Treatment of Metastatic Melanoma with an Orally Available Inhibitor of the Ras-Raf-MAPK Cascade\(^1,2\)

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Abstract

The Ras-Raf-MAPK pathway is constitutively activated in the majority of melanomas because of a mutation in the \(BRAF\) gene. It has been hypothesized that activation of this pathway is crucial for the genesis and maintenance of melanoma and therefore represents an attractive clinical target for metastatic disease. We synthesized a previously characterized MAP kinase kinase inhibitor to test the effect that blocking the Ras-Raf-MAPK pathway would have on the establishment and maintenance of melanoma metastases. Oral administration of CI 1040 inhibited formation of pulmonary metastases and caused rapid regression of established pulmonary metastases in the mouse. Our findings indicate that Ras-Raf-MAPK activation provides crucial signals for the survival of melanoma cells at ectopic sites and that the pharmacological inhibition of this pathway is a promising target for melanoma therapy.

Introduction

Melanoma is a highly aggressive and often fatal malignancy resistant to currently available therapy (1). The recent elevation in melanoma incidence (2) has highlighted the need to understand the molecular underpinnings of this neoplasm. Specific inhibition of oncogenic signaling molecules promises high efficacy with low toxicity if appropriate molecular targets are defined and blocked. This approach has been difficult in solid tumors because of, in part, heterogeneity in potential targets. A high throughput genomic screen recently identified activating mutations of the \(BRAF\) gene in the majority of melanomas (3). Thus, application of specific kinase inhibitors to melanoma is particularly attractive because of the high prevalence of \(BRAF\) mutations and the dismal clinical outcome of currently available treatment modalities.

It is now appreciated that the V599E \(BRAF\) mutation is the most common mutation in human melanoma, occurring in roughly 80% of short-term cultured tumors. Activating mutations of the \(BRAF\) gene are also present in roughly 80% of nevi (4), which are thought to be premalignant melanocytic lesions. The precise role that \(BRAF\) and \(RAS\) mutations play in the initiation and maintenance of malignant melanoma is not understood. Raf kinases play a central role in the Ras-Raf-MEK-MAPK\(^5\) signaling pathway. Raf is activated by GTP-bound ras (5). Once active,raf phosphorylates the MEK kinase (6). Active MEK phosphorylates and activates MAPK, which has multiple targets, and leads to changes in gene transcription and resistance to apoptosis (7).

Mutational activation of \(RAS\) genes occurs in human melanoma, albeit with low frequency (8). A mouse model has shown that expression of an activated \(RAS\) allele is sufficient to promote melanomagenesis in an \(INK4a\)\(^−/−\) background and that sustained ras signaling is required for melanoma maintenance (9). These studies suggest that \(BRAF\) mutations may play an important role in initiation of human melanoma, and that sustained Ras-Raf-MAPK signaling may be required for tumor survival \textit{in vivo}. To test this hypothesis, we administered a highly specific MEK inhibitor to melanoma cells \textit{in vitro} and to mice bearing pulmonary metastases of a human melanoma cell line with the V599E \(BRAF\) mutation. We targeted MEK because it is necessary for many, if not all, of the oncogenic functions of raf (5). Moreover, raf kinase inhibitors developed to date may not be fully active against V599E braf because mutations in the \(BRAF\) gene could lead to resistance as has been described for other drugs developed against kinase domains of oncoproteins (10). In this report, we show that the oncogenic potential of \(BRAF\) mutations in melanoma can be effectively diminished with adequate inhibition of MEK and that specific targeting of kinases in melanoma may be a feasible and worthwhile clinical approach to management of this disease.

Materials and Methods

\textbf{Immunohistochemistry.} Tissue samples were acquired from archival melanoma samples or tumor-bearing mice with the approval of the UCLA Office for the Protection of Research Subjects board. Sections of formalin-fixed, paraffin-embedded tissue were deparaffinized in xylene and then hydrated through a series of xylene and ethanol washes. Antigen retrieval was performed using 6.5 mM citrate buffer at pH 6.0 in a pressure cooker for 5 min, and 3% \(\text{H}_2\text{O}_2\) was used to quench endogenous peroxidase activity. After rinsing two times in PBS, the sections were blocked for 1 h at room temperature in 5% normal goat serum (Vector, Burlingame, CA). Localization of phosphorylated MAPK protein expression was examined using a polyclonal rabbit anti-phospho-p44/p42 MAPK antibody (New England Biolabs, Beverly, MA) at a dilution of 1:200. The sections were incubated in the primary antibody overnight at 25°C. Biotinylated secondary goat anti-rabbit antiserum was from KPL (Allentown, PA) and was used at 1:100. Staining was then done using 3-amino-9-ethylcarbazole and mounted with Permount.

