (2, 3). The MAPK pathway regulates many key biologic processes, including proliferation, survival, and metastasis, and thus curbing its activity is an attractive therapeutic endeavor (4). Early efforts were focused on the development of mutant BRAF inhibitors due to the presence of BRAF mutations in 50% of melanomas (5). The most common BRAF mutation (T1799A; BRAFV600E) causes constitutive kinase activity and hyperactivation of the MAPK pathway, providing a MAPK-relevant, tumor-specific target. Preclinical and clinical studies have now shown that targeting BRAF with the use of RAF-selective inhibitors results in remarkable tumor shrinkage in BRAFV600E melanomas (4, 6–9). Other activating mutations, such as V600K/D/R, also seem responsive to BRAF inhibitors (10). In a recent phase III trial in which patients with BRAFV600E melanomas were treated with the RAF inhibitor vemurafenib (PLX4032/C211), 48% of the patients had confirmed objective responses and 84% showed increased overall survival compared with those treated with dacarbazine (64%) at 6 months (11). Despite these encouraging results, responses to RAF inhibitors are transient, resistance to these compounds develops, and tumors invariably recur. Understanding the molecular mechanisms of resistance to RAF inhibitors is now critical to maximize their clinical success; achieve complete, durable responses; and improve patient outcomes.

Resistance to targeted agents, a frequent cause of therapy failure, can be mediated by diverse mechanisms, including secondary mutations or epigenetic changes in the target gene, modifications in drug metabolism, and activation of compensatory pathways, leading to increased tumor cell survival. Investigators are only now beginning to unravel what mechanisms are at play as a result of RAF inhibition and when are they engaged.

Modeling Resistance to BRAF Inhibitors—Key Findings
Our group and others have been intensively investigating the molecular mechanisms that underlie resistance to BRAF inhibitors using a variety of approaches (12–14). In our studies, we modeled the emergence of resistance to BRAF inhibitors by selecting a panel of BRAFV600E/PTEN⁺ melanoma cells that are highly sensitive to BRAF inhibition and chronically exposing them to increasing doses of SB-590885 (GlaxoSmithKline), a BRAF-selective inhibitor (15). Drug-resistant cells emerged ~6 months after persistent drug exposure and were able to proliferate and survive in the continuous presence of 1 μmol/L of SB-590885, unlike their parental counterparts. Of importance, chronic BRAF inhibition led to cross-resistance to several BRAF-selective inhibitors, including PLX4032, indicating that resistance is not likely to be easily overcome by switching to a new RAF inhibitor. All resistant clones were able to proliferate at normal rates, retain...
their anchorage-independent growth, and grow in a 3-dimensional tumor-like microenvironment even in the presence of high doses of BRAF inhibitors.

Although a frequent mechanism of anticancer drug resistance is the development of secondary mutations in the target gene, we did not identify secondary mutations in BRAF in any of our resistant cell lines, all of which retained the BRAFV600E mutation. Biochemically, our resistant melanoma cells were able to reactivate the MAPK pathway in a BRAF-independent manner. Although the parental (BRAF-inhibitor–sensitive) cells rely on BRAF for MAPK activation, the BRAF-inhibitor–resistant cells had somewhat elevated expression of CRAF and ARAF, and were able to dynamically use either of these 2 RAF isoforms to sustain MAPK activity and promote proliferation. Nevertheless, the resistant cells were still sensitive to MAP/ERK kinase (MEK) inhibitors that target downstream of RAF (Fig. 1). Treatment of BRAF-inhibitor–resistant cells with various structurally different MEK inhibitors had mostly cytostatic effects, suggesting that additional bypass mechanisms could be promoting survival. Indeed, our resistant cells displayed differential activation of several RTKs, in particular insulin-like growth factor receptor 1 (IGF-IR). Although the parental melanoma cells, like all cells of melanocytic origin, express IGF-IR, some of our BRAF-resistant melanomas expressed higher surface levels of IGF-IR. We observed that treatment of parental cells with BRAF inhibitors led to a decrease in phospho-IGF-IR levels; however, phosphorylation of IGF-IR was sustained in our BRAF-inhibitor–resistant cells. We further noted that enhanced IGF-IR–mediated signaling was not due to amplification or mutations of the IGF-IR gene. Even though the precise mechanisms of enhanced IGF-IR expression and signaling in the context of chronic BRAF inhibition are not yet completely understood, our results suggest possible cross-talk between BRAF and RTKs (particularly IGF-IR–dependent networks) that requires further investigation.

