BRAFV600E Cooperates with PI3K Signaling, Independent of AKT, to Regulate Melanoma Cell Proliferation

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Abstract

Mutationally activated BRAFV600E cooperates with PTEN silencing in the conversion of normal melanocytes to metastatic melanoma cells, but the mechanism underlying this cooperation is poorly understood. Here, the consequences of pharmacologic blockade of BRAFV600E or phosphoinositide 3-kinase (PI3K) signaling were explored using pathway-targeted inhibitors and a panel of human BRAF-mutated melanoma-derived cell lines. Blockade of BRAFV600E → MEK1/2 → ERK1/2 or class I PI3K inhibited melanoma proliferation, whereas inhibition of AKT had only modest effects, even in cells with mutated or amplified AKT. Although single-agent inhibition of either BRAFV600E or PI3K signaling elicited antiproliferative effects, combinatorial inhibition was more potent. Analysis of signaling downstream of BRAFV600E or PI3K revealed that these pathways cooperated to regulate protein synthesis through AKT-independent, mTOR complex 1 (mTORC1)-dependent effects on p70S6K, ribosomal protein S6, and 4E-BP1 phosphorylation. Moreover, inhibition of mTORC1/2 inhibited cell proliferation as profoundly as single-agent inhibition of either BRAFV600E or PI3K signaling. These data reveal a mechanism by which BRAFV600E and PI3K signaling cooperate to regulate melanoma proliferation through AKT-independent effects on protein translation. Furthermore, this study provides a potential foundation for pathway-targeted combination therapy designed to enhance the therapeutic benefit to patients with melanoma that contain combined alterations in BRAF and PI3K signaling.

Implications: PI3K, but not AKT, represent potential targets for melanoma therapy. Mol Cancer Res; 12(3); 1–17. ©2014 AACR.

Introduction

Melanoma is known for its rapidly increasing incidence, aggressive clinical behavior, and propensity for lethal metastasis (1). Mutational activation of BRAF, detected in ≥50% of patients, is one of the earliest and most common genetic alterations in melanomagenesis (2, 3). The importance of mutationally activated BRAFV600E in melanoma malignance was demonstrated by the clinical success of vemurafenib and dabrafenib, ATP-competitive inhibitors of BRAFV600E activity (4, 5).

BRAF mutations are detected at high frequency in benign nevi, nonmalignant melanocytic lesions that display hallmarks of senescence, and rarely progress to melanoma (3). Malignant progression of BRAFV600E-expressing melanocytes is frequently promoted by silencing of the tumor suppressor PTEN, a phosphatidylinositol-3,4,5-triphosphate (PIP3) phosphatase that suppresses the production of PI3'-lipids in the cell (6–10). The sufficiency for these alterations in melanomagenesis was demonstrated using genetically engineered mouse (GEM) models of metastatic melanoma built upon this same foundation (11–13).

Recently, RAF → MEK1/2 → ERK1/2 and PI3K → AKT signaling was demonstrated to cooperatively regulate protein translation in carcinomas through inhibitory phosphorylation of 4E-BP1, a negative regulator of the eIF4E–mRNA complex and cap-dependent translation (14). In this study, using pharmacologic agents and a panel of melanoma cell lines, we confirm that phosphoinositide 3-kinase (PI3K) signaling is necessary to cooperate with BRAFV600E signaling in melanoma. However, inhibition of AKT had little or no antiproliferative effects on BRAF-mutated human melanoma cell lines regardless of PTEN status. Similarly, the antiproliferative effects of pharmacologic blockade of AKT in BRAF-mutated melanoma cells expressing mutated or amplified AKT1 or AKT2, respectively, was not as profound as PI3K inhibition. Although single-agent inhibition of BRAFV600E → MEK1/2 → ERK1/2 or PI3K often displayed substantial antiproliferative activity, the effects of combined inhibition of both BRAFV600E plus PI3K signaling were significantly more potent. These data support the
hypothesis that PI3K signaling cooperates with BRAF<sup>V600E</sup> for melanoma maintenance through the regulation of protein translation and provides a biochemical basis for the simultaneous targeting of these two pathways in patients with BRAF-mutated melanoma (15–17).

**Materials and Methods**

**Cell culture and drug treatments**

Human melanoma cell lines, WM793, WM9, and A375, were kindly provided from the well-curated cell line repositories established by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA), and genomic sequencing of these cells was performed in the laboratory of Dr. Katherine Nathanson (University of Pennsylvania, Philadelphia, PA; Supplementary Table S1; refs. 18–20). The cell lines were cultured in DMEM-H16 media containing 3 mg/mL glucose, 0.584 mg/mL l-glutamine, 0.11 mg/mL sodium pyruvate, and 3.7 mg/mL NaHCO<sub>3</sub> supplemented with 10% FBS, 5 μg/mL of insulin, l-glutamine, penicillin/streptomycin, and fungizone. M249 and M262 melanoma cells were kindly provided by Dr. Antoni Ribas (University of California, Los Angeles) and authenticated by genomic sequencing as previously described (Supplementary Table S1; ref. 21). These cells were maintained in RPMI-1640 supplemented with 10% FBS, l-glutamine, penicillin/streptomycin, and fungizone. Pathway-targeted pharmacologic agents were obtained from various colleagues in the private sector or commercial sources and drug concentrations used for each treatment are listed in Supplementary Table S2.