\textbf{Western Blotting.} Immunoblotting of cultured cells was performed as described (11) with antibodies against b-raf (Santa Cruz Biotechnology, Santa Cruz, CA), Phospho-MEK (Cell Signaling, Beverly, MA), Phospho-MAPK (Cell Signaling), Total-MAPK (Cell Signaling), and actin (Sigma, St. Louis, MO).

\(5\) The abbreviations used are: MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; GFP, green fluorescent protein; SCID, severe combined immunodeficient; CCD, charge coupled device; Fluc, firefly luciferase.
Cell Lines and Genotyping. A375, SK-MEL-28, CHL, and WM-266-4 cell lines were from American Type Culture Collection. The A375 cell line was a gift from R. O. Hynes (Massachusetts Institute of Technology). All lines were maintained in DMEM (Invitrogen, Carlsbad, CA) + 10% fetal bovine serum (HyClone) and 1% penicillin/streptomycin (Invitrogen). For BRAF sequencing, 1 × 10^6 cells were pelleted, and genomic DNA was prepared using Trizol (Life Technologies, Inc.) exactly as recommended by the manufacturer. PCR-based sequencing of exon 15 was performed using the following intron-flanking primers from IDT (Skokie, IL): BRAF 15 FWD, 5′-CATGCTTGCTCTGATAGGA-3′; and BRAF 15 REV, 5′-GGCCAAAAATTTAATCAGTGG-3′.

A375M-Fluc cells were established by recombinant lentiviral transduction. Briefly, the firefly luciferase cDNA (Fluc) along with a downstream EMCV-IRES sequence was subcloned into pCS-CG lentivector using the Nhel and EcoRlIII sites upstream of the GFF gene, resulting in pCS-CG-Fluc-I-GFP. Recombinant lentivirus was produced by standard calcium phosphate cotransfection of 293T cells along with pΔVPR and pVSVG (12). A375M melanoma cells (1 × 10^5) were seeded in 10-cm plates and incubated with lentiviral supernatants filtered from virus-producing cultures in the presence of Polybrene (8 μg/ml). Stable cell pools were selected by FACS sorting for GFP fluorescence.

Soft Agar Assays. A375M cells were suspended in 0.3% Noble agar with complete DMEM, plated in duplicate in dishes coated previously with 0.5% base agar, and maintained at 37°C. Both base and suspension agar contained CI 1040 or DMSO at the indicated concentration. On day 21, colonies >0.2 mm in diameter were counted.

Cell Cycle Analysis and Apoptosis. Cells were treated with 1 μM CI 1040 or DMSO in the presence of 10% serum for 24 h and then processed for cell cycle analysis by propidium iodide staining as described (11). Apoptosis was assessed by the Annexin V assay kit (Oncogene, La Jolla, CA) exactly as described by the manufacturer.

Animal Studies. All animal studies were approved by the UCLA Animal Research Committee. Beige SCID mice, 8 weeks of age, were injected with 7 × 10^5 A375M-Fluc cells via the lateral tail vein on day 0. Mice were treated with 50 mg/kg of CI 1040 or resuspended in a 8:1:1 PBS:ethanol:Cremophore by gavage twice per day for the first 14 days after injection (n = 4, Early Treat), days 15–28 (n = 3, Late Treat), or vehicle (n = 4, Control). Mice were imaged by i.p. injection of 100 μl of t-n-luciferin (30 mg/ml; Xenogen) and imaged using a cooled CCD camera (IVIS). Imaging data were quantified as described (13) by averaging the region of interest from the maximal photon emitting exposure.

Results

To evaluate the frequency of Ras-Raf-MAPK pathway activation in melanoma, we immunostained melanomas with a phospho-specific antiserum to MAPK and found the pathway to be activated in the majority of samples tested (14 of 16; 87.5%; Fig. 1A), consistent with a recent report (14).

