The Novel ATP-Competitive MEK/Aurora Kinase Inhibitor BI-847325 Overcomes Acquired BRAF Inhibitor Resistance through Suppression of Mcl-1 and MEK Expression

Manali S. Phadke¹, Patrizia Sinì², and Keiran S.M. Smalley¹,³

Abstract

Resistance to BRAF inhibitors is a major clinical problem. Here, we evaluate BI-847325, an ATP-competitive inhibitor of MEK and Aurora kinases, in treatment-naïve and drug-resistant BRAF-mutant melanoma models. BI-847325 potently inhibited growth and survival of melanoma cell lines that were both BRAF inhibitor naïve and resistant in 2D culture, 3D cell culture conditions, and in colony formation assays. Western blot studies showed BI-847325 to reduce expression of phospho-ERK and phospho-histone 3 in multiple models of vemurafenib resistance. Mechanistically, BI-847325 decreased the expression of MEK and Mcl-1 while increasing the expression of the pro-apoptotic protein BIM. Strong suppression of MEK expression was observed after 48 hours of treatment, with no recovery following >72 hours of washout. siRNA-mediated knockdown of Mcl-1 enhanced the effects of BI-847325, whereas Mcl-1 overexpression reversed this in both 2D cell culture and 3D spheroid melanoma models. In vivo, once weekly BI-847325 (70 mg/kg) led to durable regression of BRAF-inhibitor naïve xenografts with no regrowth seen (>65 days of treatment). In contrast, treatment with the vemurafenib analog PLX4720 was associated with tumor relapse at >30 days. BI-847325 also suppressed the long-term growth of xenografts with acquired PLX4720 resistance. Analysis of tumor samples revealed BI-847325 to induce apoptosis associated with suppression of phospho-ERK, total MEK, phospho-Histone3, and Mcl-1 expression. Our studies indicate that BI-847325 is effective in overcoming BRAF inhibitor resistance and has long-term inhibitory effects upon BRAF-mutant melanoma in vivo, through a mechanism associated with the decreased expression of both MEK and Mcl-1.

Introduction

Melanoma is the most lethal type of skin cancer whose incidence continues to increase worldwide (1, 2). Around 50% of melanomas harbor BRAF mutations, with 90% of these occurring at the 600 position, leading to constitutive kinase activation (3). It is known that BRAF V600E-mutant melanomas are dependent on MAPK signaling pathway for their proliferation, invasion, and resistance to apoptosis (4–6). In the clinic, selective BRAF inhibitors such as vemurafenib and dabrafenib are associated with high levels of response, which are typically short-lived (7, 8). Most of the clinically validated resistance mechanisms reported to date involve the reactivation of MEK/ERK signaling and current strategies are focused upon combinations that vertically inhibit the MAPK signaling pathway. There is already evidence that the combination of a MEK inhibitor and a BRAF inhibitor (dabrafenib/trametinib and cobimetinib/vemurafenib) is associated with greater progression-free and overall survival compared with BRAF inhibitor alone (9, 10). Despite this, relapse and resistance also occurs in the majority of patients receiving the BRAF/MEK inhibitor combination and further therapeutic strategies are urgently needed (11, 12).

Cancer is characterized by uncontrolled cell growth and deregulation of the cell cycle (13). The mitotic cell cycle is controlled through the activity of protein–protein complexes such as the chromosomal passenger complex proteins including the Aurora kinases, survivin, borealin, and INCENP (14). Increased expression of some of these, such as Aurora kinase B, has been observed in many cancers, including melanoma (15, 16). In melanoma cells, expression of Aurora kinase B is controlled via the MAPK signaling pathway through the transcription factor FOXM1 (15). There is also evidence that melanoma cells with acquired BRAF inhibitor resistance have a greater dependency upon Aurora B (15). Aurora kinase A plays a major role in centrosome function and spindle assembly as well as cytokinesis and the telophase of mitosis (17). A potential role for Aurora kinase A in melanoma was demonstrated by the ability of the Aurora kinase A inhibitors MLN8054/MLN8237 to reduce entry into mitosis, induce senescence, and to inhibit the proliferation of patient-derived melanoma tumor xenografts (18).

¹Department of Tumor Biology, The Moffitt Cancer Center, Tampa, Florida. ²Boehringer Ingelheim RCV GmbH & Co KG, Vienna, Austria. ³Department of Cutaneous Oncology, The Moffitt Cancer Center, Tampa, Florida.

