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Minireview

Mutant BRAF Melanomas—Dependence and Resistance

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RAF inhibitors have the unique property of transactivating RAS-dependent RAF dimers in most cells but inhibit RAF/MEK/ERK signaling in cells expressing mutant BRAF, in which RAS activity is too low to support this process. These drugs thus selectively inhibit ERK signaling in tumors with BRAF mutation. RAF inhibitors have remarkable clinical activity in melanomas with BRAFV600E mutations; however, resistance invariably develops. Three recent papers reveal that acquired resistance may be due to mechanisms that cause ERK signaling to become insensitive to RAF inhibitors, or that reduce the dependence of the tumor on ERK signaling through activation of other pathways.

The discoveries that carcinogenesis depends on mutations that dominantly activate mitogenic signaling and that established tumors usually remain dependent on these pathways have led to the idea that targeted inhibition of components of these pathways would be especially effective for therapy. Recently, examples in which this has been shown to be the case have multiplied. Selective inhibitors of ABL, EGFR, HER2, KIT, and ALK kinases have unprecedented clinical activity in tumors in which their target is activated by a genetic event. However, resistance to the effects of these drugs develops and, except for the case of ABL inhibitors in chronic myelogenous leukemia, clinical responses are usually temporary.

A recent illustration of this phenomenon is the remarkable sensitivity of metastatic melanomas with BRAFV600E mutation to RAF inhibitors developed by Plexxikon (PLX4032) and GlaxoSmithKline (GSK2118436) (Flaherty et al., 2010; Kefford et al., 2010). The RAS/RAF/MEK/ERK signaling pathway regulates many key cellular processes, especially cell proliferation, and is often dysregulated in human cancer. The pathway is normally regulated by growth factor receptors that, when activated, cause RAS to adopt its active, GTP-bound state. RAS-GTP binds in the membrane to multiple effector proteins including the three members of the RAF kinase family (ARAF, BRAF, and CRAF). Binding of these kinases to RAS-GTP leads to their activation and the subsequent activation of a cascade of kinases: RAF phosphorylates and activates its substrates MEK1 and 2, which, in turn, phosphorylate and activate their two substrates, ERK1 and ERK2. Activated ERK phosphorylates a variety of nuclear and cytoplasmic substrates that mediate the pleiotypic effects of the pathway (Young et al., 2009).

RAS/RAF/MEK/ERK signaling is hyperactivated in a high percentage of tumors; this is frequently due to activating mutations of the KRAS, NRAS, or BRAF genes. Activation of ERK signaling is especially prevalent in malignant melanomas. More than half of these tumors contain BRAF mutations, almost all of which are V600E (Davies et al., 2002), and 15%–30% contain mutations in NRAS (Sekulic et al., 2008). The prevalence of ERK activation in tumors has led to the aggressive development of RAF and MEK inhibitors as anticancer drugs. Experiments with a selective allosteric inhibitor of the kinase activity of MEK show that mutant BRAF melanoma cell lines and in vivo models are especially dependent on MEK/ERK signaling (Solit et al., 2006). However, in clinical trials, only a minority of patients with metastatic melanoma with mutant BRAF undergo tumor regression when treated with these drugs (Dummer et al., 2008; Infante et al., 2010). In contrast, the two ATP-competitive RAF inhibitors that have been tested in these patients have considerably more antitumor activity. These drugs almost always cause tumor regression in patients with this notoriously difficult to treat tumor. However, tumors eventually regrow and progress in almost all patients, with a median time to progression of approximately seven months (Bollag et al., 2010; Flaherty et al., 2010). Three recent papers (Johannessen et al., 2010; Nazarian et al., 2010; Villanueva et al., 2010) reveal potential mechanisms of acquired resistance to RAF inhibitors. Understanding these mechanisms requires an understanding of the unusual biochemical effects of RAF inhibitors that account for its clinical activity.

Inhibitors of other oncogenic kinases, including MEK, inhibit their targets in all cells. The dose of these drugs is limited by the degree of pathway inhibition that causes unacceptable toxicity, and the therapeutic window, if any, is derived from hypersensitivity of the tumor compared to normal tissue. Uniquely, RAF inhibitors affect ERK signaling in a mutation-specific manner (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Hoeflich et al., 2009; Joseph et al., 2010; Poulikakos et al., 2010) due to specific features of RAF signaling (Figure 1). Activation of RAS promotes the dimerization and membrane localization of members of the RAF family. Binding of the RAF inhibitor to one member of the RAF-dimer induces an allosteric change that transactivates the other, unbound member of the dimer and activates the ERK pathway. At high concentrations, the inhibitor binds both RAFs in the dimer and results in pathway inhibition. RAS activity is crucial for multiple aspects of RAF activation, including the formation of dimers, and RAF induction by inhibitors requires levels of RAS activity adequate to support formation of enough RAF dimers capable of transactivation. In BRAFV600E melanomas, RAS-GTP levels are insufficient and RAF inhibitors inhibit RAF kinase activity and ERK signaling (Poulikakos et al., 2010).

