The Evolution of Melanoma Resistance Reveals Therapeutic Opportunities

Meghna Das Thakur and Darrin D. Stuart

Abstract

The RAS–RAF–MEK–ERK pathway is a key driver of proliferation and survival signals in tumor cells and has been the focus of intense drug development efforts over the past 20 years. The recent regulatory approval of RAF inhibitors and a MAP–ERK kinase (MEK) inhibitor for metastatic melanoma provides clinical validation of tumor dependency on this pathway. Unfortunately, the therapeutic benefit of these agents is often short lived and resistance develops within a matter of months. Preclinical models of resistance to vemurafenib have provided critical insights into predicting, validating, and characterizing potential mechanisms. A key observation has been that vemurafenib-resistant tumor cells suffer a fitness deficit in the absence of drug treatment and this led to the predication that modulating the selective pressure of drug treatment through intermittent dosing could delay or prevent the emergence of resistant tumors. Most importantly, the preclinical data are supported by observations in vemurafenib-treated patients with melanoma providing a strong rationale for clinical testing of alternative dosing regimens. Cancer Res; 73(20); 6106–10. ©2013 AACR.

Introduction

The RAS–RAF–MEK–ERK pathway transmits proliferation and survival signals from cell surface receptors to the cytoplasm and nucleus. In tumor cells this pathway is frequently hyperactivated through constitutive activation of upstream growth factor receptors or through mutations in RAS or BRAF. In melanoma, approximately 10% to 15% of tumors express a mutant, constitutively active form of NRAS and approximately 50% express mutant BRAF, with a T to A substitution at codon 1799 (BRAFV600E) being the most frequent mutation. A deep body of preclinical evidence supports the oncogenic role of mutant RAS and mutant BRAF in melanoma (reviewed in refs. 1, 2).

The frequency of oncogenic mutations in this pathway has led to intense efforts to develop inhibitors to RAS, RAF, MAP–ERK kinase (MEK), and extracellular signal–regulated kinase (ERK). Preclinical proof of concept for targeting MEK in cells expressing mutant BRAF came from Solit and colleagues who found that vemurafenib–resistant tumor cells suffer a fitness deficit compared with cells expressing wild-type BRAF, rather than suppression, as is the case with MEK inhibitors (10–12). This leads to unique and sometimes counteracting toxicities with RAF and MEK inhibitors in human patients, ultimately allowing RAF inhibitors to achieve a higher degree of pathway suppression in tumors with BRAFV600E mutations.

Reactivation of the RAS-RAF-MEK-ERK Pathway in Resistance Tumors

Although the efficacy of RAF inhibitors in metastatic melanoma represents a significant improvement over previous therapies, the vast majority of patients are not cured and patients relapse with lethal, drug-resistant disease within a few months. Understanding the mechanisms underlying resistance is crucial to develop strategies for preventing resistance using drug combinations, new drugs, or as the authors propose, alternative dosing schedules for existing drugs (13). There is a wide range of mechanisms that could mediate resistance to RAF inhibitors and recent publications have described several possibilities. For example, activating NRAS mutations (Q61K and Q61R) have been discovered in vemurafenib-resistant melanoma tumors (14, 15). That NRAS mutations are mutually...
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exclusive of BRAF^{V600E} in treatment-naïve tumors, coupled with the knowledge that vemurafenib is ineffective in RAS-activated tumor cells builds a strong case for activating NRAS mutations as a mechanism of resistance. Although drug-resistant mutations that impair drug binding have not been discovered in the BRAF gene itself, amplification, overexpression, or expression of splice variants of BRAF have all been described as mechanisms of resistance (13, 15, 16). Similarly, CRAF overexpression has been shown to drive resistance to RAF inhibitors in preclinical models (17, 18). Other possibilities include COT overexpression and MEK mutation (e.g., MEK1C121S) as mechanisms that lead to MEK activation independent of BRAF^{V600E} (18, 19). The very nature of mitogen-activated protein kinase pathway wiring and control through negative feedback provides the opportunity for adaptive resistance, whereby RAF inhibitors block ERK signaling leading to the release of ERK-dependent negative feedback, resulting in reactivation of ligand-dependent signaling, which is normally muted in BRAF^{V600E} cells (20–22). The commonality among these mechanisms is that they all lead to reactivation of ERK, suggesting that resistant tumor cells maintain their dependency on the RAF-MEK-ERK pathway and this is consistent with the central role of this pathway in proliferation and survival of melanoma tumor cells. Finally, results from a recent phase II study comparing single-agent RAF inhibitor with RAF plus MEK inhibitor combination indicate that the combination provided longer progression-free survival, suggesting that stronger, more potent blockade of the pathway delays the emergence of resistance (23). A consistent and critical observation is that drug-resistant mutations in BRAF that impair drug binding have not been observed, suggesting that the drug is still inhibiting BRAF^{V600E} and the resistant cells have used one or more of the mechanisms described earlier to compensate for the inhibitor and reactivate the pathway (24). However, mechanisms of resistance that do not result in reactivation of the RAF-MEK-ERK pathway have been proposed and could also occur (25).