IGF-IR plays an important role in survival and resistance to anticancer therapies (16) that may be mediated through activation of MAPK and phosphoinositide 3-kinase (PI3K) signaling. In the context of resistance to BRAF inhibition, we found that IGF-IR promotes activation of PI3K and phosphorylation of AKT, but it has no effect on the MAPK pathway. Pharmacologic or genetic inhibition of IGF-IR inhibited only downstream PI3K/AKT signaling: exogenous IGF-I increased PI3K-mediated signaling but was not sufficient to induce resistance. Additionally, the MAPK and PI3K pathways seemed to jointly regulate the levels of the antiapoptotic factor Mcl-1 to promote survival. Clearly, the MAPK and PI3K pathways seemed to jointly regulate the levels of the antiapoptotic factor Mcl-1 to promote survival. Thus, the MAPK and PI3K pathways seemed to jointly regulate the levels of the antiapoptotic factor Mcl-1 to promote survival. Despite this, we found that coinhibition of these 2 pathways induced apoptosis of BRAF-inhibitor–resistant cells more effectively than did inhibition of each individual pathway. The combination of MEK

![Simplified schematic of signaling pathways that drive resistance to BRAF inhibitors.](cancerres.aacrjournals.org)
inhibitors (e.g., GSK1120212 or AZD6244) with PI3K inhibitors (e.g., GSK2126458) or IGF-IR inhibitors led to striking cytotoxic effects in 3-dimensional BRAF-inhibitor–resistant melanoma spheroids. Our findings strongly support the potential use of these combinatorial approaches to treat patients who are refractory to BRAF inhibitors.

To assess the clinical relevance of our studies, we compared paired biopsies (pretreatment and postrelapse) in 5 patients with metastatic melanoma treated with PLX4032. All 5 patients initially responded to PLX4032 but relapsed after 4 to 15 months of treatment. Sequencing of all 5 paired tumor biopsies indicated that the mutation encoding BRAFV600E was present in all samples (pretreatment and postrelapse), but no secondary mutations in BRAF, CRAF, or RAS were identified. Through immunohistochemical analysis of these biopsies, we found increased levels of IGF-IR in the postrelapse samples of 2 patients, 1 of which also had increased levels of phosphorylated AKT. These findings are consistent with our in vitro data suggesting that enhanced IGF-IR expression and PI3K/AKT activity are associated with resistance to BRAF inhibitors in some patients. We also noted a homozygous loss of PTEN and increased pAKT levels in the postrelapse biopsy of 1 patient, suggesting that PTEN loss could also be linked to resistance to BRAF inhibitors in some patients; however, it is not likely to be the sole player. These findings need to be further investigated both in vitro and in clinical samples.

Multiple Resistance Mechanisms Can Bypass BRAF Inhibition

Because melanoma is a complex and heterogeneous disease composed of biologically, genetically, and histopathologically distinct subtypes, it is not surprising that multiple mechanisms of resistance can compensate for chronic BRAF inhibition. Unlike other malignancies in which the development of secondary or “gatekeeper” mutations in the target gene is a common mechanism of resistance, so far no secondary mutations have been identified in BRAF-inhibitor–resistant melanomas, despite the use of highly sensitive sequencing methods such as next-generation targeted deep and ultradeep sequencing (13, 14).

Notwithstanding the fact that a diverse array of molecular mechanisms seem to be linked to acquired resistance to BRAF inhibitors, which poses a challenge for clinical translation, some common mechanistic themes have emerged (12–14). In most instances, reactivation of the MAPK pathway is required to circumvent chronic BRAF inhibition and resume proliferation; therefore, the MAPK pathway remains a good therapeutic target. Our studies support this observation and show that melanoma cells, which are initially addicted to BRAF, can switch and use one of the other RAF isoforms (ARAF or CRAF) to reactivate the MAPK pathway and continue proliferating. Additionally, overexpression of CRAF or the MAPK3K8 COT can also lead to MAPK reactivation (14). Alternatively, treatment with BRAF inhibitors could select for minor, preexistent NRAS mutant clones that do not respond to BRAF inhibitors but paradoxically hyperactivate the MAPK pathway (17–20). In fact, Nazarian and colleagues (13) identified 2 different NRAS mutations (Q61K and Q61R) in 1 of 12 patients resistant to vemurafenib. Persistent addiction to MAPK despite BRAF inhibition renders the resistant cells partially sensitive to MEK inhibitors and provides an opportunity to use compounds such as GSK1120212 and AZD6244, which are in advanced stages of clinical investigation, to treat patients who are refractory to BRAF inhibitors.