Thus, inhibition of ERK signaling by RAF inhibitors is confined to tumor cells and this reduces toxicity and allows more potent

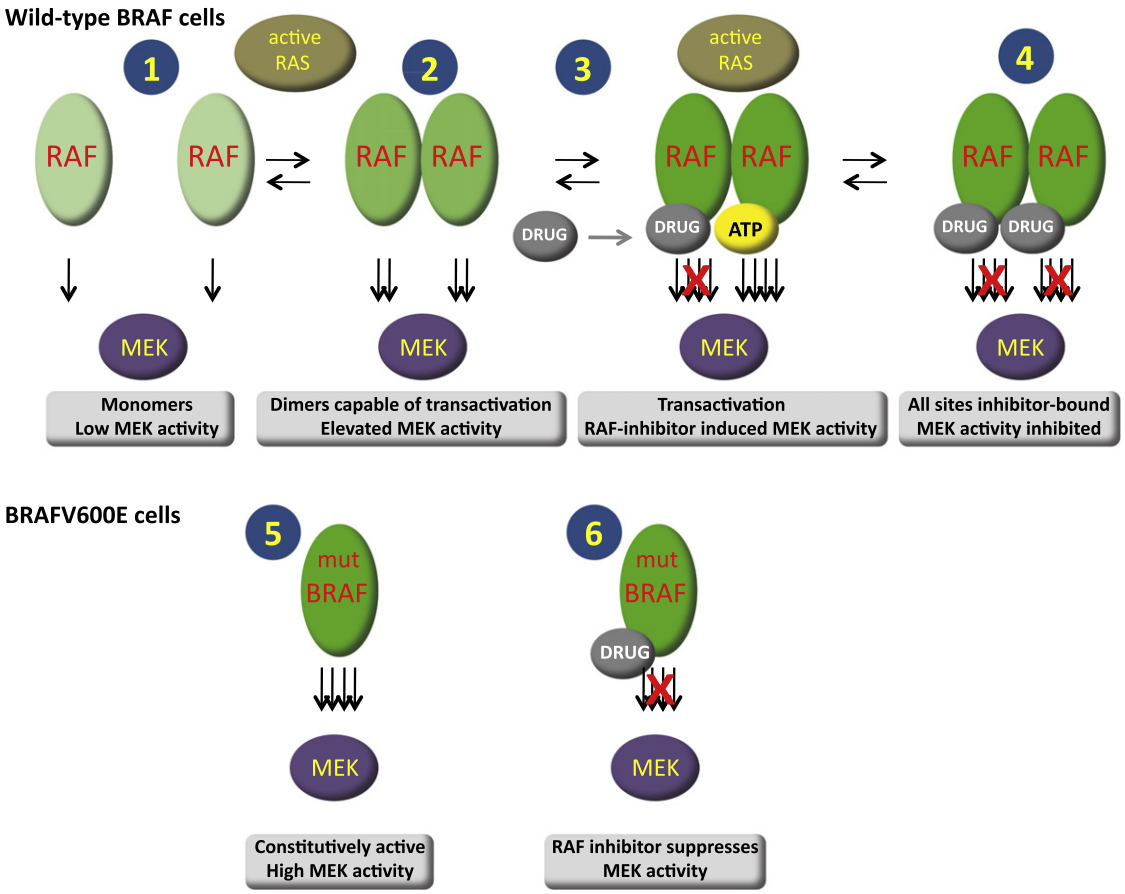

Figure 1. The Effect of RAF Inhibitors on ERK Signaling Depends on BRAF Status

In cells with wild-type BRAF (1), RAS promotes the formation of active RAF homo- and heterodimers (2). An ATP-competitive RAF inhibitor binds to RAF molecules and inhibits them, while at the same time it induces transition to the active state of the kinase, as previously reported for other kinases (Cameron et al., 2009; Okuzumi et al., 2009). However, in the case of RAF, a transition to the active state is transmitted to the inhibitor-free molecule in the dimer via direct interaction, causing a marked increase in catalytic activity (3). At higher concentrations of inhibitor, the number of inhibitor-free molecule decreases and total cellular RAF activity declines to lower than basal levels (4).