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Corresponding Author: Keiran S. M. Smalley, The Moffitt Cancer Center, 12902 Magnolia Drive, Tampa, FL 33612. Phone: 813-745-8725; Fax: 813-449-8260; E-mail: keiran.smalley@moffitt.org

doi: 10.1158/1535-7163.MCT-14-0832

©2015 American Association for Cancer Research.
Bl-847325 is a novel, ATP-competitive, orally available inhibitor of Aurora kinases and MEK. In *in vitro* studies, BI-847325 inhibited the activity of *Xenopus laevis* Aurora Kinase B with an IC₅₀ of 3 nmol/L, with IC₅₀ values for human Aurora kinase A and Aurora kinase C being 25 and 15 nmol/L, respectively. BI-847325 also inhibited human MEK1 and MEK2 with respective IC₅₀ values of 25 and 4 nmol/L. In a panel of 29 additional kinases that represented the diversity of the kinome tree, BI-847325 inhibited seven enzymes at 1 nmol/L by more than 50% (LCK, MAP3K8, FGFR1, AMPK, CAMK1D, RAF, and TBK1). The only kinases inhibited significantly in isolated kinase assays at concentrations <100 nmol/L were LCK (5 nmol/L) and MAP3K8 (93 nmol/L).

In the current study, we demonstrate BI-847325 to be highly effective at overcoming acquired BRAF inhibitor resistance mediated through multiple mechanisms in both cell lines and human melanoma mouse xenograft models. Our results reveal BI-847325 to have a novel mechanism of action involving the downregulation of both Mcl-1 and MEK.

Materials and Methods

Cell culture

The parental melanoma cell lines 1205Lu, WM793, WM39, and WM164 were a kind gift from Dr. Meenhard Herlyn (The Wistar Institute, Philadelphia, PA). Cell lines were genotyped for BRAF V600E mutations in (19). The M229, M229R, M249, and M249R were generated as described in Nazarian and colleagues (20). The A375 and RPMI-7951 melanoma cell lines were purchased from ATCC and A375R was a kind gift from Plexxikon Inc. The identity of each cell line was confirmed through STR validation analysis by Biosynthesis Inc. Vemurafenib-resistant cell lines 1205LuR, WM793R, and WM164R were generated in (ref. 21). All the naive and intrinsically resistant cell lines were cultured in RPMI complete medium with 5% FBS. All the acquired resistant cell lines were cultured in RPMI complete medium with 5% FBS with the addition of vemurafenib at the following concentrations: M229R and M249R (2 µmol/L), A375R (2.5 µmol/L), WM793R and WM164R (2 µmol/L), and 1205LuR (3 µmol/L).

Cell proliferation assay

Cells were plated at a density of 2.5 × 10⁵ cells per 100 µL and left to grow overnight before being treated with increasing concentrations of BI-847325 (Boehringer Ingelheim) for 72 hours. The metabolic activity was determined using Alamar blue reagent as per the manufacturer’s protocol.

Colony formation assay

Cells were grown overnight at a density of 1 × 10⁴ cells per mL and treated with vehicle (DMSO) or with 30 nmol/L, 100 nmol/L, 300 nmol/L, and 1 µmol/L of BI-847325 (Boehringer Ingelheim). The medium and drug/vehicle was replaced every 2 weeks. After 4 weeks of treatment, the colonies were stained with crystal violet dye, as described in (ref. 22). The percent relative clonogenic survival was determined using ImageJ software.

Flow cytometry

Cells were plated into 6-well plates at a density of 1 × 10⁶ cells per mL and left to adhere overnight. Cells were treated with 10 nmol/L, 100 nmol/L, and 1 µmol/L BI-847325 (Roehringer Ingelheim), 30 nmol/L trametinib (Selleck), 1 µmol/L VX680 (Selleck) for 48 hours. Annexin V and TMRM staining was done as described in (ref. 21).

Three-dimensional spheroid assay

3D melanoma spheroids were prepared using the liquid overlay method as described in (ref. 23) before being treated with vehicle (DMSO) or 300 nmol/L to 3 µmol/L of BI-847325 (Boehringer Ingelheim) for 48 hours. Spheroids were washed, stained with Live/Dead viability stain (Invitrogen/Life Technologies Corp.), and analyzed as described in ref. 14. The percentage of dead cells was determined using ImageJ software.