**Proliferation and growth assays**

Melanoma cell proliferation was assessed by seeding 10<sup>5</sup> cells in 12-well plates. Cells were treated with the various pharmacologic agents as described in Supplementary Table S2 for 24, 48, and 72 hours. Viable cells were enumerated using a Countess automated cell counter (Invitrogen). Data presented are representative of three independent experiments. To complement short-term proliferation assays, replicate cultures of melanoma cells were plated in 6-well plates and cultured in the absence or presence of drug for 4 to 11 days with viable cells fixed and stained with crystal violet. Cell proliferation was quantified by solubilizing the crystal violet-stained cells in 33% acetic acid and measuring the absorbance at 562 nm using a plate reader.

**Immunoblot analysis**

Cells were lysed using radioimmunoprecipitation assay buffer (50 mmol/L Tris, 150 mmol/L NaCl, 0.5 mmol/L EDTA, 10 mmol/L NaF, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40) containing protease and phosphatase inhibitors (Pierce/Thermo Scientific) and then centrifuged at 14,000 rpm for 5 minutes at 4°C to generate postnuclear lysates with protein concentrations measured using the bicinchoninic acid assay (Pierce/Thermo Scientific; ref. 22). Thirty micrograms of protein were separated using NuPAGE Novex Bis-Tris gels (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membrane using an iBlot transfer apparatus (Invitrogen). Membranes were blocked in Odyssey blocking buffer (LI-COR Biosciences) and probed with the primary antibodies as described in Supplementary Tables S3 and S4. Antibody–antibody complexes were detected using fluorescent goat anti-rabbit IRDye 800 or goat anti-mouse IRDye 680 secondary antibodies (LI-COR Biosciences) and visualized with a LI-COR infrared imaging system (Odyssey Classic or Fc). Immunoblot data were analyzed using either the Odyssey application software v3.0.30 or Image Studio v2.0 software (LI-COR Biosciences; ref. 22).

**Protein synthesis assays**

Melanoma cells, plated at approximately 6 x 10<sup>5</sup> cells in 6-well plates, were treated with the indicated agents for 18 hours and then deprived of methionine for 1 hour before culturing in DMEM-H16 media containing 20 μCi of [<sup>35</sup>S]-methionine for 1 hour. Cells were prepared as previously described and 30 μg of cell extracts were electrophoresed and then transferred to PVDF membrane. Radiolabeled proteins were visualized by exposure to X-ray film and quantified by densitometric analysis using ImageJ 1.45 software (National Institute of Health, Bethesda, MD).

**m<sup>7</sup>GTP-Sepharose cap-binding assays**

Following the indicated drug treatments for 24 hours, melanoma cells were lysed in buffer A (10 mmol/L Tris, 140 mmol/L KCl, 4 mmol/L MgCl<sub>2</sub>, 1 mmol/L EDTA, 1% NP-40 with protease and phosphatase inhibitors). Four hundred micrograms of protein were incubated with 40 μL of m<sup>7</sup>GTP-Sepharose beads (GE Healthcare) in buffer B (10 mmol/L Tris, 140 mmol/L KCl, 4 mmol/L MgCl<sub>2</sub>, 1 mmol/L EDTA with protease and phosphatase inhibitors) overnight at 4°C. ElF4E-associated complexes were washed twice with buffer C (10 mmol/L Tris, 140 mmol/L KCl, 4 mmol/L MgCl<sub>2</sub>, 1 mmol/L EDTA, 0.5% NP-40 containing protease and phosphatase inhibitors) and then twice with cold PBS containing 5 mmol/L EDTA. m<sup>7</sup>GTP-Sepharose affinity precipitated proteins were boiled in 4× LDS Sample Buffer (Invitrogen) and analyzed by immunoblotting with the indicated antibodies.

**Statistical analysis**

All quantitative data are represented as mean ± SEM. GraphPad Prism 6 statistical software was used to determine <i>P</i> values by performing either a two-way ANOVA analysis with Bonferroni multiple comparisons test for the proliferation graphs or paired, two-tailed <i>t</i> tests for the quantification of cell proliferation by crystal violet-stained cells.

**Results**

Pharmacologic blockade of either BRAF<sup>V600E</sup> or PI3K signaling inhibits proliferation and signaling of human BRAF-mutated melanoma cells

To determine the signal pathway dependencies of BRAF-mutated melanoma cells, we examined the effects

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OF2 Mol Cancer Res; 12(3) March 2014  
Molecular Cancer Research

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of pharmacologic blockade of BRAF<sup>V600E</sup> → MEK1/2 → ERK1/2 or PI3K → AKT signaling using human melanoma-derived cell lines that were selected solely on the basis of their BRAF mutation status, some of which are proficient or deficient for PTEN expression (Supplementary Table S1; refs. 18–20). Agents used to inhibit BRAF<sup>V600E</sup> → MEK1/2 → ERK1/2 or PI3K → AKT signaling were chosen because of their reported specificity and selectivity against the relevant targets (Supplementary Fig. S1 and Supplementary Table S2; refs. 23–35). Where possible, results were verified by targeting multiple components of the same pathway using structurally unrelated, mechanistically dissimilar inhibitors of the same target.

