Title: Molecular Pathways: Response and resistance to BRAF and MEK inhibitors in BRAF\textsuperscript{V600E} tumors

Running title: Molecular Pathways: Targeting BRAF\textsuperscript{V600E} tumors

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Abstract

The RAS-RAF-MEK-ERK pathway plays a central role in driving proliferation, survival and metastasis signals in tumor cells and the prevalence of oncogenic mutations in RAS and BRAF and upstream nodes makes this pathway the focus of significant oncology drug development efforts. This focus has been justified by the recent success of BRAF and MEK inhibitors in prolonging the lives of patients with BRAF$^{V600E/K}$ mutant melanoma. While it is disappointing that cures are relatively rare, this should not detract from the value of these agents to cancer patients and the opportunity they provide in allowing us to gain a deeper understanding of drug response and resistance. These insights have already provided the basis for the evaluation of alternative dosing regimens and combination therapies in melanoma patients.

Background

The RAS-RAF-MEK-ERK pathway controls cell growth, differentiation, survival and migration in normal tissues. Signals from cell surface receptors are transmitted through RAS-GTP to the RAF–MEK–ERK kinase cascade to intracellular substrates in the cytoplasm and nucleus. The RAF family of serine/threonine kinases consists of three family members: ARAF, BRAF and CRAF. All three family members share similar structural motifs, with two conserved regions in the N-terminus (CR1 and CR2) and one in the C-terminus which encodes the kinase domain (CR3). While ARAF and CRAF are ubiquitously expressed, BRAF appears to be more highly expressed in neuronal tissues (1, 2). Multiple phosphorylation sites across the RAF proteins, some of which are not conserved, may provide the basis for differentially regulation of the three family members (3). RAF kinases activate the dual specificity kinases MEK1 and MEK2 through...
phosphorylation at serine 218 and serine 222, respectively. MEK1 and MEK2 are structurally conserved, ubiquitously expressed and have a narrow substrate specificity, with ERK1 and ERK2 being the primary targets (reviewed in (4)). ERK1 and ERK2 are activated by MEK through sequential phosphorylation at threonine 202 and tyrosine 204 on ERK1 and threonine 185 and tyrosine 187 on ERK2. ERK1 and ERK2 are structurally very similar and unlike the RAF and MEK kinases, have a wide range of cytoplasmic and nuclear substrates (reviewed in (5)).

The scope of physiological responses to RAF-MEK-ERK pathway activation is partly dictated by the magnitude and the duration of the signal. For example, pathway stimulation by different growth factor receptors leads to differences in the kinetics of pathway activation which result in differences in phenotypic response (e.g. proliferation vs differentiation) (6). Furthermore, the proliferative response to RAF activation has been shown to follow a bell-shaped, rather than sigmoidal dose-response curve, with moderate levels of BRAF or CRAF activation inducing proliferation and high levels of RAF activation associated with a lack of induction of proliferation (7). These effects may not be shared equally between the two ERK isoforms as phenotypic differences have been observed in response to ERK1 versus ERK2 activation (8, 9). Therefore, fine-tuning of the RAF-MEK-ERK is achieved, at least partially by the architecture of the pathway with three modules and multiple isoforms existing at each node.

Sturm and colleagues suggest that the pathway architecture enables high signaling rates and amplification, with the negative loops providing rich dynamic properties such as oscillations and switch-like properties similar to a negative feedback amplifier (10). Negative feedback occurs at multiple levels and the transcriptional output of the pathway includes the DUSP family of ERK phosphatases as well as the SPRY proteins that are negative regulators of RAS signaling. These
qualities dictate the response to stimuli and this model provides a basis for understanding signaling in the context of normal physiological processes, as well as in cancer where the pathway is often dysregulated through oncogenic mutations such as \( \text{BRAF}^{\text{V600E}} \) which is expressed in approximately 50% of human melanomas, 35% to 60% of thyroid tumors and a lower proportion of ovarian, colorectal, and lung carcinomas (11). Figure 1A depicts a simplified model in which RAF-MEK-ERK signaling in \( \text{BRAF}^{\text{V600E}} \) cells functions independent of upstream RAS activation and is not subject to regulation by feedback that occurs at nodes upstream (12). In such a model cells expressing \( \text{BRAF}^{\text{V600E}} \) rely on the DUSP family of ERK phosphatases to regulate phospho-ERK levels.