Western blotting

Proteins extraction and blotting were performed as in (ref. 24). The primary antibodies for phospho-ERK, total-ERK, phospho-Histone 3, Mcl-1, BIM, and total MEK were from Cell Signaling Technology. The GAPDH antibody (Sigma-Aldrich) was used as a loading control to demonstrate even protein loading. The secondary antibodies, goat anti-rabbit IgG horseradish peroxidase (HRP) and sheep anti-mouse IgG HRP, were from Amersham/GE Healthcare. For mouse xenograft experiments, tumor samples were harvested and immediately stored in RNA later solution (Ambion) at 4°C before protein extraction.

Rnai

1205Lu, 1025LuR, WM793, WM793R, and WM793-Mcl-1 cells were plated at a cell density of 1 × 10⁵ and left overnight to grow in complete RPMI medium with 5% FBS. The complete medium was replaced with Opti-MEM (Invitrogen). Cells were transfected with siRNA for BIM, Mcl-1, MEK 1, or MEK 2 (Cell Signaling Technology) in complex with Lipofectamine 2000 (Invitrogen). For the Mcl-1 experiments, 25 nmol/L of siRNA concentration was used and cells were transfected overnight. In the BIM studies, cells were transfected with 50 nmol/L siRNA overnight. For MEK1 and MEK2 studies, cells were transfected with 50 nmol/L siRNA for 4 hours. Scrambled siRNAs were added as nontargeting control. Following the transfection time, cells were replaced with complete RPMI medium with 5% FBS and treated with 1 µmol/L BI-847325, 30 nmol/L trametinib, 1 µmol/L VX680 for 48 hours.

Quantitative real-time PCR

Cells were treated with 1 µmol/L BI-847325 for 48 hours before RNA isolation. Total RNA was extracted using Qiagen’s RNeasy mini kit. TaqMan Gene Expression Assays primer/probes used were: Hs00360961_m1 (MEK2), Hs01050896_m1 (Mcl-1), and P/N 4319413E (18S) for normalizing the data. Quantitative reverse-transcriptase PCR reactions were carried out as per Molecular Genomics Core Facility protocol. For mouse xenograft experiments, tumor samples were harvested and stored in RNAlater solution (Ambion) at −80°C before RNA extraction.

Mcl-1 inducible cell line

WM793-Mcl-1 cells were a generous gift from Dr. Andrew Aplin (Kimmel Cancer Center, Philadelphia). Mcl-1 expression was induced by treating the cells with 100 ng/mL doxycycline for 72 hours before treatment with 1 µmol/L BI-847325 for 48 hours.

www.aacjrournals.org Mol Cancer Ther; 14(6) June 2015 1355

Published OnlineFirst April 14, 2015; DOI: 10.1158/1535-7163.MCT-14-0832

Downloaded from mct.aacrjournals.org on June 17, 2017. © 2015 American Association for Cancer Research.
Proteasome-Glo Chymotrypsin-like cell-based assay

1205Lu and 1205LuR cells were seeded in 96-well plates at a density of 7,500 cells/50 μL per well and left overnight to grow before treatment with 1 μmol/L BI-847325 or 300 nmol/L, 1 μmol/L and 3 μmol/L MG-132 (Selleck), or BI 847325 and MG-132 in combination for 48 hours. Control cells were treated with vehicle (DMSO). Proteasomal activity was measured by luminometry using Proteasome-Glo Chymotrypsin-like cell-based assay (Promega) as per the manufacturer’s protocol.

Mouse human melanoma xenograft experiments

BALB SCID mice (Taconic) were subcutaneously injected with 2.5 × 10^6 cells. The tumors were grown to approximately 100 mm^3 size before initiation of dosing. Mice (n = 5) were treated with 70 mg/kg of BI-847325 or equivalent volume of vehicle (2-hydroxyethyl cellulose (Sigma-Aldrich), polysorbate treated with 70 mg/kg of BI-847325 or equivalent volume of control chow (Plexxikon). The mouse weights and tumor volumes (L x W^2 x 0.523) were measured twice per week. On completion of the experiment (after <24 hours for PLX4720-treated and 48 hours for BI-847325-treated mice), the treated and control tumors collected were processed for Western blotting and RNA extraction.

IHC staining

Mice xenograft tissues were formalin fixed and paraffin embedded. Slides were stained for cleaved caspase-3 as per the manufacturer’s protocol by Moffitt Tissue Core Research Histology services.

Statistical analysis

All the data show mean of three independent experiments ± SEM. Results with p value ≤ 0.05 were considered as statistically significant.