Treatment of WM793, WM9, and A375 cells with either a MAP–ERK kinase (MEK1/2; 1 μmol/L PD0325901/MEKi)<sub>1</sub>) or an extracellular signal-regulated kinase (ERK1/2; 1 μmol/L SCH772984/ERKi) inhibitor for 24, 48, and 72 hours led to robust inhibition of cell proliferation (Fig. 1A; 72 hours; P < 0.001); however, we did not observe a significant decrease in cell number, which is consistent with the negligible proapoptotic effects of MEK inhibition on cultured melanoma cells (36). Similarly, inhibition of class I PI3K (5 μmol/L GDC-0941/PI3Ki)<sub>1</sub>) inhibited melanoma cell proliferation without a measureable decrease in cell number at 72 hours (WM793; P < 0.05; WM9 and A375; P < 0.0001). Surprisingly, inhibition of AKT with either of two structurally unrelated and mechanistically dissimilar inhibitors (MK-2206/AKTi<sub>1</sub> or GSK690693/AKTi<sub>2</sub>) was largely without effect in WM793 and A375 cells. In contrast, the proliferation of WM9 cells at 72 hours was inhibited by approximately 50% by both AKT inhibitors (AKT<sub>1</sub>; P < 0.01; Fig. 1A). In addition to short-term growth assays, the effect of these agents on longer term cell proliferation (4–6 days) was examined (Fig. 1B and Supplementary Fig. S4A). These data largely confirmed the short-term assays, in which single-agent inhibition of BRAF<sup>V600E</sup>, MEK1/2, ERK1/2, or PI3K had potent anti-proliferative activity compared with modest effects of AKT inhibition.

To examine the consequences of pathway-targeted inhibition on signaling downstream of BRAF<sup>V600E</sup> or PI3K, extracts were prepared from cells treated with the various agents for 4 or 24 hours and analyzed by immunoblotting (Fig. 1C and D). Information about the significance of each phosphorylation site is included in Supplementary Table S4. Control (CTL) WM793, WM9, and A375 cells displayed readily detectable phosphorylation of ERK1/2 (P-T202/Y204), AKT (P-S473), p70S6K (P-T389), ribosomal protein S6 (pS6; P-S235/236 and P-S240/244), and 4E-BP1 (P-T37/46 and P-S65). Inhibition of MEK1/2 (1 μmol/L PD0325901/MEKi)<sub>1</sub>) or ERK1/2 (1 μmol/L SCH772984/ERKi) led to decreased P-ERK1/2 with little or no effect on P-AKT. Similarly, inhibition of class I PI3K (5 μmol/L GDC-0941/PI3Ki)<sub>1</sub>) or AKT (5 μmol/L MK-2206/AKTi)<sub>2</sub>) potently suppressed P-AKT, but had no effect on P-ERK. Thus, BRAF<sup>V600E</sup> → MEK1/2 → ERK1/2 and PI3K → AKT signaling seems to be largely insulated from the inhibitory effects of the other pathway, at least over a 24-hour exposure period.

MEK1/2 or ERK1/2 inhibition resulted in decreased P-p70S6K and P-rpS6 in all three cell lines at 24 hours, whereas P-4E-BP1 was only significantly reduced in MEK1/2-inhibited WM793 and A375 cells (Fig. 1D and Supplementary Fig. S2). Although MEK1/2 or ERK1/2 inhibition for 4 hours potently suppressed P-p70S6K, there was no effect on P-rpS6 in any of the cell lines at this time point (Fig. 1C). Interestingly, inhibition of PI3K for either 4 or 24 hours significantly reduced P-rpS6 in all cell lines, which occurred in the absence of changes in P-p70S6K (Fig. 1C and D and Supplementary Fig. S2). Inhibition of PI3K also led to reduced P-4E-BP1 at both 4 and 24 hours with the most profound effect at the later time point (Fig. 1C and D and Supplementary Fig. S2). Strikingly, AKT inhibition with either of two inhibitors had no discernible effect on either P-p70S6K or P-4E-BP1 and only reduced P-rpS6 by approximately 50% in WM9 cells at both time points (24 hours; P < 0.05; Fig. 1C and D and Supplementary Fig. S2). Taken together, these data suggest that BRAF<sup>V600E</sup> → MEK1/2 → ERK1/2 and PI3K signaling regulates mTOR complex 1 (mTORC1)-dependent phosphorylation of rpS6 and 4E-BP1 signaling events through distinct mechanisms that are largely independent of AKT activity.

**Temporal regulation of rpS6 and 4E-BP1 phosphorylation by BRAF<sup>V600E</sup> → MEK1/2 → ERK1/2 or PI3K**

To further assess differences in the regulation of rpS6 and 4E-BP1 phosphorylation downstream of BRAF<sup>V600E</sup> and PI3K signaling, WM793 or A375 cells were treated with inhibitors of either MEK1/2 (PD0325901/MEKi)<sub>1</sub>) or PI3K (GDC-0941/PI3Ki)<sub>1</sub>) for 1 to 10 hours (Fig. 2A). Both of these agents exerted robust and selective inhibitory effects against P-ERK1/2 or P-AKT after one hour of treatment. Although MEK1/2 inhibition led to a rapid decrease of P-p70S6K, this did not result in an immediate decrease in rpS6 phosphorylation as we noted a 4–to 8-hour delay between effects of MEK1/2 inhibition on P-p70S6K and dephosphorylation of rpS6. As before, MEK1/2 inhibition had negligible effects on P-4E-BP1 at all time points evaluated. Although PI3K inhibition had no effect on p70S6K phosphorylation at any time point, there was a notable decrease in P-rpS6 in both cell lines within 4 hours, which was most striking in WM793 cells after 10 hours (Fig. 2A). In addition, PI3K inhibition also led to decreased P-4E-BP1 that was detected within 1 hour in both cell lines (Fig. 2A). Thus, inhibition of either MEK1/2 or PI3K resulted in decreased phosphorylation of rpS6, but elicited their effects with different kinetics and contrasting effects on the activation status (P-T389) of p70S6K.