**Clinical-Translational Advances**

Allosteric MEK inhibitors represent the first pharmacological inhibitors of the RAS-RAF-MEK-ERK pathway, with CI-1040 being the first to be tested in human clinical trials (13). While the human efficacy of CI-1040 was likely limited due to poor drug-like properties and a lack of potency, this inhibitor provided preclinical proof-of-concept that targeting MEK can result in anti-tumor activity in preclinical models (14). This work led to the development of more potent allosteric inhibitors with improved properties, such as PD0325901 and ARRY142886/AZD6244 as well as a host of others which have demonstrated therapeutic efficacy in human clinical trials (reviewed in (15)). The most advanced MEK inhibitor, trametinib, was recently approved for the treatment of metastatic melanoma expressing the \( \text{BRAF}^{\text{V600E/K}} \) mutation. In a Phase 3 trial in patients with metastatic melanoma whose tumors express \( \text{BRAF}^{\text{V600E/K}} \), trametinib treatment resulted in a 22% response rate and 4.8 months median progression-free survival which compared favorably to chemotherapy (8% and 1.5 months) (16).
The discovery that mutant BRAF is an oncogenic driver led to a significant investment in the development of BRAF kinase inhibitors. SB590885 was the first RAF inhibitor to demonstrate preferential inhibition of the RAF-MEK-ERK pathway in tumor cells expression BRAF^{V600E} and this profile was suggested to provide a “genetic therapeutic index” (17). PLX4720 also demonstrated differential pharmacological of BRAF^{V600E} tumors versus BRAF wild-type tumors in vivo and the structural analog, vemurafenib (PLX4032), was approved in 2011 for the treatment of patients with metastatic melanoma whose tumors express the BRAF^{V600E} mutation (18, 19). In a Phase 3 trial in patients with metastatic melanoma whose tumors express BRAF^{V600E}, vemurafenib treatment resulted in a 48% response rate and 5.3 months median progression-free survival which compared favorably to dacarbazine (5% and 64%)(20). Other selective RAF inhibitors are at various stages of clinical development such as dabrafenib (FDA approved in 2013) and LGX818 (Phase III)(21, 22). A model for the pharmacological inhibition of BRAF^{V600E} is depicted in Figure 1B, where the pathway is effectively silenced by a BRAF inhibitor. It is important to note that inhibition also leads to loss of negative feedback which could be critical to the emergence of resistance as discussed in the next section (23).

An interesting aspect to the pharmacological profile of these BRAF inhibitors is that they paradoxically activate the RAF-MEK-ERK pathway in cells expressing wild-type BRAF (24-26). The lack of inhibition spares normal tissues some of the toxicities caused by pathway inhibition with MEK inhibitors, but appears to cause other toxicities (especially skin) as a consequence of pathway activation (27-29). This is supported by the observation that skin toxicities decrease in combination with a MEK inhibitor (30). An additional benefit of the BRAF plus MEK inhibitor combination is the superior efficacy compared to single agent treatment in BRAF^{V600E/K} melanoma. The combination of dabrafenib (150 mg) plus trametinib (2 mg)
resulted in a response rate of 76% with 9.4 months median progression-free survival that was superior to single-agent dabrafenib (54%, 5.8 months) (30).

As described above, BRAF inhibitors have significant clinical efficacy in BRAF\textsuperscript{V600E/K} melanoma. However, not all patients experience objective tumor response and the vast majority of patients including those who experience a robust initial tumor response, eventually relapse during the course of treatment (31-33). This pattern of drug response, with often very dramatic initial tumor regression followed by relapse within a few months, highlights the need to understand the mechanisms of resistance. In the following section we will explore the mechanisms and potential approaches to overcome acquired resistance, as well as intrinsic resistance in melanoma.