Results

BI-847325, a novel ATP-competitive MEK/Aurora kinase inhibitor, has prolonged growth-inhibitory effects on BRAF-mutant and vemurafenib-resistant melanoma cells

A panel of 14 BRAF-mutant melanoma cell lines (including some that had either acquired or intrinsic vemurafenib resistance: resistance mechanisms listed in Supplementary Table S1) was treated with BI-847325 (structure shown in Fig. 1). Concentration-dependent decreases in cell growth were noted in all cell lines evaluated (Fig. 2A). The IC_{50} values (Supplementary Table S2) demonstrate that vemurafenib-resistant cell lines were less sensitive to BI-847325 compared with the naïve cell lines. Treatment of the cell line panel with BI-847325 (30–300 nmol/L, 28 days) prevented colony formation in six BRAF-mutant melanoma cell lines (Fig. 2B and Supplementary Fig. S1A). The growth-inhibitory effect of BI-847325 (48 hours, 1 μmol/L) was also associated with apoptosis induction, with significant levels of cell death being seen at concentrations >100 nmol/L (Fig. 2C). Apoptosis induction was observed in melanoma cell lines that were both vemurafenib naïve and -resistant (Supplementary Fig. S1B). BI-847325 was also cytotoxic (>300 nmol/L) in a 3D cell spheroid model of cell lines that were both vemurafenib naïve and resistant (Fig. 2D).

BI-847325 induces apoptosis by reducing Mcl-1 expression

Western blot studies showed BI-847325 to inhibit both MEK and Aurora Kinase as shown by decreased expression of both phospho-ERK and phospho-histone-3 (Fig. 3A). BI-847325 treatment was also associated with upregulated expression of the proapoptotic protein BIM and decreased expression of Mcl-1 (Fig. 3A). Pharmacodynamic studies revealed BI-847325 to mediate concentration- and time-dependent inhibition of MEK and Aurora kinase. Although it was noted that the inhibition of Aurora kinase and MEK was equipotent, a time-dependent recovery of phospho-ERK signaling was seen following treatment with 100 nmol/L, but not 1 μmol/L, BI-847325 (Fig. 3B and C). No recovery of Aurora Kinase signaling was noted at either concentration of BI-847325. We next determined whether the effects of BI-847325 could be recapitulated through the dual inhibition of MEK and Aurora kinase. We began by determining concentrations of both the MEK inhibitor trametinib and the Aurora kinase inhibitor VX680 that supramaximally inhibited MEK and Histone H3 phosphorylation, respectively (Supplementary Fig. S2A). It was noted that 300 nmol/L of each drug inhibited MEK and Aurora kinase, respectively, to the same degree as that seen to 1 μmol/L of BI-847325, and that neither the Aurora Kinase inhibitor alone, nor its combination with trametinib, inhibited Mcl-1 expression (Supplementary Fig. S2B). A partial role for BIM induction in the cytotoxic activity of BI-847325 was confirmed through siRNA studies in which the knockdown of BIM was associated with a significant (but minor) decrease in apoptosis (Fig. 3D).

BI-847325-mediated apoptosis is associated with reduced Mcl-1 expression

We next investigated the role of Mcl-1 downregulation in the cytotoxic activity of BI-847325. qRT-PCR experiments showed BI-847325 treatment to be associated with a significant reduction in Mcl-1 mRNA in four BRAF-mutant melanoma cell lines, including two with acquired BRAF inhibitor resistance (WM793R and 1205LuR; Fig. 4A). In contrast, no downregulation of mRNA was seen in the WM793-Mcl-1 cell line where overexpression of Mcl-1 was driven under a
doxycycline-inducible promoter (the WM793-Mcl-1 cell line). The decreased expression of Mcl-1 appeared to be required for the proapoptotic activity of BI-847325, with studies showing Mcl-1 knockdown through siRNA to significantly enhance trametinib-induced apoptosis (Fig. 4B and C).

**Induction of Mcl-1 expression protects from BI-847325-mediated cell death**

We next used a BRAF-mutant melanoma cell line with inducible Mcl-1 expression (WM793-Mcl-1) to determine whether forced Mcl-1 expression protected melanoma cells from BI-847325-mediated cell death. Initial studies showed that BI-847325 was unable to decrease the expression of induced Mcl-1 in the WM793-Mcl-1 cell line (Fig. 5A). We next used Annexin-V binding/flow cytometry assays to show that induction of Mcl-1 was associated with a significant reduction of BI-847325-mediated apoptosis (Fig. 5B). The combination of the MEK inhibitor trametinib with the Aurora kinase inhibitor VX680 did not recapitulate the induction of apoptosis by BI-847325 (Fig. 5A and B). Mcl-1 overexpression also enhanced survival of the cells in a 3D collagen-implanted spheroid model treatment with little cell death seen at BI-847325 concentrations as high as 3 μmol/L (Fig. 5C).