In cells with BRAFV600E and low RAS activity (5), BRAFV600E is the dominant MEK kinase and transactivation is precluded because RAFs do not form dimers capable of transactivation: the RAF inhibitor effectively inhibits ERK signaling (6).

pathway inhibition and antitumor activity than are achieved with MEK inhibitors, which suppress ERK signaling in all cells. This model suggests that molecular lesions that enhance RAF dimerization in tumor cells by activating RAS or by other means will cause insensitivity of ERK signaling to the drug and thus tumor resistance. The demonstrations in model systems that RAS mutation (Poulikakos et al., 2010) or CRAF overexpression (Montagut et al., 2008) cause resistance to RAF inhibitors are consistent with this idea. The clinical relevance of this mechanism has now been validated in at least one patient (Nazarian et al., 2010). Cell lines resistant to the RAF inhibitor PLX4032 were derived from three melanoma cell lines that express BRAFV600E. In one resistant line, MEK/ERK signaling was insensitive to the drug. Analysis revealed that these cells contained an activating mutation of NRAS and elevated RAS-GTP. It was also shown that, in one patient treated with PLX4032, development of resistance was associated with a new NRAS mutation.

In the two other resistant cell lines, ERK signaling was at least partially sensitive to the RAF inhibitor, suggesting that the phenotype is due to activation of other pathways that reduce the dependence of the cell on BRAF signaling. Multiple receptor tyrosine kinases were shown to be overexpressed in these cells, only one of which, PDGFRβ, was also hyperphosphorylated. PDGFRβ was upregulated in 4 out of 11 PLX4032-resistant tumors from patients, suggesting that this mechanism may be clinically relevant. Introduction of PDGFRβ into BRAFV600E melanomas created some degree of resistance, and proliferation of resistant cells with elevated PDGFRβ phosphorylation was inhibited by knockdown of the receptor. These data suggest that PDGFR plays a role in mediating resistance, but the mechanism underlying its overexpression is unknown. Activation of a growth factor receptor could cause resistance by activating parallel pathways that diminish dependence on ERK signaling or by increasing RAS activity. Levels of RAS-GTP are higher in the PDGFRβ expressing clones than in the sensitive parent cells, but not nearly at the levels induced by NRAS mutation. The best argument for causation of drug resistance is reversal of the phenotype in response to inhibition of the putative mechanism. This is convincingly shown for the tumor cells that express

mutant NRAS in the context of mutant BRAF. NRAS knockdown sensitized these tumors to RAF inhibition (Nazarian et al., 2010). The PDGFRβ cells were insensitive to the PDGFR inhibitor Imatinib; it would be useful to test the effects of combining Imatinib and the RAF inhibitor. If PDGFRβ is the dominant mechanism of resistance, resistant cells should be sensitive to this combination.

Villanueva et al. describe a similar approach: resistant cell lines were selected in the context of chronic treatment with another RAF inhibitor (Villanueva et al., 2010). In these cells, ERK signaling was insensitive to the RAF inhibitor or to knockdown of BRAF, CRAF, or ARAF expression individually, but sensitive to the RAF inhibitor in cells in which ARAF and CRAF expression were knocked down as well. They concluded that ERK signaling was still under RAF control, but, in the presence of inhibitors, dependency on mutant BRAF dynamically switched to dependence on other, wild-type isoforms. The underlying mechanism is unknown, but it is tempting to speculate that this reflects drug-induced transactivation of RAF isoforms in a cell in which resistance is mediated by upstream or mutational activation of RAS or to changes in RAF isoform expression that favor dimerization.

This study also associated resistance with activation of receptor tyrosine kinases, particularly IGF1 receptor (IGF1R) and demonstrated decreased viability of melanomas exposed to IGF1R inhibitors. These results must be interpreted with some caution, as the IGF1R inhibitors used are not very selective. These inhibitors did suppress AKT activation and they enhanced cell death when given in combination with MEK inhibitors. Villanueva et al. (2010) report that in one out of five cases of melanomas that relapsed while being treated with PLX4032, the resistant tumor expressed IGF1R and phosphorylated AKT, but the original tumor did not. Homozygous loss of PTEN together with phosphorylated AKT was noted to be associated with acquisition of resistance in another tumor. Analysis of more clinical samples will be required to assess the potential importance of this mechanism for conferring resistance.