Resistant Tumors Are Less Fit in the Absence of Drug Treatment

In an attempt to model vemurafenib resistance, we used human melanoma patient-derived xenografts (PDX), which are propagated by serial transplantation in immunocompromised mice, with the goal of maintaining the heterogeneity of the original human tumor (13). As expected, these models respond to vemurafenib treatment in a dose-dependent manner, with tumor regression observed at a clinically relevant dose. However, consistent with human clinical data, continuous long-term vemurafenib treatment led to the development of drug-resistant tumors. Interrogation of the underlying mechanism of resistance led to the identification of amplification and/or overexpression of BRAF^{V600E}, which was further validated as a mechanism by siRNA knockdown in vitro using a cell line derived from the resistant PDX. An interesting observation is that only one out of nine of the resistant tumors amplified the BRAF^{p61} gene (~8 copies), whereas the remaining eight tumors simply overexpressed the gene. This is despite the fact that all of the resistant tumors were all derived from one founder resistant tumor, providing further evidence of the heterogeneity within this PDX model.

The most striking observation from these studies was that both resistant tumors in vivo and cell lines derived from resistant tumors seemed to be dependent on continued exposure to vemurafenib—upon drug withdrawal, resistant tumors transiently regressed and cell lines grew more slowly. This phenomenon was also observed in an in vitro derived vemurafenib-resistant cell line expressing the BRAF^{V600E} p61 splice variant as well as a PDX derived from a vemurafenib-resistant patient in which the mechanism of resistance was unknown. Therefore, it seems that vemurafenib-resistant tumors experience a fitness deficit upon cessation of drug treatment and the method used to generate cell lines from the resistant tumors provided a clue. To effectively establish vemurafenib-resistant cell lines, we had to grow the cells in the presence of vemurafenib. The dose-response of these cells to vemurafenib followed a bell-shaped curve with a peak rate of proliferation observed at 40 nmol/L vemurafenib. Intriguingly, this correlated very closely with the concentration of vemurafenib required to normalize pERK to the same level as the parental drug-sensitive cells. Therefore, it seems that melanoma tumor cells require a specific, set level of activated ERK for optimal growth. This was directly shown through the overexpression of ectopic BRAF^{V600E}, which led to decreased proliferation in a cell line derived from the PDX model. The effect of vemurafenib treatment on resistant tumor cell growth was also described by Tap and colleagues (26). Strikingly, this drug dependency was also observed in vivo in the resistant PDX tumors, which regressed upon vemurafenib cessation, and serial biopsies from each tumor following drug withdrawal indicated that pERK levels spiked, leading to regression or decrease in tumor growth. Although the mechanism linking pathway hyperactivation and tumor regression has not been determined, previous studies have shown that RAF–MEK–ERK hyperactivation can lead to cell-cycle arrest and senescence (27–29).

A key question is, to what degree do these observations made in preclinical models translate into human patients with vemurafenib-resistant disease. Fisher and colleagues found that tumor growth rates decreased when comparing relapse computed tomography (CT) scans with the postrelapse CT scan in 14 out of 19 vemurafenib-resistant patients following cessation of treatment (30). A significant challenge to gathering such data retrospectively is that postrelapse CT scans were not controlled for time post-vemurafenib withdrawal and were not collected at multiple time points, so minor or transient changes in tumor-growth kinetics may have been missed. These data provide evidence that vemurafenib-resistant human tumors have the same drug-dependency as observed in preclinical models. However, it should be noted that in the same study, postrelapse scans were not available for 16 patients due to rapid progressive disease, indicating that decreases in tumor growth rate following drug withdrawal may not be a universal phenomenon. Nonetheless, the observations that some human melanoma tumors experience decreases in growth rate upon cessation of RAF inhibitor treatment is consistent with our preclinical results and
suggests that resistant tumors can suffer a fitness deficit in the absence of drug treatment. This phenotypic evidence is further supported by the genetic basis for resistance in some patients; activating NRAS mutations are mutually exclusive with respect to BRAF<sup>V600E</sup> and only exist under the selective pressure of drug treatment (31).