BI-847325-mediated cytotoxicity in vemurafenib-naive and -resistant melanoma cell lines is associated with decreased MEK expression

Acquired resistance to BRAF inhibitors typically leads to the reactivation of MAPK signaling. As treatment with BI-847325 was highly effective at overcoming acquired BRAF...
inhibitor resistance, we next determined the effects the drug had upon components of the MAPK signaling pathway. Unexpectedly it was found that BI-847325 treatment decreased total MEK expression in 3 of 4 melanoma cell lines tested (Fig. 6A). The decrease in MEK expression seen was slow in onset (>24 hours; Fig. 6B) but was durable, with no recovery of expression seen 72 hours after washout of the drug (Fig. 6C, left). These effects were also concentration dependent, with differences in sensitivity observed between the naïve and resistant cell lines. In the vemurafenib-naïve 1205Lu cell lines, decreased MEK expression was seen at 1 μmol/L, the same concentration required to block reactivation of ERK signaling (Figs. 3C and 6C). We confirmed that the decrease in MEK expression following BI-847325 treatment was not mimicked by MEK inhibition (trametinib), Aurora kinase inhibition (VX680), or the MEK/Aurora kinase inhibitor combination in BRAF-mutant melanoma cell lines (Supplementary Fig. S2B).

We next performed siRNA knockdown of MEK1/2 to determine whether suppression of MEK enhanced the activity of Aurora kinase inhibitors. Knockdown of MEK1 and MEK2 was confirmed through Western blot analysis (Fig. 6D). In this instance, the downregulation of MEK1 expression enhanced the proapoptotic effects of VX680 in the 1205Lu cell line. In addition, a significant enhancement of the response was also seen in the 1205LuR cell line when both MEK1 and MEK2 were knocked down (Fig. 6D). The effects of BI-847325 upon MEK expression were not mediated through increased targeting of MEK to the proteasome, as these were not reversed following treatment with the proteasome inhibitor MG-132 (Supplementary Fig. S3A). There was also little evidence that BI-847325 consistently altered mRNA levels of MEK (Supplementary Fig. S3B).

Figure 3.
BI-847325 induces apoptosis altering the expression of pro and antiapoptotic proteins. A, Western blot analysis reveals that treatment with 1 μmol/L BI-847325 for 48 hours inhibits phospho-ERK, phospho-Histone3, decreases Mcl-1 expression, and upregulates BIM expression in four BRAF-mutant cell lines. B, BI-847325 inhibits MEK and Aurora kinase in a dose-dependent manner. Two BRAF-mutant melanoma cell lines were treated with BI-847325 (10 nmol/L–3 μmol/L; 48 hours) followed by Western blotting for pERK and pHistone-H3. C, ERK signaling recovers at lower concentrations of BI-847325. Treatment with 100 nmol/L and 1 μmol/L BI-847325 showed a time-dependent decrease in the phosphorylation of Histone-H3 and ERK. D, top, Western blot analysis showing siRNA-mediated knockdown of BIM in 1205Lu and 1205LuR cells. Cells were transfected with 25 nmol/L siRNA overnight, followed by lipofectamine washout and treatment with 1 μmol/L BI-847325 for 48 hours. Cells transfected with nontargeting siRNA were used as control. Bottom, siRNA knockdown of BI-847325-mediated BIM significantly reduces apoptosis in 1205Lu and 1205LuR cell lines. Cells were stained with Annexin V and analyzed by flow cytometry. *, P < 0.05.
BI-847325 treatment suppresses the growth of BRAF-mutant and vemurafenib-resistant human melanoma xenografts in mice

We next assessed the anti-melanoma activity of BI-847325 and its ability to overcome vemurafenib resistance in vivo. Of the four BRAF-mutant melanoma cell lines tested, the 1205Lu and 1205LuR formed xenografts in SCID mice. A 70 mg/kg/week dose schedule of BI-847325 was evaluated for efficacy against established 1205Lu and 1205LuR tumors, with significant tumor suppression being seen in the drug-naïve 1205Lu xenografts that lasted >65 days (Fig. 7A). Long-term suppression of tumor growth was also observed in the vemurafenib-resistant 1205LuR xenograft model throughout the 55-day treatment period (Fig. 7A). No significant alteration in the body weights of the mice was observed during the study period, indicating that the BI-847325 was well tolerated (data not shown).