Another emerging common theme is the activation of RTKs, in particular IGF-IR and platelet-derived growth factor receptor (PDGFR), possibly due to alterations in feedback loops or compensatory survival mechanisms. Increased expression of IGF-IR and PDGFR has been noted in 2 of 5 (12) and 4 of 11 (13) postrelapse patient samples, respectively. Both IGF-IR and PDGFR can activate PI3K/AKT signaling and modulate survival. Indeed, previous studies have shown that AKT can protect melanoma cells from PLX4720-mediated apoptosis (21). More recently, a MEK1-C121S mutation was identified in 1 patient who developed resistance to PLX4032 (22). However, how frequently MEK or RAS mutations occur and how significant their contribution to resistance is, remain to be determined in a larger cohort of patients treated with BRAF inhibitors.

Future Directions

Melanomas seem to engage a number of strategies to bypass BRAF inhibition; however, issues regarding the prevalence of each compensatory mechanism and whether other mechanisms will emerge in the clinical setting are still being investigated. Collectively, all of the studies published to date on resistance to BRAF inhibitors have put forward new strategies to potentially override tumor recurrence (Fig. 1). Reactivation of the MAPK pathway renders the resistant tumors susceptible to MEK or extracellular signal–regulated kinase (ERK) inhibitors. Although MEK inhibitors have shown mostly cytostatic effects or tumor stabilization in the clinical setting, our results suggest that combining them with BRAF or PI3K inhibitors may prove useful. Resistance associated with activation of RTKs, including IGF-IR and PDGFR, or the serine/threonine kinase COT, could be abrogated by a growing number of pharmacologic inhibitors or blocking antibodies. Encouragingly, multiple RTK inhibitors, as well as anti-IGF-IR antibodies, are now under clinical investigation and could be used in combination with RAF or MEK inhibitors. Targeting molecules downstream of the receptors is another option. PDGFR and IGF-IR can activate the PI3K pathway; therefore, it is possible that this survival mechanism can be blocked by pan-PI3K, AKT, or mTOR inhibitors (12). Meanwhile, resistance caused by RAS or MEK mutations is potentially more challenging because no effective inhibitors of RAS or mutant MEK are currently available; however, the use of combination therapies with MEK and PI3K, or BRAF and MEK inhibitors may restrain these resistant tumors.

The complexity of bypass mechanisms suggests that personalized second-line treatment strategies may be needed. Despite this challenge, however, certain commonalities indicate that some therapeutic approaches could be effective in larger cohorts of patients regardless of the specific resistance mechanism. For example, inhibitors of BRAF/MEK in combination with compounds targeting PI3K/AKT could potentially be used to treat
tumors with MAPK reactivation and enhanced PI3K-mediated survival. Additionally, it is quite possible that novel inhibitors will be developed to prevent paradoxical MAPK pathway reactivation or to keep both the MAPK and PI3K pathways in check. The drug development field has not run out of options yet, and we are optimistic about future developments.

Currently, several clinical trials are planned or in progress to help sustain the effects of BRAF inhibitors and prevent resistance, including trials combining selective BRAF and MEK inhibitors. However, to guide future trials, comprehensive preclinical studies that consider the genetic and biologic heterogeneity of melanoma should be designed to identify the most effective and least toxic combinations. Carefully designed phase I trials will also be required to determine the pharmacokinetic and pharmacodynamic profiles of the experimental agents given in combination.

The melanoma field has made tremendous progress over the past decade in elucidating the biology of this deadly disease, and the encouraging clinical results are proof that this hard work is paying off. We have many options and agents available for combination treatments, and increasing knowledge about the molecular mechanisms that underlie drug resistance will facilitate the design of new effective, long-lasting therapies. We are confident that this approach will result in sustained tumor growth inhibition and improved patient outcomes.

Disclosure of Potential Conflicts of Interest

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