Recent work suggests that in tumors with dysregulation of both PI3K and ERK signaling, both pathways must be inhibited to efficiently inhibit growth. These include tumors with RAS mutation, alone or in combination with PI3K mutation, or tumors in which EGFR activation or RAF mutation coexist with PTEN loss (Engelman et al., 2008). In these tumors, the two pathways converge on downstream targets that regulate cell survival directly (BAD) or via regulation of cap-dependent translation (4EBPs)(She et al., 2010). In mutant BRAF melanoma, the effects of inhibition of ERK signaling by MEK inhibitors on cell growth and survival have been shown to correlate inversely with basal or post-treatment level of AKT activation (Gopal et al., 2010). It is quite plausible that activation of IGF1R and PDGFRβ contributes to RAF inhibitor resistance by activating PI3K signaling. However, since PTEN has been found to be deleted in 25% of melanoma cell lines with mutant BRAF and only approximately 10% of melanomas with mutant BRAF exhibit de novo resistance to RAF inhibitors, activation of AKT signaling may not confer resistance by itself.

In a third paper, Johannessen and colleagues used a novel technique to identify mechanisms of resistance (Johannessen et al., 2010). They introduced a cDNA library encoding ~75% of annotated human kinases into a melanoma cell line with mutant BRAF and identified nine kinases that conferred resistance. Two of these, CRAF and the MAP kinase kinase Tpl2/COT (encoded by MAP3K8), were analyzed further and shown to cause ERK signaling to become insensitive to RAF inhibition. This is especially important because for the first time, it suggests the possibility that activation of alternative kinases that phosphorylate MEK can replace the requirement for RAF and maintain ERK signaling. This may be mechanistically analogous to acquisition of EGFR inhibitor resistance by activation of another tyrosine kinase, Met (Engelman et al., 2007).

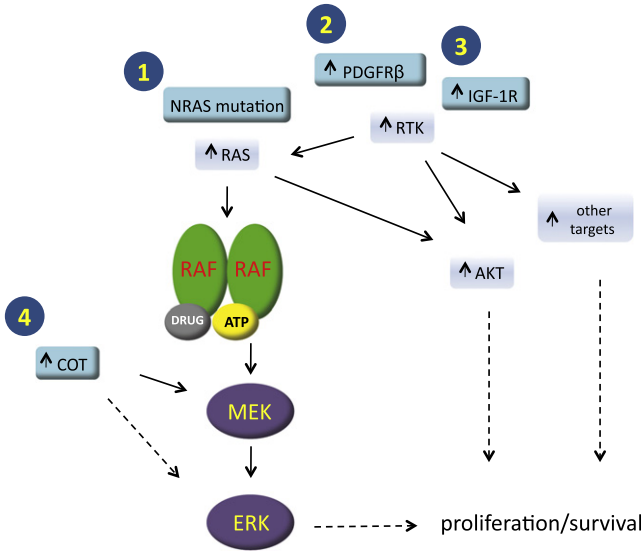

Figure 2. Potential Mechanisms of Acquired Resistance to RAF Inhibitors

BRAFV600E melanoma tumors may acquire resistance to RAF inhibitors through various mechanisms: RAS activation via RAS mutation (1) or receptor tyrosine kinase (RTK) activation (2, 3) promote RAF dimerization and subsequently compromise suppression of ERK signaling by RAF inhibitors. Activation of another MEK kinase, COT (4) would render ERK signaling refractory to RAF inhibition. RTK activation (2, 3) will in addition provide partial MEK-independence by activating other survival pathways.

COT not only activates ERK signaling in a RAF-independent manner, but its expression is also inversely correlated with that of BRAFV600E. The inference is that mutant BRAF activation downregulates COT expression and that the aberrant expression of COT causes resistance to RAF inhibitors. COT overexpression represents another mechanism for resistance: lesions that cause the mutant BRAF-independent activation of ERK signaling. Two of 38 BRAF mutant cell lines with chromosomal copy number gains that span the MAP3K8 locus were identified. These cells expressed COT and were resistant to RAF inhibitors. ERK signaling in these cell lines was insensitive to RAF inhibition but inhibited by a COT inhibitor or COT knockdown. Thus, there is some preliminary evidence for a mechanism (gene amplification) responsible for abnormal COT expression in cells with mutant BRAF. COT expression was detected in two out of three human melanomas with acquired resistance to RAF inhibitor, suggesting that this mechanism may be clinically relevant.