PLX4720 is a BRAF inhibitor that has a similar potency and pharmacologic profile to vemurafenib, but was not selected for clinical development (25). In contrast with the long-term suppression of growth seen in response to BI-847325, treatment of 1205Lu tumors with PLX4720 was only associated with a short-term inhibition of tumor growth and the onset of resistance by day 30 (Fig. 7B). A pharmacodynamic analysis of 1205Lu and 1205LuR xenograft specimens dosed with BI-847325 (70 mg/kg/week) showed significant decreases in intratumoral...
Mcl-1 and MEK and expression after 55 days of treatment (Fig. 7C). Decreases were also seen in phospho-ERK and phospho-Histone H3, indicating that both the MAPK and Aurora kinase were being inhibited (Fig. 7C). Together, our results indicate that BI-847325 is effective in overcoming vemurafenib resistance in vivo. It was further found that PLX4720 had little effect upon phospho-ERK, phospho-Histone H3, MEK, or Mcl-1 expression (Fig. 7C). Treatment of 1205Lu and 1205LuR xenografts with BI-847325 was also associated with decreased expression of MEK at the RNA level (Fig. 7D). Increased intratumoral levels of apoptosis were also seen following BI-847325 treatment as shown by the increased staining for cleaved caspase-3 (Fig. 7E).

Discussion
Despite the improvement in survival rates of patients with BRAF-mutant melanoma after vemurafenib treatment, responses still remain transient with relapse and resistance being common in most cases (7, 8). Most of the mechanisms of acquired BRAF inhibitor resistance identified thus far lead to restoration of signaling through the MAPK pathway (26). Studies have shown the MAPK pathway to be reactivated in 70% to 79% of melanomas with acquired vemurafenib and dabrafenib resistance (26, 27). The recovery of MAPK signaling can be driven through the acquisition of mutations in NRAS, MEK1/2, as well as through BRAF-splice form mutants (11, 20, 28). Although there is good evidence that vertical targeting of the MAPK pathway through combined BRAF/MEK inhibition delays therapeutic escape, this seems largely ineffective after resistance is acquired (29). In the current study, we provide evidence that the novel ATP-competitive MEK/Aurora kinase inhibitor BI-847325 overcomes acquired BRAF inhibitor resistance through a novel mechanism involving the suppression of both MEK and Mcl-1. This strategy was effective against multiple cellular mechanisms of acquired BRAF inhibitor resistance and reduced tumor growth in in vivo models of BRAF inhibitor-naïve and -resistant melanoma.

Pharmacologic studies showed BI-847325 to abrogate BRAF inhibitor resistance mediated through increased PDGFRβ

Figure 5.
Decreased expression of Mcl-1 is required for BI-847325-mediated apoptosis. A, Western blot analysis showing overexpression of Mcl-1 in WM793-Mcl-1 cell lines following treatment with 100 ng/mL of doxycycline (72 hours). WM793 and WM793-Mcl-1 cells were treated with vehicle, BI-847325, trametinib, VX680, or trametinib + VX680 for 48 hours, before being blotted for Mcl-1 and BIM. B, BI-847325-mediated apoptosis is significantly reduced in Mcl-1 overexpressed cells. Cells were treated with vehicle, BI-847325, trametinib, VX680, or trametinib + VX680 for 48 hours, before being stained with Annexin-V. Apoptosis was measured by flow cytometry. *, P < 0.05. C, Mcl-1 overexpression prevents BI-847325-induced cell death in 3D organotypic cell cultures of BRAF-mutant melanoma cell lines. Left, cultures were treated with BI-847325 (1 μmol/L, 48 hours) before being stained with ethidium bromide and calcein-AM for dead and live cells, respectively. Right, quantitative analysis of the percentage dead cells from the 3D spheroid model following BI-847325 treatment.
expression (M229R), cyclin D1 amplification (WM39), acquisition of a RAS mutation (A375R, M249R), and COT amplification (RPMI7951). It was also active in a number of resistance models for which the mechanism has not yet been determined (1205LuR, WM793R, WM164R). In all cases, BI-847325 durably suppressed the growth of multiple BRAF-mutant melanoma cell lines with little evidence of colonies emerging after 4 weeks of in vitro treatment. In contrast it was noted that the same cell lines when treated with vemurafenib, gave rise to resistant colonies in every case (22). BI-847325 was also highly cytotoxic; inducing apoptosis in 2D cell culture as well as high levels of cell death in more physiologically relevant 3D cell cultures (23) and in vivo xenograft models. In some of the cell lines, BI-847325 showed a greater potency on the resistant cell line than its drug-naïve counterpart.