Melanoma cell lines have been used extensively to study BRAF and MEK inhibitor resistance and in fact, a significant proportion of BRAF\textsuperscript{V600E} mutated melanoma cell lines are intrinsically resistant to BRAF and MEK inhibitors (32, 34). Understanding the mechanisms of intrinsic resistance in these lines could provide insight into mechanisms of acquired resistance. For instance, cell lines with genetic alterations downstream of BRAF\textsuperscript{V600E}, such as amplified cyclinD1 are resistant to BRAF inhibition because they evade cell cycle arrest (35). Genetic alterations upstream of BRAF\textsuperscript{V600E} such as NF1 loss can also confer intrinsic resistance to BRAF inhibition by activation of RAS and signaling through CRAF (36). Melanoma cell lines with PTEN deletion have an impaired apoptotic response due to an inability to upregulate BIM upon BRAF or MEK inhibition (37). Receptor tyrosine kinase (RTK) signaling through the PI3K pathway has also been shown to cause intrinsic resistance to BRAF and MEK inhibition. MEK inhibitor insensitive BRAF\textsuperscript{V600E} melanoma cell lines upregulate IGF1R-AKT pathway signaling and these cells can be sensitized by a combination of AZD6244 with IGFR1, AKT or Research.
mTORC1/2 inhibitors (38). Other BRAF<sup>V600E</sup> tumor types such as colorectal and thyroid tend to be somewhat intrinsically resistant to BRAF and MEK inhibition. In these tumors BRAF<sup>V600E</sup> dependence is not absolute and transient pathway inhibition leads to relief of feedback inhibition of receptor tyrosine kinase signaling and rapid reactivation of the pathway (39, 40). Therefore, RAF-MEK-ERK signaling in BRAF<sup>V600E</sup> tumors is more heterogeneous than depicted in Figure 1A and B and appears to differ across tumor types.

Acquired resistance is characterized by initial period of tumor response, followed by relapse and this has been noted for several targeted agents besides BRAF inhibitors. For example, BCR-ABL inhibitors in chronic myelogenous leukemia, KIT inhibitors in gastrointestinal stromal tumors, EGFR inhibitors in lung cancer and smoothened inhibitors in medulloblastoma (41-43). Analysis of the resistance mechanisms in each of these examples show that acquired drug resistance can arise from the acquisition of a secondary mutation in the kinase being targeted. The mutations occur at “gatekeeper” residues in the kinase’s ATP binding pocket, preventing the drug from binding and inhibiting the kinase activity. The T790M mutation and T315I mutation in EGFR and Bcr-ABL respectively, are gatekeeper mutations. Preclinical studies identified T529 as the gatekeeper site in BRAF<sup>V600E</sup> that confers resistance to BRAF inhibitors(44). However, this mutation has never been observed as a mechanism of acquired resistance to BRAF inhibitors in cell lines or patient biopsies. Instead a diverse array of resistance mechanisms have been published which in most cases lead to the re-activation of ERK in the presence of the BRAF inhibitor (Figure 1C).

The major ERK re-activating mechanisms of resistance discovered to date involve alterations in RAS, RAF and MEK. Activating mutations in NRAS, such as NRAS<sup>Q61K</sup>, have been shown to drive resistance to vemurafenib through activation of CRAF leading to reactivation of MEK and
ERK (45, 46). The flexible switching of signaling between the RAF isoforms has been shown in other cases to drive resistance to BRAF inhibitors, for example activation or overexpression of CRAF can re-activate p-ERK independent of BRAF<sup>V600E</sup> (47, 48). In addition the genomic amplification of <i>BRAFI</i> or the up-regulation of BRAF protein levels can also mediate resistance to vemurafenib with cross resistance to MEK inhibition as well (49, 50). Expression of a 61-kDa splice variant of BRAF<sup>V600E</sup> which lacks the RAS binding domain leads to enhanced dimerization resulting in resistance to vemurafenib(51). MEK mutations have also been identified in the context of BRAF inhibitor resistance. MEK1 (C121S) confers increased kinase activity <i>in vitro</i> and over expression of this mutant protein induced cross-resistance to both MEK (AZD6244) and BRAF (PLX4720) inhibitors (52). Two additional <i>MEK</i> mutations, MEK1 (P124L) and MEK1 (Q56P), identified from a random mutagenesis screen promote resistance to MEK inhibition and cross-resistance to BRAF inhibitor PLX4720(53). However, exposing BRAF-mutant melanoma cells containing either of the two MEK mutations to a MEK plus BRAF inhibitor combination (AZD6244 and PLX4720) prevented emergence of resistant clones. In summary, resistance to vemurafenib frequently occurs through reactivation of ERK as a result of genetic changes to RAS, RAF and MEK.