Evidence suggests that phosphorylation of rpS6 and 4E-BP1 is downstream of mTORC1 (37). However, BRAF<sup>V600E</sup> → MEK1/2 → ERK1/2 signaling can also contribute to rpS6 phosphorylation through p90RSK (38).
To determine the role of mTORC1 signaling and the S6 kinase, p70S6K or p90RSK, responsible for sustaining rpS6 phosphorylation, WM793 and A375 cells were treated with either PP242 (1 or 5 μM mTORi), a potent ATP-competitive inhibitor of mTORC1/2 signaling; FMK (1 μM RSKi), a covalent inhibitor of p90RSK; or DG2 (10 μM S6Ki), a p70S6K inhibitor (Fig. 2B and Supplementary Table S2; refs. 23, 24, 30). As expected, PP242-mediated inhibition of mTORC1/2 resulted in decreased phosphorylation of P-rpS6 and P-4E-BP1 (Fig. 2B). In addition, a mechanistically distinct mTORC1 inhibitor, rapamycin, also inhibited P-rpS6, but had no effect on 4E-BP1 phosphorylation (Supplementary Fig. S3A). Although inhibition of p90RSK had no effect on either P-rpS6 or P-4E-BP1, inhibition of p70S6K selectively reduced P-rpS6. These data are consistent with a model in which phosphorylation of both rpS6 and 4E-BP1 is mTORC1 dependent and the phosphorylation of rpS6 is p70S6K dependent but p90RSK independent (Fig. 2B).

In parallel, we assessed the effects of mTORC1/2 inhibition (PP242/mTORi) on melanoma cell proliferation...
Figure 1. (Continued.) C and D, WM793, WM9, and A375 cells were treated with DMSO (CTL) or inhibitors of MEK1/2 (MEKi1), ERK1/2 (ERKi), PI3K (PI3Ki1), or AKT (AKTi1 or AKTi2) for 4 (C) or 24 (D) hours and cell lysates were immunoblotted with the indicated antibodies.
Figure 2. Kinetics of rpS6 and 4E-BP1 dephosphorylation following inhibition of BRAF<sub>V600E</sub> or PI3K signaling. A, WM793 and A375 melanoma cells were treated with MEK1/2 (1 µmol/L PD0325901/MEKi1) or PI3K (5 µmol/L GDC-0941/PI3Ki1) inhibitors for 1, 4, 8, and 10 hours and cell lysates were analyzed by immunoblotting with the indicated antibodies. B, cells were treated with inhibitors of mTORC1/2 (1 or 5 µmol/L PP242/mTORi), p90RSK (1 µmol/L FMK/RSKi), or p70S6K (10 µmol/L DG2/S6Ki) for 24 hours and cell lysates were immunoblotted with the indicated antibodies. (Continued on the following page.)
using both short-term (1–3 days) and long-term (11 days) proliferation assays (Fig. 2C and D and Supplementary Fig. S4B). Proliferation of WM793, WM9, and A375 cells was significantly inhibited by either 1 or 5 μmol/L PP242 at all time points analyzed (24 hours: WM793 \( P < 0.05 \), WM9 and A375 \( P < 0.01 \); 48–72 hours: \( P < 0.0001 \)); however, the antiproliferative effect of 1 μmol/L PP242 was less robust against WM793 and A375 cells (Fig. 2C). These results are consistent with a longer term proliferation assay in which 5 μmol/L PP242 elicited more potent antiproliferative effects against all three cell lines than 1 μmol/L PP242 (Fig. 2D and Supplementary Fig. S4B). Similar to previous analyses of BRAF\(^{V600E} \), PTEN\(^{\text{Null}} \) melanoma cells derived from a GEM model, rapamycin was less potent in inhibiting the proliferation of all three human BRAF-mutated melanoma cell lines (Supplementary Fig. S3B; ref. 11). Thus, dual control of mTORC1 signaling by cooperative BRAF\(^{V600E} \) and PI3K signaling appears important for melanoma cell proliferation.

**BRAF\(^{V600E} \) and PI3K signaling cooperate to regulate rpS6 and 4E-BP1 phosphorylation and melanoma cell proliferation**

Given that BRAF\(^{V600E} \) and PI3K signaling cooperate to regulate mTORC1 signaling output, we tested whether combined inhibition of these pathways might elicit more striking effects on intracellular signaling events leading to more profound effects on melanoma cell proliferation. Extracts of WM793, WM9, or A375 cells were treated with inhibitors of BRAF\(^{V600E} \), MEK1/2, or PI3K, either alone or in combination, and analyzed by immunoblotting.
As previously demonstrated, MEK1/2 (PD0325901/MEKi1) or BRAFV600E (PLX-4032/BRAFi2) inhibition led to decreased P-ERK, P-p70S6K, and P-rpS6 with only modest inhibition of P-4E-BP1. PI3K inhibition (GDC-0941/PI3Ki1) robustly suppressed P-AKT and P-rpS6, had no effect on P-p70S6K, and led to a modest decrease of P-4E-BP1 in WM793 cells with a more profound inhibition in WM9 and A375 cells. Although PI3K inhibition reproducibly inhibited P-4E-BP1, we noted some experiment-to-experiment variation in the magnitude of this effect. Most importantly, combined inhibition of either BRAFV600E or MEK1/2 plus PI3K routinely resulted in a more potent suppression of P-rpS6 and P-4E-BP1 compared with the corresponding single agents (Figs. 3A and 4A).