In BRAF-mutant melanoma cells, the MAPK pathway is an important regulator of cell survival through the suppression of expression of proapoptotic BH3 family proteins as well as by increasing levels of important prosurvival proteins (30–32). BIM is a BH3-only protein that induces cell death through antagonizing the prosurvival effects of Bcl-2, Bcl-XL, Bcl-w, and Mcl-1 (33). In melanoma cell lines, levels of BIM are kept low through the MAPK-mediated phosphorylation of BIM at Ser69, leading to its proteasomal targeting (30, 34). Treatment with inhibitors of MEK and BRAF prevents the destruction of BIM by the proteasome leading to apoptosis (35, 36). Acquired BRAF inhibitor resistance is associated with increased basal phosphorylation of BIM at Ser69, as well as a suppression of its expression through epigenetic means—a state that can be reversed through treatment with inhibitors of HSP90 and epigenetic modifiers such as HDAC inhibitors (21, 37). Treatment of both BRAF inhibitor-naïve and -resistant melanoma cell lines with BI-847325 increased expression of BIM and there was evidence through siRNA knockdown that BIM induction was at least partly required for the BI-847325-mediated apoptotic response.
The antiapoptotic protein myeloid cell leukemia-1 (Mcl-1) is a member of the Bcl-2 family of proteins (38). It is known to play a critical role in cell survival and is overexpressed in many cancers (39). The prosurvival effects of Mcl-1 are mediated through its binding to and neutralizing the death-inducing effects of BIM, as well as Bak and Bax (40). Previous studies have shown Mcl-1 to be overexpressed in melanoma cells and that its expression is subject to regulation by the BRAF/MEK signaling cascade (39). Downregulation of Mcl-1 by RNAi increases the susceptibility of BRAF mutant melanoma cell lines to anoikis, a form of apoptosis induced by inappropriate adhesion (39).

BI-847325 treatment decreased the expression of Mcl-1 at both the protein and mRNA levels. In this regard, BI-847325 differed from other Aurora Kinase inhibitors such as VX680, which had little effect upon Mcl-1 expression in the BRAF-mutant cell lines. There is evidence in other systems that Aurora Kinase inhibitors alter the dependency on the expression of prosurvival BH3-only family proteins. In carcinoma cell lines, the Aurora kinase inhibitor VX680 induced apoptosis following the entry into polyploidy (41). In this context, the polyploid state was associated with neutralization of Mcl-1 function and an increased dependency upon Bcl-XL for survival (41). Down-regulation of Mcl-1 expression in the context of MEK inhibition was implicated in the proapoptotic effects of BI-847325. It was noted that the inducible overexpression of Mcl-1 conveyed complete protection to BI-847325-mediated death in both 2D cell culture assays and 3D collagen implanted spheroids. siRNA knockdown of Mcl-1 in combination with the MEK inhibitor trametinib recapitulated the cytotoxic effects of BI-847325. These findings confirm earlier studies showing Mcl-1 to be a key regulator of sensitivity to MEK inhibition in BRAF-mutant melanoma cells (42).
BL-847325 also appeared to have unique MEK inhibitory activity through the regulation of its expression. The decrease in MEK levels seen following BL-847325 treatment was slow in onset, occurring between 24 and 72 hours of treatment and was durable, with little recovery seen 72 hours after washout. This activity was in marked contrast with allosteric inhibitors of MEK such as trametinib, which inhibited MEK kinase function without altering protein expression. Mechanistically, the effects of BL-847325 upon MEK downregulation did not seem to be dependent upon its increased targeting to the proteasome, as they were not reversed upon treatment with the proteasome inhibitor MG-132. There was little evidence that BI-847325 altered MEK expression at the mRNA level, with a qRT-PCR analysis showing differing results across the cell lines. Together these data suggested that the decrease in MEK protein expression following BI-847325 treatment was unlikely to be a consequence of its downregulation at the RNA level.