Thus, multiple mechanisms for resistance to RAF inhibitors have now been identified (Figure 2). Some lead to insensitivity of RAF kinase to the inhibitor: NRAS mutation, CRAF

overexpression, and switching to dependence on multiple RAFs. The first two are consistent with the mechanism of action of the RAF inhibitor, from which it can be inferred that anything which increases RAF dimerization and transactivation will confer this pattern of resistance. Gatekeeper mutations have been shown to be the most common cause of resistance to ABL, EGFR, and KIT inhibitors. Gatekeeper mutations of BRAF that are resistant to inhibition by RAF inhibitors have been described (Whittaker et al., 2010), but, curiously, they have not been found in tumors with acquired resistance to these drugs. The reason for this is unknown; it could reflect decreased signaling or transforming function of these mutants. All of these lesions cause resistance to RAF inhibitors, but the tumor retains dependence on RAF activation. Another mechanism, COT activation, is different; it causes RAF-independent ERK kinase signaling. Thus, as is true for other tumors, it is rare for resistant tumors to become independent of the signaling pathway activated by the driver mutation.

The last mechanism is the exception; it involves activation of receptors such as PDGFRβ and IGF1R that activate multiple signaling pathways that could reduce the ERK dependence of the cell but, also, by increasing RAS-GTP, attenuates the effects of the RAF inhibitor on ERK signaling. It has been suggested that almost complete (> 80%) inhibition of ERK signaling is required for tumor responses to RAF inhibition; moderate inhibition is ineffective (Bollag et al., 2010). These data suggest that even a modest decrease in the effectiveness of inhibition of ERK signaling by the drug could cause resistance. This will be difficult to assess in tumor samples without more accurate quantitative methods for assessing inhibition of ERK output. In the case of tumors with coexistent RAS mutations and PI3K pathway mutations, tumors are sensitive to combined inhibition of MEK and PI3K signaling. In BRAF mutant tumors, overexpression of receptor tyrosine kinases or mutations in RAS will both activate PI3K and other parallel pathways and also elevate RAS-GTP, thus attenuating the ability of RAF inhibitors to inhibit ERK signaling.

One may ask why such a profusion of mechanisms for resistance to RAF inhibitors have been defined and why mutations in the target, the prevalent mechanism of resistance to inhibitors of other kinases, have not been identified. It may have to do with the mechanism underlying the unique effectiveness of RAF inhibitors. In most cells, ERK signaling is insensitive to these RAF inhibitors, so resistance may result from any lesion that leads to modest levels of RAS signaling. It also may be worth mentioning that each of these papers reports distinct mechanisms of resistance in different melanoma cell lines. It may be that different genetic backgrounds and complement of somatic mutations in each tumor predispose to selection of different mechanisms of resistance. We do have to acknowledge that these are recent discoveries and the relative importance of these mechanisms for resistance in patients has not yet been defined. The underlying molecular lesion responsible for the resistance mechanisms reported in these papers is clear only for the case of NRAS mutation and acquisition of this mutation has been identified in only one patient. Dynamic switching from BRAF to wild-type RAF dependence, as hypothesized in Villanueva et al. (2010), and feedback reactivation of COT expression after ERK pathway inhibition should occur rapidly in all mutant BRAF tumors exposed to drug. This is not consistent with the clinical data that most tumors respond for months to RAF inhibitors, so these mechanisms must require selection of molecular lesions responsible for the biochemical effects. COT overexpression may be due to gene amplification in some cases; this will require further proof. Neither the Villanueva et al. (2010) nor the Nazarian et al. (2010) papers identify the underlying lesion responsible for RAF switching or for IGF1R or PDGFRβ overexpression. The finding that multiple kinases are overexpressed and the insensitivity of these tumors to the PDGFR inhibitor Imatinib alone (Nazarian et al., 2010) suggests that the primary lesion creating resistance could be upstream of PDGFR and have effects on other signaling pathways as well. There is clearly a need to obtain much more tumor material, preferably sensitive and resistant pairs, to determine which of these mechanisms contribute significantly to clinical resistance.

The most important consequence of identifying relevant resistance mechanisms will be the development of effective therapeutic strategies for their reversal. PDGFRβ and IGF1R inhibitors are already in clinical use and COT inhibitors are in development. Resistance mediated by RAF inhibitor-refractory ERK signaling may be reversed by combined therapy with RAF and MEK inhibitors. The excitement generated by the initial unprecedented clinical activity of RAF inhibitors in melanoma has now been tempered by the realization that the tumor responses are temporary. These three papers and other recent studies (Corcoran et al., 2010; Paraiso et al., 2010) suggest a path for the development of even more effective therapies for these patients.

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