In parallel, the effects of combined MEK1/2 plus PI3K inhibition on cell proliferation were assessed in short- and long-term assays (Fig. 3B–D). In the short term, single-agent inhibition of MEK1/2 (MEKi1) or PI3K (PI3Ki1) significantly inhibited cell growth at 48 (P < 0.01) and 72 (P < 0.01) hours. As predicted by the single-agent effects, combined inhibition of MEK1/2 plus PI3K also led to a significant decrease in melanoma cell proliferation, but this was not superior to single-agent inhibition at any time point (Fig. 3B). In the longer-term assays (9 days), single-agent MEK1/2 inhibition had more potent antiproliferative activity compared with single-agent PI3K inhibition (Fig. 3C and D). However, in all three cell lines, combined inhibition of both MEK1/2 and PI3K significantly inhibited melanoma proliferation compared with the corresponding single agents with the magnitude of the observed effects being greatest in WM9 and A375 cells (Fig. 3C and D). These results were confirmed using a short-term proliferation assay (4 days), in which combined inhibition of BRAFV600E (BRAFi5) plus PI3K (PI3Ki1) more potently suppressed melanoma cell growth compared with single-agent inhibition alone (Fig. 4B and Supplementary Fig. S4C). These data suggest that, at least in vitro, BRAFV600E and PI3K signaling cooperate to promote key signaling events downstream of mTORC1, which in turn promote melanoma cell proliferation.

**Coordinate control of melanoma protein synthesis by BRAFV600E and PI3K signaling**

In conjunction with previous experiments, we assessed the effects of combined inhibition of MEK1/2 (PD0325901/MEKi1) or PI3K (GDC-0941/PI3Ki1) on melanoma protein synthesis by investigating the effects of inhibitor treatment on P-ERK1/2 T202/Y204 T-ERK1/2 P-AKT S473 P-p70S6K T389 P-rpS6 S235/236 P-rpS6 S240/244 P-4E-BP1 T37/46 P-4E-BP1 S65 T-rpS6 β-Actin in WM793, WM9, and A375 cells. The results are presented in Figure 3A and Supplementary Figure S4. The effects of inhibitor treatment were confirmed using a short-term proliferation assay (4 days), in which combined inhibition of BRAFV600E (BRAFi5) plus PI3K (PI3Ki1) more potently suppressed melanoma cell growth compared with single-agent inhibition alone (Fig. 4B and Supplementary Fig. S4C). These data suggest that, at least in vitro, BRAFV600E and PI3K signaling cooperate to promote key signaling events downstream of mTORC1, which in turn promote melanoma cell proliferation.

**Figure 3.** Effects of single-agent or combined inhibition of MEK1/2 or PI3K on melanoma cell signaling and proliferation. A, WM793, WM9, and A375 cells were treated with inhibitors of MEK1/2 (1 μmol/L PD0325901/MEKi1) or PI3K (5 μmol/L GDC-0941/PI3Ki1) either alone or in combination for 18 hours with cell lysates analyzed by immunoblotting with the indicated antibodies. B, single-agent or combined inhibition of MEK1/2 (MEKi1) or PI3K (PI3Ki1) on WM793, WM9, and A375 cell proliferation for 24, 48, and 72 hours. Results are expressed as relative cell number to the DMSO-treated controls and presented as mean ± SEM (n = 3). (Continued on the following page.)
MEKi1) plus PI3K (GDC-0941/PI3Ki1) on protein synthesis in WM793, WM9, and A375 cells (Fig. 5A and B). Single-agent inhibition of MEK1/2 or PI3K alone led to a 40% to 80% inhibition of [35S]-methionine incorporation (Fig. 5B). The effect of MEK1/2 inhibition on protein synthesis was most striking in WM793 cells, whereas PI3K inhibition had the equivalent effects on protein synthesis in all three cell lines. Importantly, combined inhibition of both MEK1/2 plus PI3K resulted in the most profound inhibitory effects on protein translation in all three cell lines. Consistent with previous observations, combined inhibition of MEK1/2 and PI3K also had the most striking effects on P-rpS6 and P-4E-BP1 compared with the corresponding single agents (Fig. 5A).

Hypophosphorylated 4E-BP1 binds to the eIF4E initiation complex at the 5’ cap of mRNAs to inhibit cap-dependent translation by preventing eIF4G from binding to this complex (39). Phosphorylation of 4E-BP1 by mTORC1 releases it from eIF4E allowing eIF4G to bind to the eIF4E–mRNA complex and initiate cap-dependent translation (40). Thus, hypophosphorylated 4E-BP1 and eIF4G directly compete for the same binding site on eIF4E, whereas hyperphosphorylated 4E-BP1 is not a strong competitor for this site (40). Because activation of BRAFV600E and PI3K signaling promotes hyperphosphorylation of 4E-BP1, we examined the effects of MEK1/2 or PI3K inhibition on cap-dependent protein translation, specifically the association between 4E-BP1 and eIF4E.
and the eIF4E complex. Extracts of melanoma cells treated with inhibitors of MEK1/2 (MEKi1) or PI3K (PI3Ki1), either alone or in combination, were used in a cap pull-down assay. In this assay, eIF4E present in melanoma lysates binds to sepharose beads coupled to the cap analog 7-methyl GTP (m7GTP) to determine whether there is an