The biology underlying these preclinical and clinical observations seem to follow basic evolutionary processes: tumor cells evolve to overcome inhibition of an oncogene-driven pathway through the selection of mutations or adaptations that result in a fitness disadvantage in the absence of drug treatment, leading to repopulation of cells with a drug-sensitive phenotype. The genetic and molecular heterogeneity within tumors provide the basis for plasticity and adaptation under the selective pressure of drug treatment and the rationale for alternative dosing regimens; however, this heterogeneity (especially intertumor) and polygenic nature of drug resistance is likely to complicate the relatively simple model proposed by our preclinical studies. With multiple potential resistance mechanisms at play within or across tumors within a given patient, drug resistance becomes a “moving target.” Tracking clonal evolution by developing sensitive methods to detect the relevant clones and using noninvasive techniques for measuring tumor burden and heterogeneity such as circulating DNA or protein biomarkers from plasma will be critical to design optimal treatment regimens in the future.

Given the fundamental nature of the biologic features of vemurafenib described earlier, drug dependency may be expected to occur in other tumors treated with agents targeting driver oncogenes. Indeed, chronic myelogenous leukemias (CML) develop resistance to imatinib through the expression of the drug-resistant mutations in BCR-ABL (e.g., T315I). The prevalence of these mutations has been shown to decrease when drug treatment is suspended, leading to the repopulation of drug-sensitive clones, which can result in a second response to imatinib treatment (32). Similar results have been described in studies of EGFR receptor (EGFR) mutant non–small cell lung cancer (NSCLC) treated with EGFR inhibitors. In preclinical studies, long-term treatment of drug-sensitive NSCLC cell lines with EGFR inhibitors led to resistance through expression of the drug-resistant T790M mutation in EGFR (33). These drug-resistant cells grew slower compared with parental cells and when grown through several passages in the absence of drug, the frequency of the T790M mutation decreased dramatically and the cells reverted to a drug-sensitive phenotype (33). These results are mirrored by clinical observations in patients with NSCLC who have developed resistance to EGFR therapy. As part of a larger translational science study, Sequist and colleagues collected serial biopsies from three patients with NSCLC and found that the T790M mutation (n = 2) or a PIK3CA mutation (n = 1) appeared in tumors that developed resistance to erlotinib, however, these mutations were not detected upon rebiopsy of the same tumors 7 to 10 months after treatment was stopped and the patients experienced a second response to erlotinib (34).

### Intermittent Dosing Can Forestall Resistance

These data led to the hypothesis that an intermittent treatment schedule in which the selective pressure of vemurafenib treatment is applied and withdrawn would prevent or delay the emergence of a resistant tumor cell population (Fig. 1). Experimental results in two different PDX models using either 4 weeks on/2 weeks off vemurafenib or an individualized regimen confirmed that tumors regressed upon

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*Figure 1. Proposed model for the dynamics of drug sensitive and resistant tumor cell populations under the intermittent selective pressure of drug treatment. Resistant tumor cells may preexist at a low frequency in treatment-naïve melanoma tumors (as shown) or may arise from the general tumor cell population. In either case, drug-sensitive cells make up a significant proportion of the tumor as evidenced by the tumor regression upon drug treatment in the majority of patients. Continued treatment leads to the enrichment or evolution of a resistant population of tumor cells that will expand on continued treatment. However, suspension of drug treatment removes the selective advantage gained by resistant cells and leads to repopulation by drug-sensitive cells. Repeated cycles of drug treatment followed by drug-free intervals will delay the emergence of drug-resistance, Tx, treatment.*
drug treatment and regrew after a short delay following suspension of treatment. Repeated cycles of vemurafenib treatment using these regimens controlled tumor growth over the course of 7 months of treatment, whereas mice treated on a continuous cycle developed resistance as soon as 2 months after initiation of therapy.