The long-term suppression of MEK expression appeared to underlie the durable antitumor responses seen in vivo when BI-847325 was administered weekly. Although concentrations >1 μmol/L were required to achieve this, this can be readily achieved in vivo, with the once weekly dose of BI-847325 (70 mg/kg) being associated with peak plasma concentrations of 2700 nmol/L in mice (Sini et al; unpublished observations). As the vast majority of therapeutic escape mechanisms involve reactivation of MAPK signaling, the direct suppression of MEK expression appears to be an excellent strategy to delay and abrogate the onset of resistance. Our group and others have already provided evidence that the combined decreased of CRAF and Mcl-1 expression and the inhibition of MAPK signaling through the use of HSP90 inhibitors may be a viable strategy to overcome and prevent acquired BRAF inhibitor resistance (21, 43, 44).

Although our study confirms the utility of combined MEK and Aurora kinase inhibition, not all the activity of BI-847325 could be recapitulated by the inhibition of Aurora Kinase and MEK. An analysis of BI-847325 against a panel of 30 kinases, showed only Lck and p38 MAPK to have an IC50 <100 nmol/L (Sini et al; unpublished observations). As yet, the potential role of these kinases in the anti-melanoma activity seen to BI-847325 remains to be determined. A more interesting explanation may be based on the fact that BI-847325 interacts with the ATP-binding site of the target protein, whereas trametinib (as well as all other MEK inhibitors currently in clinical development) are allosteric inhibitors that bind to a distant site on MEK. It is conceivable that these distinct protein–ligand interactions result in differences in MEK conformation and, consequently, its interaction with other proteins and perhaps its stability.

In BRAF-mutant melanoma cell lines, two groups have already demonstrated that the MAPK pathway directly regulates Aurora kinase B, with one group implicating the transcription factor FOXM1 (15). The Aurora protein family consists of three members Aurora A, Aurora B, and Aurora C all of which are frequently overexpressed in cancer. Of these, overexpression of Aurora A in NIH-3T3 cells is known to enhanced colony formation and solid tumor formation, as well as being implicated in the emergence of centrosome abnormalities and aneuploidy in breast cancer (45, 46). Previous studies have reported that overexpression of Aurora kinase B contributes to melanoma progression by causing genomic instability and aneuploidy (15, 47). Other work has implicated Aurora kinase in the escape from vemurafenib therapy, with two BRAF inhibitor-resistant cell lines showing sensitivity to the Aurora kinase B inhibitor AZD1152 (15). There is also some limited evidence in 3D cell culture models that the BRAF/MEK/Aurora kinase A inhibitor combination is more effective at reducing melanoma cell growth than any of these agents alone (48).

At this time, there is much focus upon pathways that can be targeted in combination with the MAPK pathway in melanoma. Although there has been interest in the dual targeting of BRAF with the PI3K/AKT/mTOR pathway, this has been associated with significant toxicity and little clinical efficacy. Here, we show for the first time that the dual targeting of Aurora kinase and MEK gives more durable effects than BRAF inhibition alone. Our study demonstrates that drugs like BI-847325 that suppress expression of both MEK and Mcl-1 are highly effective at both abrogating the onset and overcoming acquired BRAF inhibitor resistance. We suggest that strategies such as these are worthy of further clinical investigation in BRAF-mutant melanoma.

Disclosure of Potential Conflicts of Interest

K.S.M. Smalley reports receiving commercial research grant from Boehringer Ingelheim. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: K.S.M. Smalley
Development of methodology: M.S. Phadke, K.S.M. Smalley
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.S. Phadke, K.S.M. Smalley
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.S. Phadke, K.S.M. Smalley
Writing, review, and/or revision of the manuscript: M.S. Phadke, P. Sini, K.S.M. Smalley
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.S. Phadke
Study supervision: K.S.M. Smalley

Grant Support

Work in the Smalley laboratory was supported by SPORE grant 1P50CA168536-01A1 and R01 CA161107-01 from the NIH and through support from Boehringer Ingelheim.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 26, 2014; revised March 23, 2015; accepted April 6, 2015; published OnlineFirst April 14, 2015.


Molecular Cancer Therapeutics

The Novel ATP-Competitive MEK/Aurora Kinase Inhibitor BI-847325 Overcomes Acquired BRAF Inhibitor Resistance through Suppression of Mcl-1 and MEK Expression

Manali S. Phadke, Patrizia Sini and Keiran S.M. Smalley

Mol Cancer Ther 2015;14:1354-1364. Published OnlineFirst April 14, 2015.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-14-0832

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2015/04/15/1535-7163.MCT-14-0832.DC1

Cited articles
This article cites 48 articles, 22 of which you can access for free at:
http://mct.aacrjournals.org/content/14/6/1354.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/14/6/1354.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.