Parallel survival pathways may also drive acquired resistance to BRAF and MEK inhibitors in BRAF<sup>V600E</sup> melanoma. IGFR-1 has been found to be constitutively activated with a simultaneous increase in PI3K/AKT signaling in the resistant cells and combinations of PI3K and MEK inhibitors or IGFR1 and MEK inhibitors reversed the resistance (47, 48). Other studies show that vemurafenib induces FGF2 secretion which leads to the up regulation and activation of STAT3-PAX3 signaling pathway which in turn drives resistance to vemurafenib (54). Similar studies
show that hepatocyte growth factor (HGF)/c-MET and FGF/FGFR3 confer resistance to BRAF inhibition via RAF-MEK-ERK pathway reactivation (55, 56).

**Resistance can lead to a fitness deficit**

The expression of mutant NRAS and BRAF^{V600E} are normally mutually exclusive (11, 57, 58), but as described above mutant NRAS is found to reactivate the RAF-MEK-ERK pathway in BRAF^{V600E} tumors with acquired resistance to BRAF inhibitors. Such ERK activating resistance mechanisms appear to be deleterious to resistant tumor cells in the absence of BRAF inhibitor treatment. For example, vemurafenib resistant tumor cells expressing p61-BRAF^{V600E} or amplified BRAF suffer a fitness deficit in the absence of drug (49). This was demonstrated directly by expressing a BRAF^{V600E}:ER fusion protein and treating cells with 4-hydroxytamoxifen which induced dose-dependent activation of the BRAF^{V600E} kinase activity, increased phospho-ERK and a decrease in melanoma cell proliferation (49). These observations are consistent with previous studies that demonstrated when activated NRAS is expressed in cells expressing BRAF^{V600E}, the proliferation rate slows as the result of cells accumulating in G0-G1, and cells show characteristics of senescence (59). Whether this phenomenon will occur in the context of resistance mechanisms that do not involve ERK re-activation (i.e. ERK remains inhibited) is unclear.

These data are consistent with the concepts described in the first section that describe the phenotypic effects associated with differential kinetics of RAF-MEK-ERK pathway activation. In this case, melanoma cells are dependent on a precise level of \( \text{BRAF}^{V600E} \rightarrow \text{MEK} \rightarrow \text{ERK} \) pathway activation, such that too little, in response to pathway inhibition, or too much, in response to elevated flux through the pathway is deleterious to cell proliferation. Resistant tumor
cells appear to suffer a selective disadvantage over drug-sensitive tumor cells in the absence of vemurafenib treatment. This observation led to the design of a simple and effective intermittent dosing schedule that was found to delay or prevent the onset of drug resistant disease in patient-derived melanoma xenografts (49).