Although our current knowledge of the biology underlying vemurafenib resistance, coupled with these preclinical proof-of-concept studies provide a strong rationale to investigate alternative dosing regimens in human patients with melanoma, many aspects still need to be addressed such as defining optimized regimens. In one of the PDX models, we evaluated an individualized regimen that was facilitated by the ease of measurement of subcutaneous tumor dimensions using calipers. However, in patients with melanoma, repeated measures of tumor volume may be less feasible due to cost or safety concerns (e.g., CT) and indirect measures of tumor burden may be required as described earlier. Furthermore, in the preclinical models, only one tumor was evaluated in each mouse, whereas in patients with melanoma, it may be more challenging to adjust dosing schedules based on the growth of multiple metastatic lesions.

Despite these questions, evidence already exists in human patients with melanoma that some form of intermittent dosing of a RAF inhibitor may be effective. For example, Seghers and colleagues describe two patients who were successfully rechallenged with a RAF inhibitor (vemurafenib) or a RAF inhibitor plus MEK inhibitor combination (dabrafenib plus trametinib) following a treatment-free period after previously progressing on a RAF inhibitor (dabrafenib) or the dabrafenib plus trametinib combination (35). Consistent with these data, we have observed that RAF inhibitor resistant melanoma xenograft tumors grown through three generations in the absence of drug treatment respond upon rechallenge with the RAF inhibitor (unpublished results). These data are consistent with the hypothesis that drug-resistant tumor cells have a selective disadvantage in the absence of drug treatment leading to repopulation of drug-sensitive cells during a drug holiday.

Direct evidence supporting that an intermittent dosing schedule could work in human patients with melanoma comes from a recent case study (36). As discussed earlier, RAF inhibitors such as vemurafenib induce “paradoxical activation” of ERK signaling in a RAS-dependent manner and this contributes to the induction of keratoacanthomas and cutaneous squamous-cell carcinomas (37). Callahan and colleagues described a melanoma patient in which vemurafenib induced proliferation of a previously undetected NRAS-mutant chronic myelomonocytic leukemia (36). Although the patient’s melanoma initially responded to vemurafenib, treatment was stopped in an attempt to slow the growth of the leukemia. Consistent decrease in white cell counts over the following 2 weeks allowed the reinitiation of vemurafenib. However, vemurafenib treatment resulted in rising white cell counts. Thus, the patient was subsequently treated using an adjusted schedule guided by changes in white cell counts. Over the course of 5 months, white cell counts were controlled by stopping and restarting vemurafenib treatment and a partial tumor response was maintained in the melanoma tumors. The patient has continued on an intermittent schedule for over 80 weeks (Paul Chapman, personal communication).

Conclusions and Future Directions

The development of potent, selective inhibitors of oncogenic drivers such as BCR-ABL, mutant EGFR, and BRAFV600E have revolutionized cancer therapy but have also provided critical insights into tumor biology that were not apparent under treatment with cytotoxic chemotherapy. The development of resistance to these agents seems to follow basic evolutionary principles. In the case of BCR-ABL+ CML or EGFR-mutant NSCLC, the fraction of tumor cells expressing mutations that confer resistance to their respective inhibitors fluctuates with and without the selective pressure of drug treatment, suggesting that these clones are less fit than tumor cells not expressing these mutations. In the case of BRAFV600E melanoma, target mutations do not seem to confer resistance to RAF inhibitors and the pathway is reactivated through other mechanisms that cause a significant selective disadvantage in the absence of drug treatment. Although this lack of fitness can result in tumor regression or decreased growth rate in the absence of selective pressure, these observations should be used to guide alternative dosing regimens upfront, rather than after the emergence of resistant disease. Furthermore, intermittent dosing regimens could be rationally combined with other therapeutic agents to further exploit fitness deficiencies that occur following withdrawal of agents targeting key oncogenic drivers.

Disclosure of Potential Conflicts of Interest

Darrin D. Stuart is employed as Sr. Investigator in Novartis. No potential conflicts of interest were reported by the other author.

Authors’ Contributions

Conception and design: M.D. Thakur, D.D. Stuart

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.D. Thakur, D.D. Stuart

Writing, review, and/or revision of the manuscript: M.D. Thakur, D.D. Stuart

Study supervision: D.D. Stuart

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