To address the role of BRAF mutations in mediating the high level of MAPK phosphorylation in melanoma, we evaluated several melanoma cell lines with known BRAF mutations. We found that the WM-266-4, SK-MEL-28, and A375 cell lines all exhibited high levels of phosphorylated MAPK in both the presence and absence of serum, indicating that the Ras-Raf-MAPK pathway was constitutively activated in these cell lines (Fig. 1B). These findings are consistent with the reported mutations in the 599 codon of BRAF in these cell lines (3). In contrast, the CHL melanoma cell line, with wild-type Ras and wild-type BRAF alleles, down-regulated MAPK phosphorylation after serum withdrawal (Fig. 1B).

To evaluate the therapeutic potential of inhibiting the Ras-Raf-MAPK pathway, we synthesized CI 1040, a specific, p.o. available, small molecule inhibitor of the MEK kinase (11) that does not inhibit raf. We found that CI 1040 inhibited MAPK phosphorylation in all cell lines tested, indicating that a constitutively active braf required MEK activity to up-regulate MAPK phosphorylation (Fig. 1B).

To address the possibility that activating BRAF mutations contribute to cellular proliferation independent of MEK, we evaluated the ability of CI 1040 to inhibit proliferation of the above cell lines. CI 1040 potently inhibited proliferation of all three lines harboring mutant BRAF but had minimal effect on the CHL line with wild-type Ras and BRAF genes (Fig. 1C). This finding indicates that CI 1040 may be specifically potent against cells dependent on constitutive MEK activation for proliferation.

To model the effects of the V599E BRAF mutation in living animals, we sought a melanoma cell line that contained this mutation in vivo. We first confirmed that the A375M melanoma cell line (15) harbored the V599E mutation similar to its parental line (data not shown). We then

Details on the synthesis of CI 1040 are available online at: http://www.mikederm.med.ucla.edu/CI1040Synthesis.htm.

Fig. 1. The Raf-MAPK pathway in melanoma. A, immunohistochemistry of a representative primary melanoma stained for phospho-MAPK. B, immunoblots of CHL, SK-MEL, WM-266-4, and A375 cell lysates, blotted for total MAPK (T-MAPK) or phosphorylated MAPK (P-MAPK) in the presence or absence of serum and CI 1040. C, quantitation of cell proliferation in the presence (gray columns) or absence (black columns) of 1 μM CI 1040. Bars, ±1 SD.

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treated A375M cells with various concentrations of CI 1040 and observed inhibition of MAPK phosphorylation, whereas total MAPK levels were unaffected (Fig. 2 A). Of note, B-raf protein levels were not decreased by CI 1040 treatment. Phosphorylation, and thus activation of MEK, was increased by treatment with CI 1040, as reported previously (16).

Anchorage-independent growth is a hallmark of the malignant phenotype. A375M cells plated in soft agar were unable to proliferate when incubated with CI 1040, whereas untreated cells grew robustly (Fig. 2 B). Ras-Raf-MAPK signaling has been reported to increase invasion of tumor cells (7). The A375M cell line has been selected for high metastatic potential in a mouse xenograft model (15). We incubated A375M cells with CI 1040 in Matrigel invasion chambers and found that CI 1040 treatment inhibited invasion in a dose-dependent manner (Fig. 2 C). Finally, the Ras-Raf-MAPK pathway has been implicated in cell survival. Incubation of A375M cells with CI 1040 caused a dose- and time-dependent apoptosis (Fig. 2 C). These in vitro data suggest that in the A375M cell line, constitutive activation of the Ras-Raf-MAPK pathway is essential for full expression of the malignant phenotype.

CI 1040 has been reported to be a p.o. effective MEK inhibitor (11). We found that administration of a single oral dose of CI 1040 to A375M tumor-bearing SCID mice at 50 mg/kg inhibited MAPK phosphorylation within 2 h (Fig. 2 D), thereby indicating that the drug is both bioavailable and effective at this dosage and route of administration.

To further examine the sequelae of MEK inhibition on melanoma in living animals, we established A375M cells stably expressing the firefly luciferase (Fluc) and GFP genes using a bicistronic lentivirus carrying both reporters (12). The resultant A375M-Fluc cells expressed a consistent level of Fluc over a 1-month period as described (12) and performed indistinguishably from parental A375M cells in soft agar and invasion assays (data not shown), indicating that lentiviral transduction was both stable and did not appreciably alter the behavior of this cell line. A375M-Fluc cells were then injected into SCID mice via the lateral tail vein, and pulmonary metastasis formation was monitored by serial bioluminescent optical imaging with a cooled CCD over 1 month. Initial experiments revealed that light emission accurately reflected tumor burden (data not shown), as shown in similar systems (12).