**Figure 4.** Effects of single-agent or combined inhibition of BRAF<sup>V600E</sup> or PI3K on melanoma cell growth and signaling. A, WM793, WM9, and A375 cells were treated with inhibitors of BRAF<sup>V600E</sup> (5 μmol/L PLX-4032/BRAFi2) or PI3K (5 μmol/L GDC-0941/PI3Ki1) as single agents or in combination for 24 hours with cell lysates analyzed by immunoblotting with the indicated antibodies. B, melanoma cell proliferation was assessed by treating all three cell lines with inhibitors of BRAF<sup>V600E</sup> (BRAFi2) or PI3K (PI3Ki1) alone or in combination for 4 days with viable cells fixed and stained with crystal violet.
Figure 5. Cooperation of BRAFV600E and PI3K signaling on the regulation of protein translation. A, protein synthesis was measured by [35S]-methionine incorporation of newly synthesized proteins in WM793, WM9, and A375 cells treated with inhibitors of MEK1/2 (1 μmol/L PD0325901/MEKi1) or PI3K (5 μmol/L GDC-0941/PI3Ki1) as single agents or in combination for 18 hours (n = 2). These same cell extracts were also analyzed for rpS6 and 4E-BP1 phosphorylation as indicated. B, densitometric quantification of [35S]-methionine incorporation of newly synthesized proteins in all three cell lines. Results are represented as a fold-change of the DMSO-treated control. C, WM793, WM9, and A375 cells were treated with inhibitors of MEK1/2 (MEKi1) or PI3K (PI3Ki1) alone or in combination for 24 hours with cell lysates precipitated with m7GTP sepharose beads followed by immunoblotting for 4E-BP1, eIF4E, or with the indicated antibodies.
increase or decrease in 4E-BP1 bound to the eIF4E–m7GTP cap complex.

As observed previously, combined inhibition of MEK1/2 plus PI3K more potently suppressed P-rpS6 and P-4E-BP1 than the corresponding single-agent treatments in the crude cell extracts used for the cap pull-down assays (Fig. 5C). However, despite the effects of MEK1/2 inhibition on P-4E-BP1, we only observed increased association of 4E-BP1 with
the eIF4E–m7GTP cap complex in WM793 cells. In all three cell lines, PI3K inhibition led to increased association of 4E-BP1 with the eIF4E–m7GTP cap complex. Interestingly, and contrary to expectation, combined inhibition of MEK1/2 plus PI3K did not lead to a further increase in 4E-BP1 association with eIF4E–m7GTP cap complexes. These data suggest that the regulation of melanoma protein synthesis downstream of BRAFV600E and PI3K activation is more complex than the simple regulation of the stoichiometry of 4E-BP1 phosphorylation detected in cell lysates and its capacity for interaction with eIF4E.

Inhibitors of MEK1/2 and AKT display little or no cooperative effects against melanoma proliferation or signaling

The ineffectiveness of the AKT inhibitors in suppressing melanoma cell proliferation was surprising given the prominence ascribed to AKT as an effector of PI3K signaling in cancer (14, 31, 41). Because AKT inhibition had modest effects on melanoma cells, we assessed whether combined inhibition of MEK1/2 plus AKT might provide a more robust inhibition of melanoma cell proliferation and signaling. To test the effects of single-agent versus combined MEK1/2 or AKT inhibition, melanoma cells were treated with MEK1/2 (1 μmol/L GSK1120212/MEKi2) or AKT (5 μmol/L GSK690693/AKTi2) inhibitors, either alone or in combination, for 5 to 6 days with viable cells stained with crystal violet (Fig. 6A and Supplementary Fig. S4D). Similar to our previous results, single-agent MEK1/2 inhibition profoundly inhibited the proliferation of all three melanoma cell lines; however, the antiproliferative effects of AKT inhibition were modest (~35%–70% inhibition) and substantially less than that observed in response to single-agent PI3K inhibition (Fig. 1A and B). Moreover, inhibition of AKT did not substantially enhance the antiproliferative effect of MEK1/2 inhibition in these cells (Fig. 6A and Supplementary Fig. S4D).

To assess the effect of combined inhibition of MEK1/2 and AKT on melanoma signaling, melanoma cells were treated with MEK1/2 (GSK1120212/MEKi2) or AKT (GSK690693/AKTi2) inhibitors, either alone or in combination, for 24 hours with extracts analyzed by immunoblotting (Fig. 6B). As expected, MEK1/2 inhibition led to decreased P-ERK, P-rpS6, and P-4E-BP1 with no effect on P-AKT. AKT inhibition greatly enhanced AKT phosphorylation, as predicted by this agent’s mechanism of action, and reduced P-PRAS40 (T246), a direct downstream substrate of AKT. Consistent with previous data, AKT inhibition had no effect on P-4E-BP1 in any of the cell lines, but moderately decreased P-rpS6 in WM9 cells with little or no discernable inhibitory effect in WM793 or A375 cells. Combined MEK1/2 plus AKT inhibition had only a modest effect on P-rpS6 in WM9 and A375 cells, whereas P-rpS6 in WM793 cells was not further decreased compared with single-agent inhibition with MEK1/2. Likewise, the combination of MEK1/2 and AKT inhibition was also ineffective in further reducing P-4E-BP1 compared with MEK1/2 inhibition alone. Although, AKT may modestly contribute to rpS6 phosphorylation, its contribution is substantially less than what is observed following inhibition of BRAFV600E → MEK1/2 → ERK1/2 or PI3K signaling. Taken together, these data emphasize the importance of BRAFV600E and PI3K signaling in the regulation of protein translation leading to melanoma cell proliferation in a substantially AKT-independent manner.