Anecdotal evidence already exists for the successful application of intermittent dosing of vemurafenib in melanoma patients(60). As discussed above, vemurafenib induces paradoxical activation of the RAF-MEK-ERK pathway in cells expressing wild-type BRAF and a recent case study describes the induction of a previously undetected NRAS-mutant chronic myelomonocytic leukemia (CML) in a melanoma patient treated with vemurafenib. Treatment with vemurafenib caused the patient’s white-cell counts to spike leading to rapid progressive CML. Cessation of vemurafenib treatment led to decreased white-cell and monocyte counts however, treatment had to be reinitiated to control the growth of the melanoma tumor. To keep both diseases under control, the patient was maintained on an intermittent schedule of vemurafenib with the dose schedule determined by closely monitoring the white-cell counts. This patient remained on this schedule for over 80 weeks (Paul Chapman, personal communication). In another example, two patients who previously progressed on treatment with the BRAF inhibitor dabrafenib or with a BRAF and MEK inhibitor combination (dabrafenib plus trametinib), following a treatment free period were successfully re-challenged with vemurafenib or dabrafenib plus trametinib, respectively and both patients experienced tumor regression (61). While these case studies provide evidence that support intermittent dosing, a thorough testing of the efficacy, feasibility and optimization of the dosing regimen in patients is still necessary. Such testing is now underway in a Phase II clinical trial evaluating intermittent dosing with the BRAF inhibitor LGX818 in patients with BRAFV600 mutant metastatic melanoma (www.clinicaltrials.gov).
Combination therapy represents a more traditional approach to preventing resistance. As described above, the efficacy of a BRAF plus MEK inhibitor combination appears to be superior to single agent BRAF inhibitor (30). Targeting two nodes provides a more robust inhibition of the pathway and presumably delays or prevents the emergence of resistance through reactivation of downstream ERK. Given the number of potential resistance mechanisms described above, there are multiple BRAF inhibitor combinations that could be investigated and evaluating each of these clinically becomes a logistical challenge. One innovative approach to tackling this issue has recently been initiated in a Phase 2 study melanoma patients who progress on the BRAF inhibitor LGX818. Resistant tumors will be biopsied and compared to a pre-treatment biopsy in an attempt to identify the mechanism of resistance in each tumor. Based on the results, a second targeted agent from a list of MEK, CDK4/6, FGFR, PI3K, and c-MET inhibitors will be added to the treatment regimen (www.clinicaltrials.gov). Also, given the recent encouraging results with immunotherapy in melanoma, combination trials are now underway evaluating vemurafenib with anti-PD-1/PD-L1 and CTLA4 antibodies (reviewed in (62)).

**Summary**

The impressive clinical efficacy observed with BRAF inhibitors in patients with BRAF\textsuperscript{V600E} melanoma exemplifies the success of oncogene-targeted cancer therapy but as with other targeted therapies, the duration of clinical benefit is limited by the emergence of drug resistance disease. Improving the durability of response to these agents will require a deep understanding of both the genetics and the intracellular signaling pathways in drug sensitive and resistant tumor cells. These studies require a significant investment in translational approaches using both preclinical and clinical tumor samples. Recent studies using such translational models suggest
that vemurafenib-resistant tumors suffer a fitness deficit in the absence of drug treatment and intermittent dosing schedules up-front could prevent or delay the emergence of resistant disease. Such an approach should be considered along with combination therapy to improve the efficacy of BRAF inhibitors in melanoma. In the future it may be possible to rationally design intermittent treatment regimens where drug combinations are cycled on and off together or separately based on a deeper understanding of RAF-MEK-ERK signal transduction and cross-talk with other pathways in treatment naïve and resistant tumors.

References

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Figure 1. RAS-RAF-MEK-ERK signaling BRAF^{V600E} melanoma cells that are BRAF inhibitor naive and resistant. A) In melanoma tumor cells, constitutive activation of BRAF^{V600E} results in up-regulation of negative feedback loops that suppress pathway signaling at multiple levels. B) BRAF inhibitor treatment results in strong suppression of phospho-MEK and phospho-ERK leading to inhibition of proliferation and tumor regression which provides significant therapeutic benefit. However, negative feedback signals are also lost. C) A wide range of BRAF inhibitor resistance mechanisms serve to re-activate ERK, including loss of negative feedback, RTK activation, NRAS and MEK mutations, BRAF^{V600E} and COT amplification as well as expression of p61-BRAF^{V600E}.
Figure 1:

A: 
- RTK → BRAF<sup>V600E</sup> → MEK → ERK → Proliferation, survival, feedback
- RAS → MEK → BRAF
- Craf

B: 
- RTK → Vemurafenib, dabrafenib, LGX818 → BRAF<sup>V600E</sup> → MEK → ERK → Proliferation, survival, feedback

C: 
- RTK → Amplified BRAF<sup>V600E</sup> → MEK<sup>mut</sup> → Craf → BRAF → ERK → Proliferation, survival, feedback
- RAS → RAS<sup>mut</sup> → Craf → p61-BRAF<sup>V600E</sup>
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