A375M-Fluc cells injected into untreated control mice were rapidly trapped in the pulmonary vasculature. The majority of these cells died over the next 24 h, most likely because they were unable to extravasate and survive at this ectopic site. Metastases became detectable at day 7 and grew exponentially in the lungs thereafter (Fig. 3). In contrast, mice treated with CI 1040 from the day of cell injection (day 0) until day 14 showed no detectable metastases on day 14 (Fig. 3). However, after cessation of CI 1040 administration on day 14, some tumors reemerged by days 21 and 28 (Fig. 3 B). In parallel experiments, we treated established metastases with CI 1040 for 14 days, beginning at 14 days after tail vein injection. These established tumors rapidly regressed upon CI 1040 administration (Fig. 3).

Discussion

In this report, we show that Ras-Raf-MAPK inhibition by a small molecule holds promise for both the treatment and prophylaxis of metastatic melanoma. Our data support the hypothesis that specific
kinase inhibition downstream of a commonly occurring molecular defect, i.e., Ras-Raf-MAPK activation attributable to mutation of the BRAF gene, can provide effective therapy for metastatic melanoma. A well-defined murine transgenic melanoma model has shown that ras activation is sufficient to promote melanomagenesis in an INK4A/−/− background and that withdrawal of activated ras, by tet-inducible system manipulation, results in rapid tumor regression (9). Complementary work has demonstrated widespread sensitivity of human melanoma cell lines to a MEK inhibitor in vitro (17) but did not correlate this susceptibility to tumor genotype, nor was in vivo efficacy assessed. Extensive study of both ras and raf has shown that in addition to transducing signals to MEK, these molecules have additional targets (5). Our results suggest that MEK activation is necessary for the survival of melanomas harboring either activating RAS or BRAF mutations.

Previous work has shown a cytostatic effect of CI 1040 on s.c. tumors harboring RAS mutations with resumption of growth upon cessation of the drug (11). Using serial optical imaging, we document regression of metastases treated with CI 1040. However, some A375M cells survive treatment, as evidenced by the regrowth of metastases after drug withdrawal. Serial imaging of mice allowed us to differentiate rapid tumor growth at treatment cessation from slow but continuous growth in the presence of drug, with the imaging data demonstrating the prior scenario.

Our results demonstrate that pharmacotherapy targeted downstream of deregulated BRAF or RAS signaling may be a viable approach to melanoma management. Additionally, this work suggests that the malignant phenotype resulting from mutational b-raf activation might be susceptible to targeted inhibition of MEK. Approaches aimed at polarizing the cellular response to MEK inhibition toward a cytotoxic rather than cytostatic outcome hold promise. For example, combining MEK inhibition with another agent, such as the staurosporine analog UCN-01, dramatically enhances apoptosis (18). Neovascularization is also dependent on MEK signaling, and inhibition of raf in the neovascularature is an emerging antitumor approach (19). CI 1040 also may affect angiogenesis in this manner and could account in part for the efficacy of CI 1040, although we did not directly assess this possibility in the present study. Additionally, targeted inhibition of kinases in cancer has been shown to rapidly give rise to resistance in the clinic (10). The A375M cell line harbors a naturally occurring BRAF mutation and could be adapted to select for mechanisms of resistance to MEK inhibition and thus provide a framework for interpreting clinical observations with MEK inhibitors.

The V599E BRAF mutation is highly prevalent in both premalignant melanocytic nevi and melanoma, making it the earliest known genetic change in this disease. An emerging theory of cancer progression to metastasis views early events in carcinogenesis as predictive of later metastatic ability (20). The observation that metastatic-like transcriptional profiles exist within primary tumors supports the concept that the genetic changes in a primary tumor are sufficient for metastasis (21). Our findings further support this theory by demonstrating that the earliest known genetic change in melanoma is necessary for a fully metastatic phenotype, and therapy targeted against this change can reverse the metastatic progression of this disease.

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References


Fig. 3. MEK inhibition is effective in vivo. A, A37M-Fluc cells were injected into SCID mice via the lateral tail vein. Serial images of a representative mouse from each group are pictured. Mice were treated with either PBS (Control, left column), CI 1040 at 50 mg/kg, p.o. twice per day either from days 0–14 (Early Treat, middle column) or days 15–28 (Late Treat, right column). On the indicated days, mice received injections i.p. with 3 mg of 0-luciferin and placed in a cooled CCD camera for imaging. B, results were averaged from the peak light-emitting exposure from each group and graphed in logarithmic scale.


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