Effects of AKT inhibition on melanoma cells with mutated or amplified AKT1/2

A small percentage of human melanoma cells are reported to express either amplified or mutated AKT1 in addition to mutated BRAF (21). In addition, AKT1 mutation is associated with BRAFV600E inhibitor resistance in melanoma (42, 43). Consequently, we tested the effects of AKT inhibition on the proliferation of melanoma cells with alterations in AKT1 or 2 (Supplementary Table S1; ref. 21). M249 (BRAFV600E, PTENNull/AKT2Amp) or M262 (BRAFV600E, PTENWT/AKT1E17K/Amp) cells were treated with inhibitors of BRAFV600E (GSK2118436/BRAFi), MEK1/2 (PD0325901/MEKi1), PI3K (GDC-0941/P3Ki), or AKT (MK-2206/AKTi1 or GSK690693/AKTi2) with proliferation measured at 24, 48, or 72 hours (Fig. 7A). In short-term cell proliferation assays, neither of the AKT inhibitors was as effective as single-agent inhibition of MEK1/2 or PI3K at inhibiting cell proliferation (Fig. 7A). In the longer-term assays, the allosteric AKT inhibitor MK-2206 inhibited the growth of M249 cells (AKT2Amp) as effectively as single-agent inhibition of BRAFV600E, MEK1/2, or PI3K (Fig. 7B and Supplementary Fig. S4E). In contrast, the ATP-competitive AKT inhibitor, GSK690693, was substantially less effective in these same cells. Both AKT inhibitors inhibited cell growth by 60% to 65% in M262 cells (AKT1E17K/Amp), but were not as effective as single-agent blockade of BRAFV600E, MEK1/2, or PI3K (Fig. 7B and Supplementary Fig. S4E). Although these data suggest that AKT inhibition in melanoma cells with mutated or amplified AKT1/2 may predict for more robust antiproliferative effects on cell growth, these effects generally fall short of what is observed with inhibitors of BRAFV600E → MEK1/2 → ERK1/2, or PI3K signaling.

We next assessed the effects of the various agents on signaling downstream of BRAFV600E → MEK1/2 → ERK1/2 or PI3K → AKT (Fig. 7C). As previously observed with other melanoma cells, inhibition of BRAFV600E → MEK1/2 → ERK1/2 signaling in either M249 or M262 cells led to substantially decreased P-ERK, P-p70S6K, and P-rpS6 with only modest effects on P-4E-BP1. Blockade of PI3K with two different inhibitors (GDC-0941/P3Ki or BKM-120/P3Ki(2)) suppressed P-AKT and P-rpS6, modestly reduced P-4E-BP1, but did not alter P-p70S6K. Although both AKT inhibitors had their predicted effects on P-AKT (decreased with MK-2206/AKTi1, increased with GSK690693/AKTi2), these agents had only modest effects on P-rpS6 and negligible effects on either P-p70S6K or P-4E-BP1 compared with single-agent blockade of BRAFV600E, MEK1/2, ERK1/2, or PI3K.
A. Control BRAFi MEKi PI3Ki AKTi AKTi2

B. M249 (BRAF^{V600E}, PTEN^{Null}, AKT2^{Amp})

B. M262 (BRAF^{V600E}, PTEN^{WT}, AKT1^{E17K/Amp})

C. M249 (BRAF^{V600E}, PTEN^{Null}, AKT2^{Amp})

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Discussion

The conversion of melanocytes to metastatic melanoma cells is accompanied by alterations in key signaling pathways that cooperate in melanomagenesis (Supplementary Fig. S1; refs. 1, 7, 11, 44–46). The most common alterations in the PI3K α → AKT pathway are silencing of the PTEN, INPP4B, or PI3Kα lipid phosphatases and, less commonly, point mutation/amplification of PIK3CA or AKT1-3 (21, 47–51). Thus, cooperating alterations in PI3K signaling seem to be essential for progression of BRAFV600E-initiated melanoma. Here, we show that melanoma cells, regardless of their PTEN or AKT mutational status, were sensitive to single-agent inhibition of BRAFV600E or PI3K signaling and even more susceptible to combined pathway inhibition. This suggests that melanoma cells, even those lacking genetically defined alterations in PI3K signaling (such as A375 cells), have a requirement for PI3K signaling for their maintenance (52, 53).

Regulation of protein translation is a process critically required for the aberrant behavior of cancer cells (54). Considerable attention has focused on the mechanisms by which PI3K signaling regulates mTORC1 activity to initiate cap-dependent protein translation. Here, we demonstrate that, in BRAF-mutated melanoma cells, these signaling events are under the coordinate control of both BRAFV600E and PI3K signaling in a manner that appears largely AKT independent. Others have previously reported dual control of these signaling events in tumor cells with coincident KRAS and PIK3CA mutations; however, in that case AKT was reported to be a key effector of PI3K signaling (14). Moreover, in these cells, single-agent blockade of MEK1/2 or AKT had little effect on proliferation, rpS6 or 4E-BP1 phosphorylation, or cap-dependent translation, and combined inhibition of both pathways was required to suppress these processes (14). In contrast, we show that single-agent inhibition of either BRAFV600E or PI3K signaling was sufficient to inhibit both protein synthesis and melanoma cell proliferation, but that combined pathway blockade led to more robust suppression of proliferation. Interestingly, single-agent inhibition of mTORC1/2 with PP242 inhibited cell proliferation as potently as the blockade of either single-agent inhibition of mTORC1/2 with PP242 inhibition or PI3K signaling, suggesting the potential importance of mTORC1/2 signaling as an effector of cooperating BRAFV600E and PI3K activity for melanoma cell proliferation.

Mechanistically, it remains unclear how BRAFV600E or PI3K influences the phosphorylation of rpS6 and 4E-BP1. It is possible that BRAFV600E signaling through ERK1/2 regulation of the TSC1/2 complex as previously proposed (55). In contrast, PI3K blockade also led to diminished rpS6 phosphorylation, but this occurred in a largely AKT-independent manner and without any change in the key activating phosphorylation of p70S6K (P-T389). One possibility is that PI3K–lipid signaling may regulate rpS6 phosphorylation through effects on PP1, the protein phosphatase that dephosphorylates rpS6 (56). Although we propose that PI3K cooperates with BRAFV600E to regulate melanoma protein synthesis through cooperative effects on translation initiation, there are likely additional regulatory events in protein synthesis under the control of these pathways as suggested by the results of the m7GTP cap-binding experiments (57).

Despite effective suppression of AKT phosphorylation by either of the two structurally unrelated and mechanistically dissimilar agents, we observed only modest effects on melanoma cell proliferation and failed to detect the expected effects on downstream signaling events. The effects of these agents on cell proliferation or signaling in cells expressing either amplified or mutated AKT1/2 were less robust than expected on the basis of current literature (21). Moreover, the combined blockade of MEK1/2 and AKT had little or no effect on the inhibition on rpS6 and 4E-BP1 phosphorylation, which stood in contrast with the substantive cooperative effects of PI3K and BRAFV600E or MEK1/2 inhibition. Consequently, the lack of potent single-agent activity and evidence of strong cooperation with the BRAFV600E pathway inhibitors, suggest that AKT is not an attractive target for melanoma therapy. This is consistent with the inability of the allosteric AKT inhibitor (MK-2206) to prevent the growth of BRAFV600E, PTENmut melanomas in a GEM model (58). However, because evidence suggests that AKT1 mutation can promote resistance of BRAF-mutated melanomas to vemurafenib, AKT inhibitors might forestall the onset of drug resistance in patients (42, 43). Although alternative effectors of PI3K–lipid signaling in melanomagenesis remain to be elucidated, there are numerous proteins with PH, PX, or FYVE PI3K–lipid–binding domains that are potential candidates (59). Indeed, parsing out which of these effectors is important for the cooperation of oncogenic BRAFV600E and PI3K signaling will help to decipher the underlying biochemical and signaling mechanisms that exist in the melanoma cell and could lead to the identification of new potential biomarkers and molecular targets for the treatment of patients with melanoma.

Figure 7. Pharmacologic inhibition of BRAFV600E or PI3K signaling in melanoma cells with mutated or amplified AKT. A, M249 (BRAFV600E, PTENdel, AKT1E507K) and M262 (BRAFV600E, PTENdel, AKT1E17K/A357K and M652 (BRAFV600E, PTENdel, AKT1E17K/A357K) melanoma cells were treated with DMSO (CTL) or inhibitors of BRAFV600E (1 μmol/L PD0325901/BRAFi1), MEK1/2 (1 μmol/L PD0325901/MEKi1), PI3K (5 μmol/L GDC-0941/Pi3Ki1), or AKT (5 μmol/L MK-2206/AKTi1 or 5 μmol/L GSK690693/AKTi2) with cell proliferation assayed for 24, 48, and 72 hours. Results are expressed as relative cell number to the DMSO-treated controls and presented as mean ± SEM (n = 3). B, in parallel, these cells were plated at a 30% confluence and cultured in the continuous presence of DMSO or inhibitors of BRAFV600E (BRAFi1), MEK1/2 (MEKi1), PI3K (Pi3Ki1) or AKT (AKTi1 or AKTi2) for 4 (M249) or 6 (M262) days with viable cells fixed and stained with crystal violet. C, M249 and M262 cells were treated with DMSO (CTL) or inhibitors of BRAFV600E (BRAFi1), MEK1/2 (MEKi1), ERK1/2 (ERKi), PI3K (Pi3Ki) or 5 μmol/L BKM-120/Pi3Ki2 or AKT (AKTi1 or AKTi2) for 24 hours and cell lysates were analyzed by immunoblotting with the indicated antibodies.
Disclosure of Potential Conflicts of Interest

M. McMahon has commercial research grants from Novartis and Plexicon and is a consultant/advisory board member of AbbVie. No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: J.M. Silva, M. McMahon
Development of methodology: J.M. Silva, M. McMahon
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.M. Silva, M. McMahon
Writing, review, and/or revision of the manuscript: J.M. Silva, M. McMahon
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.M. Silva
Study supervision: M. McMahon

Acknowledgments

The authors thank all the members of the McMahon laboratory for advice and guidance on this project with special thanks to Victoria Marsh Durban and Marian Deuker, their colleagues Craig Trumpf and Davide Ruggero (UCSF) for assistance with protein synthesis and mGTP cap-binding assays and advice on the regulation of protein translation, and Kevan Shokat (UCSF) and Jack Taunton (UCSF) for DG2, PP242, and FMK as well as Drs. Meinhard Helin (Wistar Institute) and Antoni Ribas (UCLA) for providing melanoma cell lines. The authors also thank their many colleagues in the private sector for providing compounds and information on their use: Leisa Johnson and Lori Friedman (Genentech) for GDC-0941; Tona Gilmer and Kirin Patel (Glaxo-Smith-Kline) for GSK2118436 and GSK112021; Steven Townson, Heike Keilhack, and Ahmed Samatar (Merck & Co.) for MK-2206 and SCH772984; Janet Lyle, Darrin Snarr, and Emmanuel di Tomaso (Novartis) for BKM-120; and Fei Su (formerly of Roche) for PLX-4032.

Grant Support

This work was supported by grants from the Melanoma Research Alliance, an NIH/NCI R01 CA176839 (to M. McMahon), and an Institutional Research and Academic Career Development Award (IRA/CDA; to J.M. Silva). 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 May 8, 2013; revised December 11, 2013; accepted December 27, 2013; published OnlineFirst January 14, 2014.

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Molecular Cancer Research

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Mol Cancer Res  Published OnlineFirst January 14, 2014.

Updated version  Access the most recent version of this article at: doi:10.1158/1541-7786.MCR-13-0224-T

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